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Alcohol and related dietary effects on mouse natural killer-cell activity

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Summary. Natural killer (NK) cell activity of spleen cells from female C57BL/6 mice receiving 10% w/v alcohol solution for 4 weeks was studied in mice fed a nutritionally complete crystalline amino-acid diet and in mice fed diets moderately deficient in (i) tyrosine and phenylalanine or (ii) methionine. Natural killer cell activity was determined in a 4-hr cytolytic chromium-release assay against YAC-1 lymphoma cells. Alcohol consumption did not effect NK cell-mediated lysis irrespective of nutritional status; however, NK-cell activity was depressed in mice fed the tyrosine- and phenylalanine-deficient diet and was enhanced in mice fed the methionine-deficient diet. These data suggest that the changes in immune function often observed in alcoholics may be more closely linked to dietary and nutritional status than to the direct effects of the ingested alcohol.

INTRODUCTION

Alcoholics frequently show impaired humoral and cell-mediated immunity (Björkholm, 1980; Smith *et al.*, 1980; MacGregor, Gluckman & Senior, 1978; Lundy *et al.*, 1975), predisposing them to infectious diseases and cancer (Di Luzio & Williams, 1980; Gluckman, Dvorak & MacGregor, 1977; Smith & Palmer, 1976). Since alcoholics are often malnourished, it is unclear whether these immunological abnormalities result from generalized malnutrition, malabsorption of specific nutrients, or are the consequence of direct alcohol exposure (Bollet & Owens, 1973; Leevy, Thompson & Baker, 1970; Majumdar *et al.*, 1981; Neville *et al.*, 1968; Shaw & Lieber, 1978; Yew, Moore & Biesele, 1981). Several studies indicate that nutritional deficiencies alter both humoral and cell-mediated immune function (Bounous & Kongshavn, 1982; Gross & Newberne, 1980; McMurray *et al.*, 1981). Cell-mediated immunity is specifically enhanced by generalized protein deficiency and by certain essential amino-acid deficiencies (Bounous & Kongshavn, 1978; Good *et al.*, 1976; Kramer & Good, 1978; Jose & Good, 1973 a,b).

Natural killer (NK) cells provide an important host defence against cancer growth and metastasis (Hanna, 1982; Herberman & Ortaldo, 1981; Talmadge *et al.*, 1980). Some reports indicate an increased NK activity in human alcoholics (Saxena, Mezey & Alder, 1980), whereas others report normal activity (Ericsson *et al.*, 1980). When added to the *in-vitro* assay of NK-cell activity, lower alcohols inhibit cytotoxicity against YAC-1 lymphoma tumour cells (Ristow, Starkey & Hass, 1982). Reasons for the varying effects of alcohol on natural killer cell activity are not fully known, but may be related to the alcoholics' nutritional status, use and abuse of other drugs, and to other underlying diseases. In any event, these variables complicate

Abbreviations: CM, complete medium; E:T, effector:target; NK, natural killer.

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evaluation of the direct effects of alcohol on NK-cell activity.

In the present study, we have assessed the effect of moderate alcohol intake on natural killer-cell activity in order to eliminate impaired food intake as a variable, and to define more clearly the relationship between alcohol and nutritional status. Since alcohol lowers the blood level of tyrosine, phenylalanine, and methionine (Eriksson *et al.*, 1980; Siegel, Roach & Pomeroy, 1964) and since these deficiencies are reported to enhance delayed hypersensitivity (Bounous & Kongshavn, 1978), we also examined the effect of alcohol intake on NK-cell activity in mice fed diets selectively deficient in (i) tyrosine and phenylalanine and (ii) methionine.

MATERIALS AND METHODS

Mice and experimental diets

Female, C57BL/6 mice, 7–9 weeks old, were obtained from the breeding colony at the College of Veterinary Medicine Vivarium, Washington State University. Mice were bred from pairs originally obtained from The Jackson Laboratories, Bar Harbor, Maine, U.S.A. Experimental mice were single-housed and maintained on a 12 hr light–12 hr dark cycle at 22–24° and 60% humidity. They were fed either a nutritionally adequate synthetic crystalline amino-acid diet (basal diet) or diets deficient in tyrosine and phenylalanine or in methionine. The composition of the basal diet was previously reported, and this diet supports both growth and reproduction of mice (Meadows *et al.*, 1982). Briefly, the diet provides 3·86 Cal/g and consists of 15·7% amino acids, 10% fat, 61·9% carbohydrates, and 5% fibre. The tyrosine- and phenylalanine-deficient diet is similar to the basal diet but contains 0·04% tyrosine and 0·08% phenylalanine compared to 0·3 and 0·6% respectively in the basal diet. The methionine-deficient diet contains 0·1% methionine compared to 0·4% in the basal diet, and is cystine- and serine-free. Both deficient diets were rendered isonitrogenous to the basal diet by altering glycine and glutamic acid content. The experimental diets were obtained from BioServ Inc., Frenchtown, NJ, U.S.A.

Experimental design

Mice fed the basal diet were divided equally into two control groups and one alcohol group. The alcohol group was given 10% w/v alcohol solution *ad libitum* as its sole source of fluid and allowed free access to the diet. One control group was pair-fed the basal diet and an isocaloric glucose solution, and the other group was fed the basal diet and water *ad libitum*. In the amino acid-deficient diet experiments, all mice were allowed free access to the deficient diets and water or 10% w/v alcohol solution. Fluid and food consumption and body weight of each mouse were recorded daily. Blood alcohol levels were determined weekly at 900–1100 hours using the Sigma alcohol analytical kit number 332-UV. The method is based on oxidation of alcohol in the presence of NAD and alcohol dehydrogenase to acetaldehyde. The resulting NADH is then measured spectrophotometrically. Mice were killed at the end of 4 weeks by cervical dislocation, and their spleens were removed aseptically for use in the NK assay.

Preparation of effector cells

Spleen cells from 3–5 mice per group were pooled and used as effector cells. Spleen-cell suspensions were obtained by teasing the spleens on a sterile stainless steel fine screen into complete medium (CM) consisting of RPMI 1640 (Gibco Laboratories, Grand Island, NY, U.S.A.), 10% heat-inactivated foetal bovine serum (Sterile Systems Inc., Logan, UT, U.S.A.), 25 mM HEPES buffer, and 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cell aggregates were dispersed by pipetting, and the erythrocytes were lysed by a 4-sec exposure to sterile distilled water. Spleen-cell suspensions in CM were applied to 10-ml sterile columns of Fenwal scrubbed nylon wool, type 200, to remove B cells and macrophages (Julius, Simpson & Herzenberg, 1973). After 45 min of incubation on the nylon-wool columns, the non-adherent cells were recovered by eluting with CM.

Chromium release assay for NK-mediated cytolysis

The non-adherent cells recovered from the nylon-wool columns were centrifuged, resuspended in CM to give 1×10^7 cells/ml, and dispensed into Falcon Microtest III plates in 100-μl aliquots per well. Target YAC-1 lymphoma cells were labelled by incubation in 1 ml of CM containing 200 μCi sodium ^{51}chromate for 45 min at 37°. The labelled cells were washed three times with CM and incubated in large volumes of CM for 30 min between washes. The cells were centrifuged and the cell pellet was resuspended in CM to give 1×10^5 cells/ml. The suspensions were dispensed in 100-μl aliquots into the Microtest III plates containing 100 μl of spleen cells per well. The plates were incubated at 37° for 4 hr and then centrifuged at 200 *g* for 10 min at 4°; 100 μl of the supernatant from each well was removed and

counted in a gamma counter (Beckman Gamma 8000). Spontaneous release was determined by culturing the labelled target cells in CM without the effector cells, and the total available counts were obtained from target cells lysed with 2% sodium dodecyl sulphate. The following formula was used to compute % lysis: [(test c.p.m. − spontaneous c.p.m.)/(total c.p.m. − spontaneous c.p.m.)] × 100.

Statistical analysis

The data were analysed using analysis of variance. A Duncan's multiple range test was used to determine significant differences between experimental groups (Steel & Torrie, 1980).

RESULTS

Nutritional data

The relevant data on nutritional status of control and alcohol-consuming mice are presented in Table 1. Body weight of mice fed the basal diet increased 10·3 ± 2·0% (mean ± SEM) in both control and alcohol-consuming groups during the 4-week experiment. The tyrosine- and phenylalanine-deficient diet caused a decrease in body weight ranging from 10·8 to 12·0%, while mice fed the methionine-deficient diet lost 7·9 to 12·1% of their starting weight. The weight loss occurred during the first week of feeding the deficient diets and then mice remained relatively constant in weight thereafter. Equivalent changes occurred in the alcohol-consuming groups. Mice fed deficient diets consumed the same or a greater amount of food compared to those fed the basal diet, and intake was relatively unaffected in alcohol-consuming mice. Food consumption was slightly lower in the alcohol groups fed the basal or tyrosine- and phenylalanine-deficient diet compared to their respective *ad libitum* control groups; however, the decrease is within the limits of variability observed in other experiments (unpublished data). Clearly, mice are well nourished irrespective of alcohol consumption or specific amino-acid deficiency.

Table 2 shows the alcohol intake and blood alcohol level of mice fed the basal, tyrosine- and phenylalanine-deficient, and methionine-deficient diets. Alcohol intake increased after the first week and reached its highest level at 4 weeks in mice fed the basal diet. Intake also tended to increase in mice fed the tyrosine- and phenylalanine-deficient and methionine-deficient diets, but the values at 1 and 4 weeks do not differ significantly ($P > 0·05$). Overall, alcohol intake was

Table 1. Food and caloric intake and body weight of control and alcohol-consuming mice fed nutritionally adequate and amino acid-deficient diets for 4 weeks*

Diet group	Body weight† (g)		Diet intake (g/mouse/day)	Total caloric intake (cal/mouse/day)
	Initial	Final		
Basal				
Alcohol	19·1 ± 0·2	21·8 ± 0·3	2·5 ± 0·1	11·1 ± 0·5
Pair-fed control	17·3 ± 0·2	18·5 ± 0·6	2·3 ± 0·2	10·5 ± 0·6
Ad libitum control	18·4 ± 0·2	20·2 ± 0·3	2·9 ± 0·2	11·3 ± 0·4
Tyrosine- and phenylalanine-deficient				
Alcohol	18·7 ± 0·1 (21·5 ± 0·1)	18·1 ± 0·2	2·3 ± 0·1	9·5 ± 0·4
Ad libitum control	18·8 ± 0·2 (21·0 ± 0·1)	17·5 ± 0·2	2·5 ± 0·1	8·7 ± 0·4
Methionine-deficient				
Alcohol	21·8 ± 0·2 (23·6 ± 0·2)	21·8 ± 0·3	3·0 ± 0·1	13·2 ± 0·6
Ad libitum control	18·8 ± 0·2 (21·4 ± 0·1)	19·1 ± 0·3	3·1 ± 0·2	11·9 ± 0·7

* Mean ± SEM over the entire 4-week test period for three to five mice per group.

† Mean ± SEM during the first week of alcohol consumption (initial weight) and during the last week of the experiment (final weight). Values in parentheses represent mean weight ± SEM over the 4-day period preceding administration of the deficient diets.

Table 2. Alcohol intake and blood alcohol levels of mice fed the nutritionally adequate and amino acid-deficient diets for 4 weeks

Diet	Alcohol intake* (g/kg body weight/day)		Blood alcohol level* (mg/100 ml)	
	Week 1	Week 4	Week 1	Week 4
Basal	$10{\cdot}4 \pm 0{\cdot}6^{c}$	$12{\cdot}5 \pm 0{\cdot}5^{a,b}$	$93{\cdot}2 \pm 28{\cdot}5^{e,f}$	$18{\cdot}3 \pm 1{\cdot}8^{g}$
Tyrosine- and phenylalanine-deficient	$7{\cdot}6 \pm 0{\cdot}7^{d}$	$9{\cdot}6 \pm 0{\cdot}7^{c,d}$	$118{\cdot}6 \pm 37{\cdot}4^{e}$	$13{\cdot}2 \pm 1{\cdot}5^{g}$
Methionine-deficient	$11{\cdot}5 \pm 0{\cdot}9^{a,b,c}$	$13{\cdot}7 \pm 0{\cdot}7^{a}$	$64{\cdot}1 \pm 12{\cdot}6^{f}$	$14{\cdot}3 \pm 0{\cdot}9^{g}$

* Mean ± SEM; values with the same superscript(s) are not significantly different from each other ($P > 0{\cdot}05$).

slightly lower in mice fed the tyrosine- and phenylalanine-deficient diet than in the other dietary groups throughout the experimental period. Mice showed relatively high blood alcohol levels between 64 and 119 mg/100 ml among all dietary groups when determined after 1 week, but the levels were variable within each group. On the second week of the experiment (data not shown in Table 2), blood alcohol levels markedly decreased to $8{\cdot}7 \pm 1{\cdot}2$, $11{\cdot}1 \pm 1{\cdot}4$ and $9{\cdot}0 \pm 1{\cdot}0$ mg/100 ml in mice fed the basal, tyrosine- and phenylalanine-deficient, and methionine-deficient diets respectively. Blood alcohol levels were not significantly different ($P > 0{\cdot}05$) within the three dietary groups at two and four weeks.

NK-cell activity of spleen cells derived from control and alcohol-consuming mice

The levels of NK cell-mediated cytotoxicity in control and alcohol-consuming mice fed the basal diet were not influenced by alcohol consumption (Fig. 1), and the cytolytic activity at all effector:target (E:T) ratios was not significantly different between the groups ($P > 0{\cdot}05$). To study the effect of dietary amino-acid restriction on NK-cell activity, mice were fed a tyrosine- and phenylalanine-deficient diet or a methionine-deficient diet for 4 weeks (Fig. 2). The combined tyrosine and phenylalanine deficiency significantly decreased NK-cell activity by about 26% at an E:T ratio of 100:1 compared to the basal diet control group ($P < 0{\cdot}05$). Lower activity was also observed at the 50:1 and 25:1 E:T dilutions, but the values did not differ significantly ($P > 0{\cdot}05$). On the other hand, methionine deficiency significantly increased NK-cell

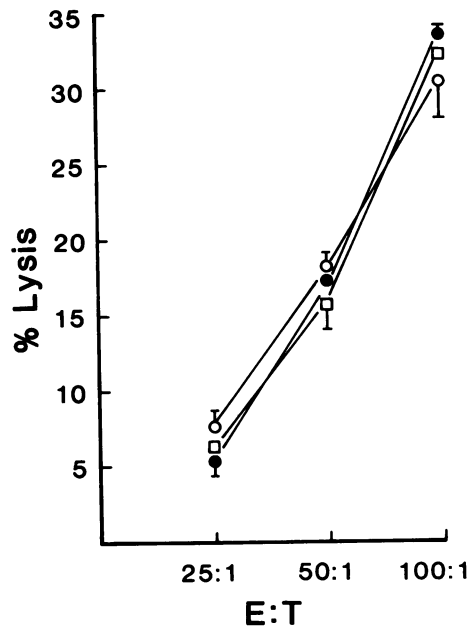

Figure 1. NK activity of spleen cells from control and 4-week alcohol-consuming mice fed the basal diet. Spleen cells (effector cells, E) pooled from three mice per group were passed over nylon-wool columns and tested in a 4-hr cytolytic assay against YAC-1 lymphoma cells (target cells, T). Values represent the mean ± SEM of quadruplicate samples from one experiment. This study was repeated three times with similar results. (○) Received 10% w/v alcohol solution and the basal diet *ad libitum*; (●) was pair-fed the basal diet and an isocaloric glucose solution; (□) received water and the basal diet *ad libitum*.

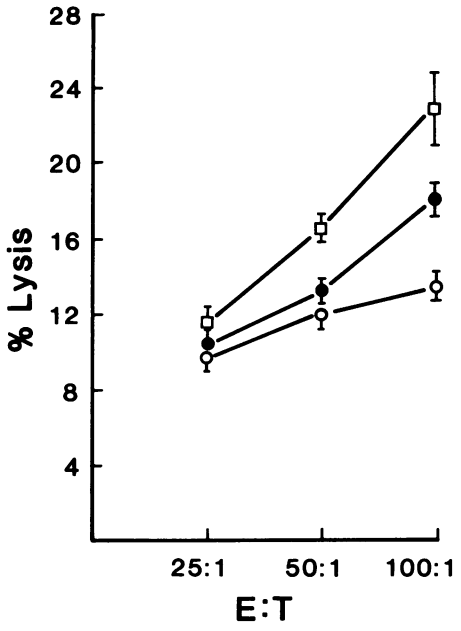

Figure 2. Effect of dietary amino-acid deficiency on NK-cell activity. The experimental conditions were identical to those described in Fig. 1. Values represent the mean ± SEM of quadruplicate samples from one experiment, and the study was repeated twice with similar results. (●) Was fed the basal diet; (○) was fed the tyrosine- and phenylalanine-deficient diet; and (□) was fed the methionine-deficient diet.

activity ($P < 0{\cdot}05$) at E:T ratios of 100:1 (by 25%) and of 50:1 (by 24%) compared to the basal diet group, but the difference was not significant at the 25:1 E:T dilution ($P > 0{\cdot}05$). Administration of alcohol to mice fed either the tyrosine- and phenylalanine-deficient diets or the methionine-deficient diets did not influence NK-cell activity (data not shown).

DISCUSSION

In this report we examined the interplay between moderate alcohol intake and nutritional status and found no effect of alcohol on natural killer-cell activity in mice that were well nourished. These data corroborate the findings of Ericsson *et al.* (1980), who observed normal NK cell activity in a group of disease-free, well fed chronic alcoholics. A major finding was that dietary tyrosine and phenylalanine restriction decreased, while methionine restriction increased, NK-cell activity. Moreover, alcohol ingestion in mice fed these deficient diets did not affect NK cell activity. Clearly, moderate alcohol intake has no effect on natural killer cell-mediated cytolysis in mice.

Blood alcohol levels were initially high in all dietary groups during the first week, they fell during the second week, and remained low throughout the remainder of the experiment, even though alcohol intake did not change or increased. Alcohol is known to activate its own metabolism and to accelerate its own blood clearance through stimulation of alcohol-metabolizing enzymes (Bleyman & Thurman, 1979; Lieber & DeCarli, 1972; Misra *et al.*, 1971). Female C57BL/6, chosen because they voluntarily consume high levels of alcohol (Unwin & Taberner, 1980), appear to metabolize alcohol readily (Kakihana, 1979). Further studies to examine higher alcohol intakes on NK-cell activity are planned, but the success of this approach will hinge upon the ability of mice to maintain adequate food intake under these circumstances.

Most studies involving the effects of nutrition on immunity have utilized moderate and severe protein restriction, while only a few studies have addressed the effect of specific amino-acid deficiency. One such study showed that cell-mediated immunity is enhanced by moderate protein restriction, but that it is depressed by severe dietary protein deprivation (Cooper, Good & Mariani, 1974). Few studies have carefully examined the potential interactions between alcohol and nutritional status on immunity. In our study we found that feeding mice diets deficient in (i) tyrosine and phenylalanine and (ii) methionine altered NK-cell activity, but that alcohol intake superimposed on dietary deficiency did not further effect this response. These data suggest that the alterations in immunity observed in alcoholics are more closely related to nutritional status than to direct activity of alcohol.

The levels of tyrosine, phenylalanine and methionine restriction used in this study are known to depress serum blocking and haemagglutinin antibody formation (Jose & Good, 1973a), and to increase cell-mediated immunity as determined by the foot-pad assay (Bounous & Kongshavn, 1978). Feeding mice a diet restricted in phenylalanine to 0·08% moderately reduces complement dependent and independent cytotoxicity of splenocytes against sheep erythrocytes and irradiated L1210 leukaemia cells, but confers resistance of host defences to irradiation (Pine, 1981). Moderate alcohol intake in combination with tyrosine and phenylalanine deficiency would also be expected to exhibit similar responses as well as moderately decrease NK-cell activity.

Methionine enhancement of NK-cell activity is interesting because methionine and other lipotrope deficiencies appear in alcoholics and further interact with alcohol in the development of cirrhosis (Rogers, Fox & Murphy, 1981). Although the underlying reason(s) for the increased NK-cell activity in human alcoholics reported by Saxena *et al.* (1980) is not known, our data suggest that methionine deficiency may be related to this increase.

By studying the relationships between alcohol and other nutritional factors it may be possible in the future to provide specific nutrients such as tyrosine and phenylalanine to ensure immune competency in alcoholics.

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