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Research Article

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In Vitro Myelosuppression and Immunosuppression by Ethanol

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ABSTRACT Concentrations of ethanol similar to those in the blood of intoxicated patients suppressed phytohemagglutinin- or streptolysin O-induced lymphocyte transformation, and inhibited bone marrow granulocyte colony growth in soft agar. Inhibition of lymphocyte transformation and granulocyte colony growth occurred despite the presence of large concentrations of folate and other vitamins. These in vitro findings may relate to in vivo effects of ethanol on myeloid and lymphoid tissue.

INTRODUCTION

Preliminary studies suggested that ethanol might have a toxic effect in vitro on human bone marrow cells and lymphocytes (1–3). The current findings extend that preliminary information, using concentrations of ethanol such as may occur in heavily intoxicated alcoholic individuals.

METHODS

Subjects. Blood and bone marrow specimens were obtained from patients on the general medical and hematology wards. The patients studied included individuals with diabetes mellitus, alcoholism, infection, or neoplasm, plus normal volunteers. None of the patients were receiving myelosuppressive therapy.

Lymphocyte collection and culture. Heparinized blood specimens (collected in 60-ml syringes containing heparin, 0.75 mg heparin/ml blood) were allowed to sediment in the original syringe placed nozzle up at a 45° angle at room temperature for 2 h. The leukocyte-rich plasma was then expressed through a Butterfly-19 infusion tube (Butterfly-19 infusion set, 4590, Abbott Scientific Products Div., Abbott Laboratories, South Pasadena, Calif.), by pressing the plunger of the syringe, into a 500 ml sterile bottle containing an equal volume of culture medium; the solution was then percolated through a sterile absorbent cotton (Acme Cotton Products Co., Inc., Valley Stream, N. Y.) or a Fenwal FT-242 scrubbed nylon fiber column (Fenwal Inc., Walter Kidde & Co. Inc., Ashland, Mass.) 12 × 1.4 cm, loosely packed, at 37°C. The yield of lymphocytes was between 25 and 50% with a lymphocyte purity of 80–90%, and 95% viability as measured by trypan blue dye exclusion. The eluted lymphocytes were washed once with 10-ml portions of the medium employed for that experiment. The washed lymphocytes were then cultured in 16 × 125-mm Falcon (Falcon Plastics, Oxnard, Calif.) plastic tubes (10^6 cells/ml) in 3 ml of pH 7.4, 0.06 M Tris-buffered Hanks balanced salt solution (containing 200 U/ml each of penicillin and streptomycin), a solution of 20 amino acids (4) plus 13 mg L-serine and 28 mg glycine/liter, plus 200 mM/liter L-glutamine (THAA),[1] plus 10% autologous serum or fetal calf serum. Alternatively, incubation was in RPMI 1640 (Roswell Park Memorial Institute 1640 incubation medium, purchased from Microbiological Associates, Inc., Bethesda, Md.) + 200 mM/liter L-glutamine + 200 U/ml of penicillin and streptomycin containing 10% serum. Difco phytohemagglutinin-P (PHA-P) (Difco Laboratories, Detroit, Mich.) was reconstituted with 5 ml THAA and 0.1 ml of the product was added to each tube except for PHA-P controls. Streptolysin O (SLO) (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) was reconstituted with 10 ml THAA, and 0.1 ml was added to each culture. Ethanol (alcohol, dehydrated analyzed reagent, U. S. Industrial Chemicals Co., Tuscola, Ill.) in varying concentrations was added to cultures which were then incubated at 37°C for 3 days, in a 7.5% CO_2-92.5% air incubator, which maintained pH unchanged, after which 0.1 ml tritiated thymidine (^{3}HTdR), 10 μCi/ml (sp act = 26 Ci/mM; Amersham/Searle Corp., Arlington Heights, Ill.) was added, and incubation continued for another 4 h. The cells were then transferred to glass tubes, centrifuged at 1500 rpm for 10 min, and the supernate discarded. 2 ml of the 10% cold (4°C) trichloroacetic acid (TCA) was added to the cell pellet, and the acid insoluble precipitate dissolved in 0.5 ml of hydroxide of hyamine (Packard Instrument Co., Inc., Downers Grove, Ill.) plus 15 ml of liquid scintillation fluid (2,5-diphenyloxazole [PPO, 17.5 g] + 1,4-bis[2-(5-phenyloxazolyl)]benzene [POPOP, 1.5 g] (Packard Instru-

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[1] *Abbreviations used in this paper:* ^{3}HTdR, tritiated thymidine; M/E, myeloid/erythroid ratio; PGA, pteroylglutamic acid; PHA-P, phytohemagglutinin; SLO, streptolysin O; THBSS, Tris-buffered Hanks-balanced salt solution; THAA, THBSS plus 20 added amino acids.

ment Co., Inc.) + 875 ml ethanol + 2,626 ml toluene [Fisher Scientific Co., Inc., Pittsburgh, Pa.]) and counted in a Beckman LS-250 refrigerated liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Morphologic evidence of mitogen-induced lymphocyte transformation was obtained by microscopic examination. Study of 3-day incubated cells, both in the presence and absence of ethanol, revealed no apparent toxicity due to presence of ethanol. Trypan blue studies after PHA-P were difficult to interpret due to clumping. However, there was no apparent difference between control cells and cells cultured in the presence of ethanol (concn. = 300 mg/100 ml).

Short-term bone marrow culture. The techniques employed were essentially those previously used in this laboratory (5, 6). 10–20 ml of bone marrow (of which most, after the 1st ml, was peripheral blood) was aspirated from the posterior superior iliac spine into a syringe containing 10 ml of pH 7.4, 0.06 M Tris-buffered Hanks balanced salt solution containing 200 U/ml each of penicillin and streptomycin (THBSS) plus 100 mg of heparin. The cells were passed through a 50 mesh, 0.003 in. gauge wire screen and washed with a 10 ml portion of THBSS. The cells were then suspended in autologous serum-enriched (25%) THBSS and 0.3-ml portions added to 10-ml B-D Vacutainer tubes (Becton-Dickinson & Co., Rutherford, N. J.). Cell count ranged between 2 and 12×10^6/ml of final culture. 0.1-ml portions of varying concentrations of ethanol were added, and the final volume adjusted to 0.9 ml with THBSS. After 1 h incubation in a Dubnoff metabolic-shaker incubator at 37°C, 0.1 ml of ^{3}HTdR (10 μCi/ml, sp act = 25 Ci/mM) was added and incubation continued for 3 more h. After incubation the cells were washed in THBSS and then shock-lysed to remove hemoglobin (7). TCA at 3°C was added as in lymphocyte cultures and the radioactivity of the acid-insoluble material determined, as an index of incorporation of ^{3}HTdR into DNA.

Bone marrow culture in soft agar. The technique here employed using bone marrow cells is essentially that used by Kurnick and Robinson (8) for culture of peripheral blood cells. 5–10 ml of bone marrow is aspirated into a heparin-wet syringe. Red cells were allowed to sediment in the original syringe standing on its plunger at room temperature for 2 h. The cell-rich plasma was then expressed and centrifuged, and the cells were washed three times in 10-ml portions of McCoys 5A medium. Peripheral blood drawn at the same time was similarly allowed to sediment. The leukocyte-rich plasma was separated and its cells used as a source of colony-stimulating factor, as had been done by Kurnick and Robinson (8). To 35-mm tissue culture dishes (Falcon Plastics) is added 1 ml underlayer containing 10^6 peripheral leukocytes, suspended in 0.5% agar in McCoys 5A medium with 15% fetal calf serum. After gelling, a 1 ml overlay containing 200,000 washed bone marrow cells, 0.3 agar and McCoys 5A medium plus 15% fetal calf serum was poured over the underlayer. Before gelling, ethanol in varying concentrations was added to the overlay; after gelling, the plates were incubated in a 100% humidity, 7.5% CO_2-92.5% air atmosphere for 10–14 days, which maintained pH unchanged. (Agar was prevented from drying out by placing the plates in a larger covered petri dish which contained a 35 mm uncovered water-containing dish.) Only colonies containing more than 50 cells were counted by use of a stereo microscope at 30 times magnification. In general, colonies consisted of both early and late granulocytes and macrophages. Many mitotic forms were present, and cell/colony varied between 50 and 1,500.

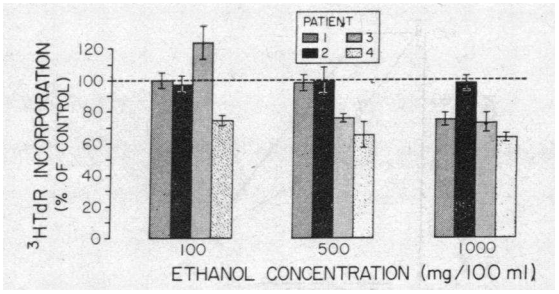

FIGURE 1 Effect of various concentrations of ethanol on incorporation of ^{3}HTdR into bone marrow DNA. Vertical lines = ±1 SD. Diagnosis: patients 1 and 2, iron deficiency anemia; 3, rheumatoid arthritis, patient 4, diabetes mellitus.

RESULTS

Experiments employing short-term (4 h) bone marrow cultures demonstrated significant ethanol inhibition of ^{3}HTdR incorporation into DNA only with large concentrations of ethanol (Fig. 1) (Controls ranged from 3–4 $\times 10^3$ cpm/10^6 cells). Bone marrow morphology from patients 1–4 varied only in the myeloid to erythroid (M/E) ratio: Patients 1 and 2 had M/E ratios of 1:1 and 1:2, patients 3 and 4 had M/E ratios of 6:1 and 4:1.

Colony growth in soft agar. Bone marrow colony growth in soft agar was sharply inhibited by concentrations of ethanol which are present in intoxicated patients. Fig. 2 reveals effects on colony count in the presence of ethanol. The addition of folinic acid (leucovorin, citrovorum factor) (10 μg/ml) to the already folate-containing McCoys 5A medium (McCoys 5A medium contains 10 mg/liter of pteroylglutamic acid [PGA]) did not reverse ethanol inhibition of colony growth (not shown in Figure). At the time colony counts were done, all colonies of any one patient consisted of either granulocytes alone or contained both granulocyte and macrophage elements.

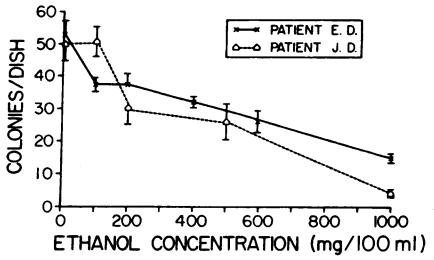

FIGURE 2 Inhibition by various concentrations of ethanol of bone marrow colony growth in soft agar of two different patients from noted in Fig. 1. Diagnosis: patient E. D., pancreatic carcinoma; patient J. D., diabetes mellitus. Underlay leukocytes were from each patient studied. Colony counts are the average of three dishes. Vertical lines = ±1 SD.

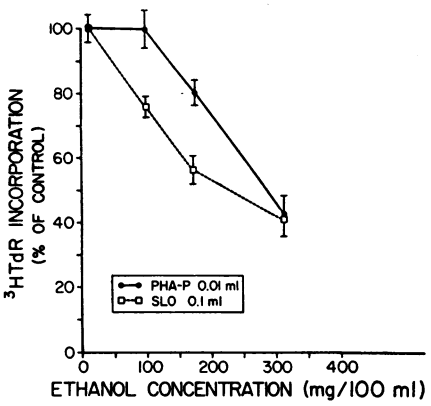

FIGURE 3 Ethanol inhibition of SLO- and PHA-P-induced lymphocyte transformation as measured by incorporation of ^{3}HTdR into lymphocyte DNA. Patient had diabetes mellitus. Culture medium was THAA plus 10% autologous serum. Vertical lines = ±1 SD.

Ethanol inhibition of lymphocyte transformation. Lymphocyte transformation response to PHA-P and SLO was markedly inhibited by ethanol (Fig. 3). (Control cpm/10^6 cells = 20×10^3 for PHA-P and 5×10^3 for SLO.)

Fig. 4 reveals lymphocyte response in counts per minute (cpm) of ^{3}HTdR incorporated into lymphocyte DNA at the end of 72 h. Note that ethanol was found again to be a potent inhibitor of lymphocyte transformation. Lymphocyte transformation using PGA-enriched medium (RPMI 1640 contains 1 mg/liter of PGA) was also inhibited by ethanol (Fig. 5).

Inhibition of granulocyte colony growth and of lymphocyte transformation was not overcome by adding to each milliliter of the incubation medium 10 μg of PGA, 2 μg of B_{12}, 0.5 μg pyridoxal HCl, and 0.5 μg pyridoxine HCl. The alcohol effect was not observed using isosmotic amounts of glucose.

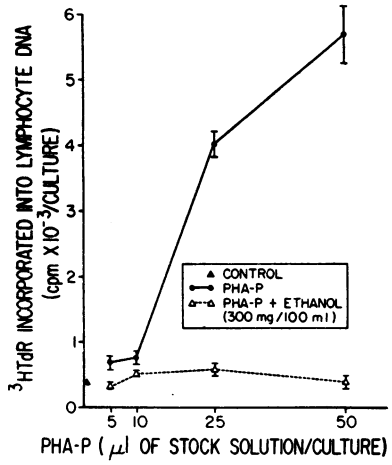

FIGURE 4 Ethanol inhibition of lymphocyte transformation induced by varying concentrations of PHA-P. Normal volunteer. Culture medium was RPMI 1640 plus 200 mM/liter L-glutamine plus 0.06 M Tris buffer pH 7.4, plus 10% fetal calf serum. Vertical lines = ±1 SD.

DISCUSSION

Alcohol ingestion has been associated with a number of hematologic disabilities (9–11) including anemia, thrombopenia, and leukopenia. The causes of anemia appear to be multifactorial including protein-calorie malnutrition, folate, B_6, and iron deficiency, liver disease and lipemia, and perhaps a direct toxic effect of ethanol on folate metabolism. Thrombopenia and leukopenia are usually attributed to splenomegaly secondary to portal hypertension or concomitant folate deficiency. Leukopenia in alcoholics without splenomegaly, significant liver disease or folate deficiency has suggested a direct toxic effect of ethanol on myelopoiesis (9, 10).

The alcoholic patient is susceptible to infection (12, 13). Contributing factors may include luekopenia, and the alcoholic's life style, i.e., "Bowery living." Guarneri and Laurenzi (13) have reported that ethanol has a toxic effect on pulmonary macrophage function. It is not known whether alcohol or one of its metabolites is the toxic agent. Since the monocyte and pulmonary macrophage have recently been reported to be sources of colony-stimulating factor (14–16), one posible ethanol effect may be to reduce the production of this factor by the leukocyte-rich underlayer of our bone marrow cultures in soft agar.

"Bone marrow culture on soft agar" as described by Kurnick and Robinson (8) is a good and sensitive technique for the study of myelopoiesis. The validity of using this technique for the study of the suppressive effect of drugs or other chemicals on myelopoiesis has also been demonstrated by Ogawa, Bergsagel, and McCulloch (21, 22).

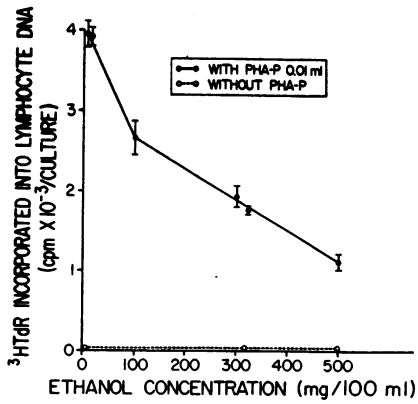

FIGURE 5 Ethanol inhibition of PHA-P-induced lymphocyte transformation. Culture medium was THAA plus 10% autologous serum. Normal volunteer. Vertical lines = ±1 SD.

The in vitro data here presented suggest that alcohol may suppress immunocompetence and inhibit myeloid tissue growth when the alcohol is present in concentrations present in the blood of chronic alcoholics, and that this suppression may not be overcome by enriching the incubation medium with up to 10 μg of folic acid/ml, 2 μg B_{12}/ml, and 0.5 μg pyridoxal HCl/ml. The question arises as to why the effects observed in vitro are not also rapidly observed in vivo. The answer may lie in part in the fact that the in vitro effects are observed after days of continuous exposure to the stated concentrations of alcohol. In vivo, such concentrations are rarely sustained over any continuous 24 h period.

T and B lymphocytes cooperate to express cellular and humoral immunity (17, 18). The inhibition of phytohemagglutinin-induced lymphocyte transformation here noted suggests that primarily T-cell function is depressed by ethanol ingestion.

In the current studies, suppressive effects on myeloid and lymphoid function occurred with in vitro concentrations of ethanol in the range of 200–300 mg/100 ml; such concentrations may occur in heavily intoxicated individuals. It should be noted that under the proposed national Uniform Act Regulating Traffic on Highways, the presence of 150 mg or more of ethanol/100 ml blood is the minimum standard for presuming drunkenness from blood ethanol evidence (19).

Colonies contained granulocytes alone, or both granulocytes and macrophages. Perturbation by ethanol of the normal proportions of each was not found. Therefore, it is possible that ethanol may inhibit macrophage as well as granulocyte production. Since macrophages may be involved in antigen processing (20), this, too, might contribute to suppression of immunocompetence in vitro.

The three in vitro effects of alcohol (inhibition of lymphocyte transformation, and inhibition of macrophage and granulocyte production) are all directed against immune defense. Whether they relate to susceptibility to infection present in vivo in chronic alcoholic patients, remains to be determined, as does the mechanism of the alcohol effect in vitro. The data presented here can be attacked on the grounds that the demonstrated myelosuppressive effect may be nonspecific, since decreased colony growth can be observed with a number of chemical compounds, some of which exhibit concentration-dependent suppression. However, alcohol is unlike these other compounds in that it is present in the intoxicated human body in concentrations such as here used in vitro. Furthermore, Ogawa et al. (21, 22) have recently reported that a system essentially identical to the Kurnick and Robinson (8) in vitro technique used in the current study is valid for the study of the suppressive effect of chemical compounds on myelopoiesis, in that it was capable of predicting drug response in vivo.

The fact that the concentrations of alcohol here used in vitro were similar to those present in vivo in intoxicated individuals does not rule out the possibility that the observed alcohol effect was nonspecific, but does suggest that even should the effect prove to be nonspecific (i.e., an osmotic or pH effect, for example), it would pertain in vivo as well as in vitro when there is similarity of alcohol concentration in both systems. Against osmotic effect as the explanation is the report that osmotic effect may actually enhance lymphocyte growth (23), and that isosmotic amounts of glucose were not inhibitory in the current study. Against pH effect is the fact that there was no measurable pH change in the medium during incubations.

It should be noted that there is also evidence supporting a toxic ethanol effect producing thrombocytopenia (24, 25).

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