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Effect of polyunsaturated phosphatidylcholine on immune mediated hepatocyte damage

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SUMMARY Studies were carried out to investigate the mechanisms underlying the reduction of hepatocellular necrosis observed when polyunsaturated phosphatidylcholine was administered to patients with HBsAg negative chronic active hepatitis. After oral administration of the agent, the susceptibility of rabbit hepatocytes to both antibody dependent cell mediated cytotoxicity and mitogen induced lymphocyte cytotoxicity was substantially reduced. Short term *in vitro* incubation of either the hepatocytes or lymphocytes with polyunsaturated phosphatidylcholine had no effect on antibody dependent cell mediated cytotoxicity. As it has been shown that orally administered polyunsaturated phosphatidylcholine can be incorporated into the liver cell membrane, it is possible that polyunsaturated phosphatidylcholine exerts its effect by blocking the interaction between immune effector cells and hepatocytes.

In a double-blind, placebo controlled randomised trial, polyunsaturated phosphatidylcholine (1·8 g/day) was used as adjunctive therapy in 30 patients with HBsAg negative chronic active hepatitis who were inadequately controlled on conventional doses of corticosteroids and azathioprine.[1] Treatment with polyunsaturated phosphatidylcholine was associated with a significant improvement in the severity of the disease as assessed histologically. A number of uncontrolled trials have shown that this agent is of therapeutic benefit in the treatment of a variety of hepatic disorders including drug induced hepatitis, chronic persistent hepatitis, and cirrhosis.[2]

One of the putative mechanisms to account for the hepatocellular damage in HBsAg negative chronic active hepatitis is an antibody dependent cell mediated cytotoxicity reaction in which 'killer' lymphocytes bind to and lyse the antibody coated target cells.[3 4] Experimental observation in both animals and man have shown that polyunsaturated phosphatidylcholine, a highly unsaturated phosphatidylcholine species extracted from soya beans, mainly dilinoleoyl phosphatidylcholine, is incorporated into hepatocyte plasma membranes.[5 6] Thus it is possible that the therapeutic action of polyunsaturated phosphatidylcholine is mediated by affecting the binding of antibody to the hepatocyte or by interfering with the interaction of the effector cells with the target cells. In the present study, the effect of polyunsaturated phosphatidylcholine on these possible interactions was studied *in vitro* using a microcytotoxicity technique.

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Methods

SERA

Sera from 10 patients with chronic active hepatitis were used for the *in vitro* cytotoxicity assay. In each case the diagnosis had been made according to internationally agreed criteria.[7] Symptoms had been present for periods ranging from six months to four and a half years, median 18 months.

The patients (nine women, one man, median age 48 years, range 25–52 years) gave no history of exposure to known hepatoxic agents and all admitted to a daily ethanol intake of less than 20 g. All patients were Caucasians. Serum from three patients was taken before the introduction of therapy and from seven who were in relapse after attempted withdrawal of prednisolone therapy, two of whom were still receiving prednisolone (2 and 2·5 mg daily). All seven had been taking azathioprine before reduction of the corticosteroids. At the time of testing the serum transferases were raised, range 220–700 IU/l, median 380 IU/l (upper limit of normal 40 IU/l), with overt piecemeal necrosis on

histological examination of the liver biopsy. One patient had suffered a relapse previously. All patients were negative for serum HBsAg, anti-HBs and anti-HBc (using Ausria, Ausab, and Corab respectively (Abbott Laboratories)). Sera were stored at −20°C and immediately before use were heated at 37°C for 30 minutes to inactivate complement.

PREPARATION OF HEPATOCYTES

Female New Zealand white rabbits (weight 2–2·5 kg) were given polyunsaturated phosphatidylcholine 100 mg/kg/day (Nattermann and Cie. GmbH, Cologne) emulsified in their drinking water throughout the day for six weeks. Control rabbits were given water. The rabbits were killed by intravenous injection of pentabarbitone and the liver removed in a sterile fashion. Isolated hepatocytes were prepared by enzyme digestion using collagenase, as described elsewhere.[3] After washing, isolated hepatocytes were seeded into microculture test wells (100 cells/well) and incubated overnight to allow the cells to adhere to the plastic.

ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY REACTION (ADCC)

Sera from patients were diluted 1 in 100 in RPMI-1640 with glutamine (Gibco Laboratories) and 10 μl added to at least 10 wells. The microtest plates were incubated for two hours at 37°C to allow any antibody present in the serum to react with normal rabbit liver cell antigens on the surface of the hepatocytes. In control wells the hepatocytes were incubated with 10% fetal calf serum (Gibco) in RPMI-1640. After washing of the hepatocytes, lymphocytes isolated from normal healthy individuals (aged 24–36 years), prepared by dextran sedimentation, cotton wool incubation, and Ficoll-Triosil centrifugation[4] were added in a ratio of 100 lymphocytes per hepatocyte. After a further 36 hours' incubation the percentage cytotoxicity was determined by comparing the number of hepatocytes remaining in the test wells with those in control wells.[4] The upper limit of normal (28%) was calculated as the mean + two standard deviations from the mean using 30 normal sera.

SHORT-TERM INCUBATION OF HEPATOCYTES AND LYMPHOCYTES WITH POLYUNSATURATED PHOSPHATIDYLCHOLINE

To determine whether polyunsaturated phosphatidylcholine exerts its effect by interference with the reaction between either the antibody and the hepatocyte or the effector lymphocyte and the antibody coated hepatocyte, short-term incubation studies were performed. The hepatocytes were isolated from normal rabbits and seeded into microculture test wells; after overnight incubation 0·1 μg polyunsaturated phosphatidylcholine homogenised in 10 μl of RPMI-1640 was added. After 18 hours' incubation, the plates were washed and the hepatocytes used in the cytotoxicity assay as described above. In the second series of experiments, normal lymphocytes were isolated as described above, and incubated in 1% polyunsaturated phosphatidylcholine in a medium of 10% fetal calf serum in RPMI-1640 in an atmosphere of 83% nitrogen, 12% oxygen, and 5% carbon dioxide to 18 hours at 37°C. The cells were washed and then used in the cytotoxicity assay as before.

MITOGEN INDUCED CYTOTOXICITY

Lymphocytes were isolated from six healthy individuals (aged 26–36 years). Lymphocytes from each individual were then divided into two aliquots at a concentration of 5×10^6 cells/ml in 10% fetal calf serum. To one culture tube was added phytohaemagglutinin (Sigma Reagents Ltd) 5 μg/10^6 cells, and the tubes were then incubated in 83% nitrogen, 12% oxygen, and 5% carbon dioxide at 37°C for 48 hours.

After washing three times, the lymphocytes were then added to the hepatocytes in a ratio of 400 lymphocytes per hepatocyte. After a further 36 hours' incubation the number of adherent hepatocytes were counted and the percentage cytotoxicity determined.

STATISTICS

Significant values were calculated using Wilcoxon's rank test.

Results

ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY

All 10 sera from the patients with chronic active hepatitis induced significant cytotoxicity to hepatocytes isolated from control rabbits (median 49%, range 31–52%). In contrast, no significant cytotoxicity was observed after the same sera were incubated with hepatocytes isolated from rabbits pretreated with polyunsaturated phosphatidylcholine for six weeks (median 12%, range 0–21%, $p<0·01$, Fig. 1). Short-term incubation of the hepatocytes with polyunsaturated phosphatidylcholine *in vitro* did not lead to any alteration in the percentage cytotoxicity induced by the sera (median 34%, range 31–58%, for six sera before treatment and median 37%, range 34–56%, after treatment). Incubation of the lymphocytes with polyunsaturated

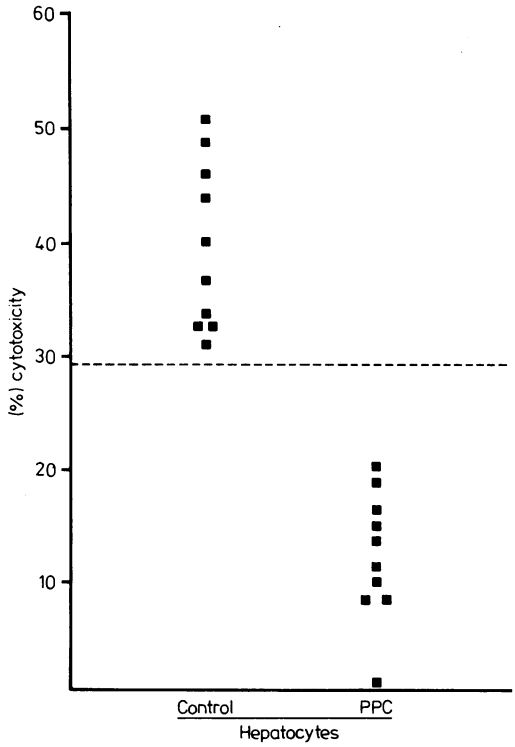

Fig. 1 *Lymphocyte cytotoxicity to isolated hepatocytes from control and polyunsaturated phosphatidylcholine treated rabbits, induced by sera from patients with chronic active hepatitis.*

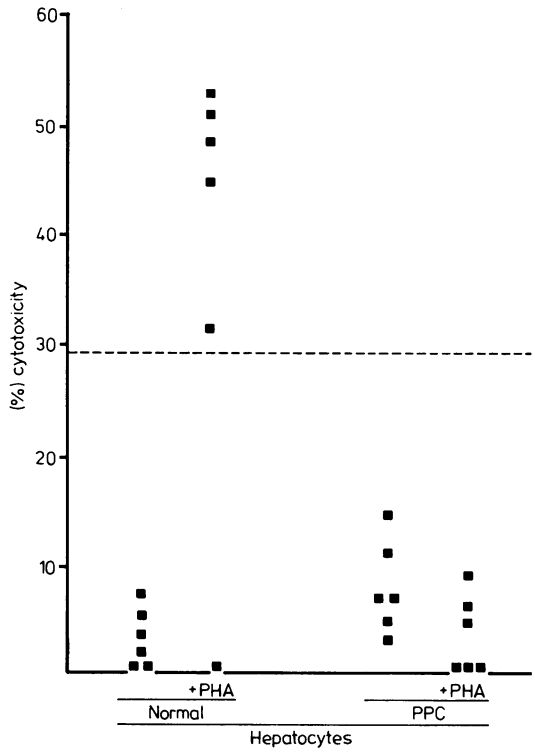

Fig. 2 *Direct cytotoxicity to untreated and mitogen induced lymphocytes to isolated hepatocytes from control and polyunsaturated phosphatidylcholine treated rabbits. PHA = phytohaemagglutinin.*

phosphatidylcholine also had no effect on cytotoxicity (median 36%, range 32–48%, before treatment and median 35%, range 30–41%, after treatment). The seeding efficiency of the treated and untreated hepatocytes was similar, showing that the adherence of the hepatocytes to the tissue culture plastic was not affected by treatment with polyunsaturated phosphatidylcholine.

MITOGEN INDUCED LYMPHOCYTE CYTOTOXICITY

None of the lymphocytes from each of six controls were directly cytotoxic to hepatocytes isolated from either the control or polyunsaturated phosphatidylcholine treated rabbits (Fig. 2). In contrast, after treatment with phytohaemagglutinin, lymphocytes from five of the six individuals were directly cytotoxic to untreated hepatocytes (median 48%, range 32–54%, for the five sera giving significant cytotoxicity). The results of the cytotoxicity of the lymphocytes from the sixth individual were excluded from the analysis as these lymphocytes did not respond to phytohaemagglutinin and therefore no effect of polyunsaturated phosphatidylcholine in the abolition of cytotoxicity would be shown. In contrast, when the hepatocytes isolated from rabbits treated with polyunsaturated phosphatidylcholine were used, none of the mitogen stimulated lymphocytes was directly cytotoxic (median 5%, range 0–10%, $p<0.01$).

Discussion

Extrapolation of the *in vitro* studies reported here would suggest that the mode of action of polyunsaturated phosphatidylcholine *in vivo* in patients with chronic active hepatitis may be to reduce the susceptibility of hepatocytes to immune mediated cell damage. These results do not support the concept that the agent exerts an effect either directly on the lymphocytes or by prevention of the interaction between the effector and antibody coated target cells.

The bioavailability of polyunsaturated phosphatidylcholine after oral administration has been

shown by extensive studies in rats, dogs, and monkeys, with up to 50% of the drug being recovered from the thoracic duct.[5] This has been confirmed in man, showing that after oral administration the radiolabelled agent is incorporated into the liver.[6 8] Furthermore, whole body autoradiographs in rats have shown that polyunsaturated phosphatidylcholine is predominantly transported to the liver.[9] More importantly, it has been shown that orally administered, the agent is incorporated into rat liver membranes and this is associated with an alteration of the membrane.[10] Such alterations in the lipids of the plasma membranes could effect effector cell target cell interaction, either directly or indirectly. For example, alteration of the lipid composition of immune lymphocytes and target cells inhibits conjugation between the two cell types.[11] Membrane lipids have also been shown to be of importance in other cell to cell interactions.[12] Indeed, it has been postulated that a specific effector cell to target cell binding is preceded by unstable and non-specific intercellular membrane interactions mediated through exposed cell surface membrane lipids.[13]

The failure of short term incubation of polyunsaturated phosphatidylcholine to modify the susceptibility of the hepatocytes to cell mediated cytotoxicity may be explicable for a number of reasons. Firstly, the incubation times may have been too short to allow incorporation of polyunsaturated phosphatidylcholine into the hepatocyte membrane. Secondly, the incorporation of polyunsaturated phosphatidylcholine into lipoprotein complexes and chylomicrons during absorption may have facilitated hepatic uptake of polyunsaturated phosphatidylcholine. Thus, polyunsaturated phosphatidylcholine may not exchange with cell phospholipids when presented directly to hepatocytes but would enter the liver incorporated in a chylomicron remnant.

Current therapy of HBsAg negative chronic active hepatitis is based on the use of corticosteroids, often in combination with azathioprine. Although good control can be obtained, attendant side effects can be severe, while in some cases, control is not obtained despite large doses of corticosteroids. Even in those cases where the disease appears to be well controlled, progression to cirrhosis may occur.[14] The addition of polyunsaturated phosphatidylcholine to the therapeutic regime could therefore be of value as an adjunct to therapy allowing a reduction in the dose of corticosteroids. If the therapeutic mechanism of polyunsaturated phosphatidylcholine is by reducing the susceptibility of target hepatocytes to cell mediated damage, then its use may be beneficial in a number of other chronic liver disorders, such as alcoholic liver disease, in which immune mediated mechanisms may play an important role.

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