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Monoclonal and Polyclonal Antibodies Recognizing Acetaldehyde–Protein Adducts

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ABSTRACT. Studies have investigated the hypothesis that metabolically derived acetaldehyde (AA) is capable of complexing with liver cell proteins to form AA–protein adducts that are capable of acting as antigens and inducing an immune response, as detected by the formation of unique antibodies. In an effort to better characterize and describe these adducts, mouse monoclonal and rabbit polyclonal antibodies specific for antigens prepared with AA under non-reducing (physiologic) and reducing (presence of sodium cyanoborohydride) conditions have been prepared. Two monoclonal antibodies were developed. The first antibody was RT1.1, which is specific to *N*-ethyl lysine (NEL); it is of the IgG2b isotype and recognizes all proteins modified with AA under reducing conditions. The other monoclonal antibody, NR-1, was of the IgG3 isotype; it recognizes proteins modified with AA under non-reducing conditions and cannot be inhibited by NEL. Affinity-purified and/or absorbed polyclonal antibodies were also produced to these epitopes. Using this panel of monoclonal and affinity-purified polyclonal antibodies, unique antigen–antibody binding occurred that: (1) detected only NEL; (2) reacted with the α-amino group on proteins prepared under reducing conditions; and (3) detected adducts on proteins prepared under non-reducing conditions. However, the only antibodies that recognized antigen(s) from alcohol-fed rat livers were those that were not specific to NEL or the α-amino group modified under reducing conditions. These data indicate that the relevant adduct in alcohol-fed rat livers is not NEL, and that it presumably is related to proteins modified with AA under non-reducing conditions. BIOCHEM PHARMACOL **56**;11:1515–1523, 1998. © 1998 Elsevier Science Inc.

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The chronic consumption of alcohol is a major cause of liver disease [1, 2]. Much evidence exists suggesting that AA,¶ the major by-product of alcohol metabolism, may be detrimental to the liver [3–5]. Numerous *in vitro* studies have shown that AA can covalently bind to a variety of proteins under physiological conditions and may interfere with biological function [6–10].

It appears that the ε-amino groups of internal lysine residues and the α-amino group of N-terminal amino acids are the major participants in AA binding, and at least two forms of these adducts may exist [11, 12]. Chemically stable adducts that are formed under physiological conditions in the absence of non-physiological reducing agents have been called "non-reduced" adducts (-NR). These are distinctly different from the unstable adducts that are mainly Schiff bases, resulting from the reaction of a carbonyl carbon with amino groups. These adducts readily dissociate when exposed to dialysis, gel filtration, and treatment with weak acids and bases, but they can be stabilized by converting them to secondary amines by treatment with reducing agents such as $NaCNBH_3$. These "reduced" adducts (-R) appear to be predominantly ethylated amines.

The current literature on the immune response to AA adducts contains experimental designs using many different types of AA–protein adducts, and there has been tremendous variability in the methods used to produce antibodies to these AA–protein adducts [11, 13–17]. Most studies have used high concentrations of AA in the presence of a strong reducing agent ($NaCNBH_3$), resulting in the predominant formation of NEL residues on proteins. Although this type of adduct is highly immunogenic, recent work in our laboratory has shown that NEL is not found on AA–protein adducts prepared under physiological condi-

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¶ *Abbreviations:* AA, acetaldehyde; H & L, heavy and light chains; h-EGF, human epidermal growth factor; m-EGF, mouse epidermal growth factor; MsPP, mouse plasma protein; -N, native adduct (e.g. BSA-N); NEL, *N*-ethyl lysine; -NR, non-reduced adduct; NR-1, monoclonal antibody to non-reduced adducts; -R, reduced adduct; RbPP, rabbit plasma protein; RT1.1, monoclonal antibody to *N*-ethyl lysine; S4Bα, Sepharose 4B beads with the α-amino groups free; S4Bε, Sepharose 4B beads with the ε-amino groups free.

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tions [18, 19]. Additionally, NEL adducts could not be found in the livers of rats chronically fed alcohol [19–21].

Recently, monoclonal and polyclonal antibodies have been produced in our laboratory that recognize AA–protein adducts prepared under non-reducing and reducing conditions. It was the purpose of this study to characterize the specificity of these antibodies and to assess their biological significance by testing their reactivity with ethanol-fed rat livers.

MATERIALS AND METHODS

Antigen Preparation

BSA (crystallized, lyophilized, and fatty acid free) was purchased from CalBiochem. AA and $NaCNBH_3$ were purchased from the Aldrich Chemical Co. RbPP and MsPP were prepared from whole serum as reported elsewhere [21, 22]. Briefly, serum proteins were precipitated by adding 70% ammonium sulfate overnight at 4°. The insoluble portion was pelleted at 10,000 *g*, the supernatant was discarded, and the pellet was rehydrated with double-distilled water. The protein was dialyzed overnight against 0.1 M phosphate buffer, and the protein concentration was determined by the method of Groves *et al.* [23].

Proteins were modified with AA under reducing conditions by adding either: (1) 5 mM acetaldehyde and 30 mM $NaCNBH_3$ (5/30), or (2) 240 mM AA for 1 hr and 100 mM $NaCNBH_3$ (240/100) for 30 min to 1 mL of protein at 1.2 mg/mL. Proteins were modified under non-reducing conditions by the addition of 100 mM AA to 1 mL of protein at 1.2 mg/mL. In each case, the solutions were incubated at 37° overnight, dialyzed against three changes of PBS for 8 hr, and used for coating ELISA plates or immunizations. All antigens were prepared fresh, as storage at 4° could result in loss of antigenicity. Native or unmodified proteins were used as a control.

Use of Animals

All animal studies were performed in accord with the legal requirements of the Animal Use Committee of the Omaha VA Medical Center.

Production of Monoclonal Antibodies

The monoclonal antibody RT1.1 has been reported previously and was used in these studies as both a positive and negative control for studying the reactivity of the other antibodies [18, 19, 21].

IMMUNIZATIONS. Balb/c mice were obtained from the National Cancer Institute via an interagency agreement with the Veterans Administration and were maintained on water and laboratory chow *ad lib*. The mice were quarantined immediately upon arrival and monitored on a monthly basis to ensure specific pathogen-free status. Five mice were injected intraperitoneally over a period of 6 weeks with 100 μg of MsPP modified with 100 mM AA in Freund's complete (week 1) or Freund's incomplete (weeks 3 and 5) adjuvant. Animals were bled 2 weeks after the last immunization and tested for antibody titers to proteins modified under native, non-reducing, or reducing conditions.

FUSIONS. Spleens from animals whose serum showed antibody to non-reduced-AA modified proteins were removed and homogenized in a Stomacher Lab Blender 80 (Tekmar Co.). A single cell suspension was prepared and mixed with an equal concentration of mouse myeloma P3.NS-1-AG4-1 (ATCC). Using the method of Kohler and Milstein [24], these cells were fused and put in 96-well plates at 1×10^5 cells/well. Selections were performed using hypoxanthine-aminopterine-thymidine (HAT) followed by hypoxanthine-thymidine (HT) in the medium. Wells were monitored for growth and tested for reactivity to bovine serum albumin modified under native, non-reducing, or reducing (5/30 or 240/100) conditions, using an ELISA system. Positive wells were subcloned twice, using limiting dilution, to ensure monoclonality.

Production of Rabbit Polyclonal Antibodies

Autologous RbPP was modified under reducing conditions by one of two methods: (1) incubating with 240 mM AA for 1 hr and adding 100 mM $NaCNBH_3$ for 0.5 hr (240/100); and (2) incubating with 5 mM AA and 30 mM $NaCNBH_3$ (5/30) overnight. To make non-reduced antigen, RbPP at 1.2 mg/mL was incubated with 100 mM AA overnight. All antigen preparations were dialyzed for 16 hr, emulsified in Freund's complete adjuvant, and injected subcutaneously. Two additional immunizations over 4 weeks were performed using Freund's incomplete adjuvant. The animals were bled 2 weeks after the last injection and tested for antibody titers by ELISA or absorbed as described below and then tested.

ABSORPTION AND ELUTION OF POLYCLONAL ANTIBODIES FROM SEPHAROSE 4B-CL LYSINE BEADS MODIFIED WITH AA. Sepharose 4B-CL was purchased from the Sigma Chemical Co., washed, and activated using 1 mM HCl. To 1 mL of swollen beads, 25 mg of either Nα-*t*-BOC-L-lysine or Nε-*t*-BOC-L-lysine (Sigma Chemical Co.) in bicarbonate buffer, pH 8.3, was added. The suspensions were rotated for 2 hr at 37°, and then overnight at 4° with 0.2 M glycine to block residual active groups. The beads were washed with 10 vol. of buffer, and the blocking groups were removed by treatment with 50% (v/v) trifluoroacetic acid/dichloromethane, and neutralized with 5% (v/v) diisopropylethylamine in dichloromethane for 45 min. The beads were washed with another 10 vol. of buffer, leaving one set of beads with a lysine attached at the ε-amino group and the α-amino group free (S4Bα), and another set of beads where the α-amino group was attached leaving the ε-amino group available for binding (S4Bε). The amount of lysine bound to the Sepharose 4B-CL was determined by assessing the number of free amino groups bound to the beads using the ninhydrin method. Using this procedure, it was determined that 1–2 μmol of lysine was

bound to each milliliter of beads, regardless of whether the attachment was through the ε-amino or the α-amino group.

These beads (20 mL) were modified under reducing (100 mM AA, with 600 mM $NaCNBH_3$) or non-reducing (500 mM AA) conditions for 16 hr at 37°. Antiserum from rabbits immunized with RbPP-R (5/30) or RbPP-NR was added to modified Sepharose 4B lysine beads and incubated with constant shaking for 1 hr. The beads were washed, and the supernatant was concentrated and dialyzed against 0.02 M Tris containing 0.01 M glycine, pH 8.2. The bound antibody was eluted with 0.1 M glycine containing 0.5 M NaCl in 0.1 M phosphate buffer, pH 4.0, and dialyzed against 0.02 M Tris containing 0.01 M glycine, pH 8.2. In some cases, either the absorbed antibodies or the affinity-purified eluted antibodies were passed through the other set of beads and treated as above. Absorbed and eluted materials were tested for antibody titers to native BSA, and to BSA modified with AA under non-reducing and reducing (5/30 and 240/100) conditions.

Antibody Activity to Modified Proteins

Proteins for coating were diluted to 20 μg/mL in bicarbonate buffer, pH 9.6, and coated on ELISA plates overnight at 37° with 100 μL of the test protein. To the washed plates, 200 μL of either serum or monoclonal supernatant was added to row B and diluted 2-fold down the plates. The plates were incubated at 37° for 45 min and washed three times with PBS-Tween 20; then either 100 μL of alkaline-phosphatase labeled rabbit anti-mouse IgG, IgM, and IgA (H & L) or 100 μL of alkaline phosphatase labeled goat anti-rabbit IgG (H & L) was added. Both antibodies were purchased from Zymed Laboratories and used at a 1:1000 dilution. Following a 45-min incubation at 37°, the plates were washed three times with PBS-Tween 20, and 100 μL of *p*-nitrophenyl phosphate in diethanolamine buffer was added. The plates were monitored at 405 nm on a DynaTech MR7000 MicroELISA Reader after 30 min of incubation. Final titers of each antibody were assessed as the point where an optical density of 0.100 or greater was detected after subtraction from the optical density on native proteins.

Preparation of NEL

Poly-L-lysine was purchased from the Sigma Chemical Co., adjusted to 1.2 mg/mL, and AA (5 mM) and $NaCNBH_3$ (30 mM final concentration) were added. The reaction was allowed to continue at 37° for 72 hr as described previously [18, 19, 21]. The purity of the NEL prepared by this method was found to be greater than 99%, as determined by HPLC [11, 25]. Unmodified lysine, which was used as a control, was prepared in the same manner as above except that no AA was added to the initial reaction mixture. The samples were then dialyzed, lyophilized, and subjected to hydrolysis at 115° for 22 hr.

Inhibition of Antibody Reactivity Determined by ELISA

NEL or lysine was adjusted to a concentration of 4000 μM and then diluted 2-fold down the plate. To every well of this plate, an equal volume of antibody preparation (adjusted to give 50% of maximum activity) was added, and the plate was incubated at 37° for 30 min. The residual antibody activity to the different adducts was determined by adding 100 μL of each sample to triplicate wells of the appropriately coated ELISA plate. These samples were incubated at 37° for 45 min, washed three times with PBS-Tween 20, and either 100 μL of alkaline phosphatase goat anti-rabbit IgG (H & L) or alkaline phosphatase-labeled rabbit anti-mouse IgG, IgM, and IgA (H & L) was added to every well. Following a 45-min incubation, the plates were washed three times, substrate was added, and the O.D. was determined at 405 nm after 30 min.

Modification of Epidermal Growth Factor

To determine whether the antibody preparations reacted with α-amino groups in addition to ε-amino-lysine groups modified under reducing conditions, m-EGF and h-EGF (Boehringer Mannheim) were modified under reducing conditions, as described previously, with $NaCNBH_3$. m-EGF has only an α-amino group available for labeling, whereas h-EGF has two ε-amino-lysine groups as well as one α-amino group. The amount of AA incorporated into these proteins was quantified using [^{14}C]AA as described previously [11, 25].

Ethanol-Fed Rat Livers

Sprague–Dawley rats (120–180 g) were fed either 36% of their caloric intake as ethanol or a pair-fed control liquid diet using the standard Lieber–DeCarli diet feeding paradigm. Animals were killed following 5 weeks of alcohol exposure. Liver cytosol fractions were prepared by methods previously described [26] then coated onto ELISA plates at 20 μg/mL, and monoclonal and polyclonal antibody binding was assayed as above.

RESULTS

Reactivity of Monoclonal Antibodies

Hybridoma supernatants were screened for activity to native, non-reduced, or reduced proteins. As shown in Fig. 1A, the monoclonal antibody RT1.1 demonstrated activity only to those adducts on proteins prepared under reducing conditions, and not on those proteins modified under non-reducing conditions. Additionally, it has been shown that RT1.1 is specific for NEL on any carrier protein. Confirmation for this specificity was demonstrated using h-EGF and m-EGF (see Table 3). [18, 19]. In these studies, it has been shown that RT1.1 can react with h-EGF (two lysines) but not m-EGF (no lysines). Since the two mole-

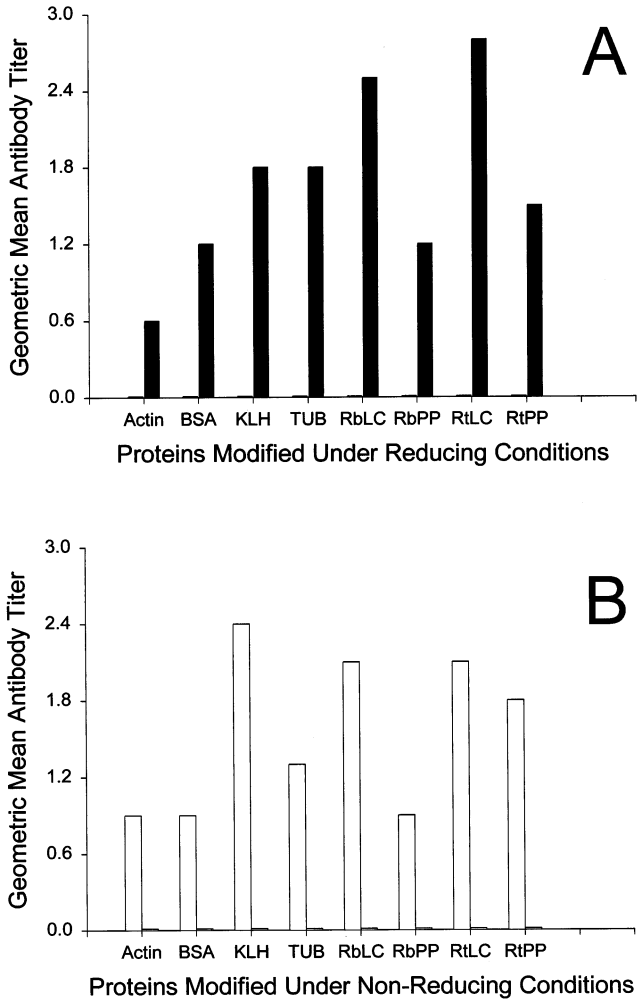


FIG. 1. Reactivity of RT1.1 (■) and NR-1 (□) to different proteins modified *in vitro* under (A) reducing conditions (5/30), or (B) non-reducing conditions. The geometric mean antibody titers of RT1.1 and NR-1 are plotted against different proteins modified with AA: RtPP = rat plasma protein, RtLC = rat liver cytosol, RbPP = rabbit plasma protein, RbLC = rabbit liver cytosol, KLH = keyhole limpet hemocyanin, and TUB = tubulin. The results are an example of experiments that were repeated 5 times.

cules are essentially identical, the binding is most probably through the modification of lysine residues by AA.

In contrast, NR-1 reacted only with proteins modified with AA in the absence of a strong reducing agent and was not carrier specific, as it reacted with all proteins modified with AA under non-reducing conditions (Fig. 1B). Isotype analysis revealed that NR-1 is an IgG3 antibody, whereas RT1.1 is an IgG2b isotype. Due to the high specificity of these antibodies to the adduct and not the carrier, these antibodies were used as controls to assess which adducts are present on proteins modified under different reducing and non-reducing conditions.

TABLE 1. Binding capacity of lysine-bound Sepharose 4B-CL beads modified with AA under non-reducing and reducing conditions

	Antibody bound (μg/mL of beads)	
Beads	Reduced (100 mM AA + 600 mM $NaCNBH_3$)*	Non-reduced (500 mM AA)
S4Bε†	188 ± 15	143 ± 12
S4Bα‡	186 ± 32	ND§

Beads were saturated with antiserum containing the appropriate antibody, eluted, and the amount of protein eluted determined. Results are the means ± SD of three separate experiments.

*$NaCNBH_3$ = sodium cyanoborohydride.

†S4Bε = Sepharose 4B with lysine bound at the α-amino group and the ε-amino group free.

‡S4Bα = Sepharose 4B with lysine bound at the ε-amino group and the α-amino group free.

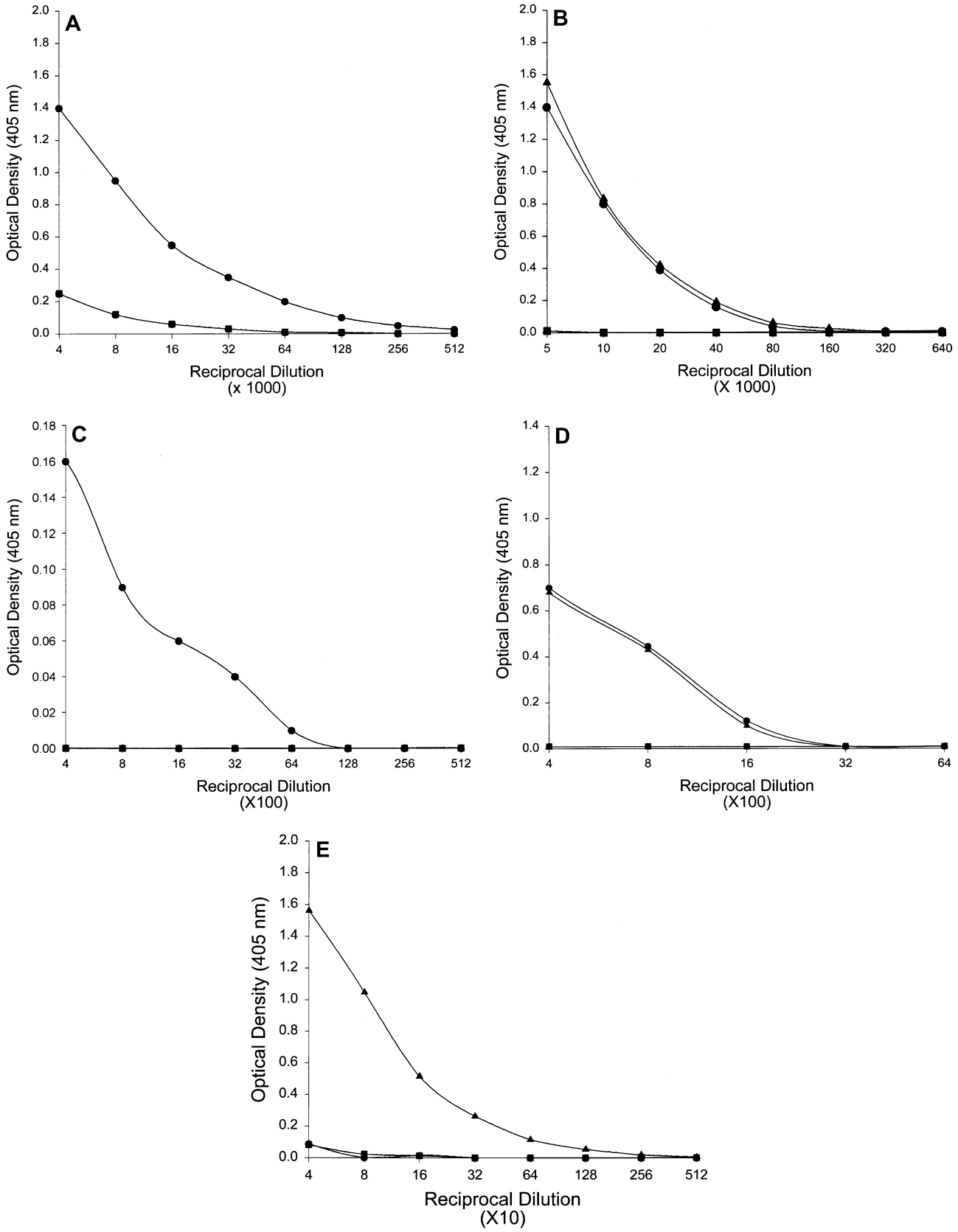
§ND = not determined.

Attachment of Lysine to Cyanogen Bromide-Activated 4B-CL Sepharose and Modification with AA

The amount of antibody bound per milliliter of beads was determined by assessing antibody activity prior to adsorption to each column. In each case, enough of each antiserum was added to ensure that the beads were saturated, as evidenced by detectable levels of antibody to the appropriately modified protein using ELISA. The antibodies were eluted and quantified by the protein determination method of Groves *et al.* [23].

As can be seen in Table 1, the amount of antibody bound was not significantly different between the reduced adducts on α-amino and ε-amino lysines (186 ± 32 and 188 ± 15 μg/mL of beads, respectively). In the case of Sepharose 4B-CL where free ε-amino lysine groups were modified under non-reducing conditions, a similar amount of antibody could be detected (143 ± 12 μg/mL of beads). However, Sepharose 4B-CL where free α-amino lysine groups were modified under non-reducing conditions never resulted in the isolation of any detectable antibody.

FIG. 2. (Opposite) Reactivity of affinity-purified and/or absorbed polyclonal antibodies: (A) antiserum to reduced adducts and affinity-purified to NEL (R = NEL); (B) antiserum to reduced adducts absorbed with Sepharose 4B (S4Bε) lysine beads prepared under reducing conditions (RbPP, R ≠ NEL); (C) antiserum to reduced adducts and then affinity-purified to Sepharose 4B lysine beads (S4Bα) prepared under reducing conditions (RbPP, R = αR); (D) unpurified antisera to non-reduced adducts (RbPP-NR); and (E) antisera to non-reduced proteins absorbed against Sepharose 4B lysine beads modified under reducing conditions (NEL Abs). All antibodies were assayed on BSA modified with AA under different conditions: native (■), non-reducing (▲), and reduced (●). All three curves are expressed in each figure. However, in some cases curves overlap, resulting in the appearance of only two curves: Panel A, (■) and (▲) are the same; Panel C, (■) and (▲) are the same. The optical density readings of the antibody against each of these preparations is shown by the y-axis and is plotted against 2-fold dilutions of the original. Shown is an example of each experiment run 5 times.


A
Optical Density (405 nm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
4
8
16
32
64
128
256
512
Reciprocal Dilution
(x 1000)
B
Optical Density (405 nm)
5
10
20
40
80
160
320
640
Reciprocal Dilution
(X 1000)
C
Optical Density (405 nm)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Reciprocal Dilution
(X100)
D
Optical Density (405 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Reciprocal Dilution
(X100)
E
Optical Density (405 nm)
Reciprocal Dilution
(X10)

Reactivity of Rabbit Polyclonal Antibodies

Serum from immunized rabbits was absorbed or eluted from Sepharose 4B lysine (S4Bα or S4Bϵ) beads as modified above. Two-fold dilutions of each antibody preparation were tested against BSA prepared under native, non-reducing, or reducing conditions. Rabbit anti-rabbit plasma protein was modified with AA under reducing conditions, and rabbits were immunized in Freund's adjuvant. The antiserum was passed through a S4Bϵ-R column, and the antibody was isolated by elution. This antibody was termed R = NEL (Fig. 2A) and reacted primarily with proteins modified under reducing conditions (1:64,000).

In contrast, antiserum that was absorbed repetitively against S4Bϵ-R beads to remove activity to NEL showed activity to both reduced (1:40,000) and non-reduced (1:40,000) BSA (Fig. 2B). Because significant activity to BSA-R remained, this suggested that there was another reduced adduct other than NEL, and this antibody was termed R ≠ NEL. To isolate this antibody, the antiserum (R ≠ NEL) was passed through a S4Bα-R column. The affinity-purified antibody eluted from this column demonstrated activity to only BSA-R (1:800) and not to BSA-NR or BSA-N and was termed R = αR (Fig. 2C).

Immunization of rabbits with RbPP-NR showed a strong antibody response (1:3200) to BSA-NR and BSA-R (Fig. 2D). This is consistent with previous reports showing that immunization with non-reduced proteins results in the production of antibodies to the reduced adducts. S4Bϵ-R columns were prepared, and activity to the reduced adduct absorbed out the resulting antisera and demonstrated reactivity to only BSA-NR (Fig. 2E).

Antibody Reactivity to Reduced Adducts

In previous studies, it was shown that NEL or the monoclonal antibody RT1.1 could be used to specifically inhibit the binding of antibodies to NEL. Additionally, using m-EGF and h-EGF, it was shown that it was possible to determine whether the antibody was binding ϵ-amino lysine groups or α-terminal groups modified under reducing conditions. The polyclonal antibody affinity purified against NEL (R = NEL) was competed out with the monoclonal antibody RT1.1 or NEL, demonstrating that its specificity is the same as that of RT1.1 (Table 2). Additionally, this antibody demonstrated activity only on h-EGF and not m-EGF, demonstrating that there is no specificity to the α-amino group (Table 3).

In contrast, antibody that was repetitively absorbed against NEL, but demonstrated activity to the proteins modified under reducing conditions, was not competed out by either RT1.1 or NEL on BSA-R (Table 2). This preparation reacted equally with m-EGF and h-EGF, suggesting that the response was to the adducted α-amino group and this antibody was termed R ≠ NEL (Table 3).

Since the antibody R ≠ NEL appeared to react with only the adducted α-amino group, this preparation was affinity-purified using S4Bα-R. This antibody preparation could not be inhibited using RT1.1 or NEL (Table 2) and reacted equally on m-EGF and h-EGF modified under reducing conditions (Table 3), strongly suggesting that there is antibody present to only the α-amino group modified under reducing conditions.

Previous studies have shown that immunizing with proteins modified under non-reducing conditions results in antibody to both the non-reduced and reduced adduct. Antiserum from rabbits immunized with RbPP-NR demonstrated good activity to the non-reduced adduct and the reduced adduct, and the response to BSA-R could be inhibited using either RT1.1 or NEL (Table 2).

TABLE 2. Inhibition of antibody binding by RT1.1 or NEL

Antibody*	RT1.1†	NEL†
RT1.1	10 μg	1000 μM
R = NEL	100 μg	500 μM
R = αR	No inhibition	No inhibition
NR	No inhibition	No inhibition
NR-1	No inhibition	No inhibition

The concentrations resulting in > 95% inhibition are reported, and are typical results from three separate assays. R = reduced; NR = non-reduced.

*Antibodies were diluted to one-half their maximum binding to BSA-R.

†Different concentrations of RT1.1 (μg/mL) or NEL (μM) were added, and the amount of inhibition was determined.

Reactivity to Ethanol-Fed Rat Livers

Other investigators have produced rabbit polyclonal antibodies that recognize AA adducts in the livers of ethanol-fed rat livers. All antibody preparations were tested for activity to pair-fed control and ethanol-fed rat livers (Fig. 3). The highest activity was detected using the monoclonal NR-1 and the polyclonal antibodies: RbPP-NR, and RbPP-NR (NEL absorbed). The monoclonal RT1.1 and the polyclonal antibodies RbPP-R (R = NEL) and RbPP-R (R = αR) did not react with anything in alcohol-fed rat livers. These data suggest that NEL and α-reduced epitopes are not present in the livers of rats chronically fed ethanol. Only those preparations that retained activity to non-

TABLE 3. Reactivity of antibodies to m-EGF and h-EGF

Antibody	h-EGF-R* (5/30)‡	m-EGF-R† (5/30)
RT1.1	1,600§	—‖
R = NEL (Affinity pure)	16,000	—
R ≠ NEL (Affinity absorbed)	16,000	16,000
R	1,600	1,600

*h-EGF modified with AA reduced conditions.

†m-EGF modified with AA reduced conditions.

‡AA (5 mM) and $NaCNBH_3$ (30 mM).

§All activities are reported as the last reciprocal dilution that results in an 0.100 optical density when activity on native carrier protein is subtracted from the reduced carrier.

‖No antibody detected.

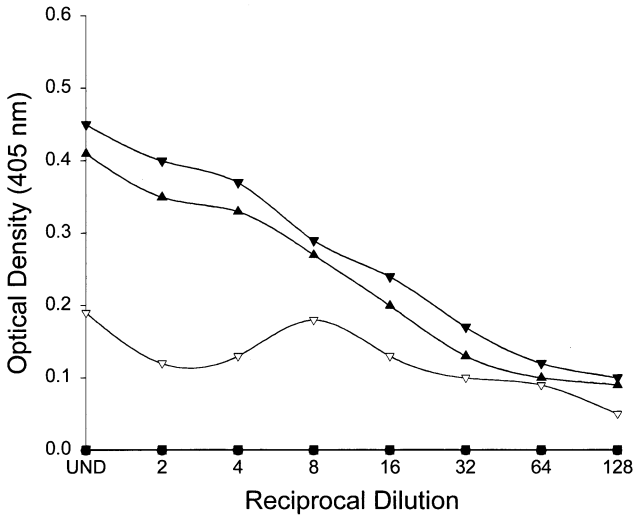


FIG. 3. Reactivity of RT1.1 (●); rabbit anti-rabbit plasma-R, affinity purified to NEL (■); rabbit anti-rabbit plasma-R, NEL absorbed (▲); rabbit anti-rabbit plasma-NR, affinity purified (▼); and NR-1 (▽). In this figure, the (●) curve is under the (■) curve. UND = undiluted. The differences in the optical density on livers from ethanol-fed and pair-fed rat livers are plotted against the dilution. Shown is an example of each experiment run 5 times.

reduced epitopes detected anything in livers from rats chronically fed ethanol.

DISCUSSION

Previous reports have shown that AA can bind to proteins and result in the production of a protein–hapten conjugate that is highly immunogenic [11, 13–17]. Lin and colleagues [27, 28] have shown the production of antibodies to a 37-kDa protein. However, Worrall *et al.* [29, 30] have shown that their antibody detects multiple proteins. In the experiments presented in this manuscript, mouse monoclonal and rabbit polyclonal antibodies were prepared to autologous plasma proteins modified under different conditions. The reactivity of the antibodies produced was tested on BSA modified under these same conditions (Table 4).

TABLE 4. Reactivity of the different antibodies to BSA modified with AA under different conditions

Antibody	Non-reduced	Reduced (5/30)*
RT1.1	—†	1,600‡
R = NEL	—	64,000
R ≠ NEL	40,000	40,000
R = αR	—	800
NR-1	1,000	—
NR	3,200	—

* AA (5 mM) + $NaCNBH_3$ (30 mM).

†No antibody higher than activity on native proteins was detected.

‡The final dilution resulting in a 0.100 optical density difference between the adducted BSA and BSA-N (unmodified) is reported. These are typical results from three separate assays.

Additionally, monoclonal antibodies to proteins prepared under reducing (RT1.1) and non-reducing conditions (NR-1) were characterized and used to describe the adducts present on proteins modified with AA under different conditions.

We have reported previously on a monoclonal antibody that detects proteins modified with AA under only reducing conditions [18, 19]. This monoclonal antibody (RT1.1) was shown to bind all proteins modified with 5 mM AA in the presence of 30 mM $NaCNBH_3$, and more precisely is specific for NEL. This antibody reacts with proteins modified with 240 mM AA followed by 100 mM $NaCNBH_3$, demonstrating that this preparation results in the production of NEL adducts on proteins. In contrast, RT1.1 does not bind to proteins reacted with AA under non-reducing conditions, and does not recognize any adducts in the livers of rats chronically fed alcohol.

The monoclonal antibody NR-1 recognizes proteins modified with AA under non-reducing conditions, but has no reactivity to proteins modified under reducing conditions. This antibody is not carrier-specific and demonstrates minimal reactivity to adducts in the livers of rats chronically fed alcohol.

This specific monoclonal was chosen for its reactivity to a broad number of proteins. However, these studies were performed using purified or semi-purified proteins that were modified *in vitro* and coated onto plates. The number and types of non-reduced adducts that are formed are maximized under these conditions (100 mM AA), and unlike reduced adducts there are probably innumerable types of non-reduced adducts that can form at low levels *in vivo*. This may make it difficult to detect non-reduced adducts on proteins modified *in vivo* and could explain the extreme variability in reactivity reported in the literature. Therefore, in probing materials that potentially have a number of different non-reduced adducts, the polyclonal antibody is probably the best antibody for detecting non-reduced adducts.

Previous studies using RT1.1 and NEL with BSA, m-EGF-R, and h-EGF-R have shown that under these conditions the predominant epitope is NEL [18, 19]. There is most likely some modification of the α-amino group with AA that is not NEL. The studies in this paper have shown that when a protein is modified under reducing (5/30 or 240/100) conditions, R = NEL and R = α groups are present and very different. Additionally, *no* reactivity to reduced-proteins was observed using the antibody NR-1 or the polyclonal Ab affinity-purified to non-reduced proteins, demonstrating that under these conditions non-reduced AA adducts are not formed.

In the case of non-reduced proteins, epitopes, as defined by the different antibodies, of either R = NEL or R = αR are not present, and only the monoclonal (NR-1) and polyclonal affinity purified to non-reduced proteins reacted. This suggests that only non-reduced epitopes are present when modified under the conditions outlined in Materials and Methods.

Antibody to NEL has been confirmed using RT1.1 and an affinity-purified rabbit polyclonal to NEL. These two antibodies appear to be identical in their activities, and neither recognize anything in alcohol-fed rat livers. Antibody to the R = α group shows reactivity to proteins modified under reducing (5/30 and 240/100) conditions. However, while this activity is different from those observed to NEL, there was still no reactivity to livers from animals chronically fed alcohol.

Antibodies to non-reduced adducts demonstrated the presence of this adduct in the livers of rats fed alcohol. However, we are no closer to understanding the structure(s) of these adducts than before these studies were initiated. Unless a structure can be proposed, synthesized, and used to demonstrate the specificity of the antibody as has been done previously for NEL and MAA [9, 31, 32] it is difficult to infer structure. This is the inherent problem in previously reported studies, thereby making it difficult to determine the role of AA adducts and the immune system in alcohol-induced liver disease. However, this study strongly suggests that the immune system does play some role and that the adduct is not NEL, but of non-reduced origin. Studies are currently underway to address the nature of non-reduced adducts using AA adducts prepared under non-reducing conditions, where the structure has been defined.

In summary, the production and careful characterization of these monoclonal and polyclonal antibodies demonstrate that (1) the immunogenicity of these adducts is highly variable; (2) there are a number of adducts that can be identified, depending upon the conditions used; (3) these antibodies are useful in assessing what types of adducts are found on different preparations; and (4) the characteristics of *in vitro* prepared antigens and their corresponding antibodies may be helpful in delineating the role of the immune system in the pathophysiology of alcohol liver disease.

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