Chapter 6
Chronic Ethanol Consumption and Generation of Etheno-DNA Adducts in Cancer-Prone Tissues

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Abstract Chronic ethanol consumption is a risk factor for several human cancers. A variety of mechanisms may contribute to this carcinogenic effect of alcohol including oxidative stress with the generation of reactive oxygen species (ROS), formed via inflammatory pathways or as byproducts of ethanol oxidation through cytochrome P4502E1 (CYP2E1). ROS may lead to lipidperoxidation (LPO) resulting in LPO-products such as 4-hydoxynonenal (4-HNE) or malondialdehyde. These compounds can react with DNA bases forming mutagenic and carcinogenic etheno-DNA adducts. Etheno-DNA adducts are generated in the liver (HepG2) cells over-expressing CYP2E1 when incubated with ethanol;and are inhibited by chlormethiazole. In liver biopsies etheno-DNA adducts correlated significantly with CYP2E1. Such a correlation was also found in the esophageal- and colorectal mucosa of alcoholics. Etheno-DNA adducts also increased in liver biopsies from patients with non alcoholic

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steatohepatitis (NASH). In various animal models with fatty liver either induced by high fat diets or genetically modified such as in the obese Zucker rat, CYP2E1 is induced and paralleled by high levels of etheno DNA-adducts which may be modified by additional alcohol administration. As elevation of adduct levels in NASH children were already detected at a young age, these lesions may contribute to hepatocellular cancer development later in life. Together these data strongly implicate CYP2E1 as an important mediator for etheno-DNA adduct formation, and this detrimental DNA damage may act as a driving force for malignant disease progression.

Keywords Cytochrome P4502E1 · Etheno-DNA adducts · Reactive oxygen species · NASH · ALD · Esophageal Cancer · Colorectal Cancer

6.1 Introduction

There is strong evidence that oxidative stress related DNA damage is induced by known inherited and acquired cancer risk factors including inflammation [1–3]. Pro-mutagenic Lipidperoxidation (LPO)-derived DNA adducts are increased significantly in chronic pancreatitis [2], ulcerative colitis, Crohn's disease [2] as well as in viral-Hepatitis [2] and other types of chronic liver disease [4]. Two major etheno-DNA adducts 1,N6-etheno-2′-deoxyadenosine (εdA) and 3,N4-etheno-2′-deoxycytidine (εdC) were quantified as marker lesions and found to accumulate in target organs over time, paralleling progression to tumor development [1–3, 5, 6]. DNA repair and cellular apoptotic processes contribute to urinary excretion of etheno-desoxyribonucleosides, which offer a non-invasive approach to monitor LPO-related pathogenic processes in vivo [7].

In this article we review formation and significance of exocyclic etheno DNA adducts and their possible role in human and experimental carcinogenesis. Major emphasis, however, will be led on the effect of chronic ethanol consumption and the generation of these adducts in the liver and other extrahepatic tissues. Finally, the relevance of etheno DNA adducts in non-alcoholic fatty liver disease (NAFLD) is discussed.

6.2 Etheno-DNA Adducts: Formation and Significance

Upregulation and overexpression of stress response enzymes such as inducible nitric oxide synthase (iNOS), lipoxygenase (LOX) and possibly cyclooxygenase (COX)-2 in inflamed tissues proceeds malignant growth. Hereby a self-perpetuating stimulation of LPO, over-production of DNA-damaging ROS and reactive nitrogen species (RNS), as well as LPO-derived exocyclic-DNA adducts takes place, acting as a driving force to malignancy [2] (Abb.1).

This cascade of events was supported by rodent models and adduct-analysis of organ tissue/ biopsy samples from cancer-prone patients. In Swiss Jim-Lambert-mice

inflammatory-related NO overproduction was found to be associated with significant increased etheno-DNA formation; both could be inhibited by the administration of an iNOS inhibitor [8]. In a multistage mouse skin carcinogenesis model etheno-DNA adducts correlated with LOX-catalyzed tumor associated arachidonic acid metabolites [9]. Similarly, increased adduct levels were found in target tissues of Apc min (multiple intestinal neoplasia) mice [10, 11] as well as in cancer-prone patients with familial adenomatous polyposis (FAP) [12].

Induction of cytochrome P-450 2E1 as in alcoholic liver disease (ALD) and NAFLD may also result in ROS and etheno-DNA adduct formation [13, 14]. Although ethanol is primarily oxidized via alcohol dehydrogenase, a small percentage is metabolized via the microsomal ethanol oxidizing system (MEOS) which is CYP2E1 dependent. This pathway increases when ethanol is consumed chronically. Besides acetaldehyde, the first metabolite of ethanol oxidation, ROS are generated which trigger lipid peroxidation (LPO), leading to DNA adduction that likely participates in tumourigenesis (Fig. 6.1).

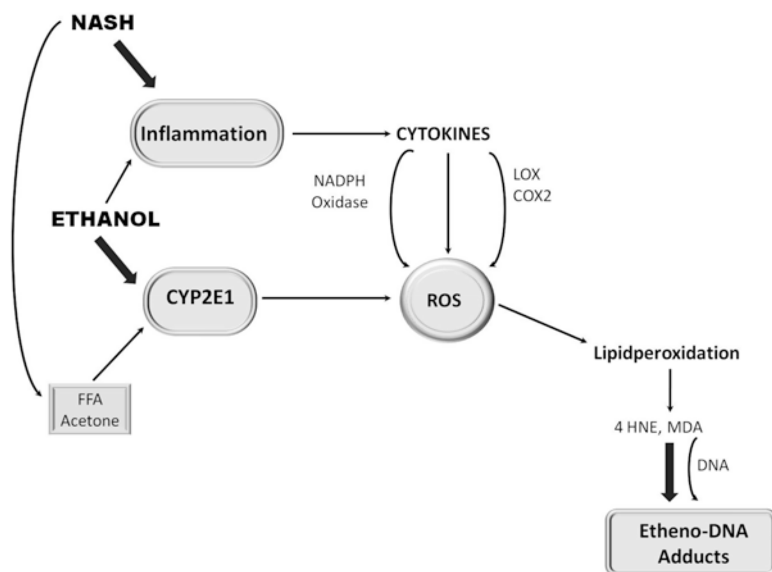

Fig. 6.1 Simplified pathophysiology of reactive oxygen species (ROS) and etheno DNA adduct formation. Inflammation driven cytokine secretion results among others in an activation of NADPH oxidase and via NFκB in an activation of lipoxygenase (LOX) and cyclooxygenase 2 (COX-2). As a result ROS are generated, which lead to lipidperoxidation with the occurrence of lipidperoxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These adducts react with DNA bases to form exocyclic etheno-DNA adducts. Chronic alcohol consumption results in the induction of cytochrome P4502E1 (CYP2E1), which is involved in ethanol oxidation via the microsomal ethanol oxidizing pathway. During this reaction ROS is generated without inflammation. To a minor degree ethanol may result in ROS formation through inflammation (alcoholic hepatitis). On the other hand, in NASH ROS is primarily formed through inflammation and to a minor degree through C YP2E1 induction via acetone (diabetes mellitus) and/or free fatty acids (FFA)

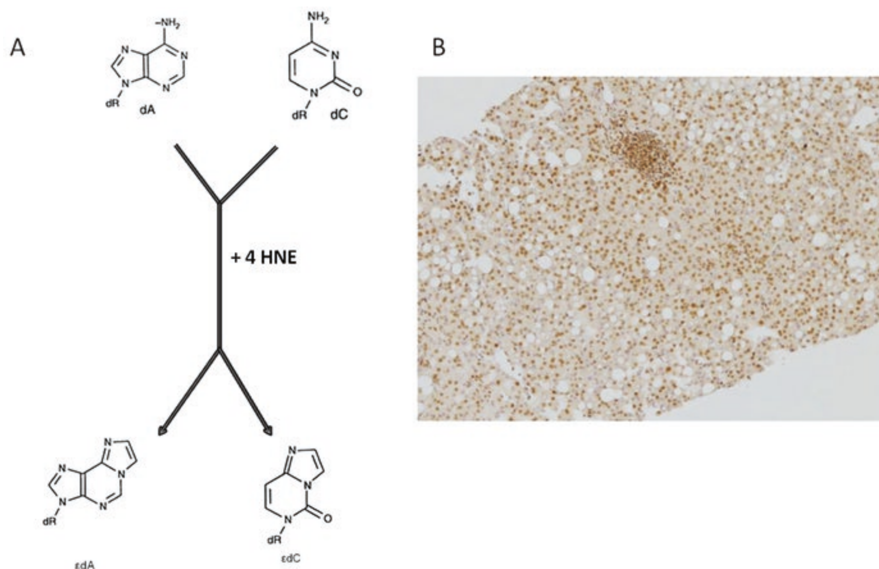

Fig. 6.2 1,N6-etheno-2′-deoxyadenosine(εdA), and 3, N4-etheno-2′-deoxycytidine (εdC), two important etheno-DNA adducts (**a**). Immunohistochemistry of εdA in the nuclei of hepatocytes in a patient with ALD (**b**)

Thus, current evidence supports the paradigm that cancer predisposing conditions (see above) lead to the ROS/RNS generation with subsequent LPO and production of by-products such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), 4-hydoxyhydroperoxy-2-nonenal (HPNE) (Fig. 6.2). These lipidperoxidation products react with DNA either directly or through bifunctional intermediates to form various promutagenic exocyclic etheno-DNA adducts [13]. LPO-products derived mainly from gamma-linoleic acid, include 4-HNE, a major LPO product and its electrophilic epoxy-, hydroperoxy-, and oxo-enal intermediates can react with the DNA bases A, C, and G. This yields the unsubstituted etheno-DNA adducts, 1,N6-etheno-2′-deoxyadenosine (εdA), 3,N4-etheno-2′-deoxycytidine (εdC), 1,N2-etheno-2′-deoxyguanosine (1,N2εdG), and N2,3-etheno-2′-deoxyguanosine (N2,3εdG). In addition, substituted base adducts are formed such as HNE-dG carrying a fatty acid chain residue. 2,N4-etheno-5-methyl-2′-deoxycytidine (ε5mdC), an endogenous LPO-derived adduct was recently identified in human tissue DNA, possibly playing a role in epigenetic mechanisms of carcinogenesis [1, 15–22]. DNA is also modified directly by ROS and RNS to yield 8-nitro-dG and 8-Oxo-dG [16]. All of these products in DNA changes have been detected in human specimens [4, 7, 23–26]. Exocyclic etheno-DNA adducts exhibit strong mutagenic properties in most organisms tested so far, producing various types of base pair substitution mutations and other types of genetic damage [27–32].

εdA can lead to AT → GC transition and AT → TA and AT → CG transversions [29, 30]. εdC cause CG → AT transversions and CG → TA transition [31, 32]. N2,3 εdA leads to GC → AT transition [32]. Incorporation of a single εdA in either DNA strand of HeLa cells showed a similar miscoding frequency and was more mutagenic than 8-oxo-dG [33].

Etheno adducts are poorly repaired in some tissues stressing their biological relevance [34]. Strong support that etheno-DNA adducts play a causal role in the initiation and progression of liver carcinogenesis comes from the formation of εdA and εdC in vivo by the human liver carcinogen vinyl chloride [35] and by the potent multiorgan, multispecies carcinogen urethane; hereby reaction with DNA occurs via their metabolic epoxy-intermediates [36]. The biological importance of etheno-DNA adducts is further stressed as they are preferentially formed in codon 249 of TP53 (which encodes p53), leading to a mutation that renders cells more resistant to apoptosis and provides them some growth advantage [37].

LPO-derived reactive products and their macromolecular interactions have been so far characterized primarily by in vitro studies, making it difficult, to pinpoint the main precursors and pathways involved in the generation of cancer-relevant DNA damage in human in vivo. For this reason, earlier studies analyzed εdA and εdC in human specimens to serve as a lead marker for other exocyclic adducts formed in vivo, but for which sensitive detection methods were not yet available at that time. Using ultrasensitive and specific detection methods [24], two miscoding etheno-DNA adducts εdA and εdC were first unequivocally identified in human DNA (Fig. 6.3) Subsequently, surgical tissue samples collected from "at risk" patients, i.e. affected by chronic inflammatory processes, persistent viral infections, iron storage- and alcohol-related diseases or exposed to inherited/acquired cancer risk factors were analyzed. Adduct levels increased 10–100-fold progressively in human cancer-prone organs including liver, bile duct, esophagus, colon and pancreas. Consistent results were also observed in rodent tumor models, that mimic human disease [3]. Taken together these data incriminate LPO-derived DNA adducts formed endogenously as strongly mutagenic and potentially cancer-causing lesions. The chemical structure of εdA and εdC as well as the immunohistochemical appearance of εdA in the liver of a patient with ALD is shown in Fig. 6.3.

6.3 Etheno-DNA Adducts in ALD and NAFLD: The Role of CYP2E1 Induction and Inflammation

Oxidative stress is a major pathogenetic factor in ALD and in NAFLD. In both diseases inflammatory driven oxidative stress occurs, which is predominant in non-alcoholic steatohepatitis (NASH) [13] as well as in alcoholic hepatitis (ASH), a clinical syndrome with high mortality [38]. In addition, CYP2E1 is found to be induced by chronic ethanol ingestion [39] as well as in NASH [40]. The intensity of ethanol mediated CYP2E1 induction differs interindividually [41]. In NASH, an

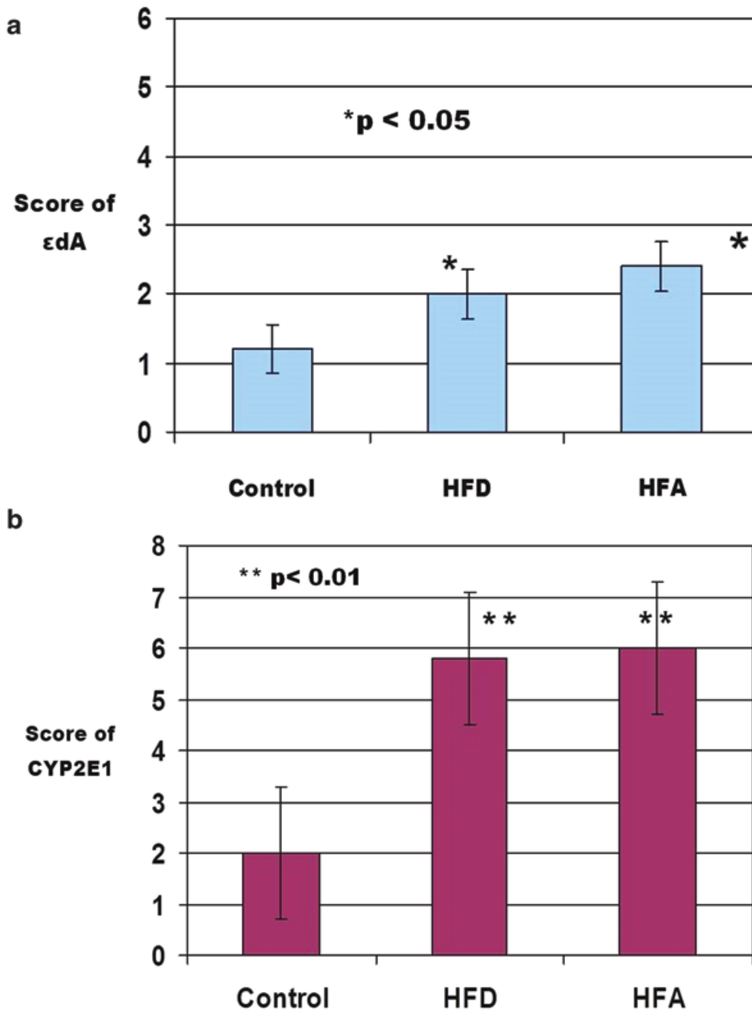

Fig. 6.3 Effect of a high fat diet with and without ethanol (16% of total calories on the level of εdA (**a**) and CYP2E1 (**b**). The high fat diet alone increased both εdA and CYP2E1 significantly, while the addition of ethanol did not further increase the two parameters. HFD = high fat diet, HFS = high fat diet plus alcohol

inflammatory liver disease associated primarily with the metabolic syndrome (overweight, diabetes mellitus, hypertension, and hypercholesterolemia) hepatic acetone (observed in diabetes mellitus) and free fatty acids (present in fatty liver) also induce CYP2E1, since there metabolism is catalyzed by CYP2E1 [42].

Ethanol metabolism through CYP2E1 generates not only acetaldehyde, but also ROS which can react with proteins and DNA affecting their structure and function. ROS can also initiate LPO which leads to formation of several byproducts such as

MDA and 4-HNE. After reaction with DNA bases exocyclic etheno DNA adducts are generated (Figs. 6.1 and 6.3). CYP2E1 induction in NASJH as compared to ALD was found to be less pronounced, whereas inflammation was predominant [13] This led to the assumption that DNA adduct formation in NASH is primarily driven by inflammatory processes, whilst in ALD, CYP2E1 induction is much stronger and inflammation generally milder. AH seems to be an exception, whereby in ALD etheno adducts are primarily formed via CYP2E1-mediated ROS formation.

6.4 Etheno DNA Adducts (εdA) in Alcohol Consuming Rodent Models and ALD Patients

Various animal experiments have underlined that in ALD CYP2E1 is responsible for the generation of ROS and DNA damage in disease causation: a) CYP2E1 knock-out mice do not develop ALD with the same severity as wild-type mice when they ingested alcohol for more than 4 weeks [43, 44]; b) inhibition of CYP2E1 by chlormethiazole (CMZ), a selective CYP2E1 inhibitor decreases ROS/RNS significantly, resulting in an inhibition of ALD [44, 45]; c) CYP2E1 knock-out mice also developed less oxidized DNA products as compared to wild type mice when both received ethanol [46]; d) Transgenic mice over-expressing CYP2E1 showed an enhancement of hepatic injury following chronic ethanol administration [43, 47, 48]; e) in (HepG2) liver cells over-expressing CYP2E1, incubation with rising ethanol concentrations led to a linear increase of εdA levels in DNA, which was inhibited by small amounts of CMZ [49].

Liver biopsies from patient with varying degree of ALD severity were immunohistochemically analyzed for CYP2E1, 4-HNE, and εdA adducts. Again, we found a significant positive correlation for CYP2E1 vs. 4-HNE as well as for CYP2E1 vs. εdA [49].

In an ongoing study analysis of liver biopsies from about hundred ALD patients confirmed at a high level of significance these correlations and the association between hepatic fibrosis, CYP2E1 and εdA (Seitz, personal communication). These data strongly implicate CYP2E1 as an important mediator for etheno DNA adduct formation and this detrimental DNA damage may act as a driving force for ALD progression.

Since chronic ethanol consumption is also a risk factor for esophageal and colorectal cancer we also measured CYP2E1 and εdA in these tissues. In 37 patients with esophageal cancer esophageal biopsies adjacent to the tumor were analyzed and were compared to control biopsies from 12 non-alcohol drinkers [50]. In the esophageal mucosa a significant correlation between the quantity of alcohol intake and CYP2E1 induction as well as etheno-DNA adduct formation was found. Both etheno-adducts εdA and εdC correlated significantly with CYP2E1 [50], while control patients did not show CYP2E1.

CYP2E1 can also induced in the colorectal mucosa. In our study in heavy and light drinkers no difference in CYP2E1 and εdA levels was observed, possibly due

to dietary modulators that affect CYP2E1, LPO, and adduct production in situ. However, when the data of all patients (controls and alcoholics) were pooled, a significant correlation between CYP2E1 and εdA became apparent [51].

6.5 Etheno DNA Adducts in Animal Models of NASH with and Without Additional Alcohol Administration and in NASH Patients

Based solely on histomorphology NASH and ASH are very difficult to distinguish. In both ALD and NASH CYP2E1 was reported to be induced [39–41], this induction in NASH is less expressed than in ALD for reasons that we investigated.

Formation of εdA in a cohort of patients with NASH has clearly been established [38], but the etheno adducts did not correlated with CYP2E1. To explain this unexpected finding we assume that in NASH inflammation predominates rather than CYP2E1 induction, and etheno adducts are formed via ROS generated during inflammatory processes (Fig. 6.1) In this context it is noteworthy that in NASH patients a significant correlation was noted between CYP2E1 and hypoxemia and ß-hydoxybutyrate [52].

In a further study we investigated etheno-DNA adducts in 21 children and adolescents who were diagnosed with NASH with and without diabetes [53]. In 3 out of 21 children etheno DNA adducts were extremely high. Since alcohol consumption even at social levels increase the risk for hepatocellular cancer in NASH it would be important to monitor these children for HCC further in life.

In a series of animal experiments where NASH was induced either in genetically modified rodents or by feeding a high fat diet we further investigated the formation of etheno-DNA adducts. When obese Zucker-rats who are leptin deficient and insulin resistant received alcohol as Lieber-DeCarli-diets etheno-DNA adducts increased to a much higher degree in obese as compared to lean Zucker-rats and this increase was further enhanced when alcohol was administered. Etheno adduct formation was highly significant and paralleled by the level of hepatic CYP2E1 [49].

When Sprague-Dawley-rats received a Lieber-DeCarli-high-fat-diet with 71% energy from fat NASH was produced within 6 weeks. Afterwards these rats were continuously fed with high fat diet (55% total energy from fat) or high fat plus alcohol diet (55% energy from fat and 16% energy from ethanol) for another 4 weeks [54]. High fat diet alone increased hepatic inflammation and apoptosis as compared to a control diet, and nearly doubled the level of hepatic etheno-DNA adducts and of CYP2E1. The addition of ethanol did not significantly affect parameters associated with lipid peroxidation, inflammation and apoptosis, and no further increase in etheno-adducts and of CYP2E1 was noted [55] (Fig. 6.4).

A similar observation was made in a mouse model [56] where multiple binge-drinking with an ethanol intake of 2 g/kg body weight twice a week for 12 weeks increased etheno-DNA adducts in the liver only to a minor degree as compared to a

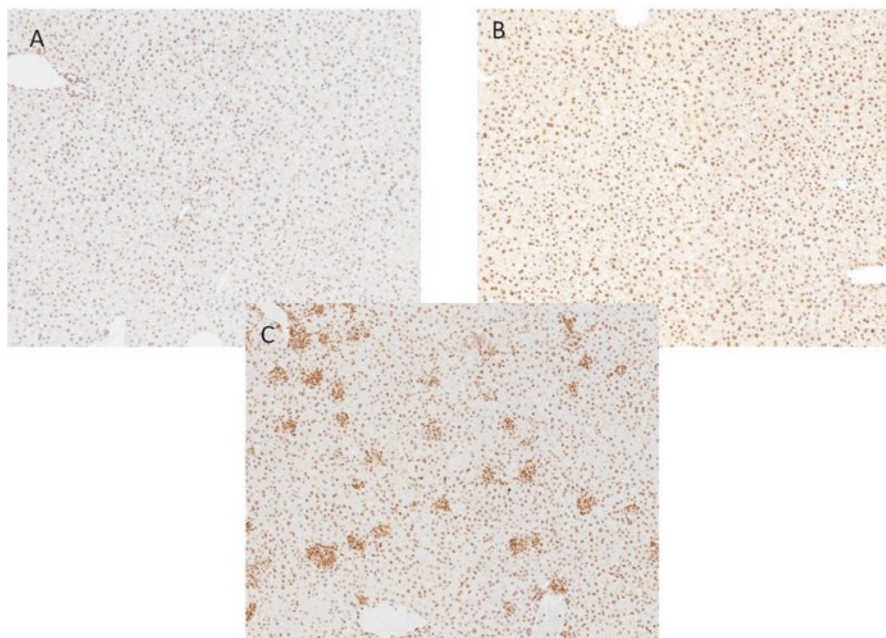

Fig. 6.4 Effect of binge drinking and high fat diet on hepatic etheno-DNA adducts (εdA). (**a**) single dose of ethanol (2 g/kg), (**b**) multiple binges (2 g/kg, 12 weeks, twice per week), (**c**) multiple binges (2 g/kg, 12 weeks, twice per week plus high fat diet (45% energy from fat). While multiple binges increase εdA moderately without reaching the level of statistical significance (**b**) as compared to a single dose of ethanol (**a**), multiple binges combined with a high fat diet significantly increase hepatic εdA which occur predominantly in clusters (**c**)

single binge of 6 g/kg body weight in an alcoholic steatosis model [56]. However, when multiple binges were combined with a high fat diet (45% of total calories from fat) a striking elevation of etheno DNA adducts was found. Interestingly, these etheno-DNA adducts occurred in clusters within the liver (Fig. 6.4).

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