0002-9270/94/8905-0767$03.00/0
THE AMERICAN JOURNAL OF GASTROENTEROLOGY

Vol. 89, No. 5, 1994
Printed in U.S.A.

T Lymphocytes from Alcoholic Cirrhotic Patients Show Normal Interleukin-2 Production but a Defective Proliferative Response to Polyclonal Mitogens

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T lymphocyte proliferation is a complex process involving intra- and extracellular molecules. T cell activation was studied in T lymphocytes from patients with alcoholic cirrhosis. A defective phytohemagglutinin (PHA)-induced T cell mitogenesis was observed in 60% of these patients. Likewise, their blastogenic response to anti-CD3 was also depressed ($p < 0.05$). However, the DNA synthesis induced by stimulation with phorbol esters (12-O-tetradecanoil-phorbol-13-acetate) + ionomycin was normal ($p > 0.05$). These alterations cannot be ascribed either to decreased interleukin-2 synthesis or to a defective interleukin-2 receptor expression after cellular activation. Moreover, supplementation of the PHA-stimulated T cell cultures with saturant concentrations of recombinant interleukin-2 did not normalize the hypoproliferative response of T cells from alcoholic cirrhotic patients. These results provide evidence that a generalized alteration in the interactions between either mitogens or interleukin-2 and their receptors can explain the T lymphocyte-defective blastogenesis found in patients with alcoholic cirrhosis.

INTRODUCTION

In alcoholic patients, several alterations of the immune system have been described, such as abnormal T and B lymphocyte functions (1–3) and hypergammaglobulinemia (4, 5). However, apparently contradictory results in T cell activation and proliferation have been reported (3, 6–9). The discrepancies in the experimental data can be explained in part by the lack of uniform study groups of patients, which occasionally have included in the same group alcoholic patients with hepatitis and cirrhosis (3, 6, 7, 9).

It has been established that several distinct steps are involved in the activation of resting T lymphocytes to proliferate in human and murine systems (10, 11). The major pathway of T cell activation occurs via the interaction of appropriately processed and presented antigen with the T cell receptor (12, 13). This interaction results in the expression of interleukin-2 receptor (IL-2R) (14) and in the synthesis and secretion of interleukin-2 (IL-2) (15). Once a critical number of T cell receptors on the T cell membrane have bound their ligands, DNA synthesis and cell division occur. The antigen-T lymphocyte interaction can be partially circumvented by the use of polyclonal activators, such as plant lectins (16), anti-CD3 monoclonal antibodies (17), and protein kinase C activators (18).

Herein we have analyzed, in a clinically and pathologically homogeneous group of alcoholic cirrhotic (AC) patients, *1)* the proliferative response of purified T cells to polyclonal mitogenic signals, *2)* the expression of IL-2R and other markers of cellular activation (DR and 4F2 molecules), before and after *in vitro* activation, and *3)* the synthesis of IL-2.

MATERIALS AND METHODS

Population

Thirty-four patients with histologically proved liver cirrhosis were studied (Table 1). To be accepted into the experimental protocol, the patients had to comply with the following conditions: *1)* more than 80 g of alcohol ingested daily for at least 5 consecutive years, *2)* alcohol abstinence for at least the preceding 3 months (family members were interviewed to corroborate this condition), *3)* absence of clinical evidence of parenchymal failure, other than hepatic, and of infectious, inflammatory or neoplastic diseases, *4)* absence of prior treatment with steroids, cytostatics, or cimetidine, *5)* absence of transfusion of blood or blood products in the patients and their families, and negative B and C virus serology (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, and anti-HCV), and *6)* liver biopsy with no sign of postnecrotic or primary biliary cirrhosis, hemochromatosis, or Wilson's disease. Likewise, to rule out the possible influence of inflammatory mediators on

Received Sept. 24, 1992; accepted Dec. 30, 1993.

TABLE 1
Characteristics of the Population Studied

Variable	Class (Child-Pugh) (ref. 19)			Overall	Control
	A	B	C		
No.	17	8	9	34	30
Age (yr)	55.0 ± 15	59.1 ± 12	53.5 ± 14	56.2 ± 14	54.1 ± 15
Alcohol (g/day)	161.5 ± 62	198.3 ± 58	226.0 v 70	180.0 ± 63	
Weight (kg)	66.0 ± 17	66.5 ± 14	60.0 ± 15	63.0 ± 15	65.0 ± 14
Height (cm)	169.0 ± 10	177.0 ± 7	165.0 ± 9	170.5 ± 11	169.0 ± 12
Hemoglobin (g/dl)	13.4 ± 1.9	11.1 ± 2.1	10.8 ± 1.9	12.4 ± 2.3	14.8 ± 1.8
Leukocytes ($\times 10^9$/L)	6.4 ± 1.6	5.3 ± 1.7	6.9 ± 1.5	6.4 ± 1.9	7.2 ± 2
Platelets ($\times 10^9$/L)	150.7 ± 58	97.6 ± 43	107.0 ± 50	130.4 ± 72	261.3 ± 75
Prothrombin activity (%)	78.1 ± 18.2	72.8 ± 19.8	50.5 ± 25.1	70.6 ± 26.4	94.0 ± 5.1
Bilirubin (mg/dl)	1.4 ± 0.5	1.7 ± 0.9	5.4 ± 2.1	2.4 ± 1.8	0.9 ± 0.2
Albumin (mg/dl)	3.7 ± 0.3	3.2 ± 0.5	2.7 ± 0.4	3.4 ± 0.8	4.7 ± 0.4
ALT (U/dl)	64.0 ± 32.1	69.1 ± 31.2	62.1 ± 21.2	64.6 ± 30.3	32.7 ± 5.7
AST (U/dl)	67.9 ± 21.3	56.7 ± 19.7	33.1 ± 18.2	55.9 ± 27.3	25.9 ± 8.3
GGT (U/dl)	53.8 ± 12.1	42.0 ± 6.4	57.5 ± 8.3	53.7 ± 11.2	17.0 ± 6.3
ALP (U/dl)	118.7 ± 23.5	104.3 ± 19.7	170.3 ± 32.3	128.2 ± 37.5	88.8 ± 20

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase.

the tests performed, those patients whose liver biopsy showed any evidence of alcoholic hepatitis were also excluded from the study group.

Thirty age- and sex-matched healthy controls were studied (Table 1). Six patients (mean age 50 yr) with a history of daily alcohol intake of more than 80 g over a period of at least 5 consecutive years and alcohol abstinence for at least the preceding 3 months were also studied as controls. These patients presented no clinical or laboratory abnormalities. Five patients (mean age 48 yr) with liver lesions histopathologically compatible with primary biliary cirrhosis were also studied. These patients fulfilled at least three of the following additional criteria: *1)* elevation of serum IgM level, *2)* increase in serum alkaline phosphatase activity to more than twice the upper limit of the normal range, *3)* presence of antimitochondrial antibody titers of 1:40 or higher, and *4)* bile duct patency demonstrated by endoscopic retrograde cholangiopancreatography, ultrasonography, or both.

The conventional determinations included: hemogram, serum biochemistry, coagulation study, liver scintigraphy with technetium-99m sulfur colloid, esophagogastroduodenoscopy, and abdominal ultrasonography. Given the poor correlation between ultrasonographic or endoscopic findings and portal pressure (20, 21), the gradation of portal hypertension was tabulated as 0 or 1, depending on the absence or presence of esophageal varices or increment of the portal vein diameter. Clinical characteristics of the cirrhotic population are shown in Table 1. All patients gave informed consent to the experimental protocol.

Material

Reagents. The following monoclonal antibodies (MoAb), that recognize specific molecules on the surface of certain cellular subpopulations, were used: OKT11 (CD2), OKT3 (CD3), OKT4 (CD4), OKT8 (CD8) (Ortho-mune, Ortho Diagnostic System, Raritan, NJ, USA), anti-B1 (CD20), anti-Mo2 (CD14), anti-TAC (CD25) (Coulter Clone, Coulter Immunology, Hialeah, FL, USA), 4F2 (donated by A. S. Fauci, NIH, Bethesda, MD, USA) and Leu 11b (CD16) (Becton Dickinson, Oxnard, CA, USA). Recombinant interleukin-2 (rIL-2) was a gift of Hoffman-La Roche (Nutley, NJ, USA).

Cell separation. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque gradient centrifugation (Nyegaard CO., Oslo, Norway). T cells were purified by double rosetting, resulting in a purity of more than 95% in every case.

Monocyte-enriched populations were obtained after double plastic adherence of PBMC and killing with the MoAb OKT3 plus Leu 11b plus complement, the purity being greater than 85% in every case.

After counting, cells were resuspended in RPMI 1640 (Flow Lab, Irvine, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 2-mercaptoethanol (5×10^{-5} M, L-glutamine (2 mM), and 1% penicillin-gentamicin, and were checked for viability by trypan blue exclusion.

Proliferation studies. T lymphocytes (50,000 cells/well) were cultured on 96 flat-bottom culture plates. Soluble phytohemagglutinin [(PHA) 10 μg/ml; Difco Lab, Detroit, MI, USA], OKT3 coupled to bead (5 μg/ml) or 12-*O*-tetradecanoil-phorbol-13-acetate [(TPA) 10 ng/ml; Sigma, St. Louis, MO, USA] plus ionomycin (5 μg/ml; Sigma) in the presence or absence of rIL-2 (100 IU/ml) were added at the beginning of proliferation studies. Each reagent was tested in dose/response titrations before use. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3,

5, 7, or 9 days. Eighteen hours before the end of the incubation, 1 μCi of ^{3}H-thymidine (Radiochemical Centre, Amersham, UK) was added. The cells were harvested and results were expressed as mean counts per minute (cpm) of triplicate cultures ± standard deviation.

Lymphokine production. Lymphokine-enriched supernatants were obtained by culturing T cells at 37°C at a density of 5×10^6/ml in complete medium. Cultures were incubated in the presence or absence of PHA (10 μg/ml), and supernatants were harvested at 24 and 72 h of incubation, sterilized by filtration through a 0.22-μm filter (Millipore Company, Bedford, CA, USA), and stored at −20°C until use. Likewise, interleukin-1 (IL-1)-enriched supernatants were obtained by culturing monocytes at 37°C at a density of 10^7 cells/ml in complete medium; LPS (25 μg/ml, Difco Lab) was added at the beginning of the culture, and supernatants were harvested at 72 h, filtered, and stored at −20°C until use.

IL-2 activity was determined in the supernatants of T cell suspensions by evaluating the dose-dependent proliferation induced in the cytotoxic murine line CTL-L2 by the lymphokine present in the cultures. Results after probit analysis were expressed in units/ml (22). IL-1 activity was detected in a thymocyte co-stimulation assay, as described by Scala *et al.* (23), and results expressed as the stimulation index.

Immunofluorescence analysis. Immunofluorescence staining of cell surface antigens was performed using a standard method as previously described (13). The fluorescence of 10,000 viable lymphoid cells stained for each different MoAb was quantified with an EPICS C flow cytometer (Coulter Immunology).

Statistical analysis. The data from the groups were compared by Mann-Whitney *U* test. For correlation studies, linear regression analysis with the least-squares method was employed. A p value of less than 0.05 was considered to indicate a significant difference between groups.

RESULTS

Two different groups of patients with alcoholic cirrhosis can be defined according to the proliferative ability of T lymphocytes

We began our study with the analysis of the blastogenic response of PBMC from AC patients and healthy controls to stimulation with PHA. As can be seen in Figure 1A, PBMC from AC patients showed a reduced ^{3}H-thymidine uptake after PHA stimulation, throughout the entire kinetic study. This hypoproliferative response cannot be ascribed to a reduction in the percentage of T lymphocytes present in PBMC from AC patients (Fig. 2A).

In patients with a history of alcohol intake, in alcohol abstinence, with no clinical or laboratory evidence of liver disease, PBMC showed a proliferative response to PHA stimulation (56, 313 ± 10, 913 cpm), with no significant differences with respect to that found in PBMC from healthy controls after 5 days of culture ($p > 0.05$). PBMC from patients with primary biliary cirrhosis showed a significantly defective proliferation in response to PHA stimulation days (41, 527 ± 12, 322 cpm), compared with that found in PBMC from healthy controls after 5 days of culture ($p < 0.05$).

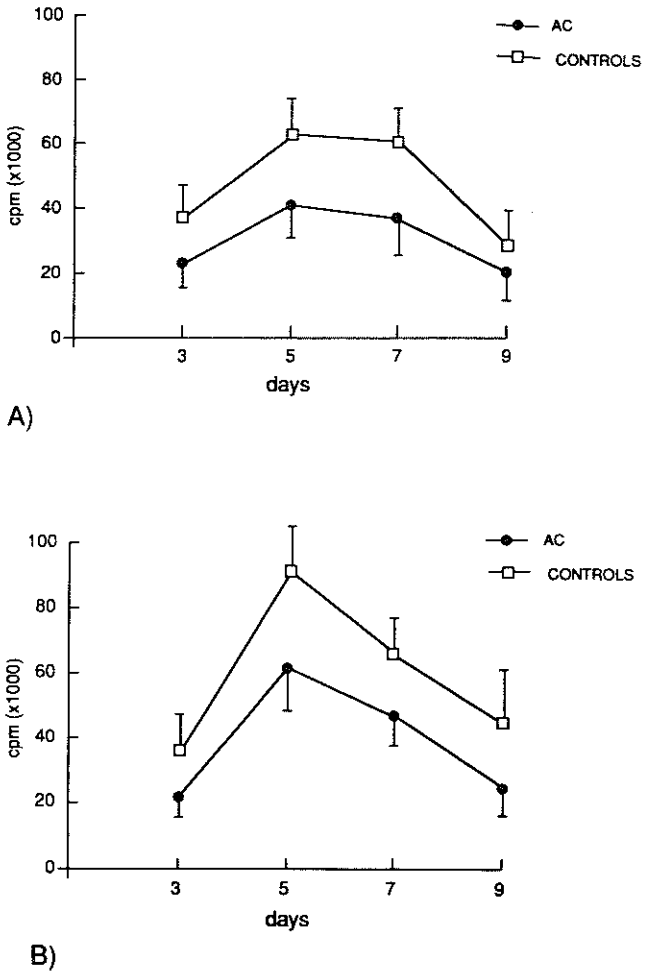

FIG. 1. Kinetics of the blastogenic response of peripheral blood mononuclear cells *(A)* and T cells *(B)* from 10 alcoholic cirrhotic *(AC)* patients and 10 healthy controls to stimulation with PHA for 3, 5, 7, or 9 days. Results are expressed in cpm as mean + standard deviation. Statistical differences between the two groups were found at 3, 5, and 7 days of culture ($p < 0.05$).

To rule out a possible inhibitory effect of the non-T cells from AC patients on the blastogenic response of the T lymphocytes present in PBMC to PHA, we studied the kinetics of the proliferative response of purified CD3+ cells to this mitogen (Fig. 1B). The assays performed also showed a marked impairment in the blastogenic response of the T cell preparation from AC patients throughout the entire kinetic study. Taking into account that maximal ^{3}H-thymidine incorporation

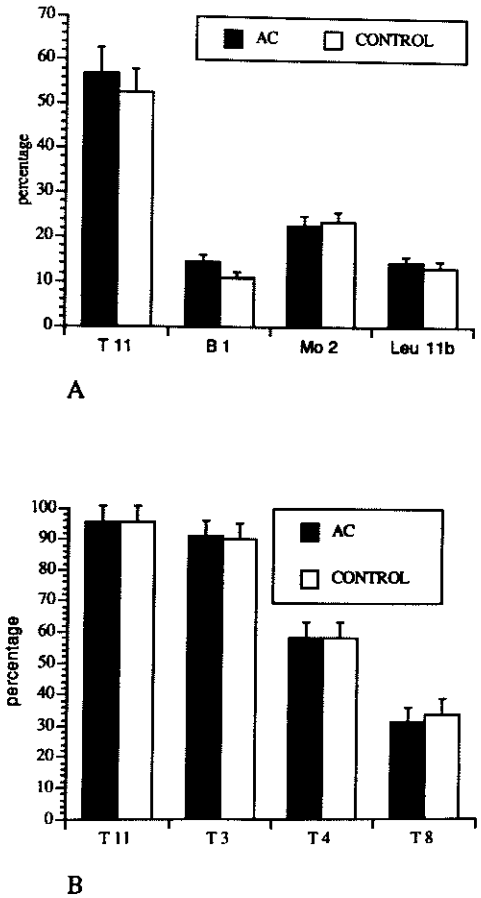

FIG. 2. Phenotypical analysis of peripheral blood mononuclear cells *(A)* and T cells *(B)* of alcoholic cirrhotic *(AC)* patients and healthy controls. Cell populations were identified by immunofluorescence staining with monoclonal antibodies which recognize specific membrane receptors: *T11* and *T3* (T cells), *B1* (B cells), *Mo2* (monocytes), *Leu11b* (NK cells), *T4* (CD4+ T cells), and *T8* (CD8+ T cells). Results are expressed as mean ± SD. No statistical differences were found between AC patients and controls with respect to the percentages of these different populations ($p > 0.05$).

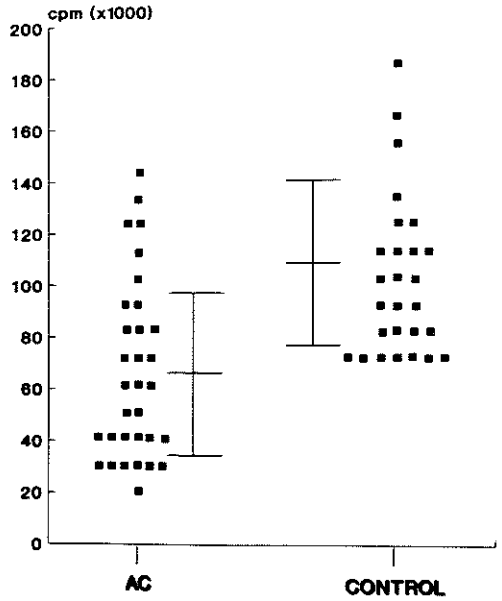

FIG. 3. Blastogenic response of T cells from alcoholic cirrhotic *(AC)* patients and controls to stimulation with PHA for 5 days. Individual values as well as mean ± SD are represented. Significant differences were found between the two groups ($p < 0.05$).

was observed at day 5 in T lymphocyte cultures from both AC patients and healthy controls, in subsequent experiments, the proliferative response was analyzed on this day of culture. We found that the proliferative response to PHA of purified T cells from the AC group as a whole was significantly less than that found in normal donors ($p < 0.05$).

We found no correlation between this mitogenic response and the clinical parameters analyzed (Child-Pugh index, indirect parameters of portal hypertension, or serum aminotransferases) (data not shown).

However, AC patients can be divided into two subsets according to the mitogenic response of T cells to PHA: *1)* those patients (40%) whose T cells proliferate within the range of normal healthy controls (normoproliferative patients), and *2)* the rest of the AC group (60%) with T cell proliferative responses below the normal range (hypoproliferative patients) (Fig. 3). It is relevant that similar percentages of CD4+ and CD8+ were found in purified T cell preparations from both groups of patients, as well as from healthy controls (Fig. 2B).

Because PHA is a lectin that interacts with multiple cell surface molecules (16, 24), we investigated the proliferative response of T cells from AC patients and healthy controls to mitogens that selectively recognize the structures of the T cell antigen receptor complex (CD3-Ti) (17). We found that those patients with hypoproliferative T lymphocyte response to PHA also have a deficient ^{3}H-thymidine uptake after stimulation with anti-CD3 ($p < 0.05$). In contrast, there were no significant differences between the anti-CD3-induced blastogenic response of T cells from normoproliferative AC patients and that of healthy controls ($p > 0.05$) (Fig. 4).

Finally, we also analyzed the proliferative response of T cells from AC patients to mitogenic signals that interact directly with protein-kinase C and intracellular Ca++ (18). As shown in Figure 4, the ^{3}H-thymidine uptake found in T cell cultures from AC patients stimulated with TPA plus ionomycin was similar to that obtained in healthy donors ($p > 0.05$).

Interleukin-2 is normally produced by either hypoproliferative or normoproliferative T lymphocytes from alcoholic cirrhotic patients

Several crucial steps can be defined in the activation and proliferation of T lymphocytes: first, the production of IL-2; second, the expression of IL-2R; and third, promotion by IL-2 of T cell proliferation upon binding to its specific receptors (10, 11).

As shown in Figure 5, IL-2 activity levels found in

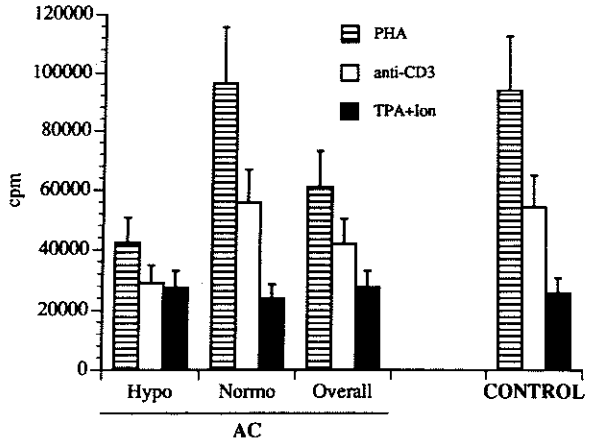

FIG. 4. ^{3}H-thymidine uptake by T cells from alcoholic cirrhotic *(AC)* patients, both hypo- and normoproliferative, and healthy controls in response to PHA, anti-CD3, and TPA + ionomycin for 5 days. Results are expressed in cpm as mean ± SD. When considered overall, statistically significant differences were found between AC patients and healthy controls with respect to the T cell proliferative responses to PHA ($p < 0.05$) and to anti-CD3 ($p < 0.05$). Likewise, significant differences were found between hypoproliferative and normoproliferative AC patients and controls when tested for the blastogenic response of their T cells to PHA ($p < 0.05$) and anti-CD3 ($p < 0.05$). However, the results were similar ($p > 0.05$) in the different groups when the mitogenic combination TPA + ionomycin was employed.

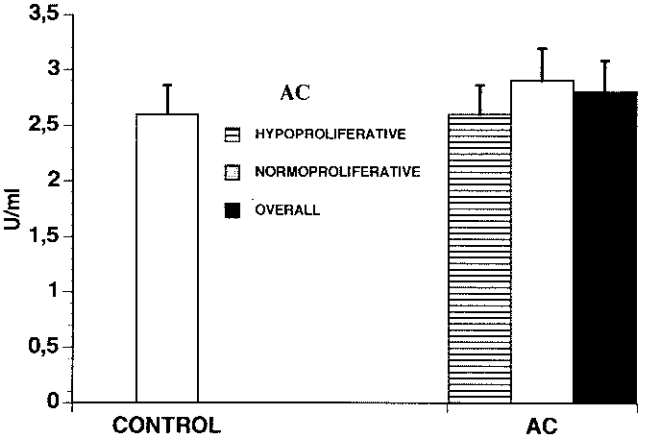

FIG. 5. Interleukin-2 production by PHA-activated T cells from alcoholic cirrhotic *(AC)* patients, either normo- or hypoproliferative, or the group as a whole, and controls. Results are expressed as mean ± SD. No significant differences were detected among the different groups ($p > 0.05$).

supernatants of cultures of PHA-stimulated T cells from AC patients and healthy controls were similar ($p > 0.05$). Furthermore, there was no difference between the IL-2 activity detected in supernatants of cultured T cells from either hypo- or normoproliferative AC patients and healthy controls ($p > 0.05$).

As could be predicted, based on the normal IL-2 production found in T cells from AC patients, IL-1 synthesis by their monocytes was also within the range found in healthy controls (data not shown).

Hypoproliferative T lymphocytes from alcoholic cirrhotic patients normally express IL-2R, but show different blastogenic responses to this lymphokine

The simultaneous findings of hypoproliferative response and normal IL-2 production after PHA stimulation in T cells from AC patients could be explained by a defective expression of IL-2R and/or an alteration in the blastogenic response induced by the interaction of IL-2 with its receptor. To address the first question, quantitative flow cytometric studies were performed after T cell staining with monoclonal antibodies specific for the IL-2R. Antibodies to the 4F2 structure and DR molecules also were employed for a broader analysis of the T lymphocyte activation process. As shown in Figure 6, T cells from AC patients normally acquire and express IL-2R after PHA stimulation for 1, 2, 3, or 5 days. Moreover, similar patterns of IL-2R expression were found in T cells from either normo- or hypoproliferative patients and healthy controls. Comparable results also were found for the T cell membrane expression of 4F2 glycoprotein and DR molecules (data not shown).

Although there was indirect evidence of a defective blastogenic response to endogenous IL-2 in hypoproliferative-activated T lymphocytes from AC patients, we tested the effect of exogenous, saturant amounts of rIL-2, added to the culture medium, on the ^{3}H-thymidine uptake of T cells from these patients. As shown in Table 2, the exogenous addition of saturant concentrations of rIL-2 to the PHA-stimulated T cell cultures from hypoproliferative AC patients neither normalized nor significantly increased the proliferative response of these T cells ($p > 0.05$). It is remarkable that this normalization was not found in any of the hypoproliferative AC patients studied.

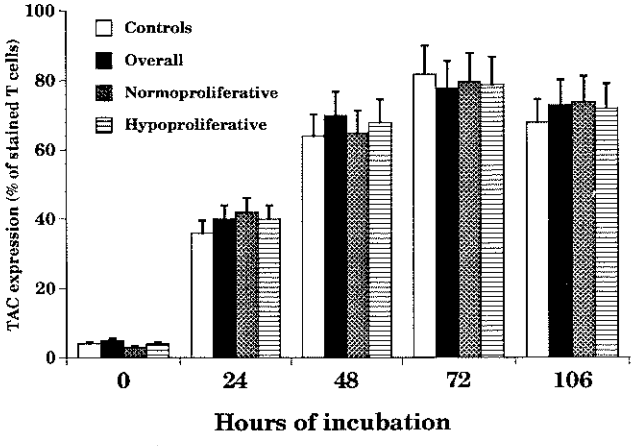

FIG. 6. Expression of interleukin-2 receptor (anti-TAC) on the cytoplasmic membrane of T cells of patients with alcoholic cirrhosis *(AC)*, either normo- or hypoproliferative, or the group as a whole, and controls, spontaneously and after activation with PHA for 24, 48, 72, and 106 h. The results detected in the different groups were similar ($p > 0.05$).

TABLE 2

Effect of Supplementation with rIL-2 on the Proliferative Response of T Lymphocytes of Patients with Alcoholic Cirrhosis and Healthy Controls in the Presence or Absence of PHA after 5 days of Incubation

Mitogen	Alcoholic Cirrhosis		Controls
	Normo-*	Hypo-*	
Basal	821 ± 745	802 ± 754	683 ± 681
IL-2	6,327 ± 3,472	5,244 ± 4,227	7,414 ± 4,373
PHA	96,373 ± 22,261	42,236 ± 16,371	93,640 ± 23,618
PHA + IL-2	97,542 ± 19,311	41,517 ± 15,314	96,348 ± 20,899

Results are expressed in cpm, as mean ± SD.

* Normo- and hypoproliferative with respect to PHA-induced blastogenesis. PHA (10 μg/dl); rIL-2 (100 U/ml).

DISCUSSION

Results from the study of immune system function in AC patients are contradictory (3, 6–9). In most reports, data on alcohol consumption or abstinence, presence of other etiological factors, or existence of pharmacological or pathological circumstances that could modify the functional characteristics of the immune system are not given (3, 6, 7, 9). To rule out the probable influence of these variables in the analysis of T cell function in AC patients, a carefully selected group of abstinent patients with alcoholic cirrhosis was studied. Likewise, to eliminate the possible effects of inflammatory processes on the tests performed, those patients whose liver biopsy showed any evidence of alcoholic hepatitis were excluded from the study group.

Our results demonstrated the presence of marked functional alterations in the activation and proliferation of T cells from AC patients. It has been proved that the process of T cell blastogenesis can be divided into two phases (10, 11, 13). First is the binding of activating ligands to specific receptors on the T cell surface (12). These include the T cell antigenic receptor coupled to its associated CD3 complex, as well as other membrane molecules (CD2, CD43) (25). The interaction of these surface receptors and their ligands results in the transduction of a transmembrane signal that induces the activation of protein kinase C and the increase in the intracellular calcium concentration (known as T cell second messengers) (26). Second, these two biochemical modifications are synergistic in inducing the production of IL-2 and the expression of its receptor, thus regulating the progression throughout the cell cycle and DNA synthesis (10, 11).

Our data clearly demonstrate that 60% of AC patients have defective T lymphocyte blastogenesis after mitogenic stimulation. It is relevant that the hypoproliferative response was not only observed with the lectin PHA, but also with direct stimulation of the CD3-Ti complex. To analyze the defect in the proliferation pathway, we examined the events that follow T cell activation by lectins or anti-CD3. The normal blastogenic response of T lymphocytes from AC patients after stimulation with TPA (a direct activator of protein kinase C) plus the calcium ionophore ionomycin suggests that the hypoproliferative T lymphocytes from AC patients have a functional defect in a step proximal to the generation of second messengers of the lymphocyte activation process.

The reported T cell functional abnormality cannot be ascribed to a defective production of either IL-1 by monocytes or IL-2 by T lymphocytes. Likewise, the expression of IL-2R, as well as other activation antigens, on the T cell cytoplasmic membrane are normal. However, a marked hypoproliferative response to IL-2 is found in T lymphocytes from AC patients. When these results are taken together, it can be hypothesized that an alteration in the metabolic process subsequent to the activation of the cytoplasmic membrane receptors of T lymphocytes could cause their defective mitogenesis.

Given the lack of correlation between the tests performed *in vitro*, both in normo- and hypoproliferative AC patients, and the clinical parameters analyzed, we find no evidence that liver function—estimated by the Child-Pugh index—or portal hypertension plays a role in the defective immune response observed in these patients. Likewise, the exclusion from the study of patients with concomitant alcoholic hepatitis makes it improbable that there inflammatory markers have any influence on the results.

At present, it is not possible to define the origin of this T cell functional abnormality. It might be an effect of the metabolic alterations associated with hepatic cirrhosis (27), a chronic and, prior to abstinence, direct toxicity of alcohol on the lymphocytes (28), or, finally, a response of the immune system to the pathological lesions induced in the liver by alcohol (29). PBMC from alcoholic patients abstaining from alcohol, without clinical and laboratory evidence of liver disease, show a normal proliferative response to PHA stimulation. This finding indicates that the defective proliferative response to PHA found in AC patients is not a direct effect of prior alcohol ingestion. PBMC from patients with primary biliary cirrhosis also show a diminished blastogenic response to PHA. However, we have ob-

served that in basal conditions, T lymphocytes from these patients show an increased expression of activation antigens on their cytoplasmic membrane that is not found in AC patients (30). These heterogeneous findings in patients with hepatic cirrhosis from different etiologies suggest that the T lymphocyte abnormalities observed in these patients may not be explained only by the metabolic alterations associated with the hepatic damage.

The T cell activation defect observed in AC patients may be implicated in the elevated incidence of certain infections found in these patients (31, 32). Future studies could outline the significance of the abnormal transduction after microbiological antigen recognition by T lymphocytes from AC patients.

ACKNOWLEDGMENTS

We thank Ms. C. Lorences and Ms. G. Peraile for their skillful technical assistance, and Ms. M. Messman for her expert editorial assistance. This work was supported in part by a grant from Fondo de Investigaciones de la Seguridad Social 91/9022.

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