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Impaired Lymphocyte Proliferative Response to Mitogen in Alcoholic Patients. Absence of a Relation to Liver Disease Activity

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Concanavalin A-induced lymphocyte proliferation was studied in 25 patients with alcoholic hepatitis or compensated alcoholic cirrhosis. Nine alcoholics without evidence of liver disease were also evaluated. A nonlinear correlation equation, which was natural logarithmic, was applied to individual dose-response proliferation curves and permitted comparisons between patient groups and controls. The proliferative response in all patient groups was significantly lower when compared to healthy controls and was independent of the presence or absence of liver disease. This suggests that some changes in immune function observed in alcoholics may be linked to the direct effects of alcohol on the immune system rather than to the associated liver disease.

LYMPHOPROLIFERATIVE RESPONSES to nonspecific mitogen stimulation have been employed to assess immunological function in healthy individuals as well as in patients with a variety of diseases. Mitogen-induced proliferation and, modulation of such reactivity, is subject to influence by multiple factors which would include the stimulating agent, the source and composition of the lymphocytes, culture conditions, and the immune status of the subject.[1,2]

Comparisons of lymphoproliferative responses between groups of disease-related patients and healthy subjects is difficult since the mitogen dose-response relationship is nonlinear. Most studies have used Student's *t* test to compare differences between groups at each mitogen dosage, however considerable individual variation exists in the magnitude of the response. In other approaches the optimal response of peripheral blood mononuclear (PBM) cells to increasing concentrations of a single mitogen is related to the response of the same PBM to a second mitogen.[3] Most frequently a single dose of mitogen is used which is derived from the peak proliferative response exhibited by control PBM.

Conflicting results have been reported on lymphocyte transformation assays in patients with alcoholic liver disease (ALD). Decreased proliferative responses to the mitogens phytohemagglutinin (PHA) and concanavalin (Con A) have been described in patients with alcoholic hepatitis (AH)[4–7] and in patients with compensated alcoholic cirrhosis (CAC) or fatty infiltration.[4,7,8] However, normal proliferative responses have also been reported in patients with AH,[9] CAC or fatty infiltration.[5,6,10] In a similar manner both decreased mitogen-induced proliferative responses[11] and normal responses[4,5,9] have been described in alcoholics without liver disease.

Recently a natural logarithmic relationship was described between mitogen stimulation and human lymphocyte response in a dose-response proliferation assay.[12] Application of a nonlinear correlation equation to individual proliferation curves may permit quantitative comparisons between groups of patients and controls.

We measured the proliferative response to increasing doses of Con A by PBM isolated from alcoholic patients and from healthy volunteers. Comparisons of the responses were accomplished using a natural logarithmic relationship equation.

PATIENTS AND METHODS

Study Groups

Twenty-five patients with ALD were studied. Fourteen patients (nine males and five females) were considered to have AH on the basis of clinical and laboratory findings to include the following criteria after at least 5 days in the hospital: Recent alcohol consumption in excess of 1 pint of whiskey per day or its alcohol equivalent; hepatomegaly; a total serum bilirubin ≥ 4 mg%; and abnormalities in at least two of the following parameters: (*a*) serum glutamic oxaloacetic transaminase (SGOT) more than 80 IU/ml (normal < 40 IU), (*b*) serum albumin ≤ 3 g%, or (*c*) prothrombin time ≥ 2 sec in excess of the control value. Coagulation deficiencies and/or the presence of ascites precluded percutaneous liver biopsy in all but four of these patients. In all cases where biopsy was accomplished, the clinical diagnosis of AH was confirmed by the presence of alcoholic hyalin, liver cell necrosis, and polymorphonuclear neutrophil infiltrate. Two of the biopsies revealed concomitant micronodular cirrhosis (Table 1).

Eleven alcoholic patients (eight males and three females) were diagnosed as having CAC following liver biopsy in which features of a superimposed hepatitis were not identified (Table 1).

Nine AWLD (all males) were studied to determine the influence of recent chronic alcohol ingestion on PBM function. These patients were selected from the inpatient alcohol rehabilitation service of the Ann Arbor Veterans Administration Medical Center. All patients were examined at least 7 days following admission and had ingested a minimum

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Table 1. Liver Function Profile of Patient Groups

Group	Age (yrs)	Total serum bilirubin (mg/100 ml)	Serum alkaline phosphatase (IU)	SGOT (IU)	Serum albumin (g/100 ml)	Prothrombin time prolongation (s)
AH*	39 ± 3	14.8 ± 2.8	244 ± 23	154 ± 22	2.4 ± 0.1	4.3 ± 0.7
CAC	54 ± 14	1.3 ± 0.3	171 ± 24	52 ± 17	3.7 ± 0.2	0.8 ± 0.3
AWLD	44 ± 3	0.4 ± 0.1	69 ± 8	42 ± 13	4.6 ± 0.1	ND†

Values, means ± SEM.

* AH, alcoholic hepatitis; CAC, compensated alcoholic cirrhosis; AWLD, alcoholics without liver disease.

† ND, not done.

of 1 pint of whiskey or its equivalent per day prior to admission. Laboratory studies showed normal or mild elevations in serum SGOT, total bilirubin, and alkaline phosphatase values. None of the patients had hepatomegaly or clinical findings to suggest acute or chronic liver disease. Liver biopsies were not obtained. Control subjects consisted of 51 healthy volunteers (39 males, 12 females; 32 ± 2 yr) not known to abuse alcohol or to be using medication. This study was approved by the Ann Arbor Veterans Administration Medical Center Human Studies Committee.

Isolation of PBM

Freshly heparinized peripheral blood was drawn from patients and controls and PBM isolated using Ficoll-Hypaque gradient as previously described (13). Cells were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) enriched with vitamins, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (FCS; Hyclone, Sterile System, Inc., Logan, UT).

Proliferation Assay

PBM, 0.15 ml (1×10^5 cells), were added to 0.05 ml of increasing concentrations of Con A (Pharmacia Fine Chemicals, Piscataway, NJ) in wells of a round-bottom microtiter culture plate (Falcon Plastics, Oxnard, CA). The final concentrations of Con A in the wells were 0, 1, 5, 10, 20, and 40 μg/ml. All experiments were performed in triplicate. The plates were incubated at 37°C for 72 h in a humidified 5% CO_2 and air atmosphere. Tritiated thymidine, 1 μCi (New England Nuclear, Waltham, MA), was added to each well 4 h prior to harvesting with an automatic multiple sample harvester (Otto Hiller Co., Madison, WI). The incorporation of [^{3}H]thymidine was measured in a Beckman liquid scintillation system. PBM from control subjects were always included in assays in which alcoholic patients were studied.

Statistical Analysis

The proliferation ratio (PR) of each condition for each subject was determined using the equation, $PR = B + K_p \ln [X]$, where $\ln = \log_e$, natural logarithm, X = Con A concentration, B = basal PR, and K_p = proliferation coefficient.

A personal computer (PC) program using BASIC language as previously described[12] enabled complete data calculations to obtain K_p, B, and r correlation coefficient values from the input of each Con A dose and the response in cpm. Comparisons of discrete variables between patient groups and controls were by Student's t test.

RESULTS

To assess for possible age-related differences in the proliferative responses of the control group,[14] the healthy volunteers were divided into two age groups: 20–40 years and 41–60 years. As shown in Table 2, there were no significant differences between the two age groups or between the two groups and the total control group in either the cpm at each Con A concentration or in the K_p values.

Significant differences in cpm were noted at several Con A concentrations when comparing the AH and CAC responses to those of the controls. A consistent decrease in proliferative response was seen in the AWLD at all mitogenic concentrations of Con A (Table 3). Although the three patient groups (AH, CAC, and AWLD) had significantly lower K_p values than the control group, all study groups displayed highly significant natural logarithmic correlations when comparing proliferation ratios and each concentration of Con A in the proliferation assay (Table 4).

DISCUSSION

The K_p values observed for the AH, CAC, and AWLD groups confirm that there is a decrease in lymphocyte proliferative responses to mitogen stimulation in these patients. Our results, in part, also provide an explanation for the conflicting observations which have previously been reported in alcoholic patients. Most studies have utilized a single concentration of PHA or pokeweed

Table 2. Age-related Comparisons of Proliferative Response and Proliferation Coefficient in Controls

Con A (μg/ml)	Total control group (51)*	Controls, 20–40 years (38)	p	Controls, 41–60 years (13)	p
0	533 ± 55†	498 ± 54	NS‡	636 ± 142	NS
1	2200 ± 450	2077 ± 396	NS	2560 ± 1326	NS
5	6144 ± 869	6174 ± 981	NS	6057 ± 1843	NS
10	10231 ± 1030	10467 ± 1139	NS	9540 ± 2276	NS
20	13711 ± 1327	14703 ± 1513	NS	10811 ± 2586	NS
40	15757 ± 1394	16721 ± 1609	NS	12942 ± 2640	NS
K_p§	12.58 ± 2.68	11.46 ± 1.86	NS	15.86 ± 8.93	NS

* Numbers in parentheses, number of subjects tested.

† Mean cpm ± SEM/10^5 PBM.

‡ NS, not significant compared to total control group and alternate age-control group.

§ K_p, coefficient of natural logarithmic difference in proliferation for each Con A condition.

Table 3. Con A Induced Proliferative Response in Alcoholic Patients

Con A (μg/ml)	Controls (51)*	AH† (14)	p‡	CAC (11)	p	AWLD (9)	p
0	533 ± 55§	1287 ± 378		586 ± 85		665 ± 101	
1	2200 ± 450	2820 ± 556		1557 ± 314		1681 ± 224	
5	6144 ± 869	5982 ± 1086		3212 ± 469	<0.01	4049 ± 343	<0.05
10	10231 ± 1030	7085 ± 1018	<0.05	9706 ± 4945		6797 ± 778	<0.01
20	13711 ± 1327	9416 ± 1525	<0.05	6932 ± 790	<0.01	9051 ± 1215	<0.01
40	15757 ± 1394	11632 ± 2087		11299 ± 2808		9597 ± 635	<0.01

* Numbers in parentheses, number of subjects tested.
† AH, alcoholic hepatitis; CAC, compensated alcoholic cirrhosis; AWLD, alcoholics without liver disease.
‡ p values, compared to controls, Student's t test.
§ Mean cpm ± SEM/10^5 PBM.

Table 4. Statistical Significance of the Proliferation Equation among Groups

	Controls	AH*	CAC	AWLD
Correlation coefficient†	0.916 ± 0.009‡	0.880 ± 0.050	0.880 ± 0.020	0.930 ± 0.010
K_p§	12.58 ± 2.68	3.16 ± 0.71 (<0.01)¶	5.06 ± 1.15 (<0.01)	4.61 ± 0.83 (<0.01)

Proliferation equation, $PR = B + K_p \ln [x]$.
* AH, alcoholic hepatitis; CAC, compensated alcoholic cirrhosis; AWLD, alcoholics without liver disease.
† Correlation coefficient (r) of the proliferation equation.
‡ Means ± SEM.
§ K_p, coefficient of natural logarithmic difference in proliferation for each Con A condition.
¶ p value, Student's t test when K_p is compared to control value.

mitogen[4–6,8,10] while two studies examined the proliferative responses to two concentrations each, of PHA and Con A.[7,9] In all, statistical analyses were accomplished using the data obtained at discrete mitogen dosages and in none were dose-response proliferation curves obtained. In one investigation,[9] the concentrations of PHA and Con A used were determined by dose-response curves in healthy subjects yielding maximum transformation responses. Previous work has shown however, that multiple doses of a mitogen are required since abnormalities in lymphocyte response may occur only at suboptimal doses.[15,16]

The Con A proliferation curve employed in the present study demonstrates a variable response to the different concentrations of the mitogen (Table 3). Selection of a single Con A dose of 40 μg/ml would have resulted in the impression that PBM from patients with AH and CAC undergo normal proliferative responses. Determination of K_p values in lymphocyte transformation assays would appear to provide a more reliable approach towards the detection of decreased cell-mediated immune function.[12]

The natural logarithmic equation has been used in a number of biological conditions where dose-response relationships are not linear.[12,17,18] In a similar manner, proliferation assays exhibit a decay in the growth rate as the concentrations of the mitogen are increased.[19,20] Although PBM from alcoholic patients are capable of responding to mitogen stimulation, with the ln correlation in cellular proliferation remaining intact (Table 4), the proliferation coefficient is significantly decreased when compared to healthy controls.

The literature is replete with descriptions of multiple immunological abnormalities associated with chronic alcohol abuse in the presence or absence of liver disease (reviewed in Ref. 21), however there is as yet no conclusive evidence to support a pathogenetic role for the host immune response in ALD.

This study provides further indication that the transformation responses in alcoholic patients may not be related to liver disease activity. Although there was no clinical indication of malnutrition in the CAC and AWLD patients reported in this study, we cannot exclude the possibility that subtle changes in nutritional status may have influenced the proliferative response to mitogens in the patient groups.[6]

The decreased proliferative response to Con A by alcoholic patients reflects a probable perturbation in cell-mediated immunity and an immune deficiency state.[2] Application of a natural logarithmic equation to a dose-response mitogen driven proliferation assay permits a more precise quantitative comparison of immunological function between patient groups and healthy volunteers.

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