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SPECIFICITY TO *N*-ETHYL LYSINE OF A MONOCLONAL ANTIBODY TO ACETALDEHYDE-MODIFIED PROTEINS PREPARED UNDER REDUCING CONDITIONS

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Abstract—A monoclonal antibody has been developed that recognizes only protein–acetaldehyde (AA) adducts prepared under reducing conditions: 5 mM AA with 30 mM sodium cyanoborohydride overnight at 37°. This monoclonal antibody is a mouse IgG2b that has been designated RT1.1. The primary adduct formed when proteins are exposed to acetaldehyde under reducing conditions is *N*-ethyl lysine (NEL). To examine the epitope specificity of RT1.1, inhibition ELISAs were developed using NEL and other possible inhibitors, such as arginine, ethylamine, lysine and proteins modified with AA under non-reducing conditions. RT1.1 (at half-maximum optical density, 50 ng/mL) was inhibited only by NEL and was independent of the carrier or the pH of the buffer used in the ELISA. Further evidence indicating that NEL is the epitope recognized by RT1.1 was obtained using mouse and human epidermal growth factor (EGF). Both proteins contain one alpha amino group but only the human-EGF contains lysine residues with epsilon amino groups. In experiments where these two proteins were modified with AA under reducing conditions, RT1.1 reacted only with human-EGF. These studies demonstrate that RT1.1 is specific for NEL that is formed by the ethylation of proteins with acetaldehyde under reducing conditions. Additionally, these studies demonstrate that the procedures and methods used herein may be useful for characterizing other antibodies prepared to AA-modified proteins under a variety of defined *in vitro* chemical conditions.

Key words: monoclonal; antibody; acetaldehyde; alcohol; adduct; ELISA

Ethanol is rapidly metabolized, primarily in the liver, to produce AA‖. During chronic ethanol ingestion, appreciable concentrations of AA can accumulate in the liver and blood [1]. A number of *in vitro* studies have shown that AA can bind to and modify proteins [2–7]. Additionally, recent reports of *in vivo* studies have demonstrated that protein–AA adducts are formed in animals or humans that chronically consume alcohol [8–14]. Although the complete chemical structures of these protein–AA adducts have not been delineated, it has been shown that, when AA binds to proteins, both stable and unstable adducts are formed [3, 4, 15]. Stable adducts are irreversible products that form in the absence of reducing agents and thus are also called non-reduced adducts. Unstable adducts are mainly Schiff bases that result from the reaction of a carbonyl carbon with amino groups and readily dissociate when exposed to dialysis, gel filtration or treatment with weak acids and bases [16]. However, they can be stabilized by converting them to secondary amines [3, 15, 16] via treatment with $NaCNBH_3$, a strong reducing agent. These adducts have been shown to be ethylated amines and are often referred to as reduced AA adducts [15]. Additionally, reduced and non-reduced adducts have different chemical properties (i.e. chemiluminescence) that further demonstrate the differences between these two preparations [16].

Previous studies by other investigators have used antibodies prepared against proteins modified under conditions that would primarily result in R-AA adducts [8, 9, 17, 18]. However, the specificities of these polyclonal antibodies have not been defined clearly. While *in vitro* treatment of AA-modified proteins with $NaCNBH_3$ primarily produces R-AA adducts, it is probable that some NR-AA adduct species are also formed. The use of monoclonal antibodies will allow the detection of specific AA adduct species formed on proteins. We have developed a monoclonal antibody that recognizes proteins modified with AA only under reducing conditions [19]. This antibody, called RT1.1, is highly specific for AA but not other aldehyde-modified proteins and is *not* carrier dependent [19]. This report characterizes the epitope structure

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‖ Abbreviations: AA, acetaldehyde; R-AA, reduced AA; NR-AA, non-reduced AA; BSA-R, reduced BSA; RT1.1, a monoclonal antibody that reacts with proteins modified under reducing conditions; NEL, *N*-ethyl lysine; m-EGF, mouse epidermal growth factor; h-EGF, human epidermal growth factor; and $NaCNBH_3$, sodium cyanoborohydride.

recognized by RT1.1 and defines other characteristics of this antibody.

MATERIALS AND METHODS

Antigen preparation. Bovine brain tubulin was prepared by the method of Shelanski *et al.* [20]. BSA (crystallized, lyophilized and fatty acid free) was purchased from Calbiochem (San Diego, CA). The purity of all proteins was monitored routinely using SDS–PAGE [21] and exceeded 98% by densitometry. Protein concentrations were determined by the method of Lowry *et al.* [22]. All proteins were labeled with acetaldehyde using methods described previously [19, 23]. Briefly, for the preparation of acetaldehyde (AA)-modified proteins under reducing conditions 120 μL of 300 mM $NaCNBH_3$ (30 mM final concentration), 60 μL of 100 mM AA (5 mM final concentration) and 20 μL of PBS were added to 1 mL of protein (1.2 mg/mL in PBS). Both AA and $NaCNBH_3$ were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Proteins were modified under non-reducing conditions by the addition of 120 μL of 1 M AA (100 mM final concentration) and 80 μL of PBS to 1 mL of protein (1.2 mg/mL). Both the reduced and non-reduced protein solutions were incubated at 37° overnight, dialyzed against three changes of PBS for 8 hr, and used for coating ELISA plates. Proteins were originally modified with 5 mM AA at 37° for 5 days followed by 8 hr of dialysis [19]. However, recent studies have shown that there is no antigenic difference between this preparation and the 100 mM overnight preparation [19]. To maintain consistency with the AA adduct prepared under reducing conditions, overnight incubations were utilized for all studies. All antigens were prepared fresh, as storage at 4° resulted in loss of antigenicity.

Preparation of NEL. NEL was prepared by the reductive ethylation of poly-L-lysine (Sigma, St. Louis, MO) as described previously [15]. Briefly, poly-L-lysine (1 mg/mL) was allowed to react with AA (15 mM) and $NaCNBH_3$(60 mM) at 37° for 72 hr. The reaction mixture was then dialyzed, lyophilized and subjected to hydrolysis at 115° for 22 hr. The purity of the NEL prepared by this method was found to be greater than 99%, as determined by high performance liquid chromatography [15]. 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.

Antibody isotyping. Immunoglobulin isotype was determined by an antigen capture technique as described previously [19]. Color changes were monitored by a Dynatech MicroELISA Reader MR7000 (Dynatech, Chantilly, VA) at 405 nm. Wells were considered positive when the O.D. of the test sample was 0.1 O.D. units greater than that of the control well, which contained conjugated second antibody alone. This procedure was also utilized for quantitating the concentration of the antibodies in solution by plotting known concentrations of a purified myeloma protein against the corresponding O.D. derived in this assay. Unknown sample concentrations were then extrapolated from their observed O.D. when compared with the standard curve.

Antibody activity to modified proteins. Carrier proteins and AA-modified proteins were diluted to 20 μg/mL in bicarbonate buffer, pH 9.6, and wells of the ELISA plates were coated overnight at 37° with 100 μL of the test protein. The plates were washed, 100 μL of either a myeloma control or monoclonal supernatant was added, and the plates were incubated at 37° for 45 min. The plates were washed three times with PBS-Tween 20, and 100 μL of rabbit anti-mouse IgG2b (1:1000) was added. Following a 45-min incubation at 37°, the plates were washed three times with PBS-Tween 20, and 100 μL of alkaline phosphatase-labeled goat anti-rabbit IgG (H & L) was added. The plates were washed three times with PBS-Tween 20, and 100 μL of *p*-nitrophenyl phosphate (substrate) was added. The plates were read as above, and wells were considered positive when the O.D. was 0.1 units greater than the activity observed with the corresponding native protein. The effects of high and low pH were examined using these procedures by replacing PBS-Tween 20 with either 0.02 M Tris-buffered saline Tween-20, pH 8.2, or 0.02 M sodium acetate-buffered saline Tween-20, pH 5.5.

Inhibition ELISA. NEL, lysine, ethylamine, arginine or proteins modified with AA under non-reducing conditions were adjusted to concentrations described in the figure legends, and diluted 2-fold, leaving 200 μL in every well of an incubation plate. An equal volume of RT1.1 supernatant or isotype control was added such that the activity of this antibody would be approximately 50% of maximum activity (50 ng/mL), and the plate was incubated at 37° for 30 min. To test for residual RT1.1 activity, 100 μL of each sample from the incubation plate was transferred in triplicate to wells of the assay plate coated as described previously using native (N; unmodified), non-reduced (NR) or reduced (R) BSA or tubulin. These samples were incubated at 37° for 45 min and then washed three times with PBS-Tween 20; 100 μL of rabbit anti-mouse IgG2b (1:1000) was added, and the mixtures were incubated at 37° for 45 min. The plates were washed three times with PBS-Tween 20, and 100 μL of alkaline-phosphatase-labeled goat anti-rabbit IgG (H & L) (Zymed Laboratories, Inc., South San Francisco, CA) was added to every well. Following a 45-min incubation at 37°, the plates were washed three times, substrate was added, and the O.D. was determined at 405 nm after 30 min. The effects of high and low pH on these inhibition studies were examined by replacing PBS-Tween 20 with either 0.02 M Tris-buffered saline Tween-20, pH 8.2, or 0.02 M sodium acetate-buffered saline Tween-20, pH 5.5, as both the wash and diluent buffer.

Effect of low and high pH buffers on binding. To determine if pH altered the ability of RT1.1 to bind to R-AA adducts, the antigen-coated plates were washed, and all incubations were performed in either 0.02 M Tris-buffered saline Tween-20, pH 8.2, or 0.02 M sodium acetate-buffered saline Tween-20, pH 5.5. Additionally, to assess the effect of pH on NEL binding, inhibition assays were performed in these same two buffer systems.

Modification of mouse and human epidermal growth factor. To determine whether the antibody preparations detected alpha amino groups in addition to epsilon amino-lysine groups, mouse and human epidermal growth factor (m-EGF and h-EGF, respectively) (Boehringer Mannheim, Indianapolis, IN) were modified under the reducing conditions described previously with $NaCNBH_3$ and in the presence or absence of [^{14}C]acetaldehyde. Mouse-EGF has only an alpha amino group available for labeling, whereas h-EGF has two epsilon amino-lysine groups as well as one alpha amino group [24]. The amount of acetaldehyde incorporated into these proteins was quantitated as described previously [3]. Based on the available groups for labeling, it was expected that the stoichiometric binding of acetaldehyde should be 3:1 (h-EGF:m-EGF).

Data analysis. All data points represent the results of at least three different experiments. Data were analyzed using either ABStat (AndersonBell) or SigmaPlot (Jandel Scientific) computer programs.

RESULTS

Inhibition assays. The observation that the monoclonal antibodies reported previously were not carrier dependent [19] is consistent with the suggestion that the major adduct formed under reducing conditions should be NEL, and that this epitope would be found on all proteins prepared in this manner. An inhibition assay was developed to show that NEL inhibited the binding of RT1.1 to proteins prepared under reducing conditions. As shown in Fig. 1A, complete inhibition of RT1.1 binding to BSA-R was obtained with 1000 μM NEL, and inhibition was linear between 25 and 300 μM NEL (Fig. 1B). Additionally, tubulin, actin and keyhole limpet hemocyanin were modified under reducing conditions and used with similar results (inhibition of binding to RT1.1 by NEL) as for BSA-R (data not shown). Substantial (>90%) but not total inhibition was achieved on tubulin-R, suggesting that RT1.1 may have a higher affinity for the modified tubulin than for NEL (data not shown).

When the epsilon amino group of lysine is ethylated to make NEL, this portion of lysine is similar in structure to ethylamine. The use of NEL at 250 μM resulted in approximately 80% inhibition of the values established when only RT1.1 was used with no inhibitor (control) (Fig. 2). Native ethylamine was used in inhibition assays with no effect. Lysine alone also did not inhibit RT1.1 binding to R-AA protein adducts. Similarly, unmodified arginine, proteins modified under non-reducing conditions or proteins exposed only to $NaCNBH_3$ without AA did not inhibit the antibody binding to the plates. Therefore, these data demonstrate that RT1.1 specifically recognizes NEL.

Effect of low and high pH buffers. When assayed in the presence of 0.02 M Tris-buffered saline Tween-20, pH 8.2, RT1.1 bound to BSA-R with results similar to those found under physiological conditions at pH 7.2 (Fig. 3A). The activity of RT1.1 on BSA-R was decreased slightly under low pH conditions compared with pH 7.2 (Fig. 3A).

Inhibition assays using NEL with RT1.1 in high, neutral and low pH conditions resulted in less inhibition at both high and low pH (Fig. 3B) with the least inhibition under low pH conditions. As previously shown, lysine did not inhibit the binding of RT1.1 to the indicator antigen, regardless of pH. Taken together, these data suggest that, under low or high pH, RT1.1 preferentially binds better to proteins modified with AA under reducing conditions than to pure NEL. This would be consistent with conformational epitopes on proteins that NEL alone cannot make. Regardless, the major binding site in all of these studies was to NEL.

Activity of RT1.1 to h-EGF and m-EGF. The amount of acetaldehyde bound to h-EGF and m-EGF fits the suggested stoichiometry of 3:1 as the molar ratio of binding was determined to be 3.12 mol of AA bound per mol of h-EGF, and 0.84 mol of AA bound per mol of m-EGF. This would suggest that the only difference between the two preparations is that the two epsilon amino-lysine groups on h-EGF, not found on m-EGF, are ethylated. In an ELISA, RT1.1 was found to bind to only the h-EGF and not to the m-EGF and strongly suggests that RT1.1 did not bind to the alpha amino group, but only the NEL groups (Fig. 4). No binding of an isotype control (IgG2b) was observed.

DISCUSSION

The major product of alcohol catabolism is AA, which has been shown to bind to proteins, resulting in the formation of new antigenic determinants [8, 9, 18, 23]. Additionally, many reports have shown that antibodies in the serum of patients with alcohol liver disease bind to AA-modified proteins *in vitro* [11–14]. Therefore, an immune response to proteins modified with AA *in vivo* appears likely.

Past reports have focused on the production of antibodies to AA–protein adducts prepared *in vitro* [8–10, 17, 18]. These adducts were prepared under conditions that were non-physiological. With the exception of one report [8], all of these antibodies were produced in rabbits [8, 9, 18, 23], and the polyclonal antibodies may recognize multiple different epitopes. The purpose of this manuscript was to characterize RT1.1. Therefore, it was imperative that we make as many NEL groups as possible. This has been shown to occur using 5 mM AA under reducing conditions, as reported previously by Tuma *et al.* [15]. While these levels are higher than those found under physiologic conditions in the liver following alcohol ingestion, they are still much lower than other investigators have reported [4, 5, 8, 9, 12]. Under non-reducing conditions, we have shown that 5 mM AA incubated for 5 days at 37° produces an adduct similar to that produced by 100 mM AA overnight at 37° [19]. Therefore, in an attempt to standardize these assays we incubated each adduct overnight.

We reported recently on a mouse monoclonal antibody that detects all proteins modified with AA under reducing conditions [19]. This monoclonal (RT1.1) is an IgG2b isotype that was shown to bind all proteins previously modified with AA in the presence of a strong reducing agent, $NaCNBH_3$. RT1.1 does not bind to proteins reacted with AA

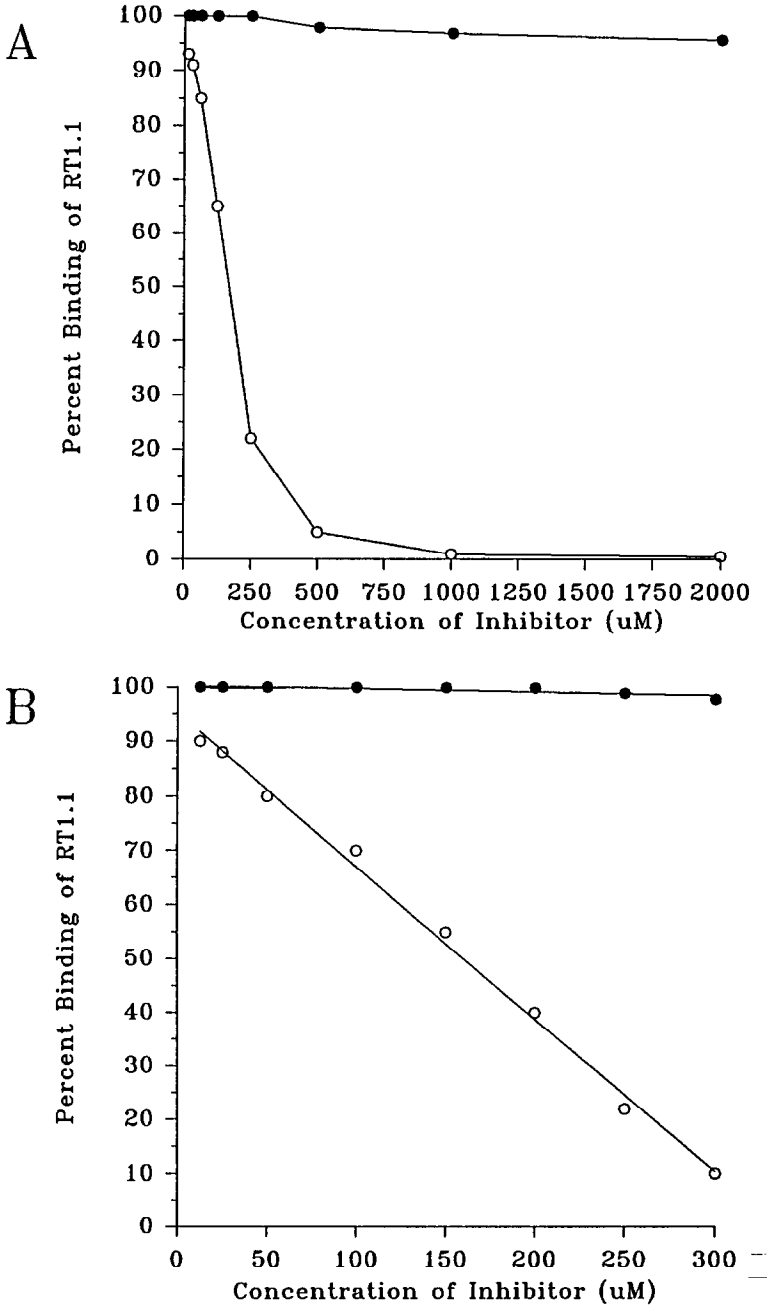

Fig. 1. Percent binding of RT1.1 to BSA-R coated plates following incubation with either lysine (●) or NEL (○). The x-axis shows the final concentration of either lysine or NEL utilized for inhibition: (A) ranging from 0 to 2000 μM; and (B) in the linear portion of the curve, 0–300 μM. RT1.1 was used at 100 ng/mL in all assays.

under non-reducing conditions, proteins prepared with $NaCNBH_3$ only, or proteins modified with other aldehydes [19].

The epitopes speculated to form under *in vitro* reducing conditions with AA are ethylated amino acids such as NEL [3, 5, 6, 15, 16]. The specificity of RT1.1 for this adduct was demonstrated, as neither lysine nor ethylamine alone inhibited the binding of RT1.1 to R-protein-coated ELISA plates but NEL caused almost complete inhibition. Unmodified lysine was used as the negative control, while ethylamine was used because it is similar in structure to the epsilon amino group of lysine when lysine is modified with AA under reducing conditions. Additionally, as opposed to lysine, ethylamine does not have an extended carbon chain that links with

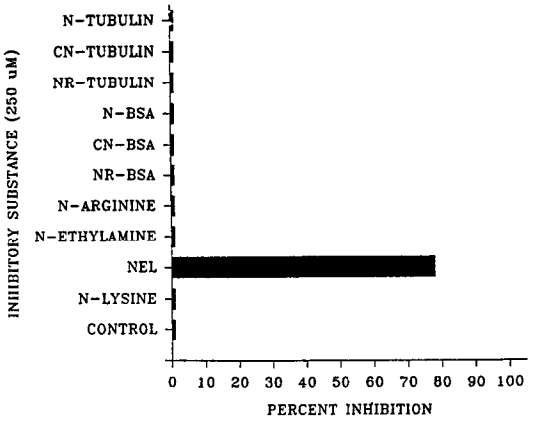

Fig. 2. Inhibition of RT1.1 by various preparations adjusted to 250 μM; NEL, *N*-lysine, *N*-ethylamine, *N*-arginine, *N*-tubulin, N-BSA, NR-tubulin, NR-BSA, CN-tubulin and CN-BSA. Materials were prepared under normal conditions (N), and in the presence of $NaCNBH_3$ (CN) only or acetaldehyde (NR) only. RT1.1 was used at 100 ng/mL in all assays.

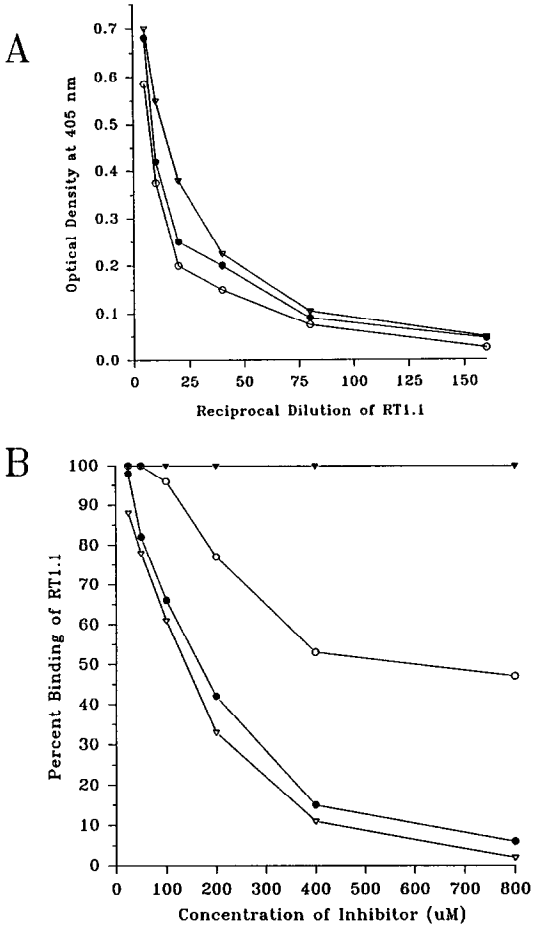

Fig. 3. (A) Binding of RT1.1 on BSA-R coated plates, expressed as optical density (405 nm). (B) Percent inhibition of RT1.1 (100 ng/mL) binding on BSA-R coated plates following inhibition with NEL. All assays were performed in (▽) PBS Tween-20, pH 7.2, (●) 0.02 M Tris-buffered saline Tween-20, pH 8.2, or (○) 0.02 M sodium acetate-buffered saline Tween-20, pH 5.5. The closed inverted triangle (▼) in panel B represents the lysine control. The x-axis shows the final dilution of RT1.1 utilized in the assay.

an alpha amino group. These data show that the reaction of AA with lysine under reducing conditions is necessary for recognition by RT1.1. Neither lysine nor ethylamine alone was recognized by RT1.1.

The alpha amino group on lysine is also available for binding AA, but since the method used to produce the NEL for inhibition assays reported in this paper prevents adducts from forming at the alpha amino group [15], it was thought not to be involved. Additional evidence that RT1.1 was not reactive to alpha amino groups was obtained with AA-modified m-EGF. Mouse-EGF has one alpha amino group and no epsilon amino groups [24], and RT1.1 did not recognize this protein modified with AA under reducing conditions. In contrast, h-EGF has the same alpha amino group as m-EGF, but in addition it also has two epsilon amino groups [24]; RT1.1 strongly reacted with h-EGF modified with AA. Therefore, these data strongly support the observation that RT1.1 is only able to bind to NEL groups.

Protein adducts prepared under physiological conditions (non-reduced, NR) did not inhibit RT1.1 from binding to reduced proteins nor did RT1.1 bind directly to these proteins coated on an ELISA plate. These data would suggest that there must be at least two different types of AA adducts formed, as has been suggested by previous studies [3, 5, 6, 15, 16]. The uniqueness of this research is that it is the first study to report at least these two different types of adducts that can be distinguished by immunological methods. It is apparent that the use of monoclonal antibodies will greatly enhance the detection of specific AA–protein adduct species formed *in vivo*.

Many investigators have utilized polyclonal antibodies prepared to R-AA adducts to demonstrate the presence of adducts in the livers of alcohol-fed rats that are not found in controls [9, 10, 17, 19]. The use of polyclonal antibodies, however, does not allow for defining the epitope or the type of AA adduct detected. It is widely assumed that NEL is a major product of AA–protein adduct formation [3–7, 15, 16]. We reported recently that RT1.1 does not detect adducts in liver cytosolic preparations from alcohol-fed rats, whereas a polyclonal antiserum does [19]. However, the lymphoid cell line P815 has been cultured in the presence of AA and/or $NaCNBH_3$. It is possible to detect AA adducts in cytosolic preparations from cells cultured under reducing conditions with RT1.1 and with the rabbit polyclonal antiserum. It is not possible to detect AA adducts using RT1.1 in cytosolic preparations from cells cultured with AA alone (at 10, 50 or 100 μM) for 15 days [25, 26]. However, the rabbit polyclonal still reacts with these cytosolic preparations. Since this report clearly shows that the specificity of RT1.1

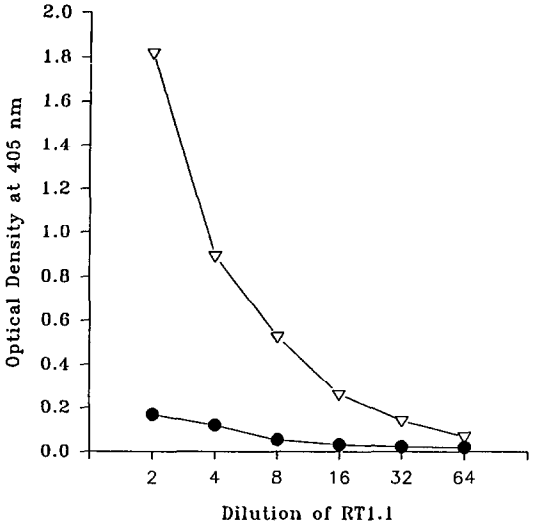

Fig. 4. Activity of RT1.1 to h-EGF (▽) or m-EGF (●) prepared under reducing conditions. Plates were coated with 20 μg/mL of either h-EGF or m-EGF prepared under native or reducing conditions. The differences between the optical density on reduced and native preparations are plotted against the dilution of RT1.1.

is to NEL, it is unlikely that ethylation of the epsilon amino group by AA occurs to any great extent in rats as a consequence of chronic alcohol exposure.

Israel *et al.* [8, 27, 28] have reported on an IgE monoclonal antibody that reacts with AA-modified proteins prepared under reducing conditions. This antibody has been used in our systems; it detects only NEL and does not react with alcohol-fed rat livers (data not shown). These data suggest that these two antibodies are similar in their binding characteristics. We are currently investigating whether they bind to the same epitope by competitive inhibition assays.

The use of similar techniques to produce and define monoclonal antibodies to other AA-modified protein adduct species will enhance the detection and identification of AA–protein adducts formed in the tissue and blood of chronic alcoholics. The identification of specific different AA–protein adduct species should help us to better understand the role of AA–protein adducts in the pathophysiology of alcoholic liver disease.

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