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Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts

(alcoholism/immunoglobulins/ethanol metabolites)

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ABSTRACT **Immunization of mice with acetaldehyde conjugated to human plasma proteins resulted in the production of polyclonal antibodies that reacted with erythrocyte protein-acetaldehyde conjugates, but not with control erythrocyte proteins. Such antibodies recognized erythrocyte protein-acetaldehyde conjugates prepared with 20–100 μM acetaldehyde, concentrations that exist in the blood of alcoholics. The antibodies also recognized acetaldehyde condensation products with synthetic poly-(L-lysine). Immunization with keyhole limpet hemocyanin-acetaldehyde conjugates resulted in antibodies against both plasma protein-acetaldehyde and erythrocyte protein-acetaldehyde conjugates, which did not cross-react with the respective unmodified carrier proteins. Immunization with human erythrocyte protein-acetaldehyde condensates led to the production of antibodies against both the protein moiety as well as the condensate. Monoclonal antibodies with affinities 50 times greater for the condensate than for the carrier protein were produced by hybridization of spleen cells from the immunized mice. Chronic alcohol administration to mice for 45–50 days led to the generation of antibodies that reacted against protein-acetaldehyde conjugates, suggesting that such adducts are formed *in vivo* and can act as neoantigens. Antibodies against acetaldehyde adducts should be of value in the identification of alcohol consumption and in the study of the biology of the adducts in relation to organ pathology.**

Acetaldehyde, a product of ethanol metabolism, has been implicated in a number of actions of alcohol (1) and is notable for its ability to bind covalently to erythrocyte and plasma proteins and to subcellular organelles (2–7). However, the identification and quantification of acetaldehyde-protein condensates have been hampered due to the small amounts of these condensates that are formed at acetaldehyde concentrations that occur following alcohol consumption (5). Thus, identification of adduct formation has relied on the use of radioactive acetaldehyde.

Studies by Stevens *et al.* (2), Donohue *et al.* (3), and Tuma *et al.* (4) have shown that acetaldehyde binding to proteins leads to the formation of both stable and unstable adducts. The latter, postulated to be Schiff bases (2–4), can be stabilized by various reducing agents, such as sodium borohydride, sodium cyanoborohydride, or ascorbate (2–4). Depending on the binding conditions, stable acetaldehyde adducts to bovine serum albumin and to human hemoglobin, prepared at concentrations of 200–300 μM acetaldehyde, comprise 15–70% of the total adducts formed (2–4). Stable and stabilized adducts are resistant to conditions such as acid precipitation, gel filtration, and extensive dialysis (2–4). Studies by Stevens *et al.* (2) have shown that three amino acid residues in hemoglobin (lysine, tyrosine, and valine) can bind acetaldehyde and can be stabilized by sodium borohydride. Spontaneous reduction and physiological reducing agents (e.g., ascorbate) have been suggested to mediate the formation of stable adducts similar to exogenous reducing agents (4). In addition, cross-linking of proteins has been shown to occur at high concentrations of acetaldehyde (8). Products formed at high concentrations of acetaldehyde have been reported not to be reducible by sodium borohydride (9).

We hypothesize that molecular structures formed in acetaldehyde-protein adducts may be recognized as foreign by the immune system, resulting in the generation of antibodies. Such antibodies could provide a basis for quantification of unlabeled condensates in erythrocytes and plasma proteins, which could in turn provide a cumulative record of acetaldehyde load, reflecting recent chronic alcohol consumption by an individual and analogous to the use of glycosylated hemoglobin as an index of mean blood glucose levels over a period of several weeks (10–12).

The detection of acetaldehyde adducts is also of interest in the study of the pathogenesis of alcohol-induced liver injury, since covalent binding of acetaldehyde to macromolecules might underlie alcoholic liver damage (13). Should such condensates be immunogenic, their presence on the surface of hepatocytes could lead to liver cell injury mediated by the immune surveillance system. In addition, circulating immune (antigen–antibody) complexes might mediate observed organic pathology induced by ethanol in tissues other than the liver.

In this report, we describe studies showing that (*i*) antibodies can be generated against epitopes containing the acetaldehyde residue in acetaldehyde-protein condensates; (*ii*) these antibodies are able to react with both stable and stabilized condensates formed at acetaldehyde concentrations comparable to those present in the blood of alcoholics; (*iii*) the antibodies can react independently of the nature of the macromolecule binding the acetaldehyde moiety; and (*iv*) chronic alcohol ingestion *per se* leads to the generation of antibodies directed against acetaldehyde-modified proteins, thus suggesting that such condensates are formed *in vivo*. Portions of this work have been previously presented (14)§.

MATERIALS AND METHODS

Preparation of Protein-Acetaldehyde Conjugates for Use as Antigens. Immunization with proteins modified at 20–100 μM acetaldehyde, concentrations that exist in the blood of alcoholics consuming ethanol (15), resulted in very low titers. Heavily conjugated condensates, on the other hand, were shown to be excellent immunogens and their preparation is described below.

Erythrocyte protein-acetaldehyde conjugates. Human erythrocytes were prepared by centrifugation of fresh blood

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containing 5 mM EDTA. The cells were washed three times with 0.9% NaCl, and the final pellet was lysed by addition of 0.25 vol of toluene, mixed for 30 min at 20°C, and centrifuged (2). The lower aqueous phase (2 ml), of which hemoglobin is the main protein constituent (300–350 mg/ml), was mixed at 20°C with 2 ml of phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4/6.0 mM Na HPO_4/0.11 M NaCl/3 mM KCl), pH 7.4, containing 480 mM acetaldehyde. After 60 min, 272 mg of sodium cyanoborohydride ($NaCNBH_3$) in 0.7 ml of water was used to reduce the linkages that had not spontaneously stabilized. After 30 min, the mixture was dialyzed against 1 mM NaH_2PO_4, pH 7.0. This treatment resulted in conversion of 96% of hemoglobin into fast hemoglobins as determined by a Bio-Rad, glycosylated hemoglobin kit. In the absence of acetaldehyde, but after $NaCNBH_3$ treatment (controls), 6–10% of hemoglobin run as "fast" (eluting) hemoglobins through a cation-exchange resin (Bio-Rad kit). In parallel experiments with $NaCNB^3H_3$, incorporation of acetaldehyde was estimated to be 44 nmol/mg of protein.

Plasma protein conjugates. Saturated ammonium sulfate (27 ml), pH 7.1, was added to 9 ml of human plasma and mixed for 2 hr at 4°C. The precipitate containing plasma proteins, of which albumin is the main constituent, was centrifuged at 10,000 × *g* for 20 min. The pellet was dissolved in 18 ml of PBS and dialyzed at 4°C against 1 liter of half-strength PBS. Final protein concentration was brought to 16 mg/ml, and 5 ml of the solution was added to 5 ml of 480 mM acetaldehyde in PBS. The reaction was allowed to proceed for 60 min at 20°C, and 700 mg of $NaCNBH_3$ dissolved in 2 ml of PBS was added. After 45 min, the mixture was dialyzed at 4°C against 1 liter of PBS. In parallel experiments with $NaCNB^3H_3$, incorporation of acetaldehyde was estimated to be 726 nmol/mg of protein.

Hemocyanin conjugates. Purified hemocyanin from keyhole limpet (Calbiochem) in saturated ammonium sulfate at a concentration of 10 mg/ml was dialyzed for 4 days at 4°C against 4 liters of 5% (wt/vol) Na_2CO_3 and for one day against PBS adjusted to pH 8.7. Two milliliters of keyhole limpet hemocyanin (5 mg/ml) was mixed with 2 ml of 480 mM acetaldehyde at 20°C. After 60 min, 63 mg of $NaCNBH_3$ in 0.5 ml of H_2O were added. The mixture was allowed to stand for 3 hr at 20°C and then dialyzed at 4°C against PBS, pH 9.5.

Preparation of Protein-Acetaldehyde Conjugates at Low Acetaldehyde Concentrations. *Poly(amino acid) conjugates.* Solutions of poly-(L-lysine) (Sigma), poly-(L-tyrosine) (Sigma) and poly-(L-valine) (Sigma) at concentrations of 0.5 mg/ml in PBS were placed in dialysis bags (2.5 ml), and immersed for 70 hr in 500 ml of PBS containing either 0, 100 μM, 1 mM, or 10 mM acetaldehyde in the presence or absence of 10 mM sodium cyanoborohydride. Subsequently, the poly(amino acid) solutions were dialyzed against PBS.

Erythrocyte protein conjugates. Erythrocyte proteins (50 μg of protein per ml) prepared as above were combined under sterile conditions with 20 μM, 100 μM, or 1 mM acetaldehyde in PBS in the presence or absence of 10 mM cyanoborohydride for 7 days at room temperature and finally dialyzed against PBS.

Immunization Procedure, Testing, and Hybridization. *Immunization.* Two mouse strains were tested, the BALB/c and the F1 generation of the cross of BALB/c × SJL strains. Optimal immunizations were obtained with the latter strain and are presented here. Four-month-old female mice were given four weekly subcutaneous injections of 50 μl of 100 μg of protein-acetaldehyde condensate in a 1:1 (vol/vol) suspension of saline and Freund's complete adjuvant. The animals were then sacrificed by bleeding, and the immune titers in plasma (in 5 mM EDTA) were determined by ELISA assays using 1–5 μg of protein to coat the wells. The secondary antibody was sheep anti-mouse immunoglobulin bound to β-galactosidase (Amersham, U.K.). The ELISA method used is that described by Eshhar (16), except that gelatin 0.2% was used to block nonspecific binding sites. For animals chronically treated with alcohol (see below) immune titers were determined by radioimmunoassay using ^{125}I-labeled goat anti-mouse immunoglobulin (16).

Affinity purification of immune plasma. Pooled plasma (400 μl) of mice immunized with human plasma protein-acetaldehyde condensate was cross-absorbed through a 10-ml column containing control human erythrocyte proteins linked to CNBr-activated Sepharose 4B (Sigma). The protein peak not retained by the column was affinity purified by absorption to a 10-ml CNBr-activated Sepharose 4B column linked to erythrocyte protein-acetaldehyde (240 mM) condensate (10 mg of protein per ml of wet column bed). The bound antibody was eluted with 0.2 M glycine·HCl (pH 2.7), dialyzed against PBS, and concentrated to 1 mg/ml.

Hybridization. Lymphocytes from spleens of animals immunized with erythrocyte protein-acetaldehyde conjugates were hybridized with myeloma cells, following the method of Eshhar (16). Clones were initially screened against the plasma protein-acetaldehyde condensate and subsequently tested for reactivity against erythrocyte protein-acetaldehyde condensates.

Chronic Administration of Ethanol. Female C57BL mice, 3–3.5 months old, were administered 0, 7.5, or 15% (vol/vol) ethanol in the drinking water for 45–50 days and then sacrificed. Plasma in 5 mM EDTA was used to test reactivity against erythrocyte protein-acetaldehyde adduct.

Other Methods. *N*-ethyllysine, obtained from David Dime at the University of Toronto, and lysine were tested for binding to the antibodies by adding the primary antibody to the ELISA wells in a solution containing 100 μM, 1 mM, or 10 mM *N*-ethyllysine or lysine in PBS after a 60-min preincubation period.

Statistical Methods. Data were analyzed by the *t*-test method for unpaired data and by analysis of variance followed by multiple comparisons using the Newman–Keuls procedure (17).

RESULTS

Immunization with the heavily conjugated erythrocyte protein-acetaldehyde condensate yielded antibodies that strongly reacted with plasma protein-acetaldehyde condensate but not with control plasma proteins (Fig. 1). Similarly, immunization with plasma protein-acetaldehyde condensate yielded antibodies that strongly reacted with erythrocyte protein-acetaldehyde condensate but not with the unmodified erythrocyte proteins (Fig. 2). The affinity-purified immunoglob-

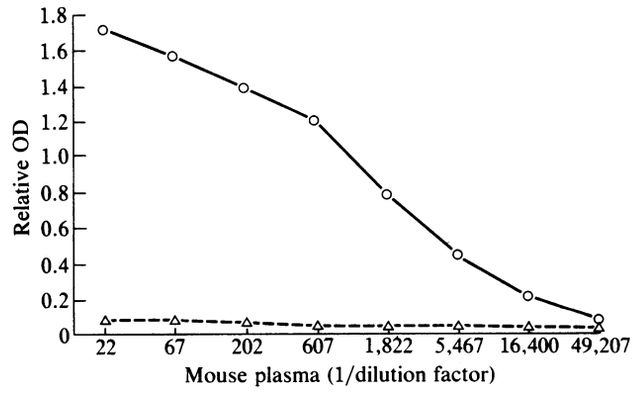

FIG. 1. ELISA of plasma from mice immunized with human erythrocyte protein-acetaldehyde condensate and tested against plasma protein-acetaldehyde condensate (○) or control (carrier) plasma proteins (△). OD was measured at 405 nm.

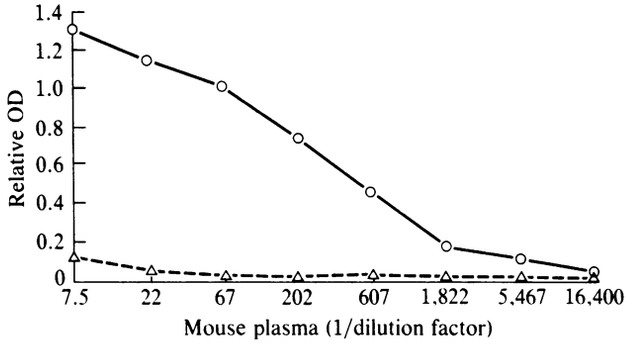

FIG. 2. ELISA of plasma from mice immunized with human plasma protein-acetaldehyde condensate and tested against erythrocyte protein-acetaldehyde condensate (○) or control (carrier) erythrocyte proteins (△). OD was measured at 405 nm.

ulins were further tested for reactivity against erythrocyte proteins modified at lower acetaldehyde concentrations including those of 20 μM and 100 μM, which exist in the blood of alcoholics (15). Fig. 3 shows that the antibodies indeed recognized erythrocyte protein-acetaldehyde condensates prepared at these concentrations. Fig. 4 shows that the antibodies recognized the erythrocyte protein-acetaldehyde condensates both in their stable and cyanoborohydride-stabilized forms, although the latter were more reactive.

When tested against poly-(L-lysine), poly-(L-tyrosine), and poly-(L-valine) condensates, the antibodies recognized only the poly-(L-lysine)-acetaldehyde adducts. The immunoreactivity toward stable poly-(L-lysine) adducts, in the absence of sodium cyanoborohydride, is as follows (mean OD at 405 nm ± SE): control polylysine, 0.150 ± 0.009; polylysine condensate with 100 μM acetaldehyde, 0.296 ± 0.040 ($P < 0.02$); 1 mM acetaldehyde, 0.348 ± 0.031 ($P < 0.001$); 10 mM acetaldehyde, 0.327 ± 0.039 ($P < 0.005$). In the presence of

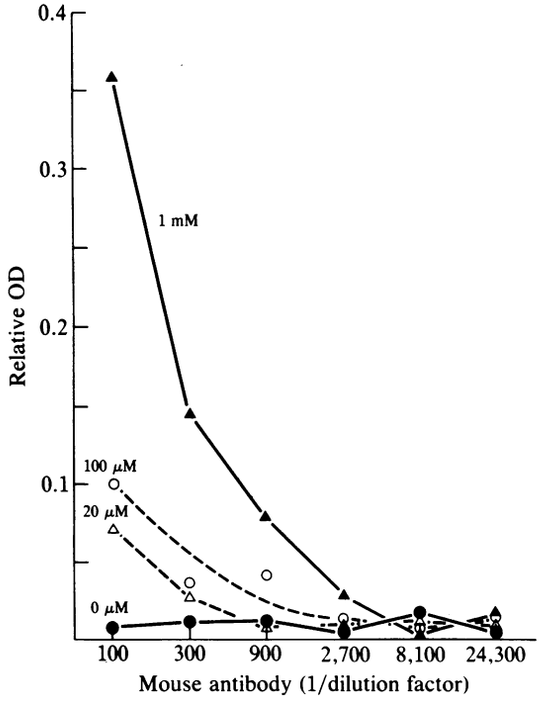

FIG. 3. ELISA of affinity-purified antibody from mice immunized with human plasma protein-acetaldehyde condensate against human erythrocyte protein-acetaldehyde conjugates (▲, ○, △) prepared at the concentrations of acetaldehyde indicated and against control (carrier) erythrocyte proteins (●).

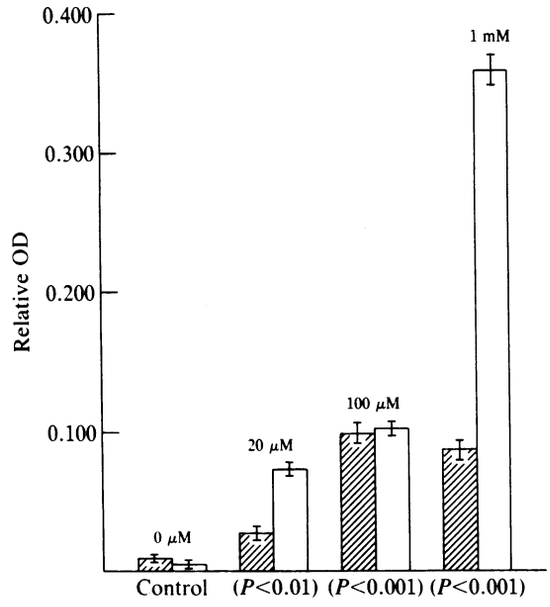

FIG. 4. ELISA of erythrocyte protein-acetaldehyde condensates prepared with (open bars) and without (hatched bars) sodium cyanoborohydride. The proteins were incubated with the different acetaldehyde concentrations, and the reaction mixtures were assayed using an affinity-purified antibody from mice immunized with human plasma protein condensate (1:200 dilution). Six samples of each were tested, and bars indicate means ± SEM.

sodium cyanoborohydride, immunoreactivity was three times higher. At 10 mM acetaldehyde in the presence of 10 mM sodium cyanoborohydride, duplicate OD readings were 0.998 and 1.020. It should be noted that high backgrounds are expected in polylysine-coated wells, independently of the type and source of the antibodies (16). No immunoreactivity was detected for either the stable or stabilized forms of poly-(L-tyrosine) and poly-(L-valine)-acetaldehyde condensates at concentrations up to 10 mM acetaldehyde. The above data suggest that the antibodies can recognize the molecular structure of the *N*-ethyllysine residue. When tested for inhibition of the immune reaction, 10 mM *N*-ethyllysine, the highest concentration tested, decreased the ELISA readings by 20–25%. At 405 nm, control OD was 0.298 ± 0.025; 10 mM *N*-ethyllysine OD was 0.232 ± 0.047 ($P < 0.05$); 10 mM lysine OD was 0.284 ± 0.020 (not significant); ($n = 4$).

The above results indicated that immunoglobulins against acetaldehyde-containing epitopes in proteins can be generated independently of the protein carriers used for immunization, suggesting that an acetaldehyde-bound nonmammalian protein (keyhole limpet hemocyanin) may also serve as an antigen. Fig. 5 shows that immunization with this condensate also generated antibodies against the plasma protein-acetaldehyde and erythrocyte-acetaldehyde condensates but not against their respective unmodified proteins.

Antibodies against the acetaldehyde-containing epitopes were also produced by an alternative approach. Specifically, lymphocytes in the spleen of erythrocyte protein-acetaldehyde immunized animals whose sera reacted both with erythrocyte proteins and with erythrocyte protein-acetaldehyde conjugate were hybridized with malignant myeloma cells. Out of 420 wells with hybridoma growth tested, 30 wells produced immunoglobulins that strongly reacted with both the erythrocyte protein-acetaldehyde condensate and with plasma protein-acetaldehyde condensate. Of these, 16 clones were subsequently selected for further cultures. The relative affinity of immunoglobulins released by the hybridoma into the culture fluids was compared at different dilutions against both the erythrocyte protein-acetaldehyde conjugate (pre-

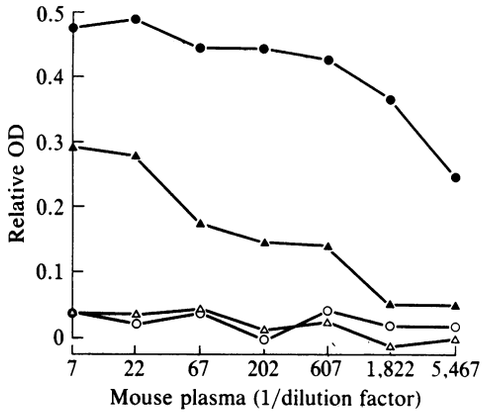

FIG. 5. ELISA of plasma from mice immunized with keyhole limpet hemocyanin-acetaldehyde condensate, against human plasma protein-acetaldehyde (●) and human erythrocyte protein-acetaldehyde condensates (▲) and against their respective carrier proteins (○, plasma protein control; △, erythrocyte protein control). OD was measured at 405 nm.

pared at 1 mM acetaldehyde) as well as the control erythrocyte protein. As is evident in Fig. 6, this approach also yields immunoglobulins that react preferentially with the acetaldehyde-containing epitope(s) in hemoglobin adducts as opposed to control hemoglobin: in four of the clones, the immunoglobulin affinity was at least 50 times higher for the erythrocyte protein-acetaldehyde condensate than for the control erythrocyte protein.

Antibody production against protein-acetaldehyde condensates was also induced by chronic administration of alcohol to C57BL mice—reputedly poor immunological responders (18) with a high preference for ethanol ingestion (19). Plasma from alcohol-treated animals showed significantly higher ($P < 0.03$) immunoreactivity against erythrocyte protein-acetaldehyde condensates than that for control animals, as measured either by radioimmunoassay (Fig. 7) or ELISA.

DISCUSSION

The studies presented here indicate that acetaldehyde conjugation to proteins results in the production of immunodominant antigenic determinants. The antibodies produced after

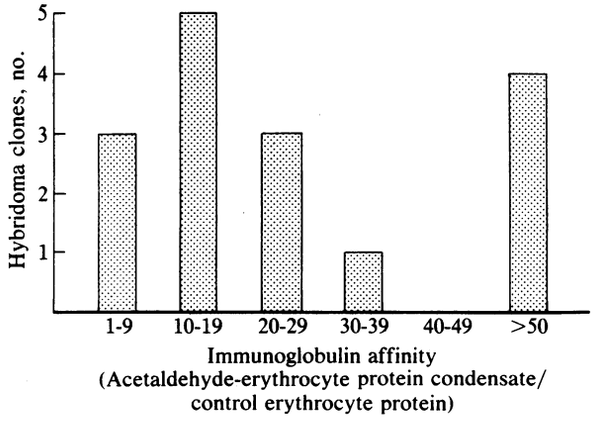

FIG. 6. Frequency distribution of hybridoma clones according to the relative affinity of immunoglobulin produced when tested against erythrocyte protein-acetaldehyde condensate as compared to control (carrier) erythrocyte protein. Hybridoma supernatants were tested at different dilutions against erythrocyte protein-acetaldehyde conjugates prepared at 1 mM acetaldehyde or against control (carrier) erythrocyte proteins.

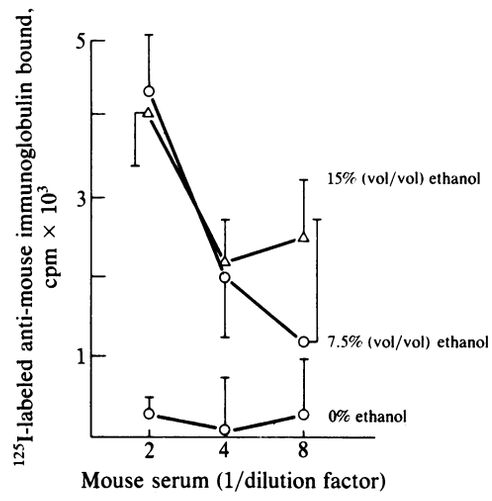

FIG. 7. Radioimmunoassay of sera of mice fed alcohol chronically, at the concentrations indicated, for 45–50 days, tested against human erythrocyte protein-acetaldehyde conjugate. Eight animals were tested at each point. Points represent means ± SEM. Background was 20,750 cpm. A 3 × 3 (groups × dilutions) analysis of variance revealed significant differences among the groups of animals [F (2,20) = 4.32, $P < 0.03$] and among the dilutions used [F (2,40) = 4.16, $P < 0.03$]. The subsequent simple-main-effect analysis indicated that the group effect occurred mainly at the 1:2 dilution [F (2,20) = 9.48, $P < 0.001$]. Finally, employing the Newman–Keuls multiple comparisons procedure, we found that both the 7.5% and 15% (vol/vol) ethanol groups differed significantly from the 0% control ($P < 0.01$).

immunization with protein-acetaldehyde condensates can recognize small acetaldehyde-containing epitopes in macromolecules. To our knowledge, this would be the smallest molecular weight hapten for which antibodies have been raised. We have found that antibodies towards acetaldehyde-containing epitopes in proteins could be raised by immunizing with acetaldehyde conjugated to a carrier protein different from the one that is used for testing. Furthermore, similar results were obtained by hybridization and selection of clones that preferentially recognize the acetaldehyde-conjugated protein rather than the unconjugated molecule.

Acetaldehyde can covalently bind to various proteins to form both stable and unstable adducts; valine, lysine, and tyrosine residues probably being the sites of reaction (2). Our data suggest that the lysine-acetaldehyde adduct, likely comprising the *N*-ethyllysine residue, is immunologically recognized by the antibodies. However, we cannot discount the possibility that an intra- or intermolecular stabilization of the lysine-acetaldehyde Schiff base also occurred and was detected by the antibodies. On the other hand, the intermolecular stabilization possibility is less likely in view of the studies by Donohue *et al.* (3), who did not observe spontaneous intermolecular cross-links at concentrations of acetaldehyde (200 μM) in the range used in our studies.

Protein-acetaldehyde adducts have been suggested as clinically useful markers for alcohol consumption (2). Separation of α and β globins of hemoglobin would increase the capabilities for the detection of acetaldehyde conjugates, as the β chains bind acetaldehyde nine times more efficiently than the α chains (20). A selective binding of acetaldehyde to some residues is also in agreement with studies by Jennett *et al.* (21), who showed that the formation of stable adducts occurs preferentially for some specific lysine residues in the tubulin dimer molecule. Our observations should provide the basis for both the development of sensitive immunological assays to monitor recent alcohol consumption and for the study of the biology of acetaldehyde condensates in relation to organ pathology in alcoholism. These suggestions are further sup-

ported by the fact that the antibodies generated recognized spontaneously stable protein-acetaldehyde adducts formed in the absence of exogenous reducing agents at concentrations of acetaldehyde analogous to those found in the blood of alcoholics.

A number of studies have suggested that alcoholic liver disease may have an immunological component (22–29). Studies by Sorrell and Leevy (22) demonstrated that, *in vitro*, acetaldehyde (190 μM), at concentrations similar to those used in our study, is able to stimulate proliferation of lymphocytes obtained from patients with alcoholic hepatitis. Based on our findings, we suggest a possible mechanism for such lymphocyte proliferation, namely that acetaldehyde may have led to the formation of adducts, which in turn are recognized by immunoglobulins present in the membrane of lymphocytes, thus leading to lymphocyte proliferation. The present data can also account for the observation that sera of alcoholics, but not of controls, can induce cytotoxicity in hepatocytes isolated from animals pretreated *in vivo* with ethanol (25, 27). The fact that such cytotoxicity was inhibited by pretreatment of the animals with 4-methylpyrazole and enhanced by disulfiram strongly suggests that acetaldehyde was implicated in this effect.

Several reports have suggested abnormalities of humoral immunity in alcoholic liver disease, resulting probably from excessive antigenic stimulation or heightened immune response (29). The increased antigenic stimulation may be due to alcohol-induced changes. The fact that in our studies chronic alcohol administration *per se* triggered the production of circulating immunoglobulins against acetaldehyde-modified proteins suggests that these condensation compounds also form *in vivo* following chronic alcohol consumption and subsequently can act as neoantigens.

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1. Salaspuro, M. & Lindros, K. (1985) in *Alcohol Related Diseases in Gastroenterology*, eds. Seitz, H. K. & Kommerell, B. (Springer, Berlin, Heidelberg), pp. 106–123.
2. Stevens, V. J., Fantl, W. J., Newman, C. B., Sims, R. V., Cerami, A. & Peterson, C. M. (1981) *J. Clin. Invest.* **67**, 361–369.
3. Donohue, T. M., Tuma, D. J. & Sorrell, M. F. (1983) *Arch. Biochem. Biophys.* **220**, 239–246.
4. Tuma, D. J., Donohue, T. M., Medina, V. A. & Sorrell, M. F. (1984) *Arch. Biochem. Biophys.* **234**, 377–381.
5. Nguyen, L. B. & Peterson, C. M. (1984) *Proc. Soc. Exp. Biol. Med.* **177**, 226–233.
6. Lumeng, L., Minter, R. & Li, T. K. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 765 (abstr.).
7. Nomura, F. & Lieber, C. S. (1981) *Biochem. Biophys. Res. Commun.* **100**, 131–137.
8. Mohammad, A., Okoh, J. S. & Fraenkel-Conrat, H. (1949) *Arch. Biochem.* **24**, 270–280.
9. San George, R. C. & Hoberman, H. D. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 1618 (abstr.).
10. Trivelli, L. A., Ranney, U. N. & Lai, H. T. (1971) *N. Engl. J. Med.* **284**, 353–357.
11. Koenig, R. J., Peterson, R. L., Jones, R. L., Sandek, M., Lehrman, M. & Cerami, A. (1976) *N. Engl. J. Med.* **295**, 417–420.
12. Gonen, B., Rochman, H., Rubinstein, A. H., Tanega, S. P. & Horwitz, D. L. (1977) *Lancet* **ii**, 734–737.
13. Sorrell, M. F. & Tuma, D. J. (1985) *Alcoholism Clin. Exp. Res.* **9**, 306–309.
14. Israel, Y., Arnon, R. & Hurwitz, E. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 570 (abstr.).
15. Nuutinen, H., Lindros, K. O. & Salaspuro, M. (1983) *Alcoholism Clin. Exp. Res.* **7**, 163–168.
16. Eshhar, Z. (1985) in *Hybridoma Technology in the Biosciences and Medicine*, ed. Springer, T. A. (Plenum, New York), pp. 3–34.
17. Winer, B. J. (1971) *Statistical Principles in Experimental Design* (McGraw-Hill, New York).
18. Goding, J. W. (1983) *Monoclonal Antibodies: Principles and Practice* (Academic, New York).
19. McClearn, G. (1972) in *Biological Aspects of Alcohol Consumption*, eds. Forsander, O. & Eriksson, K. (Finnish Foundation for Alcohol Studies, Helsinki), pp. 113–119.
20. Nguyen, L. B. & Peterson, C. M. (1986) *Proc. Soc. Exp. Biol. Med.* **181**, 151–156.
21. Jennett, R. B., Johnson, E. L., Sorrell, M. F. & Tuma, D. J. (1985) *Hepatology* **5**, 1056 (abstr.).
22. Sorrell, M. F. & Leevy, C. M. (1972) *Gastroenterology* **63**, 1020–1025.
23. Actis, G. C., Ponzetto, A., Rizzetto, M. & Verme, G. (1978) *Dig. Dis. Sci.* **23**, 883–886.
24. Cochrane, A. M. G., Moussouros, A., Portmann, B., McFarlane, I. G., Thomson, A. D., Eddleston, A. L. W. F. & Williams, R. (1977) *Gastroenterology* **72**, 918–923.
25. Neuberger, J., Crossley, I. R., Saunders, J. B., Davis, M., Portmann, B., Eddleston, A. L. W. F. & Williams, R. (1984) *Gut* **25**, 300–304.
26. Barry, R. E. & McGivan, J. D. (1985) *Gut* **26**, 1065–1069.
27. Crossley, I. R., Neuberger, J., Davis, M., Williams, R. & Eddleston, A. L. W. F. (1986) *Gut* **27**, 186–189.
28. Kakumu, S. & Leevy, C. M. (1977) *Gastroenterology* **72**, 594–597.
29. MacSween, R. N. M. (1986) *Acta Med. Scand.* **703**, Suppl. 57–65.