Ethanol Inhibition of Cell-Mediated Lysis of Antibody-Sensitized Target Cells at a Calcium-Dependent Step (42975)

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Abstract. **Ethanol inhibits antibody-dependent cell-mediated cytotoxicity in a dose-dependent manner. The inhibitory effect of ethanol was reversed by the addition of excess calcium or calcium ionophore A23187. Excess calcium at 4–8 m*M* concentrations was required to reverse 50% of the inhibition caused by ethanol. In seven of nine experiments, 16 m*M* excess calcium completely reversed the inhibition and produced greater lysis than the control. Excess calcium in the absence of ethanol induced a dose-dependent increase in lytic activity by the spleen cells. However, the reversal of inhibition by ethanol could not be attributed to a simple additive effect resulting from the increased cytolytic capacity of the lymphocytes in the presence of excess Ca^{2+}. Ionophore A23187 at 1 μM also partially reversed the inhibitory effect caused by ethanol. Ionophore alone did not potentiate lytic activity. When target cells were not sensitized with antibody, excess calcium had no effect on the lysis of target cells in the presence of ethanol-treated or untreated lymphocytes. These data suggest that ethanol inhibits antibody-dependent cell-mediated cytotoxicity at a calcium-dependent step.**

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Chronic alcohol consumption has been associated with a variety of immunologic abnormalities, including polyclonal immunoglobulin elevations (1), humoral and cellular immune responses to alcoholic hyalin (2, 3), circulating immune complexes (4), immune complex deposits in liver and kidney (5), and cell-mediated immunity to acetaldehyde (6). Alcoholics have an increased susceptibility to infection (7, 8) and have a higher than normal incidence of development of some tumors (9). *In vitro*, ethanol has been shown to inhibit several cell-mediated effector functions including phagocytosis and natural killer (NK) cell activity (6, 10–12). Our studies have indicated that ethanol inhibits antibody-dependent cell-mediated cytotoxicity (ADCC), NK activity, and alloimmune cytotoxic T lymphocyte activity (13). Concentrations of 5.5–176 m*M* ethanol produced progressive inhibition of cell-mediated cytotoxicity. Preincubation experiments revealed the ethanol-mediated inhibition of lysis to be reversible. Fifty percent inhibition of ADCC was produced by 52 m*M* (0.24%) ethanol. An inhibition constant (K_i) of 373 mM^{-2} when two ethanol molecules interact with the site of inhibition was calculated. Alcohol interferes with lysis by reacting with sites which are required for triggering the lytic event. Although the molecular details of the mechanism of inhibition are as yet undefined, we inferred that ethanol inhibits ADCC at the programming for lysis or the lethal hit stages (13).

Control of calcium flux within biologic systems is well recognized as vital for many diverse cellular functions. Calcium channels are potential sites of action for psychoactive drugs. Indeed, barbiturates, cannabinoids, and ethanol alter some aspects of calcium transport (14). Friedman *et al.* (15) have shown that some of the pharmacologic effects of ethanol may be attributable to its ability to significantly increase calcium accumulation in cells (15). A number of studies also suggests that lymphocyte activation events require Ca^{2+} participation at various levels (16). There is a strict Ca^{2+} requirement for the lethal hit stage of T cell-mediated cytolysis (17) and ADCC.

Since calcium is required for the lytic step in cell-mediated cytotoxicity and ethanol has been shown to alter Ca^{2+} flux, the present series of experiments were designed to evaluate whether the inhibitory effect of

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ethanol on cell-mediated cytotoxicity might be taking place by a calcium-dependent mechanism.

Materials and Methods

Animals. C3H/HEN male mice, 6–12 weeks of age, were used as the source of splenic lymphocytes.

Medium. RPMI 1640 (Flow Laboratories, McLean, VA) with 100 μg/ml gentamicin and 10% fetal calf serum (FCS) were used for all incubations and dilutions. Calcium-free medium was specifically prepared by Flow Laboratories for these studies.

Antiserum. IgG anti-ox red blood cells (ORBC) was obtained commercially (Cooper Biomedical, Inc., Malvern, PA). IgG was diluted 1/500 for sensitizing ORBC.

Lymphocytes. Murine spleens were dissected out under sterile conditions, placed in ice-cold RPMI 1640, and pressed through fine nylon mesh. The suspended cells were passed over a fine nylon mesh filter and pelleted by centrifugation. The cells were resuspended in a solution of tris (hydroxymethyl) aminomethane-buffered 0.75% ammonium chloride (pH 7.2) and incubated for 10 min at 37°C to lyse erythrocytes. The cells were washed three times in RPMI 1640, resuspended in complete RPMI 1640, and counted in a hemacytometer. The viability of spleen cells in the presence of various concentrations of ethanol, calcium, and calcium ionophore was evaluated by trypan blue exclusion studies.

ADCC Assays. Of a 50% suspension of ORBC, 0.2 ml was labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$, Amersham, Arlington Heights, IL) 250 μCi/1.5 × 10^7 cells; by incubating at 37°C for 1 hr. After labeling, the cells were washed three times and sensitized with a 1/500 dilution of antibody for 1 hr at room temperature. The antibody-sensitized target cells were washed again and aliquoted in triplicate at 2 × 10^5 cells/tube along with murine spleen cells to give an effector to target cell ratio of 8:1. The loosely capped tubes contained effector cells, target cells, and ethanol in the presence and absence of calcium and/or ionophore in a total volume of 0.4 ml. Controls consisted of nonsensitized target cells and sensitized target cells in the absence of ethanol, calcium, and calcium ionophore. After the tubes were incubated at 37°C in a 5% CO_2 incubator for 4 hr, 2 ml of RPMI 1640 were added to each tube, the cells were pelleted by centrifugation, and the pellets and supernatants were assayed for ^{51}Cr activity in a well-type gamma counter. The maximum releasable counts were determined by lysing nonsensitized target cells with 2 ml of distilled water.

Percentage of lysis was calculated using the following formula:

$$\% \text{ Lysis} = \frac{{}^{51}\text{Cr release (experimental)} - {}^{51}\text{Cr release (spontaneous)}}{{}^{51}\text{Cr release (maximum)} - {}^{51}\text{Cr release (spontaneous)}} \times 100$$

Percentage of inhibition was calculated as follows:

$$\frac{\text{Geometric mean of the control} - \text{Geometric mean of the experimental}}{\text{Geometric mean of the control}} \times 100$$

Percentage of recovery was calculated from the following formula:

$$100 - \frac{100 \times \%\text{ inhibition } B}{\%\text{ inhibition } A}$$

where A represents first drug (ethanol) and B represents second drug for recovery (calcium).

Chemicals. Calcium ionophore A23187 magnesium salt was obtained from Calbiochem, Behring Diagnostics, La Jolla, CA. Calcium in the form of $CaCl_2 \cdot H_2O$ was obtained from J. T. Baker, Inc. and was prepared as 100 mM (stock) in barbital buffer solution. The stock solution was sterilized through 0.45-μm Falcon Millipore filter unit. Forty-eight micromolar Ca^{2+} was prepared from the stock solution by diluting either in regular RPMI or calcium-free RPMI and adjusted to pH 7.2 with NaOH. Of 48 mM Ca^{2+}, 0.1 ml was added into a total volume of 0.3 ml in assay tubes to make a final concentration of 16 mM. A serial dilution of other concentrations of Ca^{2+} was made from this concentration.

Calcium Measurement. The exact concentration of calcium in the FCS and RPMI (Ca^{2+}-free medium) was evaluated by atomic absorption (18, 19). The concentration of Ca^{2+} in the assay tubes was monitored by calcium electrode (20).

Calcium Evaluation. Calcium concentration in regular RPMI was 1.2 mM and calcium-free medium contained no calcium. Fetal calf serum (10% FCS) contained 320 μM calcium as determined by atomic absorption.

Results

It has previously been shown that Ca^{2+} is required for the lytic step in cell-mediated cytotoxicity. However, we investigated the requirements for Ca^{2+} in ADCC under our experimental conditions. Table I shows the effect of 0.1–16 mM added calcium into the reaction mixture containing effector and target cells treated with 1 mM EGTA to chelate Ca^{2+} present in FCS and to act as a Ca^{2+} buffer. The Ca^{2+}-free medium was used for all of the dilutions of the effector and target cells. However, 10% FCS contained 320 μM Ca^{2+}. With increases in the concentration of added calcium in excess of 1 mM, there was an increase in the lytic activity. Maximum lysis was obtained with 16 mM Ca^{2+}.

To evaluate whether excess calcium could reverse ethanol-mediated inhibition of lysis, we added different concentrations of extra calcium (1–16 mM) into the reaction mixture containing antibody-sensitized target and effector cells in the presence and absence of

ethanol. As shown in Table II and Figure 1, the inhibition of ADCC by different concentrations of ethanol (22, 44, 88, and 176 mM) was completely reversed by the addition of 16 mM Ca^{2+}. The data shown in Table II are mean values from three separate experiments. In nine experiments, seven revealed an increase in lysis of the target cells in the presence of ethanol and 16 mM excess Ca^{2+} and all nine reversed the inhibition of lysis mediated by ethanol. It also appears that 4–8 mM Ca^{2+} is required for 50% reversal of lytic activity. Since increased Ca^{2+} in the absence of ethanol caused potentiated lysis of antibody-sensitized erythrocytes, it was conceivable that the reversal of ethanol-mediated inhibition of lysis might be more apparent than real. However, in Figure 1, we have taken into consideration the potentiated lysis produced by Ca^{2+} in the absence of ethanol, i.e., cultures containing the amount of excess Ca^{2+} shown were used as the positive control for calculation of zero inhibition. Excess extracellular Ca^{2+} does not induce lysis of unsensitized target cells (Table III).

As shown in Table IV addition of 1 μM calcium ionophore (A23187) to ethanol-treated cells partially reversed the inhibition; 31% when 176 mM ethanol was used and 48% recovery was obtained when 88 mM ethanol was used. This represents a significant reversal effect, although the reversal caused by excess extracellular calcium is of much greater magnitude. In the presence of 1 μM Ca^{2+} ionophore, added excess calcium did not further reverse the inhibition of lysis produced by ethanol (data not shown). By trypan blue exclusion the concentrations of calcium, ionophore, and ethanol used in our studies were not toxic to lymphocytes.

Table I. Requirement of Ca^{2+} in ADCC

Concentration of Ca^{2+} added (mM)	% Lysis (mean ± SD) Ca^{2+}-free RPMI 1640-treated with EGTA (1 mM) + 10% FCS
0	3.66 ± 0.16
0.1	3.46 ± 1.14
1	4.28 ± 0.48
2	6.72 ± 0.68
4	8.28 ± 0.53
6	7.75 ± 0.58
8	9.43 ± 0.96
10	9.08 ± 0.97
12	10.52 ± 1.41
14	13.29 ± 3.05
16	26.09 ± 1.10

Note. C3H spleen cells (effector cells) were mixed with IgG-sensitized ORBC (target cells) at 8:1 effector to target ratio in Ca^{2+}-free RPMI with 10% FCS (containing 320 μM Ca^{2+}). Controls in regular RPMI with 10% FCS showed mean lysis (mean ± SD) of 25.05 ± 4.35. Controls in nonsensitized ORBC containing extra 16 mM Ca^{2+} showed mean lysis of 0.43 ± 0.6 (mean ± SD). Values represent mean of three samples. Other experiments showed similar trend. Concentrations of Ca^{2+} above 20 mM were toxic to lymphocytes.

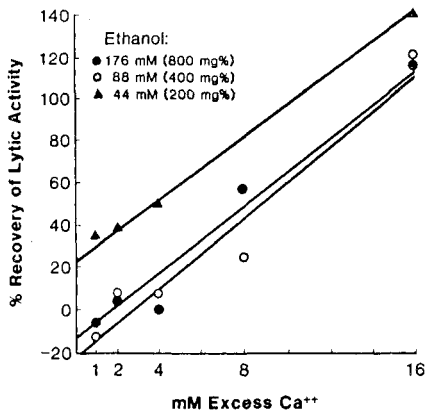

Figure 1. Percentage of recovery of ADCC activity by addition of excess calcium. Percentage of recovery was calculated from percentage of inhibition of ADCC by different concentrations of ethanol (44, 88, and 176 mM) by the following formula:

$$100 - \frac{100 \times \%\text{ of inhibition } B}{\%\text{ of inhibition } A}$$

Where A is inhibition by ethanol and B is inhibition by calcium. Data points were calculated using the percentage of lysis in the amount of excess calcium indicated but in the absence of alcohol as the control values. The data shown represents mean values from three experiments. For ADCC, C3H spleen cells as effector cells and sensitized ORBC as target cells were used at 8:1 effector to target ratio. The incubation time was 4 hr. All dilutions of ethanol and calcium were made as shown in Table II.

Table II. Inhibition of ADCC by Ethanol Dose-Dependent Effect of Ca^{2+}

Calcium concentration (mM)	% Lysis (mean ± SE) Concentration of ethanol (mM)				
	0	22	44	88	176
0	19.02 ± 2.8	17.24 ± 1.78	14.59 ± 2.23	11.21 ± 3.21	3.94 ± 2.37
1	19.57 ± 2.92	ND[a]	16.61 ± 0.8	10.53 ± 0.73	3.16 ± 1.22
2	19.39 ± 1.76	ND	16.63 ± 0.82	12.1 ± 1.62	4.65 ± 1.39
4	20.3 ± 1.03	19.68 ± 1.6	17.82 ± 1.57	12.67 ± 0.54	4.21 ± 2.23
8	27.28 ± 5.1	23.07 ± 1.5	16.68 ± 2.94	18.85 ± 4.52	17.91 ± 3.02
16	76.27 ± 7.01	87.29 ± 1.4	82.77 ± 0.55	82.25 ± 1.67	85.33 ± 2.32

Note. Data represents mean ± SE of three experiments. C3H spleen cells as effector cells and antibody-sensitized ORBC (target cells) at 8:1 effector to target ratio were used. The incubation time for the assay was 4 hr. All dilutions of ethanol were made in RPMI 1640 and dilutions of calcium and ionophore were made in phosphate-buffered saline.

[a] ND, not done.

Discussion

We have previously shown that ethanol in concentrations of 88–176 m*M* inhibited the capacity of murine lymphocytes to lyse antibody-sensitized target cells (13). Fifty percent inhibition of lysis was produced by 52 m*M* (0.24%) ethanol. The direct or indirect mechanism of inhibition is not known. However, in our previous studies we indicated that the level of inhibition may be at the effector cell during triggering or between the effector and target cells during the lethal hit stage (13). The structural basis for binding of lymphocytes to antibodies and subsequent induction of ADCC appears to be Fc receptors on the surface of lymphocytes (21–23). We found no significant inhibition of binding to antibody-sensitized erythrocytes by spleen cells in the presence of ethanol concentrations that showed inhibition of lysis. Furthermore, IgG Fc receptor binding is divalent cation independent (24, 25). Thus, it would appear that the Ca^{2+}-mediated reversal of ethanol inhibition of lysis takes place at the triggering or lethal hit stages.

Since calcium is required for the lytic step in ADCC, and ethanol is known to alter some Ca^{2+}-dependent functions, it was conceivable that ethanol might inhibit via a calcium-dependent mechanism. We evaluated ADCC of ethanol-treated spleen cells in the presence of additional calcium and calcium ionophore (A23187) and have shown that calcium is required for the lytic function (Table I). Figure 1 and Table II also show that 4–8 m*M* excess calcium produces 50% reversal of lysis. Reversal of inhibition was also obtained when 1 μM Ca^{2+} ionophore A23187 was used.

Since the presence of ethanol can disorder cell membranes which in turn can cause changes in calcium flux, it is possible that the inhibitory effect observed with ethanol may be a direct or indirect consequence of such a change in calcium flux. The disordering of cell membranes by ethanol can open Ca^{2+} channels in the membrane and the additional calcium or calcium ionophore can trigger an increase in intracellular calcium. A number of studies also suggests that lymphocyte activation events require Ca^{2+} participation at various levels (16). The nature of membrane signals involved during these intracellular calcium changes is not known. However, activation of the protein kinase C by calcium appears to be a major mechanism through which calcium signals elicit a response from the lymphocytes (26–29). It is conceivable that ethanol might inhibit diacylglycerol or inositoltriphosphate in the protein kinase C activation pathway. Protein kinase C function is dependent upon allosteric modification by calcium, diacylglycerol, and phosphatidylserine for activity (26). Diacylglycerol increases the affinity of the enzyme for calcium and in sufficient amount may activate the enzyme at nonstimulated levels of calcium. It is possible that ethanol may competitively inhibit the interaction of diacylglycerol and protein kinase C.

We have also evaluated the role of calcium and calcium ionophore on ethanol inhibition in NK and alloimmune cytotoxic T lymphocyte activity. Excess calcium and/or ionophore A23187 did not reverse the inhibition produced by ethanol on NK cell activity. Similarly, excess calcium could not reverse the inhibition produced by ethanol on cytotoxic T lymphocyte activity. However, by using 1 μM Ca^{2+} ionophore, we were able to partially reverse the inhibition caused by ethanol (Walia, unpublished observations).

Alcohol abuse has been associated with increased

Table III. Effect of Calcium on Antibody-Sensitized and Nonsensitized Target Cells

Additional Ca^{2+} (m*M*)	% Lysis (mean ± SE)	
	Antibody-sensitized target cell	Nonsensitized target cell
0	26.35 ± 2.85	3.25 ± 1.12
4	24.8 ± 0.80	2.29 ± 0.72
8	21.3 ± 1.05	2.49 ± 1.37
16	43.75 ± 8.86	1.39 ± 0.49

Note. Mean ± SE of four experiments. Effector cell: C3H spleen cells. Target cells consisted of both antibody-sensitized and nonsensitized ORBC. Effector and target cells were mixed at effector to target of 8:1 for 4 hr incubation. Content of Ca^{2+} before additional Ca^{2+} added was 1.52 m*M*.

Table IV. Effect of Calcium ionophore (A23187) on Ethanol-Treated Murine Spleen Cells: Evaluation by ADCC

Concentration of ionophore (μM)	% Lysis (mean ± SE)				
	Concentration of ethanol (m*M*)				
	0	88	% Recovery	176	% Recovery
0	27.09 ± 2.83	14.89 ± 3.01		8.46 ± 1.73	
0.1	34.17 ± 4.13	14.81 ± 2.48	0	10.53 ± 1.92	11
1	28.30 ± 5.58	20.75 ± 1.44	48	14.20 ± 1.61	31

Note. Mean ± SE of three experiments. One micromolar ionophore is not toxic to murine lymphocytes by trypan blue exclusion. Ten micromolar ionophore is, however, toxic to lymphocytes. Effector cells were C3H spleen cells and target cells. Antibody-sensitized ORBC were used at effector to target ratio = 8:1 for 4 hr of incubation. Regular medium RPMI with 10% FCS was used for dilutions. However, dilutions of calcium and ionophore were made in phosphate-buffered saline. An 0.01 μM ionophore had an effect similar to that of 0.1 m*M* ionophore.

susceptibility to infection and increased incidence of some tumors (7, 9). Conceivably, the increased risk factors for these diseases may be related to the documented alteration in immune function produced by ethanol. Decreased lytic effector function by lymphocytes is only one part of the demonstrated alteration. However, the demonstration that ethanol inhibits ADCC at a calcium-dependent step suggests a broader significance, in terms of the cell biology of ethanol-mediated toxicity, than a single *in vitro* model of cell-mediated lysis of antibody-sensitized target cells. Ca^{2+} is a protean mediator involved with near ubiquity in cellular activation and regulation at multiple steps.

Thus, ADCC provides a convenient model for evaluation of the alcohol-mediated alteration of a calcium-dependent function. The receptor ligand requirements for initiation of this lytic event are well defined, i.e., Fc-Fc receptor interaction. The lytic event occurs rapidly following combination of lymphocytes and sensitized target cells; lysis can be detected within 15 min and proceeds to plateau levels within 8 hr (30). Also the stoichiometry of the alcohol-mediated inhibition of ADCC has been defined (13). Finally, the calcium-dependent (ethanol inhibitable) lytic event is quantifiable by ^{51}Cr release.

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