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Binding of Anti-Acetaldehyde IgG Antibodies to Hepatocytes with an Acetaldehyde-Phosphatidylethanolamine Adduct on Their Surface

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We have previously shown that antibodies raised against acetaldehyde adducts of protein cross-react with an acetaldehyde adduct of dioleoylphosphatidylethanolamine, *N*-ethyl-dioleoylphosphatidylethanolamine, when the latter is incorporated into hexagonal phase phospholipid micelles. In the present study we demonstrate that these same IgG antibodies cross-react with *N*-ethyl-dioleoylphosphatidylethanolamine when this adduct is incorporated into the surface of hepatocytes. Hapten-specific IgG antibodies were purified from the sera of rabbits sensitized to an albumin-acetaldehyde conjugate that had been reduced with sodium cyanoborohydride (*N*-ethyl-RSA). The *N*-ethyl-RSA was coupled to an Affi-Gel-10 column to affinity purify the IgG. Liposomes containing *N*-ethyl-dioleoylphosphatidylethanolamine were fused with isolated hepatocytes, the affinity purified primary IgG antibodies were added, then fluorescein-conjugated second antibodies were added, and antibody binding to hepatocytes was measured by flow cytometry. The fluorescence of these hepatocytes was significantly greater ($p < 0.01$) than control hepatocytes prepared with (1) pre-immune primary IgG antibodies with fluorescein-conjugated second antibodies, (2) no primary antibody but with fluorescein-conjugated second antibodies, and (3) no fluorescein-conjugated second antibodies.

Key Words: Alcohol, Hepatocytes, Acetaldehyde, Antibodies, Phospholipid.

METABOLISM OF ethanol produces acetaldehyde[1,2] that forms Schiff bases with proteins. These Schiff bases can be reduced to form stable *N*-ethyl-lysine adducts.[3-5] Specific antibodies against acetaldehyde adducts appear in the serum following ethanol ingestion[1] and synthetic adducts of acetaldehyde with proteins such as hemocyanin or albumin have been shown to elicit production of polyclonal antibodies in rabbits and monoclonal antibodies in mice.[6] It has been suggested that these antibodies may be involved in antibody-mediated hepatotoxicity.[1,7-9]

We have recently shown that antibodies raised against acetaldehyde adducts of protein cross-react with an acetaldehyde adduct of dioleoylphosphatidylethanolamine (*N*-ethyl-DOPE) when this phospholipid adduct is incorporated into hexagonal phase phospholipid micelles.[10] In the present study we used flow cytometry to test whether the same IgG antibodies bind to *N*-ethyl-DOPE when it is incorporated into the membrane surface of isolated hepatocytes in primary culture.

MATERIALS AND METHODS

Materials

Fluorescein-conjugated goat antibody raised against rabbit-IgG was purchased from Caltag (South San Francisco, CA) and phospholipids were purchased from Avanti Polar Lipids, Inc. (Pelham, AL).

We have previously described in detail the synthesis of the adduct of acetaldehyde with RSA after reduction with sodium cyanoborohydride (*N*-ethyl-RSA), the adduct of dioleoylphosphatidylethanolamine with acetaldehyde after reduction with sodium cyanoborohydride (*N*-ethyl-DOPE), as well as the preparation and purification of the IgG antibodies that were affinity purified from serum of rabbits sensitized to *N*-ethyl-RSA (anti-*N*-ethyl-RSA IgG).[10] Therefore, only brief descriptions of the syntheses are included here.

Synthesis of N-ethyl-RSA

Synthesis of *N*-ethyl-RSA was performed by a modification of the techniques used by Means and Feeney[11] and Tuma et al.[3] RSA was dissolved at a concentration of 10 mg/ml in a 10 mM solution of sodium cyanoborohydride in phosphate-buffered saline (PBS). Redistilled acetaldehyde was then added to the mixture (final concentration 100 mM) and incubated at 37°C for 18 hr. The samples were then removed from the incubator and dialyzed against 3 × 2 liters of Tris-HCl buffer, pH 8.3.

Synthesis of N-Ethyl-DOPE

Synthesis of *N*-ethyl-DOPE was carried out essentially as described by Kenney.[12] Twenty milligrams of dioleoylphosphatidylethanolamine (DOPE) were dissolved in 250 μl CH_2Cl_2 and in a 3-ml Reacti-vial that was flushed with N_2 and chilled in ice. Then 20 μl pyridine and 10 mg of freshly re-distilled acetaldehyde dissolved in 200 μl of CH_2Cl_2 were added to the phospholipid for 30 min at 0°C. A solution of 50 mg $NaCNBH_3$ dissolved in 250 μl MeOH was added through the septum, the mixture was allowed to come to room temperature for 10 min, and the *N*-ethyl-DOPE was extracted. It was purified by HPLC on a 25 × 1 cm Si 60-199 Brownlee cartridge column at a flow rate of 2 ml per min using a binary gradient of hexane: isopropanol: 0.1 M NH_4HCO_3, pH 7.0; (A) 40:58:2 and (B) 40:50:10. The elution program was 100% A until 5 min and then a linear gradient to 100% B from 5 to 35 min. The *N*-ethyl-DOPE eluted in 38.2 min and the *N,N*-diethyl-DOPE adduct eluted at 16.5 min. The structure was confirmed by direct chemical ionization mass spectrometry.[10]

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Preparation of Anti-N-Ethyl-RSA IgG Antibodies

The anti-*N*-ethyl-RSA IgG antibodies were purified from the serum of rabbits sensitized to *N*-ethyl-RSA by precipitation with caprylic acid[13] followed by affinity chromatography on Affi-Gel 10 (Biorad, Richmond, CA) using 4 M KSCN for elution. Purified samples of IgG from pre-immune rabbits (pre-immune IgG) were also prepared to be used as controls.

Preparation of Liposomes Containing N-Ethyl-DOPE

Liposomes containing 50% by weight *N*-ethyl-DOPE and 50% dioleoylphosphatidylcholine were prepared by mixing the lipids in $CHCl_3$ in 6 ml flat-bottomed Vari-Clean vials (Pierce Chemical, FL), removing the solvent with a stream of nitrogen, and then removing traces of solvent in a vacuum desiccator.[14] The lipids were hydrated in PBS at a concentration of 200 μg/ml of 1 hr and then sonicated in a bath sonicator for 3 × 1 min at room temperature.

Preparation of N-Ethyl-DOPE Labeled Hepatocytes

Rat hepatocytes were isolated by in situ perfusion with collagenase as previously described.[15] It was estimated from previous data[15] that 2.5×10^5 hepatocytes contain approximately 0.5 mg of lipid. In order to obtain a concentration of approximately 1% *N*-ethyl-DOPE in the hepatocytes, liposomes containing 5 μg of *N*-ethyl-DOPE were allowed to fuse with the surface of 2.5×10^5 hepatocytes in 350 μl PBS for 45 min. We have shown that similar liposomes containing spin-labeled phospholipids rapidly equilibrate with the surface of other protein-containing membranes during in vitro incubations[16] and the same technique and conditions were used.

Flow Cytometry

The anti-*N*-ethyl-RSA IgG, the pre-immune IgG, and the fluorescein-conjugated goat anti-rabbit IgG were dissolved to a concentration of 1 μg/10 μl of 1% nonfat dry milk in PBS. The final concentration of nonfat dry milk was always adjusted to 0.35%. Three sets of samples were prepared: Hepatocytes that had been fused with *N*-ethyl-DOPE liposomes were placed in 13 × 100 mm test tubes and 1,5, or 10 μg of either anti-*N*-ethyl-RSA IgG (A) or pre-immune IgG (B) were added. A control set of hepatocytes that had not been fused with *N*-ethyl-DOPE was also prepared (C). After 30 min of incubation at 22°C, the corresponding 1, 5, or 10 μg of fluorescein-conjugated secondary antibody were added to each of the three sets of hepatocytes (A–C), and the mixtures were lightly agitated. After 30 min incubation at 22°C, an equal volume of 1% paraformaldehyde in PBS was added and the samples were stored at 4°C overnight before being analyzed by flow cytometry. As a control for autofluorescence of the phospholipids or the anti-*N*-ethyl-RSA IgG, a forth set of hepatocytes was prepared in which both the fusion with *N*-ethyl-DOPE liposomes and exposure to the fluorescein-conjugated secondary antibody was omitted (D). Flow cytometry was performed on a Facstar II (Becton Dickinson, Palo Alto, CA). Analysis of the hepatocytes was gated on forward scatter and 10,000 cells were counted from each sample. All samples were prepared and run in triplicate; the entire experiment was repeated once with identical results. The fluorescence intensity, forward scatter, and obtuse scatter were recorded for each sample and the fluorescence intensity was integrated.[17]

RESULTS

The results of the flow cytometry analysis are shown in Fig. 1. In each case, forward scatter (FSC, a qualitative measure of the size and refractive index of a particle) is plotted versus the log of fluorescein fluorescence intensity (F1).[17] The letters (A–D) in the figures correspond to the four sets of hepatocytes described in "Methods." In Fig. 1A it can be seen that anti-*N*-ethyl-RSA IgG antibodies bind strongly to hepatocytes that contain *N*-ethyl-DOPE on their surface. In contrast, the fluorescent intensity was much less ($p < 0.01$) in the controls in which pre-immune IgG was substituted for the primary antibody (Fig. 1B), in which the primary antibody was omitted (Fig. 1C), or in hepatocytes without *N*-ethyl-DOPE and exposed only to the primary antibody (Fig. 1D).

Since there was no great alteration in the distribution of particle sizes as measured by forward scatter (FSC), neither fusion with the liposomes nor binding of the antibodies caused aggregation of the hepatocytes.[17] Comparison of Fig. 1C with Fig. 1D shows that much of the background fluorescence is due to nonspecific binding of the fluorescein-conjugated goat anti-rabbit second antibody to the hepatocytes, whereas relatively little background is contributed by autofluorescence of the hepatocytes themselves. Comparison of Fig. 1B with Fig. 1C shows that initial exposure to pre-immune IgG antibodies did not increase the fluorescence intensity above that seen with only the second antibody. Therefore the anti-*N*-ethyl-RSA antibodies were effective in binding to *N*-ethyl-DOPE on the hepatocyte surface and the increased fluorescence was not due to nonspecific binding of the primary IgG antibody to the surface.

The integrated fluorescent intensities of triplicate sets of 2.5×10^5 hepatocytes exposed to 1, 5, or 10 μg of primary and secondary antibodies are shown in Table 1. The fluorescence intensity of anti-*N*-ethyl-RSA IgG bound to hepatocytes that had been fused with *N*-ethyl-DOPE is significantly greater ($p < 0.01$) than those exposed to either pre-immune IgG or only secondary antibody when either 5 or 10 μg of IgG antibodies per sample was added. In addition, there is a clear dose response to increased amounts of antibodies. Under these conditions the fluorescence intensity of unexposed hepatocytes alone was 2.6 ± 0.02.

DISCUSSION

In the present study we have demonstrated that anti-*N*-ethyl-RSA IgG antibodies will bind to hepatocytes that contain *N*-ethyl-DOPE on their surface to the same extent that they bind to *N*-ethyl-DOPE in hexagonal phase micelles. Previous investigators have suggested that anti-acetaldehyde antibodies may be involved in antibody-mediated hepatotoxicity[7–9,11]; however, no direct immune mechanism has been demonstrated. Our present results suggest that antibodies elicited by *N*-ethyl-protein adducts could be directly involved in ethanol-related hepatotoxicity by binding to the hepatocyte surface and attracting or activating either macrophages or complement. In addition, these results provide evidence that there are domains on the hepatocyte surface that allow antibodies access to binding to *N*-ethyl-DOPE. In this respect these domains

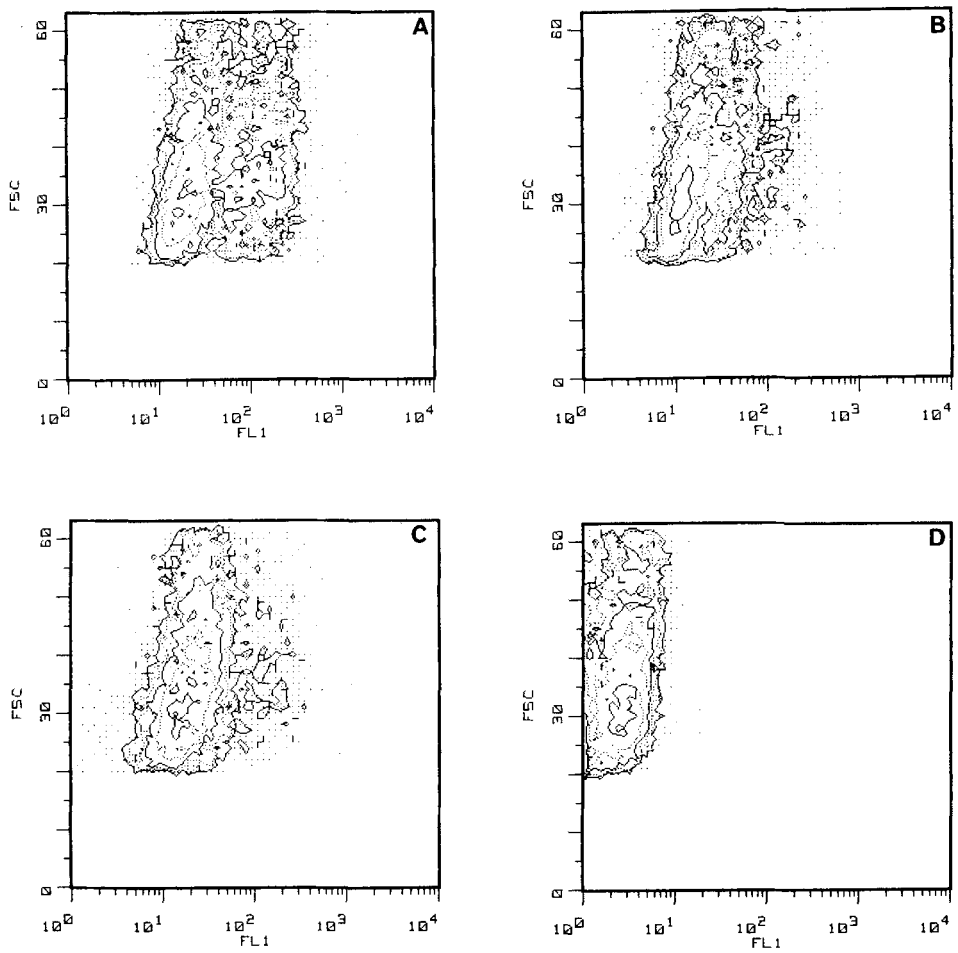

Fig. 1. Flow cytometry analysis of 2.5×10^5 hepatocytes that had been: A, fused with liposomes containing 5 μg *N*-ethyl-DOPE, then exposed to 10 μg of anti-*N*-ethyl-RSA IgG followed by 10 μg of fluorescein-conjugated goat anti-rabbit IgG; B, fused with liposomes containing 5 μg *N*-ethyl-DOPE, then exposed to 10 μg of pre-immune IgG followed by 10 μg of fluorescein-conjugated goat anti-rabbit IgG; C, exposed to 10 μg of fluorescein-conjugated goat anti-rabbit IgG; D, no treatment. In each case, forward scatter (FSC, a qualitative measure of the size and refractive index of a particle) is plotted versus the log of fluorescence intensity (F1). The contour plot intervals are binary (1, 2, 4 to 128).

Table 1. Integrated Fluorescence Intensities of Antibody-Bound Hepatocytes

	(A) hepatocytes + N-ethyl-DOPE + anti-N-ethyl-RSA IgG + G-anti-R IgG	(B) hepatocytes + N-ethyl-DOPE + pre-immune IgG + G-anti-R IgG	(C) hepatocytes + no addition + no IgG + G-anti-R IgG
1 μg IgG	10.5 ± 1.8	5.4 ± 1.5	7.0 ± 1.1
5 μg IgG	39.9 ± 1.4*	20.0 ± 3.5	17.5 ± 0.6
10 μg IgG	67.6 ± 1.2*	32.0 ± 1.2	29.8 ± 1.1

Integrated fluorescence intensity of 10,000 out of 2.5×10^5 hepatocytes that had been either: (A) fused with liposomes containing 5 μg *N*-ethyl-DOPE, then exposed to 1, 5, or 10 μg of anti-*N*-ethyl-RSA IgG followed by a corresponding 1, 5, or 10 μg of fluorescein-conjugated goat anti-rabbit IgG (G-anti-R IgG); (B) fused with liposomes containing 5 μg *N*-ethyl-DOPE, then exposed to 1, 5, or 10 μg of preimmune IgG followed by a corresponding 1, 5, or 10 μg of fluorescein-conjugated goat anti-rabbit IgG; or (C) exposed to 1, 5, or 10 μg of fluorescein-conjugated goat anti-rabbit IgG. The values are means ± SEM for triplicate samples.

* $p < 0.01$ for (A) versus (B) by an unpaired Student's *t* test.

are similar to the surface of a hexagonal phase micelle and are very different from that of a phospholipid bilayer in a lamellar liposome.[10]

We have previously shown that anti-*N*-ethyl-RSA IgG antibodies have a strong preference for binding to *N*-ethyl-DOPE when it is incorporated into hexagonal phase phospholipid micelles as compared to lamellar phase liposomes. This selective binding to phospholipid haptens in the hexagonal phase is consistent with previous studies on the preference of lupus anti-phospholipid antibodies for hexagonal phase lipid.[18] The observed preference for binding of antibodies to haptenic phospholipids in hexagonal phase host membranes may be a function of the tighter packing of the phospholipids and the resulting higher surface pressure of phospholipids in the lamellar phase.[19] In the case of lamellar phase phospholipid host membranes, haptenic groups on phospholipids require a spacer to extend them several carbon atoms beyond the phosphate group in order to be recognized by antibodies.[20,21] In the present study the anti-*N*-ethyl-RSA antibodies bound to the surface of the hepatocytes that had incorporated *N*-ethyl-DOPE as well as they had to *N*-ethyl-DOPE in hexagonal phase micelles. Of course, this does not show that hexagonal phase micelles exist within the surface of the hepatocyte membrane. However, the presence of phospholipid domains in hepatic microsomes with motional averaging similar to hexagonal phase micelles has been inferred by Stier et al. from ^{31}P-NMR studies.[22] Similar phospholipid structures are thought to occur sandwiched within cellular membranes and at junction points between bilayers where they are known as lipidic particles or interlamellar attachments.[14,23-26] These structures could provide domains on the hepatocyte surface in which *N*-ethyl-phosphatidylethanolamines are accessible to binding by circulating antibodies.

It is important to consider the possibility that fusion of the liposomes containing *N*-ethyl-DOPE with the surface of the hepatocyte membrane was incomplete. This could result in locally high concentrations of *N*-ethyl-DOPE or even alter the structure of the hepatocyte membrane. Several lines of evidence argue against these possibilities. First, in our studies of the fusion of liposomes containing spin-labeled phospholipids with acceptor liposomes of reconstituted cytochrome P-450, we used ESR to measure the rate of fusion. The initial spectrum of the concentrated spin-labeled phospholipids was very broad due to spin-spin exchange; it sharpened as they diluted into the acceptor membranes. The appearance of a sharp three-line ESR spectrum at the conclusion of the experiments demonstrated that the spin-labeled phospholipids were homogeneously dispersed.[16] Second, we have incubated ^{14}C-labeled phospholipids with liver homogenates and have observed a homogeneous dispersion of the label into all hepatocyte membranes.[27] Third, Knoll et al. have recently used autoradiography to show the homogeneous redistribution of labeled phospholipids following fusion of liposomes with the plasma membrane of epithelial cells.[28] Forth, Hesketh et al. have used a similar fusion technique to add phospholipids to sarcoplasmic reticulum membranes and have found it results in homogeneous lamellar membranes.[29]

In summary, these results demonstrate that antibodies raised against an *N*-ethyl-protein adduct can cross-react with an *N*-ethyl-phosphatidylethanolamine adduct. In addition, they provide evidence that there are domains on the hepatocyte surface that allow antibody binding to *N*-ethyl-DOPE in a manner similar to that provided by the surface of a hexagonal phase micelle. These results suggest several future experiments that would help relate the present in vitro findings to chronic in vivo exposure to alcohol. For example, it will be important to determine if *N*-ethyl-phosphatidylethanolamines are formed during in vivo ethanol metabolism and if antibodies bind to them in sufficient density to initiate activation of neutrophils or complement. These in vivo studies would aid in evaluating the possible role of these cross-reactive antibodies in ethanol-related hepatotoxicity.

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