Suppressive effect of alcoholic liver disease sera on lymphocyte transformation¹

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SUMMARY The effect of alcoholic patient sera on *in vitro* lymphocyte transformation was studied using mitogen-induced uptake of ³H-thymidine to measure blastogenesis. With pokeweed mitogen as the stimulus, transformation of normal lymphocytes in sera of alcoholics with either normal or fatty livers was not significantly different from that obtained in pooled human serum (PHS). However, in sera of patients with either alcoholic hepatitis or inactive cirrhosis mean transformation was significantly reduced ($P < 0.001$, < 0.02 respectively). With phytohaemagglutinin-P or concanavalin A as mitogens, suppression of transformation was not as marked but followed the same pattern. A significant negative correlation was observed between the magnitude of transformation and serum bilirubin and aspartate aminotransferase levels. An intra-patient comparison of the effects on transformation of normal lymphocytes by simultaneously collected peripheral and portal venous sera, and of peripheral sera obtained before and after portasystemic shunt surgery, indicated that the factor(s) responsible did not originate in the splanchnic circulation nor did it accumulate in the serum because of failed hepatic clearance. By performing transformation experiments in the presence of inhibitory patient sera diluted with PHS it was possible to show that these sera caused true inhibition of transformation rather than suppression due to failure to sustain cell culture because of nutritional deficiencies. Inhibitory sera did not contain high levels of the enzyme thymidine phosphorylase and did not significantly inhibit binding of ¹²⁵I-labelled mitogens to the lymphocyte surface. These findings indicate that the inhibitory effect of sera from alcoholics is of potential *in vivo* importance, that the effect increases with the degree of hepatocyte damage, and that it is unrelated to the nonhepatic metabolic affects of chronic alcoholism.

Although it has been demonstrated that the sera of patients with a variety of hepatic disorders including viral hepatitis, primary biliary cirrhosis, chronic active hepatitis, and alcoholic liver disease can suppress *in vitro* lymphocyte transformation (Mella and Taswell, 1970; Hsu and Leevy, 1971; Fox *et al.*, 1973; Newberry *et al.*, 1973; Macsween and Thomas, 1973; Wands *et al.*, 1975; Nakao *et al.*, 1975; Brattig and Berg, 1976), the nature of this phenomenon is poorly understood in the majority

of instances. In chronic alcoholic patients it is not clear whether this effect is a consequence of liver damage or of the accompanying metabolic effects of alcoholism, including nutritional deficiency. This inhibitory effect of serum might also be an artefact in that it may be peculiar to the conditions of culture and hence of no possible relevance to *in vivo* lymphocyte function. Furthermore, existing reports of this phenomenon in alcoholic liver disease are conflicting and do not relate it to the spectrum of alcoholic liver damage (Hsu and Leevy, 1971; Newberry *et al.*, 1973; Thestrup-Pedersen *et al.*, 1976).

In an attempt to define the incidence and characteristics of this immunosuppressive phenomenon we have analysed the effect of sera from chronic alcoholics, with or without liver disease, on mitogen-induced transformation of normal lymphocytes. In addition, we attempted to identify the source of the factor(s) responsible for this suppression and ex-

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amined the possibility that this effect might be an *in vitro* artefact of no *in vivo* significance.

Methods

PATIENTS

Hospitalised patients suffering from chronic alcoholism, defined as a daily consumption of more than 100 g of alcohol for 10 years, were considered for admission to the study. Patients suffering from uraemia, pregnancy, uncorrected nutritional deficiency, suspected or confirmed autoimmune or viral liver disease, severe infection, neoplasia, or those receiving immunosuppressive therapy were excluded. Of those included, 39 were hospitalised because of suspected liver disease and the remainder for non-hepatic complications of chronic alcoholism. On the basis of clinical and biochemical findings and liver histology these patients have been arbitrarily divided into four groups:

- 1 Alcoholics: normal liver function with normal histology or fatty change only on liver biopsy (10 patients).
- 2 Alcoholic hepatitis: defined as patients with histological or biochemical evidence of alcoholic hepatitis (Sherlock, 1975), with or without cirrhosis (14 patients)
- 3 Alcoholic cirrhosis: defined as patients with features of alcoholic cirrhosis (Sherlock, 1975) without evidence of hepatitis (15 patients).
- 4 Alcoholic cirrhosis and a surgical portasystemic shunt (12 patients).

Serum was obtained from each patient by venepuncture and then stored under sterile conditions at -24°C . Normal human pooled serum (PHS) was obtained from 100 normal blood donors and stored under identical conditions. Peripheral and splanchnic venous sera were simultaneously obtained from four patients with alcoholic cirrhosis undergoing portasystemic shunt surgery and from four non-alcoholic patients with normal liver function undergoing cholecystectomy. Approval to carry out this study was obtained from the Alfred Hospital Research Advisory Committee.

LYMPHOCYTES

Citrated peripheral venous blood samples, obtained from 18 healthy medical students and laboratory personnel, were centrifuged at 170 *g* for 20 minutes and the platelet rich plasma discarded. The buffy coat was suspended in Hanks' balanced salt solution (HBSS) and the mononuclear cells isolated by Ficoll-Hypaque gradient centrifugation (Böyum, 1968). After centrifugation at 800 *g* for 20 minutes the lymphocyte rich interface was removed and washed

twice with HBSS. The cell pellet was suspended in Eagle's minimum essential medium (MEM), incubated at 37°C for one hour in a 5% CO_2 /air mixture and washed once with MEM. The final cell preparation had a platelet to mononuclear ratio of approximately 4:1 with a cell viability, as determined by trypan blue exclusion, of greater than 98%. On stained smears of the lymphocyte preparations more than 90% of the cells were lymphocytes. For mitogen binding studies the mononuclear preparations obtained in this way were centrifuged through a discontinuous sucrose gradient (Perper *et al.*, 1968) reducing the platelet to mononuclear ratio to 1:4.

E-ROSETTES

E-rosettes were measured in the presence of 20% PHS or patient serum according to Chisari and Edgington (1975). The lymphocyte:sheep erythrocyte ratio was 1:40.

LYMPHOCYTE STIMULATION

Lymphocyte stimulation was performed in microtitre plates containing 1.25×10^5 normal donor cells per well in 0.26 ml of MEM containing penicillin and streptomycin, 100 units/ml, and supplemented with 20% patient or PHS. All tests were performed in triplicate and the mitogens used were purified phytohaemagglutinin (PHA-P; CSL, Melbourne), pokeweed mitogen (PWM; Grand Island Biological Co., New York), and concanavalin A (Con A; Calbiochem, Sydney). The concentrations of PHA-P used were 11.5, 46, and 185 $\mu\text{g/ml}$; PWM, 1, 4, and 10 $\mu\text{g/ml}$; and Con A 10 $\mu\text{g/ml}$. The cultures were incubated at 37° for 72 hours in a humidified 5% CO_2 /air atmosphere. Four hours before the end of the culture period, 0.5 μCi of (methyl- ^3H)-thymidine (The Radiochemical Centre, Amersham, 5 Ci/mmol) was added to each well in 0.01 ml of the culture medium. Trichloroacetic acid (TCA) precipitable material was collected on glass fibre filters by harvesting the cells with the aid of a multiple automated sample harvester (Titertek, Skatron, Norway) and radioactivity was determined in a Packard liquid scintillation counter. Results were expressed as mean cpm of triplicate cultures. The results obtained in 20% PHS for each batch of lymphocytes acted as the control. Patient groups were compared with controls using Student's *t* test (paired).

LYMPHOCYTE-MITOGEN BINDING

Con A and PWM were iodinated with ^{125}I (Na^{125}I , Australian Radiation Laboratory) using the lactoperoxidase method (Phillips and Morrison, 1970). Both compounds retained their mitogenic properties after this procedure. The labelled mitogens, at concentrations used in the transformation experi-

ments, were added to lymphocytes suspended in MEM and 20% human serum. After incubation at room temperature for 15 minutes the cells were centrifuged at 1000 *g* for two minutes and washed twice with phosphate buffered saline. All experiments were performed in triplicate, and in the case of ^{125}I -Con A, with and without 0.2 M α methyl-D-glucopyranoside (α MG). ^{125}I binding was determined in a Nuclear Chicago gamma spectrometer. Results were expressed as $\text{cpm}/4 \times 10^5$ cells, the binding of mitogen to the plastic incubation tubes being

subtracted in all cases. In the case of ^{125}I -Con A, α MG inhibited binding to cells by up to 90% and the effect of serum on this specific binding only was considered.

THYMIDINE PHOSPHORYLASE ASSAY

These were carried out under the conditions applied in lymphocyte culture using a modified microassay technique (Pauly *et al.*, 1977) in which the chromatography papers were developed by ascending chromatography. Conversion of tritiated thymidine

Fig. 1 Transformation of normal lymphocytes measured by ^3H -thymidine uptake by trichloroacetic acid precipitates in individual test sera using optimal dosage of (A) pokeweed mitogen and (B) phytohaemagglutinin-P. Solid horizontal bars represent the mean for each group and the dotted lines the standard error of the mean. P values refer to comparison between alcoholic sera sub-groups and pooled human serum ('controls') (see text). 'Alcoholics' refers to chronic alcoholics with normal or fatty livers.

to tritiated thymine was determined by measuring radioactivity in the appropriate areas by liquid scintillation counting. Enzyme activity was expressed as the percentage tritiated thymidine degraded to tritiated thymine.

Results

The effects of sera from patients suffering from chronic alcoholism on PHA- or PWM-induced transformation of normal peripheral blood lymphocytes are shown in Fig. 1. With optimal concentration of PWM (Fig. 1a), transformation of normal lymphocytes in sera from patients with alcoholic hepatitis (4526 ± 1051 cpm/ 1.25×10^6 lymphocytes, mean $\pm$ SE, $P < 0.001$), alcoholic cirrhosis (10457 ± 1772 cpm, $P < 0.05$), and post-shunt alcoholic cirrhosis (10595 ± 2004 cpm, $P < 0.05$) was significantly reduced compared with normal controls (16469 ± 1508 cpm). In contrast, sera from alcoholics with normal liver function did not suppress transformation (17337 ± 1134 cpm). Using PHA-P suppression was less marked than with PWM (Fig. 1b). In non-shunted cirrhotics suppression with PHA-P was significant when compared with alcoholics with normal liver function ($P < 0.05$) but not when compared with control PHS ($0.10 > P > 0.05$). In data not shown here, the same pattern of response was obtained using optimal concentration of Con A. Unstimulated cultures in PHS gave 134 ± 75 cpm ($\pm$ SE) and did not differ significantly from results in patient sera.

The observed inhibitory effect of sera on transformation was independent of the duration of culture, for similar findings were obtained in experiments in which cells were harvested three, four, and five days after mitogen stimulation. Supraoptimal concentrations of PHA-P or PWM failed to overcome observed suppression in the large majority of patients (Fig. 2). Transformation in sera from the group of cirrhotic patients with surgical portacaval shunts did show significant improvement with increasing doses of PHA-P, although this did not reach a significantly higher level than transformation with supraoptimal doses in sera from cirrhotics without surgical shunts.

A significant degree of negative correlation was observed between the magnitude of transformation using either mitogen and the serum bilirubin level ($r = -0.5138$, $P < 0.001$ for PHA-P; $r = -0.4909$, $P < 0.01$ for PWM), between serum aspartate aminotransferase levels and PWM-induced transformation ($r = -0.3301$, $P < 0.01$), and between γ -globulin levels and PHA-P-induced transformation ($r = -0.3650$, $P < 0.05$). No significant correlation (positive or negative) could be demonstrated

Fig. 2 Mean phytohaemagglutinin (PHA-P) stimulated transformation of normal lymphocytes in sera from each subgroup of chronic alcoholics and in pooled human serum (PHS). Vertical bars represent standard error of the mean. Alcoholic hepatitis ●; alcoholic cirrhosis ○; post-shunt cirrhotics ▼; PHS △; alcoholics without liver disease ■.

between the magnitude of transformation and serum levels of albumin, alkaline phosphatase, suppression of E-rosette formation, fibrin degradation products, and very low density lipoproteins. No patient was α -foetoprotein positive.

EFFECT OF PORTACAVAL SHUNTING ON SERUM INHIBITION

Peripheral blood sera obtained from nine patients with cirrhosis and portal hypertension just before and one month after portacaval shunt surgery were studied. With optimal concentration of PHA-P, transformation of normal lymphocytes (mean $\pm$ SE) using preshunt sera was $19\,102 \pm 3039$ cpm and with postshunt sera was $21\,435 \pm 3848$ cpm ($P > 0.2$). Results obtained with other concentrations of PHA-P, and with PWM as the mitogen, confirmed that the suppressing effect of these sera was not accentuated by an increase in degree of portacaval shunting. All patients appeared to have patent shunts at the time of study on the basis of

Lymphocyte transformation in sera from alcoholics

clinical findings and scintisplenoportography in some patients.

In either controls or alcoholic cirrhosis patients, comparison of the effects of corresponding portal versus peripheral sera on transformation revealed no significant differences.

MECHANISM AND NATURE OF INHIBITION BY PATIENT SERA

Thymidine phosphorylase assays

Thymidine phosphorylase activity was measured in PHS and 11 suppressing sera. Mean enzyme activity ($\pm$ SE) in 20% PHS was $14.0 \pm 1.0\%$. The mean level in 20% patient sera was $9.0 \pm 1.1\%$ (range 1.4–27.8%) with only one patient having a mean value in excess of that for PHS.

Lymphocyte-mitogen binding

Eleven patient sera most suppressive for Con A-induced transformation (mean suppression of 40.4%) failed to cause a significant reduction of specific Con A binding—mean binding in control PHS was 5713 ± 429 cpm/ 4×10^5 cells ($\pm$ SE) against 5369 ± 428 in patient sera. Similarly, eight sera suppressive for PWM (mean suppression of 57.2%) gave a mean binding of 2095 ± 180 cpm/ 4×10^5 cells ($\pm$ SE) against 2597 ± 407 in PHS. These differences are not significant.

Effect of dilution and dialysis

To ascertain if the inhibitory effect of patient sera was principally a consequence of a nutrient deficiency, transformation experiments were simultaneously performed in two sets of culture conditions which were then compared: (1) PHS only in the cultures, in proportions of 5%, 10%, 15%, and 20%; (2) PHS mixed with individual patient sera in ratios of 3:1, 2:2, 1:3, and 0:4 respectively, the total serum concentration being maintained at 20%. The results are shown in Fig. 3. Using sera from five patients, with PWM as the mitogenic stimulus, the addition of 10% or 5% patient serum to 10% or 15% PHS respectively, reduced transformation. Seven patients were similarly tested with PHA-P and five with Con-A and similar patterns were observed.

Dialysis of sera against culture media did not reduce their inhibitory effect and heating to 56°C for one hour did not significantly alter the degree of inhibition of transformation.

Discussion

Previous reports of the effect of sera from patients with alcoholic liver disease on lymphocyte transformation are conflicting. Whereas Hsu and Leevy (1971) and Newberry *et al.* (1973) found that sera

from patients with alcoholic cirrhosis suppressed *in vitro* lymphocyte transformation, a more recent study using heat-treated sera (Thestrup-Pedersen *et al.*, 1976) failed to find such an effect. In the present report it has been demonstrated that the sera of patients with chronic alcoholism have wide-ranging effects on lymphocyte transformation, possibly explaining these conflicting reports. In addition, we found significant inhibition to occur only in sera from those with cirrhosis or alcoholic hepatitis, suggesting that alcoholic liver damage is responsible rather than nonhepatic metabolic effects of alcoholism. The slight possibility that suppression resulted from failure to correct subtle nutritional deficiencies despite clinical and biochemical normality seems unlikely, as the results of serum dilution experiments (Fig. 3) can be explained only by the presence of an active inhibitor(s) of tritiated-thymidine uptake.

The mechanism by which these sera cause suppression remains obscure but a number of alternatives peculiar to *in vitro* conditions, and thus of no *in vivo* consequence, have been excluded. The enzyme thymidine phosphorylase has been shown to be present in plasma and has prompted the suggestion that alteration in its activity may lead to erroneous interpretation of these effects of differing sera on lymphocyte transformation (Pauly *et al.*, 1977). However, assay of this enzyme in control and

Fig. 3 The effect on pokeweed mitogen-induced lymphocyte transformation of varying percentages of pooled human serum (PHS) only (●—●) or mixing with patient serum (○—○) in varying proportions to keep total serum concentration at 20%. The patient sera curve (○—○) represents the mean for five different sera. Vertical bars represent SE of the mean.

inhibitory sera demonstrated that changes in its activity could not account for the observed suppression. It is also possible that sera may interfere with mitogen-lymphocyte interaction by causing steric hindrance with surface receptors for mitogen (Boldt *et al.*, 1972; Nicolson, 1974) or by serum protein binding of large amounts of the mitogenic stimulant (Humphrey *et al.*, 1974). These effects appear unlikely in this study, however, as ^{125}I -PWM and ^{125}I -Con A binding experiments showed that quantitative mitogen binding to lymphocytes was not significantly reduced by inhibitory sera; many of the suppressing sera suppressed transformation to all three mitogens, each having different receptor specificity (Nicolson, 1974); and with only occasional exceptions could inhibition be overcome by increasing the dose of mitogen.

As inhibition is most marked in sera from patients with alcoholic hepatitis, and as it correlates with bilirubin and AST levels, hepatocyte damage appears important in the development of inhibitory factors in the serum (SIF). It is not clear whether SIF is released from damaged hepatocytes or not. The effects of peripheral and portal venous sera on transformation were similar for both patients and non-alcoholic controls, and, as SIF did not increase after portasystemic shunt surgery, it is unlikely that SIF originates in the splanchnic circulation and gains access to the peripheral circulation either because of failed clearance by the damaged hepatocyte or because of portasystemic shunting.

In hepatic cirrhosis failure of hepatic clearance of gut antigens results in an increased antigenic challenge, which appears to be a major factor leading to the hypergammaglobulinaemia seen in many patients (Zinneman, 1975). *In vitro* animal studies have shown that sensitised lymphocytes exposed simultaneously to multiple antigens, produce soluble factors inhibitory to antibody production (Kempf and Rubin, 1977) and lymphocyte proliferation (Thomas *et al.*, 1975). SIF may thus be a non-specific immunoregulatory factor released by lymphoid cells of patients with alcoholic liver disease as a consequence of hyperstimulation of the immune system.

These observations, that suppression of lymphocyte transformation does not appear to result from (1) nutrient deficiencies in the sera, (2) increased serum thymidine phosphorylase levels, or (3) interference with lymphocyte-mitogen interaction, are of some importance. They suggest the possibility that SIF acts at a point common to both lectin- and antigen-induced blastogenesis and thus could modify *in vivo* function of lymphocytes with immunological consequences. Both the susceptibility of alcoholics, particularly with liver disease, to various infections

(Straus and Berenyi, 1971, 1973; Berenyi *et al.*, 1974; Leevy *et al.*, 1976; Isselbacher, 1977) and the impairment of delayed sensitivity reactions observed in alcoholics with liver disease (but not in those without liver disease) (Berenyi *et al.*, 1974) may be in part a consequence of inhibition of *in vivo* lymphocyte function by SIF. This effect of SIF on nucleotide incorporation may inhibit lymphocyte turnover and explain the lymphopenia encountered in patients with alcoholic liver disease (Young *et al.*, 1979).

The presence of SIF in the sera of subjects with alcoholic liver disease represents a further metabolic abnormality in these patients, which may have important immunological clinical consequences. Further characterisation of the nature of SIF, the mechanism by which it affects lymphocyte function, and its *in vivo* consequences seems warranted.

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