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Interleukin 2 activity in chronic liver disease and the effect of *in vitro* α-interferon

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SUMMARY

To investigate mitogen induced helper interleukin 2 (IL-2) production in patients with chronic liver disease (CLD), IL-2 activity was assessed by an IL-2 bioassay using phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMNC). IL-2 activity was significantly reduced in patients with autoimmune chronic active hepatitis, primary biliary cirrhosis and alcoholic hepatitis with or without cirrhosis ($P < 0{\cdot}01$), and was comparable to controls in those with alcoholic cirrhosis alone. *In vitro* preincubation of PBMNC with lymphoblastoid α-interferon (α-IFN) before stimulation with PHA, led to a significant increase in IL-2 activity in all subjects ($P < 0{\cdot}01$), except those with alcoholic hepatitis, but in none of the groups did the levels of IL-2 activity reach those seen in normal subjects. The decrease in IL-2 activity in patients with CLD may be due to low IL-2 production or presence of an IL-2 antagonist(s). Such an abnormality may occur, not only as a result of liver damage, but may also be important in determining immunological disturbances involved in the pathogenesis of the liver disease.

Keywords interleukin 2 chronic liver disease α-interferon

INTRODUCTION

Interleukin 2 (IL-2), a soluble protein, is present in cultures containing mononuclear cells stimulated by mitogen (Smith *et al.*, 1980), or antigen (Gillis & Smith, 1977). It has been demonstrated that this entity has a potent immunoregulatory action which enhances thymocyte mitogenesis (Watson *et al.*, 1979), stimulates the generation of cytotoxic T cells in response to alloantigens (Morgan, Ruscetti & Gallo, 1976), and promotes the anti-sheep red blood cell response of spleen cell culture (Gillis, Smith & Watson, 1980).

Patients with chronic liver disease (CLD) have several immunological abnormalities, e.g. low proliferative response to mitogens, skin anergy (Fox, Dudley & Sherlock, 1970; Snyder *et al.*, 1978), together with an increase in both antibody dependent cytotoxicity (ADCC) (Mieli-Vergani *et al.*, 1979) and cell-mediated cytotoxicity (CMC) (Actis *et al.*, 1983). Some of these abnormalities may result from the disturbances in the balance between T-helper and T-suppressor cell populations. Whilst suppressor T cell function has been found to be defective (Nouri-Aria *et al.*, 1982), and B cell activity was shown to be unaltered (Kakumu, Kazuaki & Kashio, 1980), the role of T-helper cells has received limited study.

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In the present study we have examined the activity of *IL-2* using a bioassay in patients with CLD. We have recently demonstrated that *in vitro* α-interferon (α-IFN) treatment of peripheral blood mononuclear cells (PBMNC) from patients with hepatitis B virus (HBV) related CLD significantly increased IL-2 activity (Saxena *et al.*, 1985). Since α-IFN has immunomodulatory effects, in addition to its well known anti-viral effects, we also studied IL-2 activity following *in vitro* incubation of PBMNC with α-IFN in patients with CLD.

MATERIALS AND METHODS

Patients. Forty-five patients with chronic liver disease and 17 normal controls were studied. The patients were divided into three subgroups. Biochemical and immunological data of the patients are shown in Table 1.

(a) *Autoimmune chronic active hepatitis (AI.CAH).* The diagnosis was confirmed histologically in all 12 patients (10 females, 2 males). Serum autoantibodies (SMA & ANA) in titres greater than 1:40 had been present in all patients at some time during the course of their illness. The median age was 39 years (range 21–60). Ten patients had cirrhosis and three of them were untreated at any time of the study. Nine patients were receiving immunosuppressive therapy (Prednisolone 7·5–12·5 mg/day and Azathioprine 50–10 mg/day), six of whom were in clinical remission as assessed clinically and biochemically. The remaining three patients showed moderate to severe disease activity on liver biopsy despite immunosuppressive therapy.

(b) *Primary biliary cirrhosis (PBC).* Sixteen patients were included in this group (14 females, 2 males). The median age was 51 years (range 36–66). Liver biopsies showed histological changes consistent with PBC in all patients, and all had anti-mitochondrial antibodies in serum. Cirrhosis was present in five. Histological staging showed one stage I, seven stage II, three stage III and five stage IV. Ten were on no specific treatment, and the remaining six on Cyclosporin A (300 mg/day).

(c) *Alcoholic Liver Disease (ALD).* The diagnosis was confirmed histologically in seven patients. In the remaining patients in whom a liver biopsy was not possible, all had oesophageal varices with clinical and isotope scan evidence of severe, longstanding chronic liver disease. Cirrhosis was present in 12, of whom six had alcoholic hepatitis in addition. The remaining five patients showed the features of alcoholic hepatitis alone. None was on any specific treatment.

Table 1. Biochemical and immunological data of patients with chronic liver disease

Groups		Serum aspartate amino-transferase (iu/l)	Smooth muscle antibody	Anti-nuclear antibody	Anti-mitochondrial antibody	IgG	IgA	IgM
Autoimmune CAH ($n=12$)		131 (28–410)	1:40 (−ve–1:160)	1:10 (−ve–1:40)	–	19·2 (11·7–31·6)	2·5 (1·8–5·2)	2·4 (1·0–5·5)
PBC ($n=16$)		77 (25–214)	1:10 (−ve–1:160)	1:40 (−ve–1:256)	1:450 (1:16–1:640)	16·1 (11·6–25·0)	2·6 (1·3–5·0)	2·8 (1·6–11·4)
ALD	AH ($n=11$)	98·3 (31–347)	–	(−ve–1:20)	–	12·8 (7·8–21)	3·3 (1·1–9·7)	1·6 (1·0–4·1)
	AC ($n=6$)	66 (29–170)	–	(−ve–1:40)	–	17·0 (9·9–27·3)	5·4 (1·6–9·7)	1·8 (0·84–4·4)

Data are expressed as median (range).
AH = alcoholic hepatitis ± cirrhosis.
AC = alcoholic cirrhosis.

Mononuclear cell preparation. Peripheral blood mononuclear cells (PBMNC) were isolated by centrifugation of leucocyte enriched plasma, obtained from heparinized blood (10 units per ml of dextran 6% w/v in 0·15 M NaCl) on Ficol-Triosil for 20 min at 400 *g* (Bøyum, 1968). The interface layer was incubated in plastic Petri dishes for 1 h at 37°C to remove adherent cells. The non-adherent cells containing 3–7% monocytes as assessed by peroxidase were washed three times in Hank's balanced salt solution (HBSS) and adjusted to a concentration of 1×10^6/ml in 10% heat inactivated fetal calf serum in RPMI 1640 containing 2 mM glutamine, 200 μg/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml amphoteracin B.

IL-2 production. Non-adherent PBMNC, contained in Falcon tubes, were stimulated with 2 μg PHA per 10^6 cells and incubated at 37°C in humidified air with 5% CO_2 for 48 h. After centrifugation, the supernatant was filtered through a 0·22 μm Millex-GS filter and frozen at −20°C until used. In additional experiments, 11 supernatants from patients with various chronic liver disease were absorbed overnight with chicken or sheep red cells to remove contaminating PHA (Miyawaki *et al.*, 1985).

Assay of IL-2 activity. The assay was based on the capacity of the supernatants to promote DNA synthesis of cultured cells (Karttunen *et al.*, 1984). Short-term lectin stimulated blast cells, obtained by stimulation of PBMNC, obtained from a healthy donor, with 10 μg/ml PHA (Sigma Chemical Co., St Louis, MO, USA) for 5 days at 37°C, as described above, were utilized in this assay (Karttunen *et al.*, 1984). 10^4 blast cells per well were cultured in a flat bottomed microplate in the presence of serial dilutions (1:2, 1:4, 1:8 & 1:16) of IL-2 containing supernatants with RPMI 1640 from both patients and normal controls. Each sample was assayed in triplicate. Reference IL-2 (Biotest-Diagnostic) was originally diluted 1:20 in RPMI 1640 and kept frozen. At the time of study, a serial dilution of this IL-2 was used as a standard, and at least two samples from healthy individuals were included in each experiment. Cells were incubated for 72 h at 37°C as above. 1 μCi tritiated thymidine was added to each well 4 h before the end of the culture period, and the cells were harvested (Titertek, Flow. Lab.) and counted in a liquid scintillation counter. The results are expressed as a percentage of that with standard and IL-2 containing supernatants which were assayed concomitantly as follows (Nakayama *et al.*, 1983):

$$\frac{\begin{array}{c}H^3\text{-TdR uptake at 1:2 dilution of test} \\ \text{sample stimulated with PHA}\end{array} - \begin{array}{c}H^3\text{-TdR uptake of short term} \\ \text{lectin stimulated PBMNC}\end{array}}{\begin{array}{c}H^3\text{-TdR uptake at 1:2} \\ \text{dilution of standard}\end{array} - \begin{array}{c}H^3\text{-TdR uptake of short term} \\ \text{lectin stimulated PBMNC}\end{array}} \times 100$$

Initial experiments were carried out to obtain optimal conditions for IL-2 production, which was achieved at a concentration of 1×10^6 cells/ml when stimulated with 2 μg PHA. IL-2 production was maximal at 48 h and then gradually declined. Partial depletion of monocytes increased IL-2 activity. In some experiments, PBMNC were treated with lymphoblastoid α-IFN (Wellferon—as characterized elsewhere) (Zoon *et al.*, 1984) (1,000 units/10^6 cells) for 1 h at 37°C. The cells were washed three times with HBSS and then cultured in the presence and absence of PHA for IL-2 production as described previously.

Statistical analysis. Statistical significance was calculated by Wilcoxon's Rank sum test for non parametric values. The paired rank test was used to analyse IL-2 activity before and after IFN treatment. Linear regression analysis was performed to evaluate the correlation between IL-2 activity and serological, biochemical and histological parameters.

RESULTS

Maximal IL-2 activity was demonstrated at a PBMNC supernatant dilution of 1:2 for both normal individuals and patients with CLD (Table 2), and the results are presented as units of IL-2 activity per millilitre at this dilution.

To demonstrate whether residual PHA, present in the IL-2 containing supernatants, would influence proliferative responses of PHA blast cells, 11 supernatants from patients with chronic liver

Table 2. The proliferative responses (median (ct/min) and range) of PHA blast cells to four different dilutions of lymphocyte IL-2 containing supernatants from normal subjects and patients with chronic liver disease

	Dilutions of Supernatants containing IL-2			
	1:2	1:4	1:8	1:16
Normal subjects ($n=17$)	3143 (806–12688)	2046 (487–10060)	1463 (612–5950)	962 (303–2508)
Autoimmune chronic active hepatitis ($n=10$)	1139 (599–2079)	801 (411–1317)	668 (256–1469)	518 (210–1117)
Primary biliary cirrhosis ($n=16$)	1402 (250–3700)	1035 (274–2620)	725 (223–2669)	598 (154–1292)
Alcoholic liver disease ($n=15$)	1223 (345–8936)	913 (315–2934)	768 (185–1691)	506 (103–1266)

disease were tested for IL-2 activity both before and after absorption overnight with chicken or sheep red cells. The results showed that following absorption with red blood cells, the median activity of IL-2 decreased by only 17% (43·5 before and 36·0 after absorption).

IL-2 activity in normal subjects ranged from 46·5 to 331·6 units/ml (Fig. 1), with a median of 151·1 units/ml. This wide range of IL-2 activity in our normal subjects is in agreement with previous findings and was similar in both sexes (female range 131–293, median 165·2 and male range 49–331, median 147·1 units/ml).

IL-2 activity in patients with autoimmune CAH (range 6–88·4, median 26·2 units/ml), was significantly less than that observed in normal controls ($P<0·01$), and did not differ in those with untreated active CAH (range 8–45, median 24·7 units/ml), or those with corticosteroid treated CAH (range 6–88·4, median 26·3 units/ml) (Fig. 1). Nor was there any difference between those in clinical

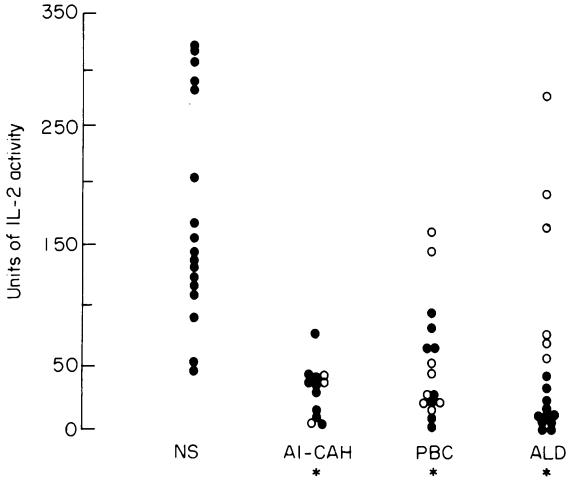

Fig. 1. The activity of IL-2 (units/ml) on the proliferative responses of cultured lymphocytes.
Normal subjects (NS).
Patients with:
Autoimmune chronic active hepatitis (AI.CAH) (○) untreated and (●) corticosteroid treated.
Primary biliary cirrhosis (PBC) (○) stages I & II and (●) stages III & IV.
Alcoholic liver disease (ALD) (○) alcoholic cirrhosis and (●) alcoholic hepatitis.
* $P<0·01$ when compared with normal subjects.

remission (range 31–38, median 34·3 units/ml), and those showing no response to prednisolone (range 6–88·4, median 25·0 units/ml).

In patients with PBC, IL-2 activity (range 3–168, median 40 units/ml) was also significantly less than that of normal subjects ($P < 0{\cdot}01$). There was no significant difference between the untreated (range 11–156, median 47·0 units/ml), and treated (range 3–168, median 29·7 units/ml) patients with PBC. Similarly, there was no significant difference between those patients with early disease (stages I & II) (range 16–168, median 46·1 units/ml), and those with late disease (stages III & IV) (range 3–91, median 34·7 units/ml).

The median IL-2 activity in alcohol related liver disease with cirrhosis alone was similar to that seen in normal subjects (range 59–289, median 124 units/ml) (P = ns). In contrast, IL-2 activity in patients with alcoholic hepatitis with or without cirrhosis (range 2·6–47·3, median 11·8 units/ml) was markedly and significantly reduced ($P < 0{\cdot}01$) (Fig. 1).

Kinetic studies using PBMNC from four patients with CLD when cultured with different concentration of PHA (1, 2 & 4 μg/ml) at different time periods (1, 2 & 3 days), showed very little change in IL-2 activity with incubation time ranging from 24 to 72 h. The optimum dose of mitogen was not consistent, being 1 μg in two patients, 4 μg in another and no clear-cut effects at different doses in the remaining case.

There was no correlation between serum aspartate aminotransferase, alkaline phosphatase, total bilirubin, serum Ig levels, autoantibodies or treatment, and IL-2 activity in any of these groups of patients studied.

In vitro effect of α-IFN on IL-2 activity. In vitro treatment of PBMNC from eight normal subjects with α-IFN before incubation with PHA, produced a significant fourfold increase in IL-2 activity (median 132·0 units/ml before and 479·5 units/ml after α-IFN treatment) ($P < 0{\cdot}01$) (Fig. 2).

IL-2 activity was also increased when PBMNC from patients with autoimmune CAH (12·3 before and 59·5 units/ml after α-IFN treatment) and PBC (78·1 before and 246·7 units/ml after α-IFN treatment) were incubated with α-IFN, but this increase was less than that seen in normal controls.

Patients with AC showed an increase in PHA induced IL-2 activity similar to that observed in normal subjects following preincubation of their PBMNC with α-IFN (80·2 before and 305·7 units/ml after α-IFN-IFN preincubation) (Fig. 2). In contrast, preincubation of PBMNC from patients with AH with α-IFN did not change IL-2 activity (21·0 before and 16·3 after α-IFN treatment).

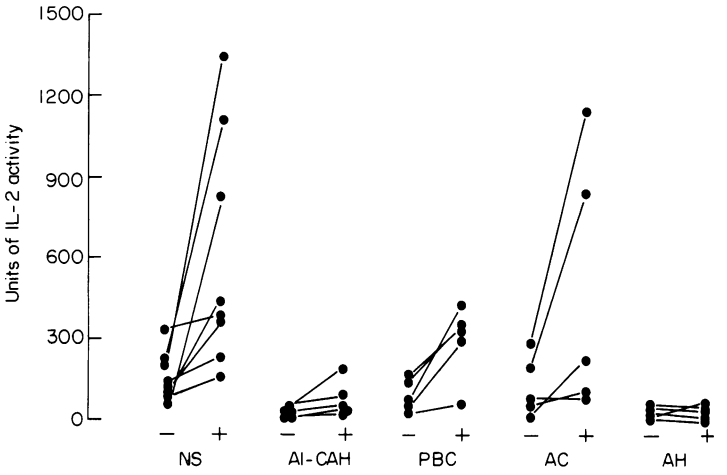

Fig. 2. *In vitro* effect of α-IFN on IL-2 activity (units/ml) from normal subjects and patients with autoimmune chronic active hepatitis (AI.CAH), primary biliary cirrhosis (PBC), alcoholic cirrhosis (AC) and alcoholic hepatitis (AH).
(−) In the absence of α-IFN treatment.
(+) In the presence of α-IFN treatment.

Table 3. *In vitro* effect of α-IFN and PHA on IL-2 activity in patients with chronic liver disease and normal subjects

	Unstimulated	α-IFN	PHA	α-IFN + PHA
Normal subjects ($n=8$)	10·4 (4–38)	46·2 (11·3–270)	132·0 (46·5–331)	479·5 (151–1363)
Autoimmune CAH ($n=5$)	1·7 (1–4)	4·6 (1·2–12·2)	12·3 (6–42)	59·5 (27–184)
PBC ($n=5$)	21·7 (4·7–166)	48·8 (43–90)	78·1 (29–168)	246·7 (57–359)
Alcoholic cirrhosis ($n=5$)	12·6 (1–43)	23·7 (8–64)	80·2 (59–289)	305·7 (103–1162)
Alcoholic hepatitis ($n=5$)	5·2 (2·7–7·5)	10·9 (6–17)	21·0 (6·8–47)	16·3 (2–57)

Data are expressed as median (range) in units/ml.

To further investigate whether the increase in IL-2 activity following exposure to *in vitro* α-IFN is due to mitogenic effects of interferon, some α-IFN treated PBMNC were cultured in the absence of PHA. The results in all groups clearly demonstrated that α-IFN itself does not have a significant stimulatory effect (Table 3).

DISCUSSION

We emphasize that we have measured IL-2 activity throughout this study rather than IL-2 production. Previous workers have used mainly an IL-2 dependent cytotoxic T lymphocyte line (CTLL) (Linker-Israeli *et al.*, 1983) for the measurement of IL-2 activity, although some studies in humans have used a short-term lectin stimulated blast cell assay, similar to that used in the present studies (Karttunen *et al.*, 1984). Recent evidence suggests that both assays give comparable results (Nouri, Panayi & Goodman, 1984) and the present results with red cell absorption show that carry-over of PHA into the assay system is not a major contribution to the proliferative response of the assay cells.

The results presented in this study clearly demonstrated that the activity of IL-2 containing supernatants from PHA stimulated PBMNC is reduced in the majority of patients with CLD. Similar observations were presented by Yoshioka *et al.* (1984). However, in the present study, IL-2 activity in patients with alcoholic cirrhosis was found to be comparable to normal subjects. The mechanism for the poor IL-2 activity in patients with CLD is unknown. One possible explanation for the deficiency of IL-2 production may be due either to reduced numbers of IL-2 producing cells in the peripheral circulation, or to reduced production of IL-2 by normal numbers of IL-2 producing cells. Thus, Schafer, Scribner and Klassan (1984) have shown that the proliferative responses of PBMNC to exogenous IL-2 in patients with PBC is significantly reduced.

An alternative explanation for the decrease in IL-2 activity may be a decrease in activity of IL-1 or overactivity of prostaglandin producing monocytes (e.g. PGE_2) in patients with CLD, since the inhibitory effects of PGE_2 on IL-2 production have been clearly demonstrated (Tilden & Balch, 1982). However, our preliminary studies using a radioimmunoassay to measure PGE levels in lymphocyte supernatants have shown that there is no correlation between IL-2 activity and PGE levels in patients with CLD (data not shown).

In vitro incubation of PBMNC with α-IFN produced a rise in the activity of IL-2 following PHA stimulation in all groups except in patients with alcoholic hepatitis. As we were able to exclude the possibility that α-IFN had direct mitogenic effects, it seems that there are at least two possible

explanations for this observation. Firstly, α-IFN treatment may induce the expression of IL-2 receptors. Thus, in a recent study, Johnson *et al.* (1984) showed that T cells activated by either Concanavalin A or γ-IFN for 24 h, absorbed increased amounts of IL-2 and they attributed this to an increase in the number of IL-2 receptors. Our results are consistent with a similar receptor induction following exposure to α-IFN.

An alternative explanation for the enhancement of IL-2 activity, may be that α-IFN treatment activates monocytes to secrete monokine(s) and that the relevant monokines and/or IL-1, stimulate IL-2 production.

The lack of correlation between liver function tests, immunological parameters and disease activity and IL-2 activity, and on the other hand the normal activity of IL-2 in patients with alcoholic cirrhosis alone, may suggest that the depression of IL-2 activity in CLD is not secondary to cirrhosis. However, further studies are required to determine whether the abnormalities in IL-2 activity described here are induced by a chronic inflammatory process in the liver or are part of the immunological disturbances which predispose to organ damage.

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