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Analysis of T cell activation pathways in patients with liver cirrhosis, impaired delayed hypersensitivity and other T cell-dependent functions

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SUMMARY

Patients with cirrhosis of the liver frequently demonstrate anergy in intracutaneous tests and fail to respond to vaccination, suggesting impaired delayed hypersensitivity and other T cell-dependent functions *in vivo*. T cell activation through the coordinated interaction of different cells of the immune system (B cell, antigen-presenting cells (APC)) is an important step in the induction of cellular and humoral immune responses. Impaired T cell-dependent functions in patients with liver cirrhosis may thus be explained by defective T cell activation. We prospectively investigated T cell activation pathways in 12 patients (nine males, three females) with alcoholic liver cirrhosis (seven Child Pugh stage A and B (CP A + B), five Child Pugh stage C (CP C)) and five healthy controls and compared the *in vitro* results of T cell activation with data obtained *in vivo*, e.g. intracutaneous tests and vaccination against hepatitis B surface antigen (HBs-Ag). Five out of eight patients who completed vaccination against hepatitis B virus infection were non-responders; one of the three responders had a non-protective anti-HBs titre. Moreover, three of five patients with alcoholic liver cirrhosis CP A + B, and two out of three with CP C were anergic in intracutaneous tests to a set of diverse antigens. All parameters of T cell activation were normal, including proliferation mediated by CD2, CD3–T cell receptor (TCR) complex, and CD28; acquisition of responsiveness to exogenous IL-2 and IL-4; activation of proteinkinase C (PKC) by phorbol ester and calcium influx by addition of ionomycin. The ability of monocytes to deliver costimulatory signals was preserved in patients with alcoholic cirrhosis. In addition, serum of patients with alcoholic liver disease did not inhibit T cell proliferation. We conclude that, although in patients with alcoholic liver cirrhosis T cell-dependent functions are impaired *in vivo*, T cell activation pathways are not responsible for the observed immune defect.

Keywords liver cirrhosis T cell activation delayed hypersensitivity vaccination with HBs-Ag in liver cirrhosis

INTRODUCTION

Patients with liver cirrhosis have an impaired immune response. They are frequently anergic in intracutaneous tests to a set of diverse antigens, have an increased incidence of infection, and often fail to respond to vaccinations such as hepatitis B virus (HBV) vaccine [1–9]. A coordinate interaction of different cell types (T cells, B cells, antigen-presenting cells (APC)) with their respective cell surface receptors is required to mount an immune response against a given antigen such as a vaccine [10]. Since both antibody production and T cell functions depend on an appropriate interaction between T cell receptor (TCR), antigen, and MHC molecule, we ask in the present study whether the underlying defect in patients with alcoholic liver cirrhosis may be explained by defective T cell activation.

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With MoAbs directed against certain epitopes of surface molecules it was possible to study activation events necessary for T cell proliferation. This led to the discovery of activation pathways (mediated by the antigen/MHC-reactive CD3–TCR complex, CD2 or CD28) [11–20]. Activation of T lymphocytes results in responsiveness to exogenous interleukins. IL-2 augments DNA synthesis via CD2, CD3–TCR complex and CD28, whereas responsiveness to IL-4 in CD2-pretreated T cells can be achieved by triggering the LFA-3 (CD58) binding site of the CD2 molecule [21,22]. A disturbed interaction of growth factors/cytokines with their respective cell surface receptors has been shown in malignant transformed T lymphocytes, leading to autonomous growth [23].

The aim of this study was to elucidate in more detail mechanisms of T cell activation in patients with liver cirrhosis. Unlike

other clinical diseases with accompanying immune deficiencies (e.g. common variable immunodeficiency (CVID) syndrome, where the alternative pathway of T cell activation is defective [24,25]), our data provide evidence that T cell activation mediated by CD3–TCR complex, CD2, or CD28 and the ability of monocytes to deliver costimulatory signals are normal in patients with liver cirrhosis and thus not responsible for the impaired immune response.

MATERIALS AND METHODS

Patients

Twelve patients (nine males, three females) with alcoholic cirrhosis (seven Child Pugh stage A and B (CP A+B), five Child Pugh stage C (CP C)) and five healthy controls were investigated. The clinical background and laboratory data are summarized in Table 1. Diagnosis was made by a history of alcohol abuse, biopsy, and typical findings on ultrasound or computed tomography (CT) scan of the abdomen. There was no evidence for viral, autoimmune, metabolic or drug-associated liver disease. Patients abstained from alcohol for at least 9 months before the study was performed. Patients, their spouses or other relatives were regularly interviewed about their alcohol abstinence. When there was any doubt about the drinking habits of patients, serum alcohol levels were determined.

Liver disease was classified by the Pugh′s modification of Child′s grading as described previously [26]. Briefly, encephalopathy, ascites, bilirubin, albumin, and prothrombin time were assessed and 1–3 points were scored for increasing abnormality. Addition of the points for the five variables gave a score ranging from 5 to 15. Patients with up to 6 points were determined as grade A, 7, 8, or 9 as grade B, and 10–15 as grade C.

Patients were vaccinated with 20 μg of recombinant HBV-surface antigen (HB-Vax; Merck, Sharp, and Dohme (MSD), Munich, Germany) at 0, 4, and 8 weeks. Two months after the last booster the anti-hepatitis B surface (HBs) titre was measured by conventional ELISA technique.

The Multitest Mérieux (Institut Mérieux, Leimen, Germany) was used for intracutaneous testing with the following antigens: tetanus toxoid, diphteria toxoid, Streptococcus antigen, tuberculin, Candida antigen, Trichophyton antigen, *Proteus mirabilis* antigen and control (glycerine). The test procedure was applied at the inner side of the forearm. Subcutaneous infiltration was measured after 48 h; an induration of $\geq$ 2 mm was considered positive.

Cell preparation

Peripheral blood mononuclear cells (PBMC) of patients and healthy individuals were isolated from heparinized blood by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. PBMC were plated onto plastic dishes at 3×10^6 cells/ml and incubated for 1 h at 37°C, 7% CO_2 and 100% humidity. Non-adherent cells were separated into E-rosette-positive (E^+) and E-rosette-negative (E^-) populations by Ficoll–Hypaque gradient centrifugation. E^+ cells were 98% pure and used at a concentration of 50 000 cells/well.

In order to isolate monocytes, PBMC were plated onto plastic dishes at 3×10^6 cells/ml and incubated for 1 h at 37°C, 7% CO_2 and 100% humidity. Non-adherent cells were removed, and dishes were carefully washed three times in order to remove all non-adherent cells. After this procedere all adherent cells were incubated for another 2 h at 37°C, 7% CO_2, and 100% humidity and carefully removed by moving a stamp over the ground. Purity was assessed by FACS analysis. Monocytes were used at a concentration of 5000 cells/well.

Monoclonal antibodies

MoAbs reactive with human CD2 (M1 and M2) were provided by Dr S. Meuer (German Cancer Research Centre, Heidelberg, Germany). They were used at a final dilution of 20 μg/ml and 40 μg/ml, respectively. Anti-CD3 OKT-3 was purchased from Ortho Diagnostics (Neckargmünd, Germany). Round-bottomed microtitre plates (Costar, Cambridge, MA) were coated with MoAb OKT-3 by overnight incubation at 4°C at 50 μl (0·1 mg/ml in PBS), and subsequently washed (three times) to remove uncoated MoAb. Anti-CD28 15E8 was a gift of Dr E. Wierenga (Departments of Cell Biology and Histology, University of Amsterdam, The Netherlands) and used at a final dilution of 1:1000 of ascites fluid.

Table 1. Clinical data of patients with liver cirrhosis

Patient	Age, years	Sex	Alcohol abstinence/months	Bilirubin, mg/dl	Child Pugh grade	Complications	α-HBs tire, U/*l*	Lymphocyte, G/*l*
1	47	F	15	1·9	B	Eso. bleed.	Neg.	0·4
2	58	M	9	1·5	B	DM, Eso. bleed.	NC	0·3
3	45	F	12	1·5	B	Eso. bleed.	76	1·2
4	50	M	10	1·8	A	Eso. bleed.	Neg.	1·4
5	55	M	18	1·4	B	Eso. varices	38	1·9
6	34	M	9	1·1	B	Eso. varices	5·8	2·1
7	46	M	10	1·0	B	Eso. varices	NC	0·6
8	49	M	15	4·0	C	Eso. bleed.	NC	0·8
9	46	M	18	3·1	C	Eso. varices	NC	1·0
10	42	F	9	7·1	C	SBP, Eso. varices	Neg.	1·5
11	45	M	10	6·0	C	DM, SBP, Eso. bleed.	Neg.	1·4
12	64	M	15	5·8	C	SBP, Eso. bleed.	Neg.	1·0

Eso. bleed., Bleeding from oesophageal varices; Eso. varices, oesophageal varices; DM, diabetes mellitus; SBP, spontaneous bacterial peritonitis; NC, vaccination schedule not completed.

Normal range: lymphocyte count 1·0–4·8 G/*l*; bilirubin < 1 mg/dl. An anti-HBS titre >10 U/*l* is considered protective [28].

Lymphokines and mitogens

Recombinant IL-4 was provided by Dr W. Ewald (Theodor-Boveri-Institute for Bioscience, Würzburg, Germany) and highly purified natural IL-2 by Dr U. Schwulera (Biotest, Dreieich, Germany). Phorbol ester (phorbol myristate acetate (PMA)), and calcium ionophor (ionomycin) were purchased from Sigma (Deisenhofen, Germany). IL-4 was added at a final concentration of 20 ng/ml, IL-2 at a concentration of 20 U/ml. PMA and calcium ionophor were used at concentrations of 1 ng/ml and 1 μg/ml, respectively.

Proliferative assay studies

Purified T cells ($5{\cdot}0 \times 10^4$) were cultured in round-bottomed microtitre plates (Costar) in 200 μl RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2% L-glutamine, 1% penicillin/streptomycin and 10% fetal calf serum (FCS) or patients' sera in selected experiments. Monocytes were added at a concentration of 5000 cells/well. After 96 h, individual wells were pulsed with 2 μCi ^{3}H-thymidine (specific activity 80 μCi/mmol; Radiochemical Centre, Amersham International Ltd, Aylesbury UK) for 16 h and harvested (Skatron LKB (Skatron, Inc., Sterling, UK) cell harvester). ^{3}H-thymidine incorporation was measured by a liquid scintillation spectrometer (LKB). Results were expressed as means of triplicates and s.d. were < 15%.

Flow cytometric analysis

Triple immunofluorescence staining was performed with the following combinations of conjugated antibodies: CD3 (MT301-FITC) and CD14 (M-M42-FITC) provided by Dr E. P. Rieber (Institute for Immunology, Munich, Germany), CD4 (Leu-3a-PE; Becton Dickinson, Hamburg, Germany), CD8 (3B8-TRI-colour; Medac, Hamburg, Germany), CD16 (Leu-11c-PE; Becton Dickinson), CD19 (CD19-Tricolor; Medac), CD45RA (2H4-PE; Coulter, Hialeah, FL), CD56 (NKH-1-PE; Coulter).

EDTA-blood (100 μl) was incubated for 30 min at 4°C with MoAbs at optimal concentration. Erythrocytes were lysed and probes fixed with 1% paraformaldehyde employing an automated lysing instrument (Q-Prep; Coulter). The cell suspension was diluted with 300 μl distilled water and analysed by FACScan (Becton Dickinson), as described previously [27].

RESULTS

Response to intracutaneously applied antigens and HBs-antigen vaccination

Three of five patients with liver cirrhosis CP A + B, and two of three with CP C were completely anergic in the intracutaneous tests (Table 2). For example, six of eight patients (three with liver cirrhosis CP A + B and three with cirrhosis CP C) showed no reaction to tetanus toxoid, while only two had a normal response. In contrast to patients, all healthy controls reacted to a number of intracutaneously applied antigens. Eight patients completed the vaccination schedule against HBV, and only three of them (all with liver cirrhosis stage CP B) showed seroconversion. However, one of the responders did not develop a protective anti-HBs titre (Table 1), which is considered above 10 U/*l* [28]. In contrast, 80–90% of healthy indviduals produce a protective anti-HBs titre [29]. Taken together, these data demonstrate the marked impaired T cell-dependent function in patients with liver cirrhosis.

Expression of surface makers on T cells

Expression of surface markers on T lymphocytes of patients with alcoholic liver disease was determined by flow cytometry (Table 3). Blood was available from nine of 12 patients. A group of healthy donors (age 18–34 years, 26 female, 16 male) served as controls [27]. We found an increased number of $CD4^+$ cells in patients with liver cirrhosis in comparison with healthy control individuals (mean 57·1% *versus* 41·1%; $P < 0{\cdot}05$ (Wilcoxon test)) which is an accordance with data of Müller *et al.* [30]. This group also observed a slightly elevated number of $CD3^+$ and a reduced

Table 2. Intracutaneous tests in patients with liver cirrhosis and healthy controls

Patient	Child Pugh grade*	Control	Tetanus toxoid	Diphteria toxoid	Streptococcus	Tuberculin	Candida antigen	Trichophyton	Proteus antigen
1	B	–	++	–	–	–	–	–	–
3	B	–	–	–	–	–	–	–	–
4	A	–	–	–	–	–	–	–	–
5	B	–	–	–	–	–	–	–	–
6	B	–	++	–	–	–	–	–	–
10	C	–	–	–	–	–	–	–	–
11	C	–	–	–	–	–	–	–	–
12	C	–	–	–	–	++	–	–	–
HC		–	++	++	–	++	–	–	++
HC		–	++	++	++	++	++	–	–
HC		–	++	–	–	++	++	–	–
HC		–	++	++	–	++	–	++	++
HC		–	++	++		++	–	++	–
HC		–	++	–	–	++	++	–	++
HC		–	++	++	–	++	–	–	–
HC		–	++	++	–	–	++	–	–

* For Child Pugh classification, see Materials and Methods.

The results of intracutaneous tests are classified as: –, no induration; ++, positive as described in Materials and Methods.

Table 3. FACS analysis of peripheral blood mononuclear cells (PBMC) from patients with liver cirrhosis and healthy controls

Patient	Lymphocyte count, G/*l*	CD3* (%)	CD4 (%)	CD4/45RA (%)	CD8 (%)	CD8/45RA (%)	CD16/56 (%)	CD19 (%)	CD45 (%)	CD45RA (%)
1	0·4	83·4	71·2	54·8	12·1	10·7	4·1	6·3	96·8	66·3
2	0·3	60·8	57·5	49·2	13·1	10·2	18·3	11·4	95·9	60·9
3	1·2	81	61·7	9·2	19·3	10·9	15·1	3·8	99·9	20·4
4	1·4	70	51·4	36·8	21·6	16·3	8·7	8·6	99·7	57·2
5	1·9	67	52·5	21·2	21·7	15·5	22·8	6·6	99·5	35·9
8	0·8	59	46·3	34·8	11·6	10·2	28·1	2·8	99·3	45·7
9	1·0	31	40·1	26·6	26·9	19·1	53·1	9·6	99·5	49·5
10	1·5	81·6	68·4	49·1	13	5·3	7·1	5	99·8	54
12	1·0	75	64·9	48·2	17·7	11·2	11·3	6	96·5	58
Mean	1·05	67·6	57·1	36·6	17·4	12·2	18·7	6·6	ND	ND
Control		69	41·1	ND	22·9	ND	17·6	9·2	ND	ND
Min/max	0·3–1·5	56/78	31/48	ND	11/38	ND	6/36	4/15	ND	ND

* For details, see Materials and Methods.
ND, Not done. Normal range: lymphocyte count 1·0–4·8 G/*l*.

percentage of $CD8^+$ T lymphocytes, which we could not confirm. In one patient (patient 9) an increased number of cells expressing natural killer (NK) markers CD16 and CD56 was found. Taken together, these changes in the composition of PBMC subpopulations of patients with liver cirrhosis are minor and probably not responsible for the immune defect.

Proliferation of T cells

Mediated by CD2. Results of the response mediated by CD2 are shown in Fig. 1 for healthy controls, patients with liver cirrhosis CP A + B, and CP C, respectively. Responders ($n = 3$) and non-responders ($n = 5$) to HBs-antigen (HBs-Ag) vaccination were grouped. Triggering with mitogenic MoAbs directed at the CD2 molecule resulted in comparable proliferation of T lymphocytes from healthy control individuals and from patients. Acquisition of responsiveness to exogenous IL-2 and IL-4 was also not different between patients with cirrhosis and healthy controls (HC). There was a trend for a lower responsiveness to IL-4 in CP C patients (44 398 ± 17 966 ct/min (mean ± s.e.m.) *versus* 84 843 ± 14 075 ct/min in HC; $P > 0{\cdot}05$, Wilcoxon test).

Only one other study [31] has investigated T cell activation mediated by CD2 in patients with alcoholic liver cirrhosis. The authors reported a decreased proliferative response and reduced IL-2 responsiveness during active alcohol intake. However, after a 6-month period of ethanol abstinence most patients showed a normal proliferation, as did our patients.

Mediated by MHC-reactive CD3–TCR complex. Analysis of the proliferative response of T lymphocytes stimulated by the CD3–TCR complex revealed no defect in the signal transducing capacity (Fig. 1). Triggering of the CD3 molecule alone did not result in clonal expansion. Addition of IL-2 to anti-CD3-pretreated T lymphocytes resulted in DNA synthesis which was equally high in HC and patients, regardless of their cirrhotic stage and whether or not they responded to HBs-Ag vaccination. Since the responder group was small ($n = 3$), statistical analysis was performed between healthy controls and patients with cirrhosis stage CP A + B and CP C. The analysis revealed no significant difference.

A decreased proliferative response of T cells to stimuli mediated by the MHC-reactive CD3–TCR complex was observed

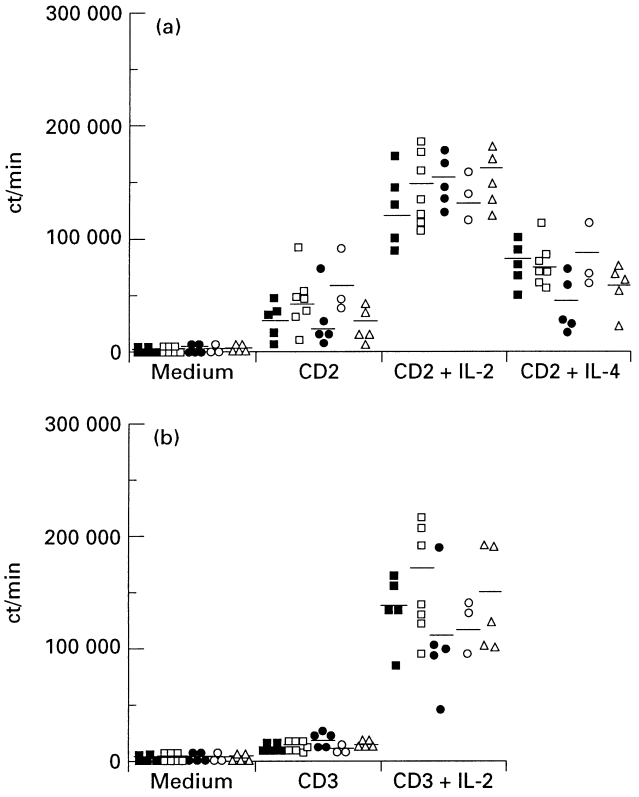

Fig. 1. Proliferation of T cells mediated by CD2 (a) and MHC-reactive CD3–T cell receptor (TCR) complex (b). Incorporation of ^{3}H-thymidine in response to the indicated stimuli. Results are grouped for healthy controls (■) and patients with liver cirrhosis Child Pugh stage A and B (□) and Child Pugh stage C (●). Furthermore, patients were subdivided into responders (○) and non-responders (Δ) to hepatitis B surface antigen (HBs-Ag) vaccination, respectively. Proliferation mediated by the CD2 molecule, the CD3–TCR complex and responsiveness to exogenous interleukins showed no significant difference.

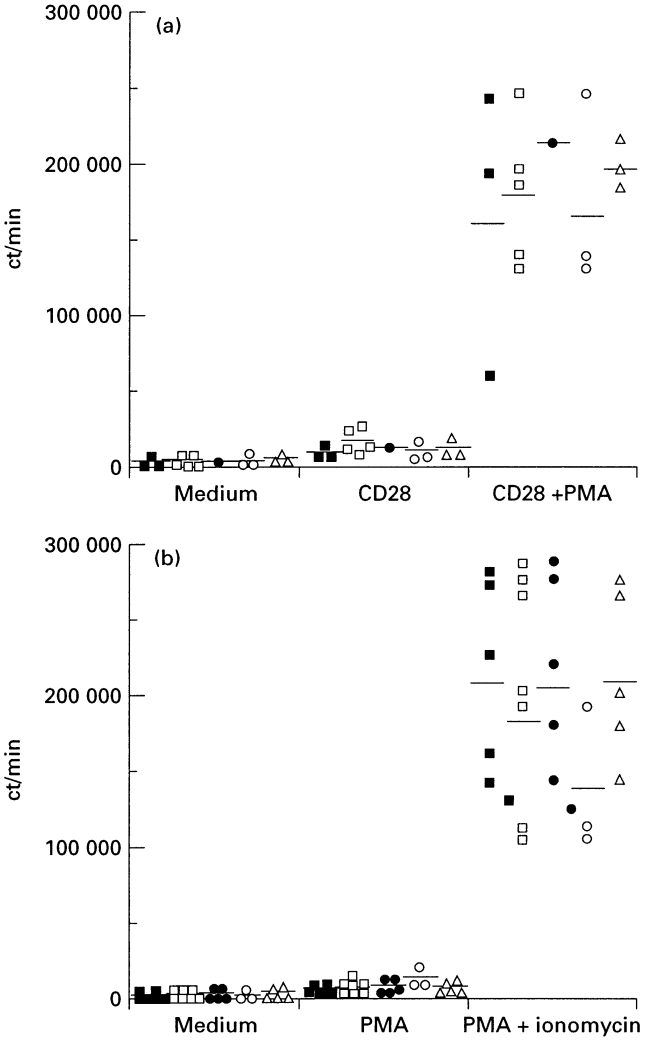

Fig. 2. Proliferation of T cells mediated by CD28 (a) and phorbol myristate acetate (PMA) plus ionomycin (b). Incorporation of ^{3}H-thymidine in response to the indicated stimuli. The results are grouped as described in Fig. 1 (■, healthy controls; □, Child Pugh stage A and B; ●, Child Pugh stage C; ○, responders; Δ, non-responders). Proliferation mediated by CD28, and PMA plus ionomycin showed no significant difference.

by Spinozzi *et al.* and Girón-González *et al.* [31,32]. However, the first group observed a normalization of proliferation mediated by the CD3–TCR complex in most patients after a 6-month period of ethanol withdrawl, and the second group performed the study after an alcohol abstinence of 3 months.

Mediated by CD28. The CD28 glycoprotein expressed on T lymphocytes serves as a ligand for the B7 molecule which is expressed on B lymphocytes and other APC. Together with phorbol ester it is capable of inducing proliferation of T lymphocytes. Since CD28 may play an important role in T and B lymphocyte interaction, we investigated its signal transducing capacity.

No significant decrease in T cell proliferation following triggering of this accessory molecule was found in patients with liver cirrhosis compared with HC (Fig. 2). At present there are no other data on the proliferative response of T cells to CD28 stimulation in a group of patients with liver cirrhosis.

Mediated by PMA plus ionomycin. Phorbol esters like PMA which activate protein kinase C (PKC) and ionomycin which leads to release of intracellular calcium act synergistically on T cell activation by inducing gene activation events leading to DNA synthesis and proliferation. Moreover, this combination of activators bypasses early activation defects in the signalling cascade, and may therefore unmask proliferation defects.

In a low concentration as used in our study, phorbol ester PMA alone was not capable of inducing proliferation in healthy individuals and in patients (Fig. 2). However, additional release of intracellular calcium by ionomycin led to a considerable growth of T lymphocytes in HC and patients with liver cirrhosis. Statistical analysis was performed between HC and cirrhosis stage CP A + B and CP C, revealing no significant difference. This has been confirmed by Girón-González *et al.* [32].

APC function

In order to investigate the capacity of monocytes to deliver costimulatory signals to T lymphocytes, purified monocytes (5×10^3 cells/well) were added to T lymphocytes (5×10^4 cells/well) pretriggered by immobilized anti-CD3 antibody (Fig. 3). The proliferative response of T cells, induced by either autologous monocytes or allogeneic monocytes from one healthy control, did not differ significantly between controls and the various subgroups of cirrhotic patients. When monocytes of patients with liver cirrhosis were added to T lymphocytes of one healthy donor pretriggered with immobilized anti-CD3 antibody, the proliferative response was also not diminished, indicating a preserved capacity of monocytes to deliver costimulatory signals to T lymphocytes.

Influence of serum from patients with liver cirrhosis

To exclude the possibility that a factor derived from the cirrhotic liver may inhibit T cell activation, PBMC of one healthy donor

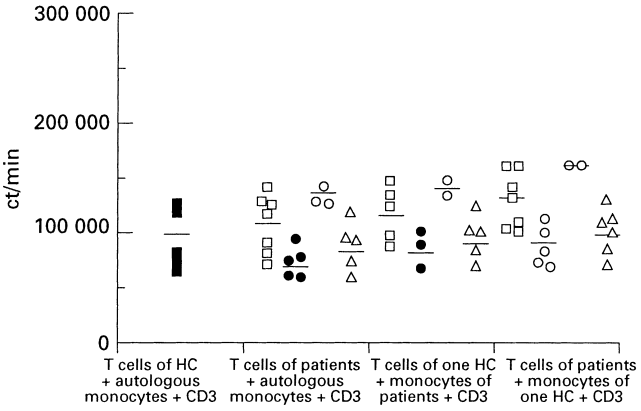

Fig. 3. Capacity of monocytes to deliver costimulatory signals. Anti-CD3-pretreated T cells from healthy controls (HC; ■) and patients with alcoholic liver cirrhosis Child Pugh stage A and B (CP A + B; □), Child Pugh stage C (CP C; ●), responders (○) and non-responders (Δ) to anti-hepatitis B surface antigen (HBs-Ag) vaccination proliferated equally strongly in the presence of autologous monocytes (10% or 5000 cells/well). If monocytes of patients with liver cirrhosis (□, CP A + B; ●, CP C; ○, responders; Δ, non-responders) were added to anti-CD3-pretreated T cells of one HC, DNA synthesis was not statistically different between the groups, indicating a largely preserved ability of monocytes to deliver costimulatory signals. Last row: if monocytes of an HC were added to anti-CD3-pretreated T cells of patients with liver cirrhosis (□, CP A + B; ●, CP C; ○, responders; Δ, non-responders), proliferation was comparable (Wilcoxon, $P > 0{\cdot}05$) to that of cells from HC.

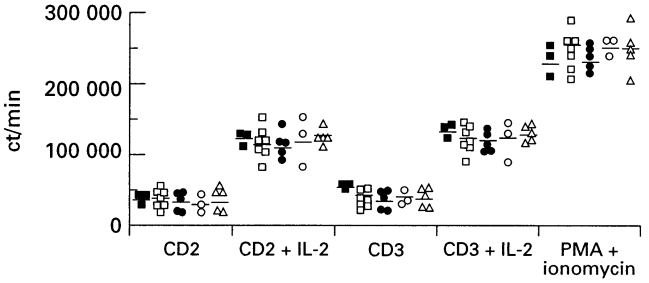

Fig. 4. Influence of serum on T cell activation via CD2, CD3 and phorbol myristate acetate (PMA) plus ionomycin is shown. T cells of one healthy control (HC) were activated via the indicated stimuli in the presence of serum (10% v/v) from patients with liver cirrhosis (Child Pugh stage A and B (□), Child Pugh stage C (●), responders (○) and non-responders (Δ)). Autologous serum (■), fetal calf serum (■), and AB-serum (■) served as controls. There was no significant difference in proliferation (Wilcoxon, $P > 0{\cdot}05$), excluding the possibility that a factor(s) derived from the liver might inhibit T cell proliferation.

were triggered via CD2, MHC-reactive CD3–TCR complex and with PMA plus ionomycin in the presence of serum of patients with alcoholic liver cirrhosis. Autologous serum, AB serum and FCS (10% v/v) served as controls. The results are shown in Fig. 4. Serum of patients with alcoholic liver cirrhosis had no influence on T cell proliferation via CD2, MHC-reactive CD3–TCR complex and PMA plus ionomycin (Wilcoxon, $P > 0{\cdot}05$).

DISCUSSION

The majority of patients with alcoholic liver cirrhosis have a critically impaired immune response which frequently represents a serious clinical problem [1–9]. The underlying mechanisms responsible for this defect are unkown. In several diseases such as CVID or Hodgkin's disease the observed immunodeficiency can be explained by defects in T cell activation pathways [24,25]. To elucidate whether the immune defect in patients with alcoholic liver cirrhosis may be explained by impaired T cell activation, we triggered T cells with MoAbs directed against the CD3–TCR complex, CD2 and CD28 molecule, and measured the DNA synthesis of proliferating cells.

Two T cell-dependent functions studied *in vivo*—cellular immune response against a set of diverse antigens and antibody production against HBs-Ag—were substantially impaired in patients with alcoholic liver cirrhosis. This can not be explained simply by an altered composition of T lymphocyte subpopulations in the peripheral blood, since 'marker' analysis revealed a normal expression of surface molecules in patients with liver cirrhosis, with the exception of a slight increase in $CD4^+$ cells. This finding is in accordance with previous publications, which showed only minor deviations in T cell subpopulations of these patients [32,33].

Our data indicate that in patients with alcoholic cirrhosis all parameters of T cell activation were normal, including proliferation mediated by CD2, CD3–TCR complex, and CD28; acquisition of responsiveness to exogenous IL-2 und IL-4; activation of PKC by phorbol ester- and ionomycin-induced calcium influx. Grouping the patients with regard to their cirrhotic stage and response to HBs-Ag vaccination revealed no difference in the signal transducing capacity of the pathways investigated, leading to an obvious discrepancy between the data obtained *in vivo* and *in vitro*. Such a discrepancy was recently observed in a mouse model, designated split tolerance, and explained by the existence of various levels of peripheral tolerance, to which T cells can be driven in consecutive steps by repeated contact with antigen [34]. However, the importance of these mechanisms for the maintenance of tolerance in our patients' group cannot easily be established and remains unclear.

Up to now, only two groups have published studies [31,32] which investigated activation pathways in patients with alcoholic liver cirrhosis. Spinozzi *et al.* [31] found that alcohol itself is capable of down-regulating anti-CD3 and anti-CD2 response *in vitro* by a decrease in intracellular Ca^{2+} mobilization and impaired inositol 1,4,5-triphospate (IP3) generation. However, the concentrations used in that study were relatively high (40–200 mmol/*l*). Moreover, after a 6-month period of alcohol abstinence, most patients showed a normal response to stimuli mediated by MHC-restricted CD3–TCR complex and CD2 molecule. Our patients abstained from alcohol for 9 months and reacted similarly. Spinozzi *et al.* did not investigate the immune response *in vivo*. The impaired proliferative response to polyclonal mitogens and triggering of the CD3–TCR complex observed by Girón-González [32] could perhaps be explained by a too short period of alcohol withdrawal (3 months).

APC play a pivotal role not only in antigen presentation but also in T cell activation, by delivering costimulatory signals. One study with uraemic patients who are non-responders to hepatitis B vaccination reported a defect in monocyte function resulting in deficient IL-2 production of T cells. Consequently, these patients were treated by addition of a low dose IL-2 to the HBs-Ag vaccine in order to bypass the deficient IL-2 production and thereby to mount antibody production. Several studies have addressed this issue, but reached inconclusive results [35,36]. Therefore, we investigated the role of APC (monocytes) in our patients, but found no defect in the ability of monocytes to deliver costimulatory signals to CD3-pretreated T cells.

Hypothetically, a factor(s) derived from the cirrhotic liver may alter or modify T cell activation and thereby the immune response. In one example, T cell activation mediated by MHC-reactive CD3–TCR complex of lamina propria T lymphocytes of the normal gut is down-regulated by an intestinal mucosa-derived factor(s), whereas alternative pathways mediated by CD2 and CD28 are largely preserved [37,38]. To address this issue we activated T cells of one healthy donor in the presence of serum of patients with alcoholic liver cirrhosis. The results show that serum of our patients did not inhibit T cell proliferation.

Our data represent the first study of T cell activation pathways in patients with alcohol liver cirrhosis, who clearly demonstrate a substantial defect of delayed hypersensitivity and other T cell-dependent functions. The results obtained in *in vitro* studies in these patients, irrespective of their stage of cirrhosis, do not reveal a defect in either T cell activation pathway, which could explain the failure of the immune response. Also, there was no difference in the proliferative response whether patients were responders or non-responders to HBs-Ag vaccine. The ability of monocytes of patients to deliver costimulatory signals in CD3-triggered T lymphocytes was also not impaired. Serum of patients did not inhibit T cell proliferation mediated by CD2 and MHC-reactive CD3–TCR complex. Thus, we speculate that the failure of these patients to mount an equivalent immune response may be due to impaired or altered lymphokine profiles secreted by T cells upon stimulation. Further investigations should address this issue in

order to develop strategies to bypass the defect and, perhaps, to find a rational basis for a therapy with immune modulators.

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