0026-895X/94/040786-06$03.00/0

MOLECULAR PHARMACOLOGY, 46:786–791, 1994

Ethanol-Derived Immunoreactive Species Formed by Free Radical Mechanisms

C. MONCADA, V. TORRES, G. VARGHESE, E. ALBANO, and Y. ISRAEL

Department of Pharmacology (C.M., V.T., Y.I.), University of Toronto, Toronto, Canada; Department of Experimental Medicine and Oncology, University of Turin, Turin, Italy (G.V.); Addiction Research Foundation, Toronto, Canada (E.A.); and Department of Anatomy, Pathology, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania (Y.I.)

Received May 12, 1994; Accepted August 2, 1994

SUMMARY

Recent studies have shown that the α-hydroxyethyl radical ($CH_3\dot{C}HOH$), a metabolite of ethanol, is produced *in vitro* and *in vivo*. We report studies that establish the immunogenicity of α-hydroxyethyl radical-derived protein adducts. Rat liver microsomes incubated in the presence of [^{14}C]ethanol and NADPH (under aerobic conditions) incorporate ^{14}C into acid-stable adducts. Incorporation was markedly inhibited by the free-radical scavenger α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone. Rabbits immunized with rat liver microsomes that had been preincubated with ethanol and NADPH generated antibodies that recognized polylysine-acetaldehyde adducts and adducts formed by incubation of proteins with an α-hydroxyethyl radical-generating system (ethanol plus H_2O_2 plus Fe^{2+}). Rabbits immunized with microsomes that had been preincubated with ethanol and NADPH plus α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone generated antibodies that recognized polylysine-acetaldehyde adducts. However, their reactivity against α-hydroxyethyl-derived protein epitopes was greatly reduced or was virtually abolished. Data indicate that microsomes metabolizing ethanol generate two types of adducts, acetaldehyde-derived adducts and α-hydroxyethyl radical-derived adducts, both of which are immunogenic. Immunization of rabbits with α-hydroxyethyl-bovine serum albumin adducts led to the production of antibodies that recognized α-hydroxyethyl-rabbit serum albumin adducts but did not recognize the native protein. Chronic alcohol feeding of rats led to the production of antibodies that recognized α-hydroxyethyl-rat serum albumin adducts but did not recognize rat serum albumin. The study (i) indicates that α-hydroxyethyl radical-derived protein adducts are immunogenic, (ii) supports earlier work that proposed that α-hydroxyethyl radicals generated in different systems bind covalently to proteins, and (iii) demonstrates the formation of antibodies to α-hydroxyethyl-derived protein adducts after chronic alcohol ingestion *in vivo*. The findings may have implications in the identification of chronic alcohol abuse and the pathogenesis of alcohol-induced organ damage.

Classically, the first product of ethanol oxidation has been considered to be acetaldehyde, produced when two electrons and two protons are removed from the ethanol molecule. Recent studies have demonstrated that a one-electron oxidation product also exists; ethanol reacts efficiently with hydroxyl free radicals, generating the α-hydroxyethyl radical ($CH_3\dot{C}HOH$). Hydroxyethyl free radicals have been demonstrated to occur *in vitro* in a number of biological systems, including liver microsomes (1–8) and activated neutrophils (9), and in the liver *in vivo* (7, 10). Both acetaldehyde and the α-hydroxyethyl radical are highly reactive molecules (11–13).

Studies by Peterson and co-workers (11) and by Tuma, Sorell, and associates (12, 14) demonstrated that acetaldehyde binds to proteins and generates two types of adducts, a stable adduct and an unstable adduct that can be stabilized by reducing agents such as sodium borohydride and sodium cyanoborohydride. Studies by Steinbrecher *et al.* (15) and Israel *et al.* (16) demonstrated that animals immunized with acetaldehyde-protein adducts stabilized with sodium cyanoborohydride generate antibodies that recognize reduced acetaldehyde adducts. The studies showed that the epitopes generated were recognized independently of the protein used. It was further shown that mice fed alcohol chronically generated antibodies against reduced acetaldehyde-protein adducts (16), suggesting that reduced acetaldehyde adducts are formed *in vivo*. The existence of this type of antibody was also observed in human alcoholics (17–22) and in rats fed alcohol chronically (23, 24).

Studies by Schuessler *et al.* (13) have indicated that α-hydroxyethyl radicals, formed after X-ray irradiation of [^{14}C]ethanol-containing solutions, react with proteins, forming covalent bonds and promoting cross-links between protein molecules, both of which incorporate the alcohol carbon moiety (13, 25, 26). Evidence for the binding of α-hydroxyethyl radicals to proteins in a physiological system has also been obtained by Albano *et al.* (27), who observed that covalent binding of ^{14}C from [^{14}C]ethanol to rat liver microsomes (incubated in the

We acknowledge the support of the National Institute on Alcohol Abuse and Alcoholism (Grant 5R01-AA09331) and the Medical Research Council of Canada (Grant MT-8928).

ABBREVIATIONS: POBN, α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

presence of NADPH and O_2) was markedly reduced by the spin-trapping agent POBN, which is known to be a scavenger of α-hydroxyethyl radicals. POBN did not affect the production of acetaldehyde in this system.

The aim of the present study was to assess the immunogenicity of the adducts formed in microsomal preparations metabolizing ethanol and to determine whether these adducts can be recognized differentially by the immune system. Data presented constitute the first demonstration that haptens formed in the reaction of α-hydroxyethyl radical plus a protein are immunogenic, and they support earlier studies that proposed that α-hydroxyethyl radicals generated in different systems bind covalently to proteins. Studies also show that, *in vivo*, animals fed alcohol chronically generate antibodies against epitopes formed in the reaction of α-hydroxyethyl radicals with proteins.

Materials and Methods

Microsomal preparations. Rat liver microsomes were prepared as described by Johansson *et al.* (28). In short, the livers were homogenized in 2 volumes of 10 mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% (w/v) KCl. The microsomes were isolated after centrifugation of the homogenate at 10,000 × *g* for 10 min and ultracentrifugation of the resulting supernatant at 100,000 × *g* for 60 min. The microsomal pellet was washed once in the aforementioned buffer and was then suspended in 50 mM potassium phosphate buffer, pH 7.4. The microsomes were stored at −70° at a concentration of about 30–50 mg of protein/ml. To increase the cytochrome P450 2E1 content of microsomal preparations used for the studies, male Wistar rats (body weight, 160–190 g) were fed a liquid diet containing 36% of calories as ethanol (11–14 g of ethanol/kg/day) for 30 days (29, 30). Ethanol was withdrawn from their diet 24 hr before sacrifice. Induction of cytochrome P450 2E1 activity was evaluated by measuring oxidation of *N*,*N*-nitrosodimethylamine to formaldehyde (31, 32) and measuring the level of cytochrome P450 2E1 by Western blotting of microsomes using anti-cytochrome P450 2E1 antibodies (30). The relative density of immunostained bands was quantitated by computerized densitometry. Both methods showed an induction of cytochrome P450 2E1 (2- and 3-fold, respectively) in the alcohol-fed group, compared with the isocaloric carbohydrate-fed control group (data not shown).

[1-^{14}C]Ethanol binding to proteins. Microsomal proteins (0.5 mg) were incubated with [1-^{14}C]ethanol (specific activity, 21 mCi/mmol; NEN-DuPont Canada) (final ethanol concentration, 50 mM) in 0.3 M potassium phosphate buffer, pH 7.7, under atmospheric oxygen, in the presence or absence of 2 mM NADPH and in the presence or absence of 20 mM POBN (Sigma Chemical Co., St. Louis, MO), in a final volume of 25 μl. The reaction was allowed to proceed for 60 min at 22°, and 1 ml of 15% trichloroacetic acid was added to the mixture. The precipitated protein was centrifuged at 5000 rpm in an Eppendorf centrifuge. The supernatant was discarded, another 1 ml of 15% trichloroacetic acid was added, and the pellet was loosened by vortex mixing. This washing procedure was repeated six times. The pellet was dissolved in 0.1 ml of 1 N NaOH and was transferred to a counting vial. The Eppendorf tubes were thoroughly washed with six 0.1-ml aliquots of 0.3 M potassium phosphate buffer. The contents of the counting vials were neutralized with 0.1 ml of glacial acetic acid, reaching a final pH of about 6.5, and 10 ml of liquid scintillation fluid (Aquasol-2; NEN-DuPont Canada) were added. Radioactivity was measured in these vials, as well as in the last two neutralized washing supernatants (to verify the absence of counts), in a scintillation counter (Beckman LS5801). Small aliquots were used for the measurement of proteins by the method of Lowry *et al.* (33). Radioactivity is expressed as nanomoles of [1-^{14}C]ethanol/milligram of microsomal protein.

Acetaldehyde production by microsomes. Acetaldehyde production was measured as described by Lieber and DeCarli (34). A mixture containing 1 mM sodium azide, 5 mM $MgCl_2$, 1 mM Na_2EDTA, and 50 mM ethanol, in 50 mM phosphate buffer, pH 7.4, was preincubated in the main well of a tightly sealed, 25-ml, center-well Erlenmeyer flask, in a total volume of 3 ml. The center well contained 0.6 ml of 15 mM semicarbazide HCl in 100 mM potassium phosphate buffer, pH 7.4. Three milligrams of microsomal proteins in 0.2 ml of 50 mM potassium phosphate buffer were added to the main well and preincubated in a shaking water bath at 37° for 5 min. A solution (0.5 ml) of NADPH (final concentration, 1 mM) was added by injection to the main well and the reaction was allowed to proceed for 5 or 10 min. The reaction was stopped by addition of 15% (w/v) trichloroacetic acid. The flasks were removed from the water bath and allowed to stand overnight at room temperature, to trap in the center well the acetaldehyde generated. In some flasks standards were included by replacing the microsomes with acetaldehyde (Aldrich, St. Paul, WI). A 1/5 dilution of the center-well solution was used for absorbance readings at 224 nm. The rate of acetaldehyde production is expressed as nanomoles of acetaldehyde/milligram of protein/minute.

Preparation and purification of acetaldehyde adducts. Protein-acetaldehyde adducts were prepared as described elsewhere (16). Four milliliters of a 16 mg/ml solution of bovine serum albumin or poly-L-lysine (M_r 102,000–109,000; both from Sigma) were added to 4 ml of 480 mM acetaldehyde (in PBS, pH 7.4). The reaction was allowed to proceed for 60 min at 22°, and 2 ml of 0.5 M $NaCNBH_3$ in PBS were added. After 4 hr the mixture was dialyzed at 4° against 1 liter of PBS. No acetaldehyde was added for the controls. The resulting adducts were stored at −70°.

Preparation and purification of hydroxyethyl radical adducts. The Fenton reaction was chosen as an α-hydroxyl radical-generating system (35). Hydroxyl radicals react with ethanol, forming α-hydroxyethyl radical. α-Hydroxyethyl radicals were produced by the reaction of ethanol plus H_2O_2 plus Fe^{2+} (the Fenton reagent plus ethanol) (4). The complete reaction mixture contained 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.2 mM EDTA, and 0.1 mM H_2O_2, in 0.3 M potassium phosphate buffer, pH 7.8. The components were mixed with or without 50 mM ethanol or 50 mM acetaldehyde, in the presence of 2 mg/ml bovine, rabbit, or rat serum albumin. The reaction was initiated by addition of H_2O_2 and was allowed to proceed for 60 min.

Incubation of microsomes for immunization. Four milligrams of microsomal proteins were incubated for 4 hr at 37° in the presence of atmospheric oxygen, in the presence or absence of 50 mM ethanol, 2 mM NADPH, and 20 mM POBN, in 0.3 M potassium phosphate buffer, in a final volume of 2 ml, in a shaking water bath. After incubation the reaction mixtures were placed on ice for 10 min. One milliliter was taken and mixed with 1 ml of Freund's complete adjuvant, according to the method of Moncada *et al.* (36). One milliliter of this emulsion (containing 1 mg of protein) was used for immunization of each rabbit. The remaining reaction mixture was diluted 1/2 and stored at −70°. At 2 weeks rabbits were given a booster injection of an emulsion containing 0.5 ml of the diluted reaction mixture and 0.5 ml of Freund's incomplete adjuvant. Sera from the animals were obtained by ear venipuncture before immunization (preimmune sera) and 2 and 4 weeks after immunization.

ELISA. The ELISA method used has been previously described by Israel *et al.* (16). One to 5 μg of protein were used to coat the wells. Powdered skim milk (2.5%) was used to block nonspecific binding sites. The secondary antibody was an affinity-purified goat anti-rabbit immunoglobulin bound to horseradish peroxidase (Dimension Labs, Mississauga, Ontario).

Measurement of ethanol production. Gas chromatography was used to measure the amount of acetaldehyde converted to ethanol in the reaction of acetaldehyde plus $NaCNBH_3$ at 22°. An HP7620A gas chromatograph equipped with a 5% Carb 20M, 30/60 mesh column (Chromatographic Specialties, Brockville, Canada) was used. The conditions were as follows: column temperature, 100°; injector and flame detector temperature, 200°; air flow rate, 60 ml/min; hydrogen flow rate, 45 ml/min. Under these conditions, the retention times for acetaldehyde, ethanol, and isopropanol (the internal standard) were 79 sec, 106 sec, and 133 sec, respectively.

Antibodies in sera of alcohol-treated and control animals. Rats were maintained for 30 days with an alcohol-containing diet or an isocaloric carbohydrate control diet, as described above. After laparotomy under ether anesthesia, blood was extracted from the inferior vena cava in the absence of anticlotting agents. Blood was allowed to clot and serum was used as the antibody source for the ELISA method. The secondary antibody was a horseradish peroxidase-linked rabbit anti-rat IgG (Dimension Labs).

Results

Production of acetaldehyde and ^{14}C incorporation in microsomes. In the presence of 50 mM [^{14}C]ethanol, 2 mM NADPH, and oxygen (air), microsomes incorporated ethanol-derived radioactivity (25.6 ± 0.8 nmol/mg of protein in a 4-hr incubation period at 37°). Incorporation was reduced to 8.05 ± 1.2 nmol/mg of protein ($p < 0.001$) by the addition of 20 mM POBN and to 2.8 ± 0.6 nmol/mg of protein ($p < 0.001$) by omission of NADPH. The production of acetaldehyde was not significantly affected by POBN (control, 14.6 ± 4.2; POBN, 12.3 ± 2.7 nmol of acetaldehyde produced/min/mg of protein).

Immunogenicity of adducts formed in microsomes incubated with ethanol. Rabbits were immunized with microsomes that had been incubated at 37° for 4 hr in medium containing oxygen (air) and (i) ethanol, (ii) NADPH, (iii) NADPH plus ethanol, or (iv) NADPH plus ethanol plus POBN. Sera of all of the rabbits injected with microsomes displayed immunoreactivity against rat microsomal proteins, with little variation between groups (data not shown). Fig. 1 shows the marked immunoreactivity against acetaldehyde-polylysine conjugates of sera of rabbits immunized with microsomes incubated with NADPH plus ethanol. Virtually no immunoreactivity was observed when ethanol was omitted from the microsomal incubation medium. Reactivity was also markedly reduced when NADPH was omitted. Addition to the microsomal incubation system of the spin trap POBN plus NADPH plus ethanol did not significantly alter the immunoreactivity developed against the acetaldehyde-polylysine adduct. Fig. 2 shows that marked immunoreactivity also developed against acetaldehyde-bovine serum albumin adducts. This immunoreactivity was greatly reduced by addition of POBN to the microsomal system. Thus, a second type of epitope, present in protein-acetaldehyde adducts, is also formed in microsomes metabolizing ethanol, and its synthesis is largely reduced by the spin trap POBN. This observation is in line with the marked reduction produced by POBN of incorporation of [^{14}C]ethanol into microsomes. Both ethanol and NADPH were also required for maximal production of this second type of adduct (Fig. 2).

We subsequently tested the hypothesis that antibodies could be generated against hydroxyethyl-protein adducts that might be formed in the microsomal preparations in the presence of ethanol. This was done by determining the immunoreactivity of the antibodies, produced after immunization with microsomes incubated with NADPH plus ethanol, against hydroxyethyl-derived bovine serum albumin adducts. The latter adducts were prepared by incubating bovine serum albumin with an hydroxyethyl radical-generating system (H_2O_2 plus Fe^{2+} plus ethanol). Fig. 3 shows an experiment typical of four to six animals, in which marked immunoreactivity was elicited against bovine serum albumin modified with ethanol plus the Fenton reagent (H_2O_2 plus Fe^{2+}). No immunoreactivity was developed against bovine serum albumin treated with acetaldehyde plus the Fenton reagent, compared with the reactivity against bovine serum albumin plus Fenton reagent in the absence of acetaldehyde. Minor reactivity was, however, ob-

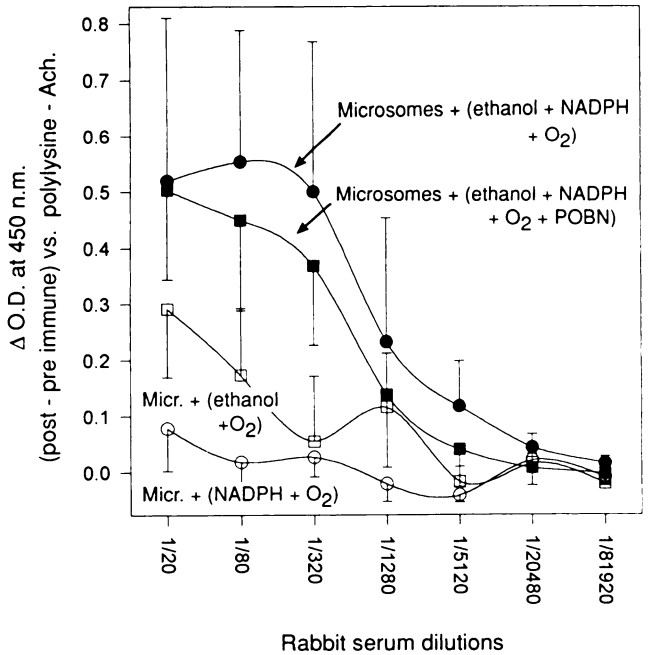

Fig. 1. Antibody reactivity against reduced polylysine-acetaldehyde adducts. ELISA results show the immunoreactivity of sera of rabbits immunized with rat liver microsomes (*Micr.*) that had been incubated for 4 hr at 37° with (i) 2 mM NADPH, (ii) 50 mM ethanol, (iii) 50 mM ethanol plus 2 mM NADPH, or (iv) 50 mM ethanol plus 2 mM NADPH plus 20 mM POBN. Basal reactivity against polylysine was subtracted from that observed for reduced polylysine-acetaldehyde (*polylysine-Ach.*). Each *point* is the average ± standard error for four or five rabbits. Each *curve* results from subtracting preimmune serum reactivity from postimmune serum reactivity.

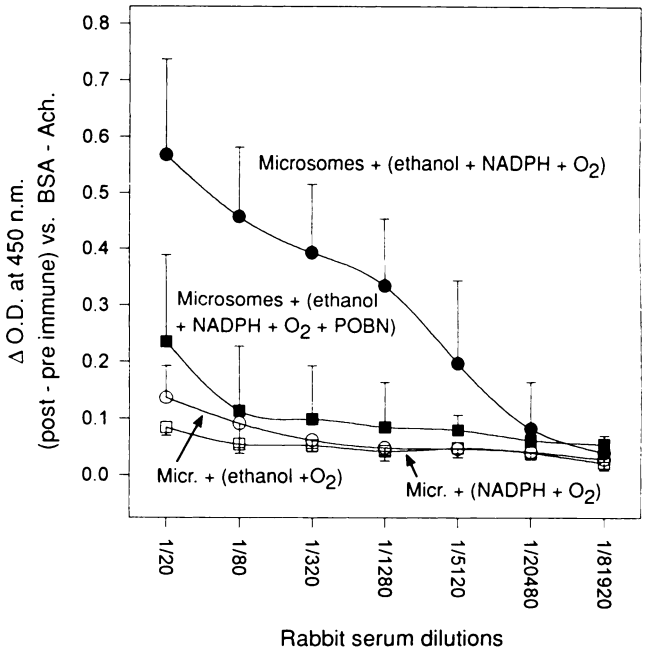

Fig. 2. Antibody reactivity against reduced bovine serum albumin-acetaldehyde adducts. Conditions were as for Fig. 1. The sera from the same rabbits were tested against reduced bovine serum albumin-acetaldehyde adducts. Basal reactivity obtained against control bovine serum albumin (treated with sodium cyanoborohydride) was subtracted from absorbance values obtained for reduced bovine serum albumin-acetaldehyde (*BSA-Ach.*).

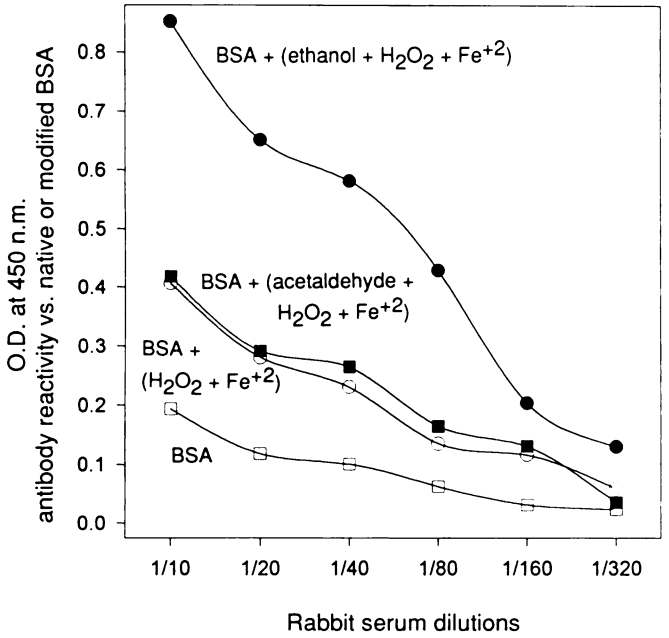

Fig. 3. Antibody reactivity against products of ethanol-Fenton and acetaldehyde-Fenton incubations with bovine serum albumin. A typical ELISA for serum from a rabbit immunized with microsomes that had been incubated under aerobic conditions with 50 mM ethanol plus 2 mM NADPH is shown. Reactivity is shown against bovine serum albumin (*BSA*) (i) as a native protein or incubated with (ii) Fenton reagent (100 μM H_2O_2 plus 100 μM Fe^{2+}), (iii) Fenton reagent plus acetaldehyde, or (iv) Fenton reagent plus ethanol.

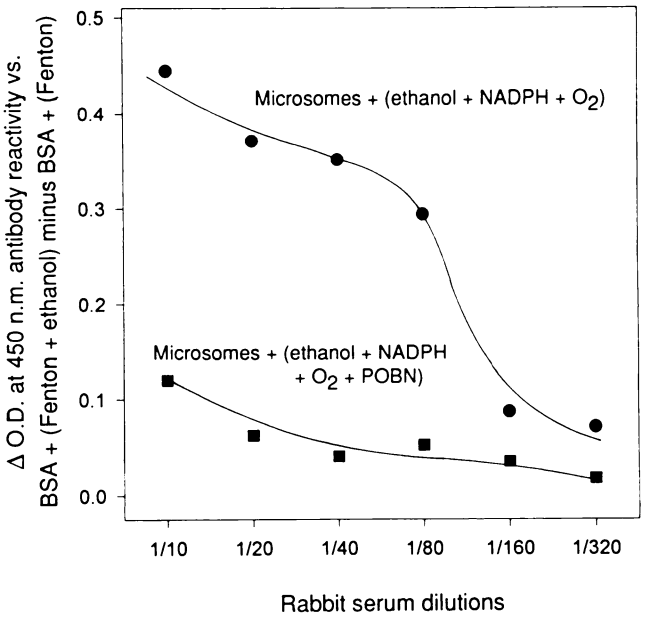

Fig. 4. Antibody reactivity against hydroxyethyl-derived bovine serum albumin adducts. A typical ELISA shows the immunoreactivity of sera from a rabbit immunized with microsomes that had been incubated under aerobic conditions with 50 mM ethanol plus 2 mM NADPH and from a rabbit immunized with microsomes that had been incubated with 50 mM ethanol plus 2 mM NADPH plus 20 mM POBN. Each *curve* represents the immunoreactivity of each rabbit against bovine serum albumin (*BSA*) reacted with 50 mM ethanol plus the Fenton reagent minus the reactivity against bovine serum albumin reacted with the Fenton reagent alone.

served against proteins treated with the Fenton reagent alone, likely reflecting immunoreactivity against oxidized groups in the proteins. None of the rabbits immunized with microsomes treated with NADPH plus ethanol plus POBN developed significant immunoreactivity against bovine serum albumin incubated with ethanol plus Fenton reagent. An experiment typical of such animals is shown in Fig. 4. Experiments were conducted to determine the incorporation of [^{14}C]ethanol into bovine serum albumin incubated for 60 min in the presence of the Fenton reagent [control ^{14}C incorporation (50 mM [^{14}C] ethanol), 7.8 ± 0.5 nmol of [^{14}C]ethanol/mg of protein; ethanol-Fenton reagent (50 mM [^{14}C]ethanol plus 100 μM Fe^{2+} plus 100 μM H_2O_2), 22.5 ± 1.3 nmol of [^{14}C]ethanol/mg of protein ($p < 0.001$)].

The experiments described above suggested that it would be possible to immunize rabbits with a protein modified under ethanol-Fenton conditions. Fig. 5 shows data from experiments in which antibodies were generated by immunization with bovine serum albumin treated with the hydroxyethyl radical-generating system (Fenton reagent plus ethanol). These antibodies recognized rabbit serum albumin treated under the same conditions but not rabbit serum albumin treated with the Fenton reagent in the absence of ethanol. The immunoreactivity against rabbit serum albumin treated with the Fenton reagent plus acetaldehyde was only 1/100 of that observed against rabbit serum albumin treated with the Fenton reagent plus ethanol.

It was of interest to determine whether chronic alcohol treatment would be able to activate the immune system to produce antibodies against ethanol-Fenton reagent-derived adducts. Fig. 6 shows that chronic alcohol administration to rats indeed led to the production of antibodies that recognize rat

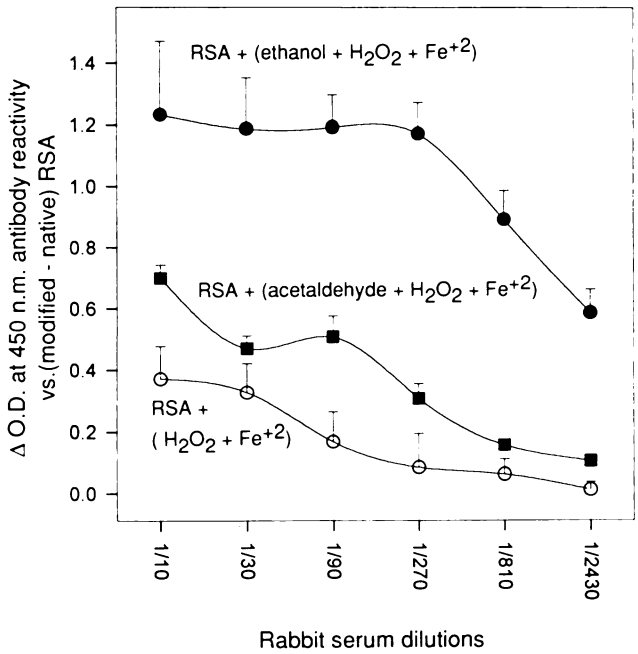

Fig. 5. Antibody reactivity of rabbits immunized with hydroxyethyl-derived bovine serum albumin adducts. An ELISA shows the antibody reactivity of sera from rabbits immunized with bovine serum albumin that had been reacted with the Fenton reagent plus ethanol against rabbit serum albumin (*RSA*) reacted with (i) the Fenton reagent, (ii) the Fenton reagent plus 50 mM acetaldehyde, or (iii) the Fenton reagent plus 50 mM ethanol. Each *curve* results from subtracting the reactivity observed for the native protein from the specific reactivity.

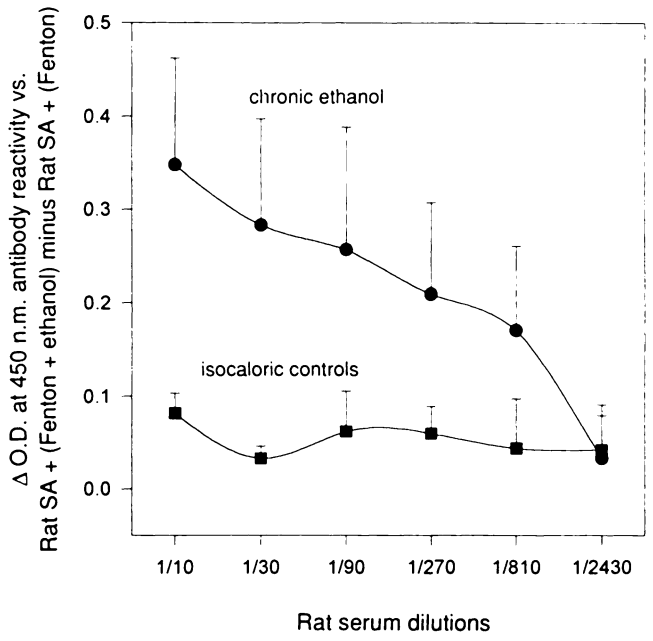

Fig. 6. Antibody reactivity of chronically ethanol-fed rats against hydroxyethyl-derived rat serum albumin adducts. An ELISA shows the immunoreactivity of the sera of ethanol-fed and control rats. Rat serum albumin (*SA*) was reacted with 50 mM ethanol plus the Fenton reagent (100 μM H_2O_2 plus 100 μM Fe^{2+}). Each *curve* represents the absorbance values obtained for the serum reactivity from seven animals (mean ± standard deviation), from which the absorbance values for the native protein have been subtracted.

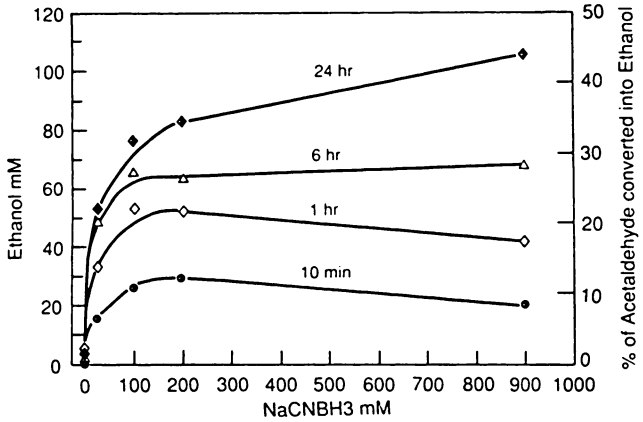

Fig. 7. Ethanol production in the reaction of acetaldehyde with $NaCNBH_3$. Ethanol produced during the incubation of acetaldehyde (240 mM) with different concentrations of $NaCNBH_3$ is shown for different times. The concentration of ethanol produced in these reactions was quantified by gas chromatography, as indicated in Materials and Methods.

serum albumin-hydroxyethyl adducts. These antibodies were not observed in carbohydrate pair-fed control animals.

Discussion

Considerable effort has been devoted to elucidating the role of acetaldehyde in the actions of alcohol. There is considerable evidence that acetaldehyde binds to proteins, producing adducts that render these proteins neoantigenic (15, 16, 19, 24). Chronic alcohol consumption leads to the production of antibodies that recognize acetaldehyde adducts (17–23). Although the different types of haptens formed by acetaldehyde remain to be determined, one such type is *N*-ethyl-lysine (37–40).

In the presence of ethanol, NADPH, and oxygen, rat liver microsomes oxidize ethanol to the highly reactive α-hydroxyethyl radical (CH_3CHOH) and to acetaldehyde (1–3, 5, 6, 8, 34). Data presented suggest that at least two immunogenic types of adducts are formed in this system, (i) adducts whose synthesis is not affected by the presence of a free-radical scavenger, which generate antibodies that recognize acetaldehyde-lysine conjugates, and (ii) adducts whose synthesis can be virtually abolished by a free-radical scavenger, which yield antibodies that react with adducts that can be generated by the reaction of proteins with the Fenton reagent plus ethanol (Fe^{2+} plus H_2O_2 plus ethanol), a reaction known to generate hydroxyethyl radicals. These antibodies also react with an adduct formed in the reaction of bovine serum albumin and acetaldehyde in the presence of sodium cyanoborohydrate (see below).

Studies by Schuessler *et al.* (13) have indicated that α-hydroxyethyl radicals bind readily to proteins. The present studies also show that [^{14}C]ethanol is readily incorporated into proteins under Fenton reaction conditions. Thus, it is likely that the second type of antibodies indicated above are against α-hydroxyethyl-protein adducts, which have been reported to be formed in microsomal systems (27). Such a view is in line with data showing that immunization of rabbits with protein adducts produced in the presence of an inorganic α-hydroxyethyl radical-generating system (the Fenton reagent plus ethanol) results in the production of antibodies that react with other proteins modified in the presence of the α-hydroxyethyl-producing system. Thus, data indicate that immunoreactivity can be elicited against α-hydroxyethyl radical-generated haptens in the proteins. It should be noted that we have not identified the chemical nature of the adducts formed. Also, it is not possible from the present studies to determine whether other reactive species formed under α-hydroxyethyl radical-generating conditions mediate the formation of the adducts.

A question that arises involves the mechanism by which bovine serum albumin treated with acetaldehyde plus $NaCNBH_3$ forms epitopes that are similar to those produced in the reaction of bovine serum albumin with ethanol plus the Fenton reagent or by a microsomal system metabolizing ethanol in the absence of POBN. It has been observed that incubation of proteins with aldehydes under aerobic conditions promotes the synthesis of a number of active oxygen radicals, including hydroxyl radicals (41), and of protein oxidation products (42). If ethanol is generated in the acetaldehyde plus $NaCNBH_3$ reaction mixture, then ethanol-derived radicals could conceivably also be formed. Fig. 7 shows that ethanol was vigorously produced in the reaction of acetaldehyde plus $NaCNBH_3$. Additional studies should be conducted to determine whether ethanol generated in this system is transformed into hydroxyethyl radicals or into other moieties able to form protein adducts.

The ability of hydroxyethyl radicals to bind to proteins and to be immunogenic is of biological interest. Hydroxyethyl radical-protein adducts or their degradation products in urine may be of value in the diagnosis of chronic alcohol use. It is also of note that oxygen radicals in general, and hydroxyl radical in particular, can be produced extracellularly, for example by activated neutrophils (9, 43–45). Thus, an antigen-antibody reaction involving hydroxyethyl radical adducts and neutrophil-bound immunoglobulins might serve as an amplification system for neutrophil degranulation and organ damage. Hydroxyethyl adducts on the external surface of cell membranes

might also constitute a target for antibody-mediated cell-dependent cytotoxicity or for complement-mediated lysis. As shown by the studies presented here, rats fed alcohol chronically developed antibodies against hydroxyethyl radical-derived adducts.

In conclusion, data presented indicate that hydroxyethyl radical-derived protein adducts are immunogenic and are formed in physiological preparations. Furthermore, chronic alcohol administration leads to the production of antibodies that recognize hydroxyethyl-protein adducts. The findings may have diagnostic applications and pathogenic implications.

Acknowledgments

We thank Mr. Aron Ross for the treatment of animals with the liquid diet and the preparation of microsomal fractions and Dr. Magnus Ingelman-Sundberg for his kind donation of anti-cytochrome P450 2E1 antibodies.

References

1. Reinke, L. A., E. K. Lau, C. M. DuBose, and P. B. McCay. Reactive free radical generation *in vivo* in heart and liver of ethanol-fed rats: correlation with radical formation *in vitro*. *Proc. Natl. Acad. Sci. USA* **84**:9223–9227 (1987).
2. Albano, E., A. Tomasi, L. Gatti-Goria, and M. U. Dianzani. Spin trapping of free radical species produced during the microsomal metabolism of ethanol. *Chem. Biol. Interact.* **65**:223–234 (1988).
3. Reinke, L. A. Possible roles of free radicals in alcoholic tissue damage. *Free Radical Res. Commun.* **9**:205–211 (1990).
4. Gonthier, B., A. Jeunet, and L. Barret. Electron spin resonance study of free radicals produced from ethanol and acetaldehyde after exposure to a Fenton system or to brain and liver microsomes. *Alcohol* **8**:369–375 (1991).
5. Rashba-Step, J., N. J. Turro, and A. I. Cederbaum. Increased NADPH- and NADH-dependent production of superoxide and hydroxyl radical by microsomes after chronic ethanol treatment. *Arch. Biochem. Biophys.* **300**:401–408 (1993).
6. Rashba-Step, J., N. J. Turro, and A. I. Cederbaum. ESR studies on the production of reactive oxygen intermediates by rat liver microsomes in the presence of NADPH or NADP. *Arch. Biochem. Biophys.* **300**:391–400 (1993).
7. Knecht, K. T., B. U. Bradford, R. P. Mason, and R. G. Thurman. *In vivo* formation of a free radical metabolite of ethanol. *Mol. Pharmacol.* **38**:26–30 (1990).
8. Knecht, K. T., R. G. Thurman, and R. P. Mason. Role of superoxide and trace transition metals in the production of α-hydroxyethyl radical from ethanol by microsomes from alcohol dehydrogenase-deficient deermice. *Arch. Biochem. Biophys.* **303**:339–348 (1993).
9. Ramos, C. L., S. Pou, B. E. Britigan, M. S. Cohen, and G. M. Rosen. Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. *J. Biol. Chem.* **267**:8307–8312 (1992).
10. Reinke, L. A., Y. Kotake, P. B. McCay, and E. G. Janzen. Spin-trapping studies of hepatic free radicals formed following the acute administration of ethanol to rats: *in vivo* detection of 1-hydroxyethyl radicals with PBN. *Free Radicals Biol. Med.* **11**:31–39 (1991).
11. Stevens, V. J., W. J. Fantl, C. B. Newman, R. V. Sims, A. Cerami, and C. M. Peterson. Acetaldehyde adducts with hemoglobin. *J. Clin. Invest.* **67**:361–369 (1981).
12. Donohue, T. M., D. J. Tuma, and M. F. Sorrell. Acetaldehyde adducts with proteins: binding of ^{14}C-acetaldehyde to serum albumin. *Arch. Biochem. Biophys.* **220**:239–246 (1983).
13. Schuessler, H., G. Schmerler-Dremel, J. Danzer, and E. Jung-Körner. Ethanol radical-induced protein-DNA crosslinking: a radiolysis study. *Int. J. Radiat. Biol.* **62**:517–526 (1992).
14. Tuma, D. J., T. M. Donohue, V. A. Medina, and M. F. Sorrell. Enhancement of acetaldehyde-protein adduct formation by L-ascorbate. *Arch. Biochem. Biophys.* **234**:377–381 (1984).
15. Steinbrecher, U. P., M. Fisher, J. L. Witztum, and L. K. Curtiss. Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: generation of antibodies specific for derivatized lysine. *J. Lipid Res.* **25**:1109–1116 (1984).
16. Israel, Y., E. Hurwitz, O. Niemelä, and R. Arnon. Monoclonal and polyclonal antibodies against acetaldehyde containing epitopes in acetaldehyde-protein adducts. *Proc. Natl. Acad. Sci. USA* **83**:7923–7927 (1986).
17. Hoerner, M., U. J. Behrens, T. M. Worner, I. Blacksberg, L. F. Braly, F. Schaffner, and C. S. Lieber. Humoral immune response to acetaldehyde adducts in alcoholic patients. *Res. Commun. Chem. Pathol. Pharmacol.* **54**:3–12 (1986).
18. Niemelä, O., F. Klajner, H. Orrego, E. Vidins, L. Blendis, and Y. Israel. Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. *Hepatology* **7**:1210–1214 (1987).
19. Hoerner, M., U. J. Behrens, T. Worner, and C. S. Lieber. Humoral immune response to acetaldehyde adducts. *Hepatology* **8**:569–574 (1988).
20. Worrall, S., J. de Jersey, B. C. Shanley, and P. A. Wilce. Antibodies against acetaldehyde modified epitopes: an elevated IgA response in alcoholics. *Eur. J. Clin. Invest.* **21**:90–95 (1991).
21. Koskinas, J., J. G. Kenna, G. L. Bird, G. J. M. Alexander, and R. Williams. Immunoglobulin A antibody to a 200-kilodalton cytosolic acetaldehyde adduct in alcoholic hepatitis. *Gastroenterology* **103**:1860–1867 (1992).
22. Teare, J. P., A. J. Carmichael, F. R. Burnett, and M. O. Rake. Detection of antibodies to acetaldehyde-albumin conjugates in alcoholic liver disease. *Alcohol Alcohol.* **28**:11–16 (1993).
23. Worrall, S., J. de Jersey, B. C. Shanley, and P. A. Wilce. Ethanol induces the production of antibodies to acetaldehyde-modified epitopes in rats. *Alcohol Alcohol.* **24**:217–223 (1989).
24. Worrall, S., J. de Jersey, B. C. Shanley, and P. A. Wilce. Detection of stable acetaldehyde-modified proteins in the livers of ethanol-fed rats. *Alcohol Alcohol.* **26**:437–444 (1991).
25. Schuessler, H. Reactions of ethanol and formate radicals with ribonuclease A and bovine serum albumin in radiolysis. *Int. J. Radiat. Biol.* **40**:483–492 (1981).
26. Puchala, M., and H. Schuessler. Radiation-induced binding of methanol, ethanol and 1-butanol to haemoglobin. *Int. J. Radiat. Biol.* **50**:535–546 (1986).
27. Albano, E., M. Parola, A. Comoglio, and M. U. Dianzani. Evidence for the covalent binding of hydroxyethyl radicals to rat liver microsomal proteins. *Alcohol Alcohol.* **28**:453–459 (1993).
28. Johansson, I., G. Ekstrom, B. Scholte, D. Puszycki, H. Jornvall, and M. Ingelman-Sundberg. Ethanol-, fasting-, and acetone-inducible cytochromes P450 in rat liver: regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies. *Biochemistry* **27**:1925–1934 (1988).
29. Lieber, C. S., and L. M. DeCarli. The feeding of ethanol in liquid diets: 1986 update. *Alcohol Clin. Exp. Res.* **10**:550–553 (1986).
30. Eliasson, E., I. Johansson, and M. Ingelman-Sundberg. Ligand-dependent maintenance of ethanol-inducible cytochrome P-450 in primary rat hepatocyte cell cultures. *Biochem. Biophys. Res. Commun.* **150**:436–443 (1988).
31. Yang, C. S. Interactions between solubilized cytochrome P-450 and hepatic microsomes. *J. Biol. Chem.* **252**:293–298 (1977).
32. Tu, Y. Y., J. Sonnenberg, K. F. Lewis, and C. S. Yang. Pyrazole-induced cytochrome P-450 in rat liver microsomes: an isozyme with high affinity for dimethylnitrosamine. *Biochem. Biophys. Res. Commun.* **103**:905–912 (1981).
33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
34. Lieber, C. S., and L. M. DeCarli. Hepatic microsomal ethanol oxidizing system: *in vitro* characteristics and adaptive properties *in vivo*. *J. Biol. Chem.* **245**:2505–2512 (1970).
35. Leffler, J. E. The hydroxyl radical, in *An Introduction to Free Radicals*. Wiley-Interscience, New York, 116–125 (1993).
36. Moncada, C., V. Torres, and Y. Israel. Simple method for the preparation of antigen emulsions for immunization. *J. Immunol. Methods* **162**:133–140 (1993).
37. Tuma, D. J., M. R. Newman, T. M. Donohue, and M. F. Sorrell. Covalent binding of acetaldehyde to proteins: participation of lysine residues. *Alcohol Clin. Exp. Res.* **11**:579–584 (1987).
38. Israel, Y., A. Macdonald, O. Niemelä, D. Zamel, E. Shami, M. Zywulko, F. Klajner, and C. Borgono. Hypersensitivity to acetaldehyde-protein adducts. *Mol. Pharmacol.* **42**:711–717 (1992).
39. Lin, R. C., and L. Lumeng. Further studies on the 37 kD liver protein-acetaldehyde adduct that forms *in vivo* during chronic alcohol ingestion. *Hepatology* **10**:807–814 (1989).
40. Klassen, L. W., D. J. Tuma, M. F. Sorrell, T. L. McDonald, J. M. DeVasure, and G. M. Thiele. Dectection of reduced acetaldehyde protein adducts using a unique monoclonal antibody. *Alcohol. Clin. Exp. Res.* **18**:164–171 (1994).
41. Mullarkey, C. J., D. Edelstein, and M. Brownlee. Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem. Biophys. Res. Commun.* **173**:932–939 (1990).
42. Reznick, A. Z., C. E. Cross, M.-L. Hu, Y. J. Suzuki, S. Khwaja, A. Safadi, P. A. Motchnik, L. Packer, and B. Halliwell. Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem. J.* **286**:607–611 (1992).
43. Bautista, A. P., and J. J. Spitzer. Platelet activating factor stimulates and primes the liver, Kupffer cells and neutrophils to release superoxide anion. *Free Radicals Res. Commun.* **17**:195–209 (1992).
44. Jaeschke, H., A. P. Bautista, Z. Spolarics, and J. J. Spitzer. Superoxide generation by neutrophils and Kupffer cells during *in vivo* reperfusion after hepatic ischemia in rats. *J. Leukocyte Biol.* **52**:377–382 (1992).
45. Williams, A. J. K., and R. E. Barry. Free radical generation by neutrophils: a potential mechanism of cellular injury in acute alcoholic hepatitis. *Gut* **28**:1157–1161 (1987).

Send reprint requests to: Yedy Israel, Department of Anatomy, Pathology, and Cell Biology, Thomas Jefferson University, Room 275, Jefferson Alumni Hall, 1020 Locust Street, Philadelphia, PA 19107.