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Cultured lymphocytes from alcoholic subjects have altered cAMP signal transduction

(adenosine/ethanol/adenosine receptor)

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ABSTRACT **Previous work has shown that freshly isolated lymphocytes from alcoholic subjects show significantly reduced basal and adenosine receptor-stimulated cAMP levels. This decrease could be due to ethanol-induced cellular adaptation or to a genetic difference in the regulation of cAMP signal transduction. Therefore, we cultured human lymphocytes in defined medium without ethanol for 7–8 days and then examined differences in receptor-dependent cAMP accumulation between lymphocytes from alcoholic and nonalcoholic subjects. After four to six generations in culture without ethanol, lymphocytes from alcoholic subjects have significantly higher cAMP levels than do cells from nonalcoholic subjects. Thus, a difference in cAMP signal transduction is demonstrable in cells from alcoholic subjects grown without ethanol. We also found that cultured lymphocytes from both alcoholic and nonalcoholic subjects show a decrease in receptor-stimulated cAMP levels after exposure to 200 mM ethanol for 48 hr. To determine whether alcoholic subjects have increased sensitivity to ethanol, lymphocytes were exposed to only 100 mM ethanol for 24 hr. Under these conditions, receptor-dependent cAMP levels did not change in cells from nonalcoholic subjects. However, lymphocytes from alcoholic subjects showed a 39% decrease ($P < 0.003$) in adenosine receptor-stimulated cAMP levels. Taken together, the results show that (*i*) chronic ethanol treatment in culture reproduces the suppression of cAMP levels found in circulating lymphocytes from alcoholic subjects and (*ii*) despite four to six cell divisions in culture without ethanol, lymphocytes from alcoholic subjects exhibit significantly increased adenosine receptor-dependent cAMP levels and increased sensitivity to chronic exposure to ethanol. These findings suggest that the suppression of cAMP levels observed in freshly isolated lymphocytes from alcoholic subjects results from both a direct effect of chronic exposure to ethanol and a genetic difference leading to altered cAMP signal transduction.**

Receptor-stimulated cAMP production is altered after acute and chronic exposure to ethanol (1–12). Although ethanol acutely stimulates receptor-dependent cAMP production, chronic exposure of cultured NG108-15 neural cells to ethanol decreases adenosine receptor-stimulated cAMP levels when measured in the absence of ethanol (8, 9). Similar findings have also been described for β-adrenergic receptor stimulation of cAMP production in S49 mouse lymphoma cells (6) and prostaglandin E_1 receptor stimulation in both NIE cells (7) and NG108-15 neural cells (9).

We have suggested that decreased receptor-stimulated cAMP levels have pathophysiologic significance in chronic alcoholism (13). Freshly isolated lymphocytes from alcoholic subjects show a 75% reduction in basal and adenosine receptor-stimulated cAMP levels when compared with age- and sex-matched control subjects and patients with nonalcoholic liver disease (13). The results of that study indicated that reduced cAMP levels in lymphocytes could be used to distinguish a population of alcoholic from nonalcoholic subjects. A recent study in platelets (14) has confirmed the observation that stimulated cAMP levels are significantly decreased in alcoholic subjects.

Reduced levels of cAMP in cells from alcoholic subjects may reflect an acquired abnormality due to chronic alcohol abuse similar to the direct effect of ethanol on cAMP signal transduction observed in cultured cells (6–9). However, it is also possible that cells from alcoholic subjects have a genetic difference in their response to ethanol. To distinguish between these possibilities, we have grown human lymphocytes from alcoholic and nonalcoholic subjects in defined medium without ethanol and have determined how cAMP levels change when the cells are challenged by chronic exposure to ethanol.

METHODS

Cell Culture. Peripheral blood was collected into heparinized Vacutainers, and lymphocytes were isolated as described (13). Lymphocytes were seeded into T-25 flasks at 1.0–1.5 × 10^5 cells per ml and maintained at 37°C in humidified 5% CO_2/95% air. The growth medium was 1:1 (vol/vol) Dulbecco's modified Eagle's medium/Ham's F-12 medium (15, 16) containing 2 mM glutamine, 25 mM Hepes, 20 nM sodium selenite, 2 nM testosterone, 6.3 μM linoleic acid complexed to fatty-acid-free bovine serum albumin, insulin at 6.3 μg/ml, transferrin at 30 μg/ml, fatty-acid-free bovine serum albumin at 2.4 mg/ml, phytohemagglutinin at 1 μg/ml, penicillin at 50 units/ml, streptomycin at 50 units/ml, and adenosine deaminase at 0.02 unit/ml. This concentration of adenosine deaminase is approximately that found in serum (17). Cell counts and viability (13) were routinely determined on days 2, 6, 8, and 9 of culture.

To study the chronic effects of ethanol on cultured lymphocytes, lymphocytes were cultured for 6 days in defined medium, after which 100–200 mM ethanol was added to the lymphocyte cultures where indicated, and the flasks were shut tightly to prevent evaporation of ethanol. Cells are withdrawn from ethanol by centrifugation for 10 min at 100 × *g* and resuspended in fresh medium for subsequent culture without ethanol.

Monoclonal antibodies to cell surface antigens [antibody T101 to the 65-kDa antigen (anti-T), anti-IgG (anti-B), anti-CD4, and anti-CD8; BioRad] were used to determine subpopulations of lymphocytes before and after growth in culture and after chronic exposure to ethanol.

Assay for Adenosine Receptor-Stimulated cAMP Levels and cAMP-Phosphodiesterase Activity. After centrifugation for 10

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min at 100 × *g*, lymphocytes were resuspended at 0.5–1.0 × 10^6 cells per ml in Dulbecco's phosphate-buffered saline containing 0.2% glucose and 25 mM Hepes (pH 7.2) (13). Cells were preincubated in triplicate for 5 min in polypropylene tubes with 1 unit of adenosine deaminase per ml and 10 μM ZK-62711 (a gift from Berlex Labs, Cedar Knolls, NJ), an inhibitor of phosphodiesterase. cAMP production was stimulated by adding 100 μM N^6-(*R*-phenylisopropyl)adenosine in the presence and absence of 150 mM ethanol in a final volume of 0.60 ml. Reactions were terminated after 30 min by addition of 50 μl of 2% Nonidet P-40 in 1 M HCl and subsequent incubation on ice for 10 min. cAMP levels then were determined in a 700 × *g* supernatant by radioimmunoassay as described (18).

Activity of cAMP-phosphodiesterase was measured in fresh lymphocytes and in lymphocytes cultured in the presence or absence of ethanol by the method of Thompson *et al.* (19). The high-affinity enzyme was measured in the presence of 1 μM cAMP, and the low-affinity enzyme was measured in the presence of 20 μM cAMP.

Description of Alcoholic and Nonalcoholic Subjects. Seven volunteer, actively drinking alcoholic subjects with a lifetime ethanol consumption greater than 1000 kg were drawn from a pool of alcoholic subjects followed at the Ernest Gallo Clinic and Research Center. They appeared to fit the type II classification as described by Cloninger (20). Experiments with lymphocytes from alcoholic subjects were conducted simultaneously with lymphocytes from nonalcoholic subjects (laboratory staff and members of a local Mormon church group). Both populations were the same age; alcoholic subjects ranging from 31 to 59 years of age and nonalcoholic subjects from 23 to 54. Informed consent was obtained from all subjects in accordance with the Human Use Committee of the University of California, San Francisco.

Statistical Analysis. Repeated measures of analysis of variance and Student's *t* test were performed by the Statistical Analysis System (SAS) program for personal computers (*n* = number of subjects, each sample assayed in triplicate). Results are expressed as means ± SEM.

RESULTS

Growth of Lymphocytes in Culture in the Absence of Ethanol. *Lymphocytes from nonalcoholic subjects.* From day 2 to day 8 in defined medium, lymphocytes grew with a doubling time of 24 hr. During the first 24 hr in culture, the cell number decreased by ≈50%. There was no selective loss of either T or B lymphocytes after 8 days in culture (Table 1) as Darfler and Insel (21) also have reported. However, there was a shift in the population of helper (CD4) and suppressor (CD8) T cells during the culture period (Table 1) (16).

We observed a significant reduction in the stimulation of cAMP production by 100 μM phenylisopropyladenosine in cultured lymphocytes (2.95 ± 0.60 pmol of cAMP per 10^6 cells, *n* = 9) compared with freshly isolated cells (20.4 ± 3.30, *n* = 11; $P < 0.001$). This decreased cAMP production was not due to an increase in the activity of either high- or low-affinity phosphodiesterase. cAMP hydrolysis by the high- and low-affinity enzymes was, respectively, 7.7 ± 0.9 pmol of cAMP per mg of protein per min and 68.2 ± 13.7 pmol of cAMP per mg of protein per min (*n* = 6) in cultured cells compared with 6.8 ± 1.8 and 52.7 ± 25.0 (*n* = 6) in freshly isolated cells. Since lymphocytes release adenosine (22, 23), extracellular accumulation of adenosine could desensitize and/or down-regulate the adenosine receptor during culture (24, 25). This might account for the reduction in adenosine receptor-dependent cAMP levels we observed in cultured lymphocytes relative to freshly isolated lymphocytes. Treatment with adenosine deaminase, which converts adenosine to inosine, should reduce the accumulation of adenosine and thus limit the decrease in phenylisopropyladenosine stimulation of cAMP production in culture. Indeed, cells grown with increasing amounts of adenosine deaminase during the first 6 days in culture showed greater phenylisopropyladenosine stimulation of cAMP levels (Fig. 1). Moreover, when the cell culture medium was changed 24 hr prior to assay to completely remove accumulated adenosine, PIA-stimulated cAMP levels (13.04 ± 3.06 pmol of cAMP per 10^6 cells) were significantly higher when compared with levels in cells grown for 24 hr in conditioned medium (2.08 ± 0.09, *n* = 6; $P < 0.01$).

Lymphocytes from alcoholic subjects. Basal and phenylisopropyladenosine-stimulated cAMP levels were significantly lower in freshly isolated lymphocytes from alcoholic subjects as compared with levels in lymphocytes from nonalcoholic subjects (Fig. 2), thus confirming our earlier results (13). Phenylisopropyladenosine-stimulated cAMP levels, after acute exposure to ethanol, also were significantly lower in alcoholic subjects (Fig. 2). However, after growth in culture for 7 days in the absence of ethanol, lymphocytes from alcoholic subjects not only recovered but also showed increased stimulation of cAMP levels relative to lymphocytes from nonalcoholic subjects (Fig. 3). This difference was not due to differences in the percentage of T and B cells: 80 ± 3% (*n* = 7) of the cells from nonalcoholic subjects were T-cells compared to 79 ± 2% (*n* = 5; $P = 0.82$) in alcoholic subjects. There also was no difference in doubling time between alcoholic subjects (26.9 ± 1.8 hr) and nonalcoholic subjects (23.7 ± 1.7 hr; $P = 0.10$).

Table 1. Lymphocyte cell populations of nonalcoholic subjects

Cell type	Fresh lymphocytes	Cultured lymphocytes (day 8)	
		Control	200 mM EtOH
T-cell	74 ± 3	77 ± 5	81 ± 6
B-cell	12 ± 4	9 ± 3	8 ± 3
T helper	53 ± 5	27 ± 8	27 ± 11
T suppressor	23 ± 1	52 ± 6	56 ± 11

Cell populations were determined by using monoclonal antibodies to cell surface antigens [antibody T101 to the 65-kDa antigen (anti-T), anti-IgG (anti-B), anti-CD4, and anti-CD8; BioRad] of freshly isolated and cultured lymphocytes. Cells were cultured for 8 days either in the absence of ethanol or with 200 mM ethanol added for the last 48 hr of culture.

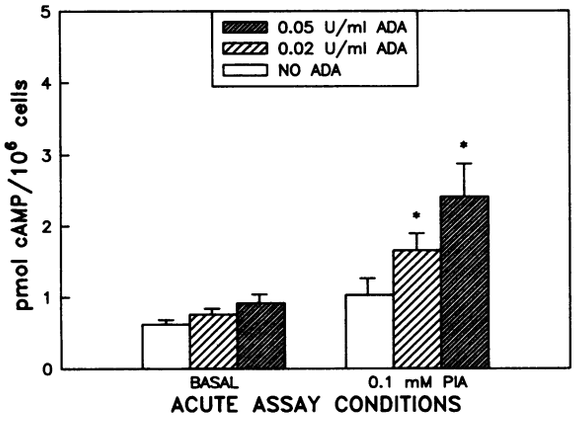

FIG. 1. Effect of adenosine deaminase (ADA) in cell culture media on adenosine receptor-stimulated cAMP levels. Cells were grown for 8 days without adenosine deaminase (*n* = 4) or with 0.02 unit (U) (*n* = 6) or 0.05 unit (*n* = 4) of adenosine deaminase per ml and were assayed for adenosine receptor-stimulated cAMP levels as described. *, $P < 0.04$ (compared with cells grown without adenosine deaminase).

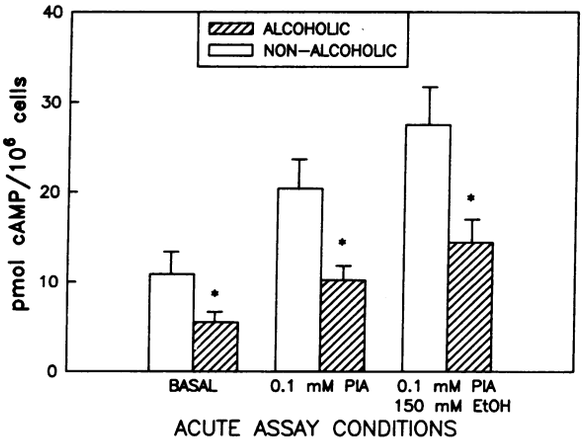

FIG. 2. Adenosine receptor-stimulated cAMP levels in freshly isolated lymphocytes from alcoholic and nonalcoholic subjects. Lymphocytes from alcoholic (n = 7) and nonalcoholic (n = 9) subjects were assayed as described. *, $P < 0.006$ (compared with nonalcoholic subjects).

Chronic Exposure to Ethanol. Addition of 200 mM ethanol to the medium for 48 hr significantly reduced adenosine receptor-stimulated cAMP production by 66% in cultured lymphocytes from nonalcoholic subjects (Fig. 4). Basal and receptor-stimulated cAMP levels in the presence of ethanol also were reduced significantly. Similar reductions in cAMP levels also were observed in cells from alcoholic subjects after 48 hr of exposure to 200 mM ethanol (data not shown). The decreases in both cell types were reversible; cells recovered their initial response to adenosine stimulation within 24 hr after the removal of ethanol (data not shown). The ethanol-induced reduction in cAMP levels in cells from nonalcoholic subjects was not due to changes in lymphocyte population (Table 1), cell growth, or activity of phosphodiesterase. Activity of the high-affinity phosphodiesterase in lymphocytes from nonalcoholic subjects cultured without ethanol was 7.7 ± 0.9 pmol of cAMP hydrolyzed per mg of protein per min. After exposure to 200 mM ethanol for 48 hr, the activity was 9.5 ± 1.1 (n = 6; P = 0.22). Activity of the low-affinity phosphodiesterase was 52.8 ± 25.0 pmol of cAMP per mg of protein per min in cells cultured without ethanol and 60.0 ± 15.8 (n = 5; P = 0.81) after chronic exposure to ethanol.

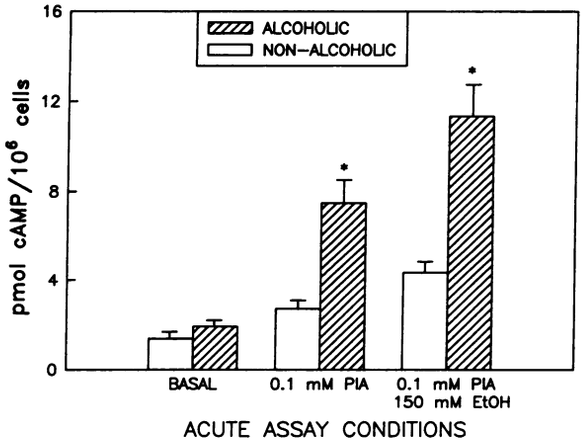

FIG. 3. Adenosine receptor-stimulated cAMP levels in cultured lymphocytes from alcoholic and nonalcoholic subjects. Lymphocytes from alcoholic (n = 7) and nonalcoholic (n = 9) subjects were grown for 7 days in the absence of ethanol and assayed as described. *, $P < 0.001$ (compared with nonalcoholic subjects).

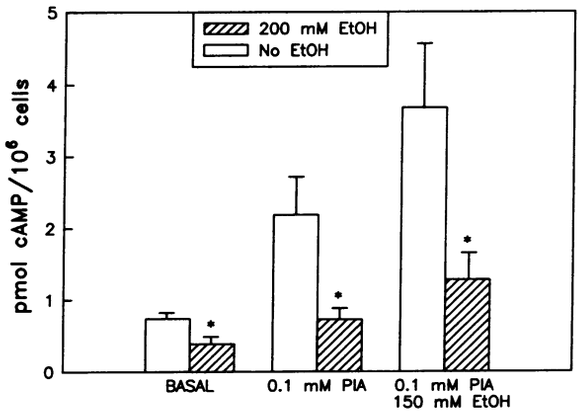

FIG. 4. Cells from nonalcoholic subjects chronically exposed to 200 mM ethanol for 48 hr. Lymphocytes from nonalcoholic subjects (n = 7) were grown in culture for 8 days in the absence of ethanol; 200 mM ethanol was added to half of the culture flasks for the last 48 hr, and adenosine receptor-stimulated cAMP levels were determined. *, $P < 0.01$ for basal, 0.009 for phenylisopropyladenosine (PIA), and 0.02 for the latter agent with ethanol (compared with cells grown without ethanol).

To determine whether there is also a difference between lymphocytes from alcoholic and nonalcoholic subjects in their response to chronic exposure to ethanol, we grew cells for 7 days in the absence of ethanol and then challenged them with lower ethanol concentrations for shorter times. After treatment with 100 mM ethanol for 24 hr, phenylisopropyladenosine-stimulated cAMP levels were reduced by 39% in lymphocytes from alcoholic subjects, whereas cells from nonalcoholic subjects showed no response (Fig. 5). Basal cAMP levels were unaffected in both groups.

DISCUSSION

Sustained intake of ethanol can produce physical dependence in man and animals. When lymphocytes from nonalcoholic subjects are grown in defined medium without ethanol and subsequently treated with 200 mM ethanol for 48 hr, adenosine receptor-stimulated cAMP levels decreased by 66%. These results in culture reproduce the suppressed cAMP

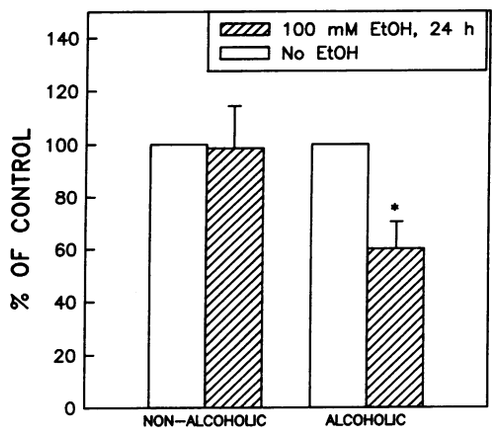

FIG. 5. Difference in sensitivity to chronic ethanol exposure between cells from alcoholic and nonalcoholic subjects. Lymphocytes from alcoholic (n = 7) and nonalcoholic (n = 9) subjects were grown in culture for 7 days; 100 mM ethanol was added to half of the culture flasks for the last 24 hr, and phenylisopropyladenosine-stimulated cAMP levels were determined. Values represent cAMP levels in ethanol-treated cells as a percentage of cAMP levels in cells grown without ethanol. *, $P < 0.003$ (compared with nonalcoholic subjects).

levels observed in freshly isolated lymphocytes from alcoholic subjects (13). Therefore, we conclude that the suppression in cAMP levels in alcoholic subjects is due in part to a direct effect of ethanol.

Similar decreases in receptor-dependent cAMP levels after chronic ethanol treatment have been described for stimulation of the adenosine receptor in NG108-15 neural cells (8, 9), the β-adrenergic receptor in both mouse S49 lymphoma cells (6), and cultured human lymphocytes (unpublished observations), and the prostaglandin E_1 receptor in N1E (7) and NG108-15 neural cells (9). These data suggest that heterologous desensitization and/or down-regulation of hormone receptors coupled through G_s may be a common mechanism of adaptation to chronic exposure to ethanol (9, 10, 12).

Alterations in adenosine receptor-dependent cAMP levels may be of particular importance in the development of dependence and tolerance because of evidence that adenosine, acting through its membrane receptor, may mediate some of the effects of ethanol in the central nervous system (26, 27). Lymphocytes release adenosine (22, 23), and we find that endogenously released adenosine appears to regulate the response of the adenosine receptor system in cultured lymphocytes (Fig. 1). In other systems, adenosine has also been shown to cause homologous and heterologous desensitization of membrane receptors (24, 25). Therefore, if ethanol acutely increased the accumulation of extracellular adenosine, the chronic effects of ethanol could involve adenosine-dependent desensitization and/or down-regulation of the adenosine receptor system.

There is compelling evidence that genetic factors play a role in the development of alcoholism (20). Since the cAMP signal transduction system is altered by ethanol, genetic changes affecting this system might lead to a susceptibility to alcoholism. We determined that lymphocytes from alcoholic subjects, after growth for four to six generations without ethanol, do exhibit differences in signal transduction. Cells from alcoholic subjects not only recover their responsiveness to adenosine receptor stimulation when grown in the absence of ethanol but also have a 2.8-fold greater stimulation of cAMP accumulation by phenylisopropyladenosine than do lymphocytes cultured from nonalcoholic subjects. In addition to this difference in the absence of ethanol, lymphocytes from alcoholics are also more sensitive to ethanol than are nonalcoholic subjects. After challenge with only 100 mM ethanol for 24 hr, lymphocytes from alcoholic subjects exhibit a 39% decrease in adenosine receptor-stimulated cAMP levels, whereas lymphocytes from nonalcoholic subjects show no change under these conditions. Since these differences have been observed after growth in the absence of ethanol, it is possible that they reflect genetic differences between alcoholic and nonalcoholic subjects.

Goldstein and Goldstein (28) have proposed that drug dependency develops as a cell or organism makes homeostatic adjustments that compensate for the primary effect of a drug. In the case of ethanol, acute stimulation of cAMP levels by ethanol appears to be countered by a chronic decrease in receptor-stimulated cAMP levels (6–9). In the presence of acute ethanol and phenylisopropyladenosine, cells from alcoholic subjects exhibit a 2.8-fold increase in cAMP levels compared to cells from nonalcoholic subjects (Fig. 3). It is therefore possible that these higher amounts of cAMP could cause the cells of alcoholic subjects to adapt at lower concentrations of ethanol (Fig. 5).

Our results suggest that suppressed adenosine receptor-stimulated cAMP levels in freshly isolated lymphocytes from alcoholic subjects are caused by long-term exposure to ethanol. In addition, a genetically determined alteration in cAMP signal transduction could contribute to this response. It should be possible to determine which component(s) of the signal transduction pathway (receptors, G-protein, adenylate cyclase, or cAMP-dependent protein kinase) is altered by chronic exposure to ethanol. In addition, by growing lymphocytes in culture for many generations without ethanol, it will also be possible to identify which component(s) of the adenosine receptor-mediated cAMP signal transduction system is genetically altered in alcoholic subjects.

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