# Decreased Natural Killer Cytotoxic Activity in Chronic Alcoholism Is Associated With Alcohol Liver Disease but Not Active Ethanol Consumption

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**Chronic alcohol intake is associated with an increased incidence of certain neoplasms. Natural killer (NK) cells have been considered to be involved in control tumor development and growth. The goal of the present study was to contribute to a better understanding of the effects of ethanol (EtOH) *per se* on the NK-cell population. Both patients with chronic alcoholism without liver disease (AWLD) and subjects with alcohol-induced cirrhosis (ALC) were carefully selected for this study. Immunophenotypical and functional studies of peripheral blood (PB) NK-cells were performed during active EtOH intake and after 3 months of a withdrawal period. In the AWLD group a significant increase in number of NK-cells (CD3−/CD56+) ($P < .05$) associated with a parallel increase in NK-cell lytic activity ($P < .01$) was observed. In addition, the number of cytotoxic T cells displaying the CD3+/CD56+ phenotype as well as CD8−/CD57+ NK-cell subset was also increased ($P < .01$ and $P < .001$, respectively). By contrast, in ALC patients with active EtOH intake (ALCET group), although a significant increase in the number of NK PB lymphocytes was observed ($P < .05$), NK lytic activity was depressed ($P < .05$), suggesting the existence of a decreased lytic activity/NK-cell. After 3 months of EtOH withdrawal, PB mononuclear cells (PBMC) from the AWLD group patients still displayed an increased NK cytolytic activity; in addition, the number of PB NK-cells (CD3−/CD56+ and CD8−/CD57+) and CD3+/CD56+ PB T cells continued to be increased. Independently of the duration of withdrawal period, in ALC patients EtOH withdrawal was followed by a slight decrease in the NK lytic activity of PBMC with respect to the values in active alcoholism phase; slight differences observed in the NK lytic activity in ALC patients who quit drinking could be related to the tendency to decrease of the number of NK-cells toward normal values. Furthermore, although an increase in NK cytotoxic activity after stimulation of PBMC with interleukin-2 (IL-2) was observed in ALC, it did not reach the levels observed in healthy subjects. Overall, our results show that the behavior of PB NK-cell population in chronic alcoholism is different according to both the moment of EtOH consumption and the existence or not of ALC. Alcohol by itself induced an increase in the number and lytic activity of NK-cells. By contrast, the NK cytolytic activity is constantly depressed in the stage of alcoholic cirrhosis, supporting the notion that immunosurveillance mechanisms may be affected in these patients. (Hepatology 1997;25:1096-1100.)**

Chronic alcohol intake is associated with an increased incidence of larynx, esophageal, liver, and pancreas neoplasms.[1] This feature has been related, at least partially, to an ethanol (EtOH)-induced enzymatic activation of the conversion of procarcinogens into carcinogens.[2,3] In addition, previous reports suggest that an alteration of the immunosurveillance could also contribute to the higher incidence of neoplasms observed in these patients.[4] Natural killer (NK) cells have been classically considered to be involved in control of tumor development and growth.[5]

The study of NK-cells in chronic alcoholism has provided conflicting results in the literature.[6] Several variables can contribute to these discrepancies: 1) the time-point at which the study was performed (active alcoholism vs. withdrawal period), 2) the criteria used for patient selection (alcoholic liver disease vs. alcoholism without liver damage), 3) the occurrence of associated factors such as malnutrition or chronic infection by hepatitis B and C viruses, and 4) the use of different methodological approaches to analyze either the distribution of lymphoid subsets and/or their NK-cell cytotoxic activity.[7-9]

The goal of the present study was to contribute to a better understanding of the effects of EtOH *per se* on the NK-cell population. Thus, both patients with chronic alcoholism without liver disease (AWLD group) and individuals suffering from alcohol-induced cirrhosis (ALC group) were carefully selected for this study to explore both the immunophenotypical and functional changes of peripheral blood (PB) NK-cells were performed in parallel during active EtOH intake and after 3 months of a withdrawal period.

## PATIENTS AND METHODS

This study included a total of 55 alcoholic patients referred to the Alcoholism Unit of the University Hospital of Salamanca, who had consumed at least 90 g of EtOH per day for more than 5 years. From them, 20 were AWLD and 35 were ALC patients. In addition, 25 age- and sex-matched healthy volunteers were included in the study. All patients and control subjects gave their informed consent to participate, and the study was approved by the Ethical Committee of the University Hospital of Salamanca. In all patients and controls PB

Abbreviations: EtOH, ethanol; NK, natural killer; AWLD, alcoholism without liver disease; ALC, alcohol-induced cirrhosis; PB, peripheral blood; ALCET, ALC with active ethanol intake; ALCAW, ALC with at least 1 year of alcohol withdrawal; Mab, monoclonal antibodies; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells.

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**TABLE 1. Comparison of Clinical and Biological Characteristics of Alcoholic Cirrhotic Patients**

| | Control (n = 25) | ALCET (n = 20) | ALCAW (n = 15) |
|---|---|---|---|
| Mean age (yr) | 51 ± 7 | 54 ± 2 | 57 ± 2 |
| Sex (M/F) | 17/8 | 18/2 | 14/1 |
| Bilirubin (mg/dL) | 0.8 ± 0.1 | 2 ± 0.5 | 2 ± 0.8 |
| AST (IU/L) | 27 ± 6 | 71 ± 10 | 52 ± 10 |
| GGT (IU/L) | 28 ± 5 | 149 ± 24 | 37 ± 9 |
| Prothrombin time (%) | 96 ± 2 | 75 ± 5 | 62 ± 3 |
| Albumin (g/L) | 43 ± 4 | 34 ± 2 | 35 ± 2 |
| Gamma globulin (g/L) | 12 ± 1 | 20 ± 2 | 12 ± 0.6 |
| Hemoglobin (g/dL) | 14 ± 1 | 13 ± 0.5 | 13 ± 0.3 |
| Leukocytes ($\times 10^6$/L) | 7,870 ± 1,332 | 7,921 ± 1,232 | 5,712 ± 707 |
| Lymphocytes ($\times 10^6$/L) | 3,826 ± 921 | 2,162 ± 282 | 1,542 ± 360 |
| Child-Pugh score (A/B) | | 13/7 | 12/3 |

Abbreviations: AST, aspartate transaminase; GGT, $\gamma$-glutamyl transferase.

samples were obtained between 9:00 and 10:00 AM under condition of fasting.

***AWLD Group (n = 20).*** All AWLD patients were actively drinking until the day of entering the study. Subjects who were polydrug abusers, active smokers, as well as those displaying the physical stigmata of chronic liver disease (i.e., cutaneous signs, hepatosplenomegaly, gynecomastia, testicular atrophy, and muscle wasting) were excluded from the study; all patients had normal hemoglobin concentrations, prothrombin time, and albumin serum levels; alanine aminotransferase and aspartate aminotransferase serum levels were required to be less than twice the upper normal limits (<40 IU/L); hepatitis B surface antigen, antibodies to hepatitis C virus, and human immunodeficiency virus were negative in all alcoholic patients. Liver biopsy was not performed in this group of patients for ethical reasons. To avoid enrolling patients with malnutrition, a nutritional status evaluation was made, both using laboratory parameters (serum albumin concentration and transferrin levels) and anthropometric analysis (height, weight, triceps skin fold, and midarm muscle circumference measurements) that were compared with published criteria to derive percents of standard values.[10] Only subjects with anthropometric tests >90% of expected normal values were entered in the study protocol.

***ALC Patients (n = 35).*** According to the alcoholic intake state at the moment of this study, ALC patients were divided into two groups: 1) patients with active EtOH intake (ALCET group, n = 20), and 2) patients who underwent at least 1 year of alcohol withdrawal (ALCAW group, n = 15). Liver histopathological examination in all the 24 patients in which it was performed showed a micronodular cirrhosis, and in none of them signs of alcoholic hepatitis were observed. In the remaining 11 patients, liver biopsy was not performed because of blood coagulation abnormalities, the diagnosis of ALC being established in these cases on the basis of the presence of physical stigmata of chronic liver disease, history of ascites, variceal bleeding, or hepatic encephalopathy, as well as gastroscopic and/or ultrasonographic findings.[11] The most relevant clinical and biological data at the moment this study was performed are shown in Table 1. Active smokers were excluded from the study. All patients included were hepatitis B surface antigen (−), antibody to hepatitis C virus (−), and human immunodeficiency virus (−), and none of them had malnutrition, according to the above-mentioned parameters. None of ALC patients was diagnosed with acute alcoholic hepatitis before the moment of entering the study, none had ascites, jaundice, encephalopathy, or gastrointestinal bleeding within 1 year before the study, nor during the withdrawal period; none of them had received blood transfusion or was under treatment with steroids or immunosuppressive therapy. In the ultrasonographic studies performed during the withdrawal follow-up period no evidence of hepatocellular carcinoma was detected.

***Control Subjects.*** Twenty-five age- and sex-matched healthy volunteers were included in the study. All of them reported to drink <10 to 15 g of EtOH per day. Liver function as well as routine hematological and biochemical tests were within the normal range. The analysis of these individuals was performed along the study in parallel to that of the patients. Some controls have been followed at different periods of the study for the determination of PB lymphoid subsets distribution and no major differences were detected as compared with the first analysis.

***Reagents.*** Monoclonal antibodies (Mab) against the CD56, CD3, CD8, and CD57 antigens were purchased from Becton Dickinson (San José, CA). RPMI 1640 (Microbiological Associates, Walkersville, MD) supplemented with 10% fetal calf serum (Flow Lab., Irvine, UK), 1% glutamine (Flow Lab.), 0.5% HEPES (Flow Lab.), and 1% penicillin-streptomycin (Difco Lab, Detroit, MI) was used for cell culture studies; this will be referred as complete medium. Human recombinant interleukin-2 (IL-2) was kindly provided by Dr. J. Farrar and Dr. P. Surler (Hoffman-La Roche Inc, Nutley, NJ).

***Isolation and Culture of Effector Cells.*** Peripheral blood mononuclear cells (PBMC) were obtained in all cases from heparin anticoagulated venous blood by Ficoll-Hypaque (Lymphoprep Nyegaard, Oslo, Norway) density gradient centrifugation. PBMC, were resuspended in complete medium after 3 washes in saline serum (3 mL each). In a set of experiments 1 million/mL of PBMC were incubated in 24-macrowell plates (Costar, Cambridge, MA) in complete medium, in the presence or absence of different concentrations (10 U/mL and 100 U/mL) of IL-2. The cultures were performed for 18 hours at 37°C in a 5% $CO_2$-humidified atmosphere.

***Cytotoxicity Assays.*** Cytotoxicity was quantified by a $^{51}$Cr-specific release assay using the erythromyeloid K 562 leukemia cell line as a standard NK target. The effector cells were resuspended in complete medium. Target cells were labeled by incubating 1.5 to $2 \times 10^6$ cells, resuspended in 0.5 mL of complete medium, with 150 $\mu$Ci of $^{51}$Cr for 90 minutes at 37°C in a water bath, washed twice, and resuspended in complete medium at a concentration of $5 \times 10^4$ cells/mL. Cells were then placed in round-bottom microwell plates (Costar) by diluting 0.1 mL of the target-cell suspension ($5 \times 10^3$ cells) in 0.1 mL containing the effector cells at final effector:target ratios of 50:1, 25:1, 12:1, and 6:1. All experiments were performed by triplicate. Controls included 0.1 mL of target cells at the same concentration incubated, under the same conditions, with 0.1 mL of complete medium (spontaneous release) or 0.1 mL of detergent (total release). Plates were incubated for 4 hours at 37°C in a 5% $CO_2$ atmosphere. Once the incubation period was finished, 0.1 mL of supernatants from each microwell was collected and counted in a gamma counter. The percent of specific cytotoxicity was calculated as follows: Mean experimental radioactivity release − mean spontaneous release/mean total release − mean spontaneous release × 100.

In all the experiments performed, the mean radioactivity of spontaneous release was always <10% of the value obtained for the total release. Lytic units were calculated from the cytolytic data at the reference lysis level of 10% for $10^7$ effector cells, according to Pross et al.[12] Basal NK cytotoxicity assays were performed in all patients. Additionally, in six patients of ALCAW group NK cytotoxicity was assessed in the same way after PBMC incubation (18 h) with different amounts of IL-2. In all experiments, patient and control samples were run in parallel. No significant differences were observed among control for the NK cytotoxic activity, according to the time of the year they were analyzed.

***Immunological Marker Analysis.*** PB samples were analyzed by direct immunofluorescence using double staining combinations of Mab directly conjugated with fluorescein isothiocyanate and phycoerythrin. The following panel of Mab combinations was used: CD3/CD56 and CD57/CD8. Cells were stained by incubating 200 $\mu$L of ethylenediaminetetraacetic acid anticoagulated PB containing between 0.5 and $1 \times 10^6$ nucleated cells for 10 minutes at room temperature with each of the Mab combinations described above. Erythrocytes were lysed by incubating the stained sample for 10 minutes (room temperature) with 2 mL/tube of FACS lysing solution (Becton Dickinson). For data acquisition the FACScan flow cytometer (Becton Dickinson) and the Lysys II software program (Becton Dickinson) were used. Information on at least 15,000 events/tube was stored. For data analysis the Paint-A-Gate Plus software (Becton Dickinson) was used according to previously described methods.[13] The percentage of the different lymphoid subsets analyzed was obtained upon specifically gating on the low forward scatter/low side scatter population (lymphoid gate). The absolute count of each lymphocyte subset was calculated by multiplying their percentage from the total PB lymphocytes by the PB lymphocyte count obtained by means of an automated cell counter (Coulter Corporation, Miami, FL). No significant differences were detected between the groups under study as regards the number of nonlymphoid PB leukocyte populations.

***Study Schedule.*** In all patients the immunological tests were performed at the moment of entering the study protocol, and in the AWLD and ALCET groups they were repeated after 3 months of withdrawal. No significant changes in the patients' behavior were observed during withdrawal.

**TABLE 2. PB Distribution of Lymphoid Subsets Expressing NK-Associated Antigens Both in the AWLD and ALCET Groups During Active Alcoholism**

| Cell Subset | Control | AWLD | ALCET |
|---|---|---|---|
| CD56+ | 255 ± 35 | 548 ± 53* | 445 ± 36† |
| CD3−/CD56+ | 185 ± 31 | 302 ± 47† | 298 ± 54† |
| CD3+/CD56+ | 69 ± 10 | 246 ± 44‡ | 143 ± 37† |
| CD8−/CD57+ | 24 ± 4 | 78 ± 14§ | 48 ± 8† |
| CD8+/CD57+ | 86 ± 15 | 102 ± 28 | 48 ± 8‡‖ |

NOTE. Results are expressed as n. cells × $10^6$/L.
* $P = .001$ vs. control group.
† $P < .05$ vs. control group.
‡ $P < .01$ vs. control group.
§ $P < .001$ vs. control group.
‖ $P < .05$ AWLD vs. ALCET.

***Statistical Analysis.*** Results are expressed as mean ± SEM. Nonparametric one-way ANOVA Kruskall-Wallis test was used for overall differences. Pairwise comparisons were analyzed using Wilcoxon or Mann-Whitney $U$ nonparametric tests for paired or unpaired observations, respectively.

## RESULTS

The overall distribution of the different PB NK-cell subsets analyzed as well as the results of the basal NK cytotoxic assay, before the incubation with cytokines, are shown in Tables 2 and 3 and in Fig. 1.

***Active Alcohol Intake.*** The AWLD group of patients displayed a parallel increase in both NK lytic activity ($P < .01$) and in the absolute number of NK-cells (CD3−/CD56+) ($P < .05$) as compared with healthy controls. In addition, the number of T cells displaying a phenotype that has been associated with major histocompatibility complex unrestricted cellular cytotoxicity (CD3+/CD56+) as well as the CD8−/CD57+ NK-cell subset were also increased ($P < .01$ and $P < .001$, respectively). By contrast, in ALCET patients, although the number of NK-cells also significantly increased ($P < .05$), the NK lytic activity was depressed ($P < .05$).

***Withdrawal Period.*** After 3 months of EtOH withdrawal, PBMC from the AWLD group patients still displayed an increased NK lytic activity. In addition, the number of PB NK-cells (CD3−/CD56+ and the CD8−/CD57+) and PB CD3+/CD56+ T cells continued to be increased. In ALCET patients, EtOH suppression during a 3-month period was followed by a slight decrease in NK lytic activity of PBMC with respect to values in the active alcoholism phase, in parallel to the decrease of the number of NK-cells toward normal values.

Patients with ALC who underwent a withdrawal period of more than 1 year (ALCAW group) displayed normal number of PB NK-cells, with a NK-cell lytic activity significantly decreased as compared with that observed for the healthy individuals ($P < .05$).

**TABLE 3. PB Distribution of Lymphoid Subsets Expressing NK-Associated Antigens Both in the AWLD Group and Cirrhotic Patients During Withdrawal**

| Cell Subset | Control | AWLD + 3 Months Withdrawal | ALCET + 3 Months Withdrawal | ALCAW |
|---|---|---|---|---|
| CD56+ | 255 ± 35 | 730 ± 148*,§ | 354 ± 76 | 270 ± 58 |
| CD3−/CD56+ | 185 ± 31 | 400 ± 40†,‖ | 249 ± 52 | 192 ± 27 |
| CD3+/CD56+ | 69 ± 10 | 177 ± 98‡ | 103 ± 31 | 78 ± 21 |
| CD8−/CD57+ | 24 ± 4 | 121 ± 24*,§ | 37 ± 19 | 31 ± 18 |
| CD8+/CD57+ | 86 ± 15 | 110 ± 35 | 157 ± 36‡ | 166 ± 29‡ |

Results are expressed as n. cells × $10^6$/L.
* $P < .001$ vs. control group.
† $P < .01$ vs. control group.
‡ $P < .05$ vs. control group.
§ $P < .01$ AWLD vs. ALCET.
‖ $P < .05$ AWLD vs. ALCET.

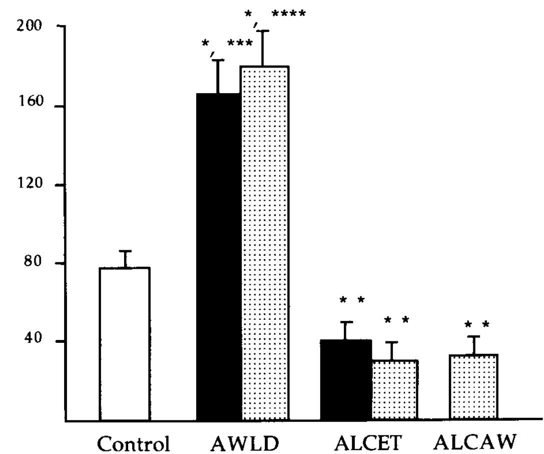


FIG. 1. NK cytotoxic activity in chronic alcoholism: comparison between active alcoholism and the withdrawal periods. Results are expressed in lytic units (LU)/$10^7$ effector cells. AWLD, alcoholism without liver disease; ALCET, alcohol-induced liver cirrhosis with active alcohol intake at the moment of entering in the study; ALCAW, alcohol-induced liver cirrhosis with at least 1 year of alcohol withdrawal. *$P < .01$ vs. control group; **$P < .05$ vs. control group; ***$P < .01$ AWLD vs. ALCET (active alcoholism); ****$P < .01$ AWLD vs. ALCET (withdrawal period). ■, Active alcoholism; ⊠, withdrawal period.

To gain further insights into the possible effects of cytokines on NK-cell lytic activity in ALC patients, basal NK activity was compared with NK enhanced activity using IL-2 in a group of six ALCAW patients. As observed in Fig. 2, IL-2 promoted a significant increase ($P < .05$) in the cytotoxic activity of PBMC from both ALC patients and healthy controls. Furthermore, a direct relationship between the IL-2 concentration and the lytic activity induced was clearly observed. However, the IL-2–induced cytotoxicity of PBMC from ALC patients continued to be depressed with respect to that obtained for healthy subjects, at the two concentrations of IL-2 used ($P < .05$).

## DISCUSSION

Overall, the results of the present study show that the behavior of PB NK-cell population in chronic alcoholism is

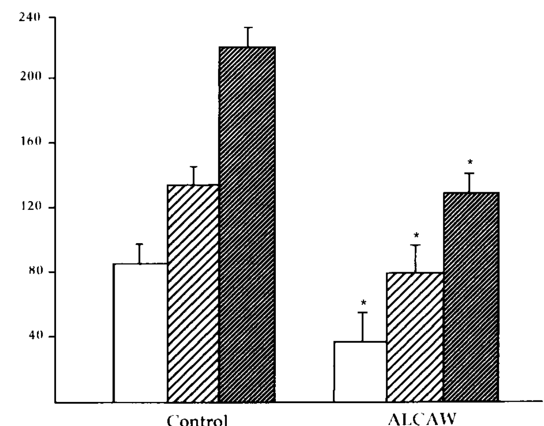


FIG. 2. NK cytotoxic activity in cirrhotic patients (ALCAW group) on activation with different concentrations of IL-2. Results are expressed in lytic units (LU)/$10^7$ effector cells. *$P < .05$ vs. control group. □, Basal NK cytotoxicity; ▨, NK activity in the presence of 10 U/mL of IL-2; ▩, NK activity in the presence of 100 U/mL of IL-2.

different according to both the moment of EtOH consumption and the existence or not of ALC; in addition, discrepancies are observed between the number of PB NK-cells and the NK cytotoxic activity of PBMC in ALC patients.

During the alcohol intake period, patients included in the AWLD group showed an increased NK cytotoxic activity together with a significant expansion of both PB NK-cells and cytotoxic T cells displaying the CD3+/CD56+ phenotype. The influence of EtOH itself on the NK-cell population is complex and remains largely unknown. *In vitro* studies suggest that low levels of alcohol may enhance NK-cell cytotoxic activity, a suppressive effect being observed at higher concentrations.[14,15] Results from animal studies have shown either an increase,[16,17] no changes,[18] or a decrease in NK lytic activity.[19] Blank et al.[20] have reported the existence of both an enhancement and a suppression of NK lytic activity, depending on the time of exposure to EtOH. Studies in humans are still scanty and affording contradictory results. Accordingly, while some groups have found an increased NK lytic activity,[21] these findings could not be confirmed in other series, in which decreased NK lytic activity has frequently been specifically associated with the existence of alcoholic liver disease.[22] The present study clearly shows increased NK cytotoxic activity as well as expansion of PB NK-cells. This could be related to an elevated antigenic stimulation[23] linked to EtOH consumption in the absence of alcoholic liver disease, which would be because of increased translocation of gut-derivated toxins such as endotoxin[24,25] and/or decreased hepatic antigen clearance[24] as well as an increased expression of modified hepatic antigens including acetaldehyde-modified proteins.[26] Surprisingly, in our study after a 3-month withdrawal period both the number of PB NK-cells and the NK cytotoxic activity remained significantly increased, in accordance with recent findings regarding other PB lymphoid subsets.[27]

Upon comparing the results of cytotoxicity assays in the ALC patients with those obtained for the AWLD group, important differences were observed. Although during active EtOH consumption an expansion of PB NK-cells was observed in both cirrhotic patients and in the AWLD group, the former patients showed an important depression in their NK cytotoxic activity, suggesting the existence of a decreased lytic activity/NK-cell. Moreover, independent of the duration of withdrawal period, in ALC patients EtOH withdrawal was followed by a slight decrease in the NK lytic activity of PBMC with respect to values in the active alcoholism phase. The results about the cytotoxic activity of PBMC in alcoholic cirrhosis are in accordance with other reports,[22,28,29] although in none of them the state of EtOH consumption is specifically mentioned. The cause for the decreased NK lytic activity found in ALC patients remains largely unknown. The possible existence of supressor cells or serum inhibitors that could influence the cytotoxic activity of NK cell subsets has been ruled out in previous studies.[22,28] Nevertheless, a significant increase of the PB CD8+/CD57+ lymphocytes has been found in the present study in ALC patients, and it has been shown that this lymphoid subset may release a factor that supresses the killer activity.[30] Moreover, slight decrease observed in the NK lytic activity in ALC patients who quit drinking could be related to the tendency to decrease the number of NK-cells with respect to values in the active alcoholism phase. Cytokines involved in the process of hepatic fibrogenesis, in particular the transforming growth factor $\beta$, could also contribute to the suppression of NK-cell activity.[31] Interestingly, in the present study it is shown for the first time that in ALC patients EtOH consumption *per se* do not exert an inhibitory effect in NK lytic function because the decreased NK cytotoxic activity of PBMC is observed in both patients with active EtOH intake and during withdrawal, independent of the duration of this period.

To gain further insights into the defect of NK-cells, we have analyzed the cytotoxic activity of PBMC on activation with IL-2 in ALC patients with a withdrawal period of at least 1 year. As compared with basal levels, IL-2 induced an increase of the cytotoxic activity of PBMC in ALC patients, although it remained significantly lower than that observed under the same conditions for healthy individuals. It should be noted that we have also observed a decreased production of interferon gamma in ALC patients on culturing PBMC enriched in NK-cells (Laso FJ et al., Unpublished observations, December, 1995). These findings altogether point to the existence of an intrinsic NK-cell defect.

In summary, our results show that in spite of an increased number of PB NK-cells during active chronic alcoholism, a depressed NK cytotoxic activity is constantly observed in alcoholic patients in the stage of alcoholic cirrhosis, supporting the notion that immunosurveillance mechanisms may be affected in these patients.

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