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INHIBITION OF PHA-STIMULATED LYMPHOCYTE TRANSFORMATION BY PLASMA FROM PATIENTS WITH ADVANCED ALCOHOLIC CIRRHOSIS

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SUMMARY

Studies of lymphocyte transformation in patients with alcoholic cirrhosis of the liver indicate lymphocytes of such patients respond normally to PHA stimulation; however, their plasma or serum contains factors which block expected response to PHA. This effect was diminished by heating at 56°C for 60 minutes but was not affected by freezing and thawing. It could be overcome by increasing the dose of PHA. Blocking was also encountered in the response to pokeweed mitogen and Candida antigen stimulation but was not seen in the mixed lymphocyte reaction. Indentical results were obtained with plasma from patients with allergic rhinitis, uraemia, carcinomatosis, and granulomatous hepatitis. The magnitude of blocking effects in all of these conditions was negatively correlated with the serum γ-globulin levels.

INTRODUCTION

The peripheral blood lymphocytes can be stimulated *in vitro* by phytohaemagglutinin (PHA) to initiate histone acetylation, synthesize RNA, protein and DNA, increase a variety of enzyme activities, transform into large pyroninophilic 'blast' cells, and undergo mitosis (Ling, 1968; Naspitz & Richter, 1968). Blast cells so induced closely resemble lymphoblasts which appear in the course of graft rejection (Gowans & McGregor, 1965). Impaired lymphocyte response to PHA has been noted in patients with Hodgkin's disease (Hersh & Oppenheim, 1965), sarcoidosis (Hirschhorn *et al.*, 1964), ataxia telangiectasia (Naspitz, Eisen & Richter, 1968), thymic aplasia (Kretschmer *et al.*, 1968) and Sjögren's syndrome (Leventhal, Waldorf & Talal, 1967), where there is impaired delayed hypersensitivity. It has been suggested that lymphocytes may play a significant role in the course of certain liver diseases since lymphocytes from patients with primary biliary cirrhosis and chronic active hepatitis may be stimulated to transform by autologous liver tissue homogenate (Tobias, Safran & Schaffner, 1967).

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In the present study a comparison was made of the effects of plasma and serum from normal subjects, patients with randomly selected disease states and patients with advanced alcoholic cirrhosis on PHA-stimulated lymphocyte cultures.

MATERIALS AND METHODS

Culture of circulating lymphocytes

Venous blood (30–50 ml) was obtained with a B-D disposable plastic syringe containing 500 units of Pan-heparin (Abbott Laboratories) and allowed to stand at 37°C at a 75° angle for 1½–2 hr. The lymphocyte rich plasma was separated through a bent 18 gauge needle. The cell-free plasma was separated by centrifugation at 300 *g* for 10 min. The cells were washed three times with medium 199 (Grand Island Biological Company) containing 200 units of penicillin and 200 μg of streptomycin/ml. Total and differential cell counts were performed and the cells were suspended in medium 199 with antibiotics at a lymphocyte concentration of 2×10^6/ml. A second culture medium containing 40% of the serum or plasma to be tested was prepared and the cell suspension and the culture medium were mixed in equal amounts to make a final volume of 0·5 ml per tube. The following stimulants were added: 0·01 ml of PHA-M (Difco, Lot No. 483161), 0·01 ml of pokeweed mitogen (PWM) (Grand Island Biological, Lot No. 271110), both diluted to 20 ml per vial in the suggested diluent (1 : 4 dilutions of reconstituted volume), Candida extract (Hollister-Stier, Lot No. L9674501) in 1 : 50 dilutions. The same lots of stimulants and calf serum were used throughout the experiment. Cultures were run in triplicate, except in fourteen sets in which a fourth replicate tube was prepared to rule out cytolysis. All culture tubes were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 days in the PHA and PWM studies and 6 days in the Candida and mixed lymphocyte cultures.

Lymphocyte harvest and processing

24 hr before harvesting the lymphocytes, 1 μCi of tritiated thymidine (H^3T) (Schwartz Bioresearch, specific activity 1·9 Ci/mM) was added to each tube. An aliquot smear of culture fluid from one of the replicate tubes was stained with Trypan blue and Wright-Giemsa-stain. Smears were free of microorganisms. Cell morphology, viability and percentage of blast transformation was evaluated by scanning 1000 cells.

Radioactive analysis of H^3T-incorporated DNA

After labelling with H^3T for 24 hr, 1 ml of 0·6 M perchloric acid (PCA) was added to terminate the culture. The precipitate was washed twice further with 1 ml of 0·2 M PCA. It was then hydrolysed by adding 1 ml of 5% trichloroacetic acid (TCA) and heated in 96°C for 45 min. The supernatant was transferred to a counting jar containing 15 ml of Bray's solution. The radioactivity of H^3T incorporated into DNA was read in a liquid scintillation spectrometer (Packard Tricarb, Model 3003) and expressed in counts per minute with background counts subtracted automatically. Throughout the experiment 0.05 μCi of H^3T in medium carrier was processed and used as the radioactive blank. This gave counts/min of about 1000 throughout the experiment.

Calculation of stimulation index and per cent stimulation by PHA

The results of the H^3T uptake were expressed as mean counts/min of three replicate tubes, stimulation index (SI) or per cent stimulation by PHA. SI is calculated as:

$$SI = \frac{\text{Counts/min of stimulated cultures in the serum or plasma}}{\text{Counts/min of unstimulated cultures in the same serum or plasma}}$$

On the 3-day cultures of unstimulated cells approximately the same counts/min were obtained despite the different sources of plasma or sera except that there was a tendency for the plasma from patients with cirrhosis to give slightly higher counts/min. The unstimulated cultures in calf serum were used as the denominator. Per cent stimulation by PHA was calculated as:

$$\frac{\text{SI in the plasma tested} - 1}{\text{SI in the calf serum in the same experiment} - 1} \times 100$$

Serum and plasma protein studies

Serum protein electrophoresis was performed using cellulose acetate media (Microzone, Beckman Instruments). Immunoglobulin levels were measured in plasma using Hyland's Immunoplate.

Patient material

Four groups of patients were studied. Group I: ten healthy controls, Group II: nine patients with advanced alcoholic cirrhosis, Group III: five subjects with allergic rhinitis, and Group IV: seven subjects selected with various diseases.

Each of the patients in Group II had advanced cirrhosis of the liver without evidence of other diseases and the absence of drug intake for at least 4 days prior to the plasma collection. A diagnosis of alcoholic cirrhosis was based on the long history of heavy alcohol intake (20–40 years) with poor dietary history; clinical features including hepatomegaly, jaundice, ascites, palmar erythema and spider angiomata; abnormal liver function tests and/or histological evidence of cirrhosis.

Each of the patients in Group III had allergic rhinitis with one or more of the following symptoms: paroxysms of sneezing, profuse watery rhinorrhea, nasal obstruction and/or itching of the nose and eyes with seasonal onset and occured intermittently during the day.

Patients in Group IV consisted of two (Ko and Ba) with acute viral hepatitis, two (Cu and Vi) with extrahepatic biliary obstruction, one (McK) with liver metastasis from adenocarcinoma of the sigmoid colon, one (Li) with granulomatous hepatitis and one (Wi) with uraemia due to interstitial nephritis (blood urea nitrogen 127 mg%).

RESULTS

The influence of plasma or serum on lymphocyte transformation was studied in the lymphocyte cultures which gave a stimulation index of over 6 after addition of PHA in calf serum regardless of whether the lymphocyte donor was a normal or cirrhotic individual. Lymphocytes from patients with cirrhosis responded to PHA to the same degree as lymphocytes from healthy controls. Viability of unstimulated lymphocytes was 90–95% and of stimulated lymphocytes 85–90% in 3 day cultures.

PHA produced a 170% ± 63% (SD) stimulation of lymphocytes cultured in plasma from healthy controls; it produced a 17% ± 10% stimulation of lymphocytes cultured in plasma from patients with alcoholic cirrhosis ($P < 0{\cdot}001$ by t-test) (Fig. 1). Serial testing of plasma from patients with cirrhosis showed the same degree of suppression. Increasing proportions

of cirrhotic plasma using the calf serum to make up for the deficient amount showed a progressive decrease of H^3T uptake with an increasing percentage of the cirrhotic plasma (Fig. 2, left). Heating the cirrhotic plasma at 56°C for 60 min produced an increase in lymphocyte stimulation (Fig. 2, right). Normal plasma treated similarly did not produce significant changes in the H^3T uptake. The inhibitory factor(s) in cirrhotic plasma was not affected by

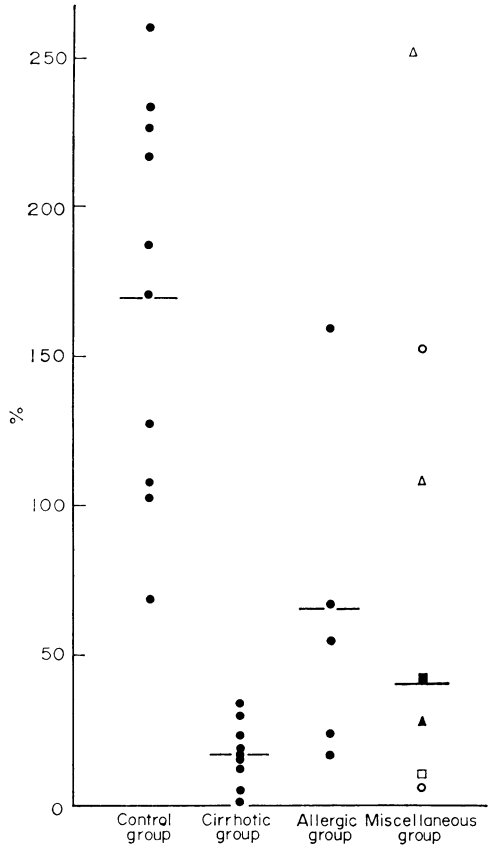

FIG. 1. Scattergram of the results of the per cent stimulation by PHA in the plasma of different groups of patients. Each dot represents the mean of from one to four determinations. The horizontal bar represents the mean of the group. ○, Viral hepatitis; △, obstructive jaundice; □, metastatic cancer; ▲, granulomatous hepatitis; ■, uraemia.

freezing and thawing for up to 16 cycles or by incubating in 37°C for up to 5 days (Fig. 3). Simultaneous testing of cirrhotic serum and plasma from patients Su and Mo showed that the serum gave 32% and 20% stimulation by PHA, respectively, while the plasma gave 20% and 23% stimulation, respectively.

The lymphocyte response was suppressed by plasma from a patient with viral hepatitis without a positive test for hepatitis associated antigen, while lymphocytes from the patient with a positive hepatitis antigen gave an SI of only 2·7 when stimulated by PHA in calf serum. Plasma from two patients with extrahepatic biliary obstruction did not suppress

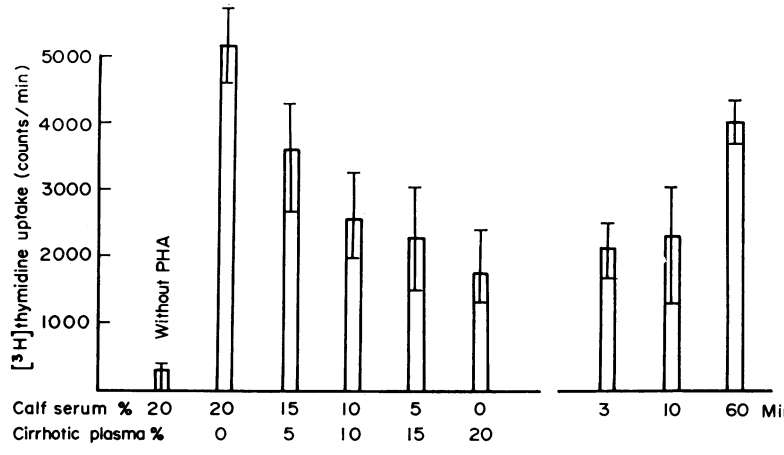

FIG. 2. Left columns: the influence of various concentrations of cirrhotic plasma on PHA-stimulated lymphocytes. Right columns: the influence of heating the cirrhotic plasma at 56°C for various time periods.

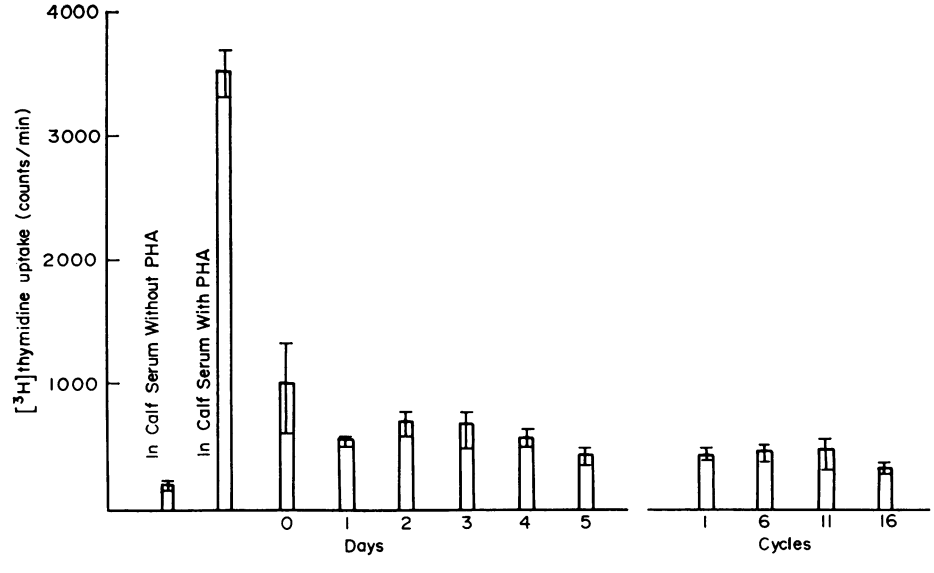

FIG. 3. Left columns: the influence of heating cirrhotic plasma at 37°C for various time periods on PHA-stimulated lymphocytes. Right columns: the influence of freeze-thawing the cirrhotic plasma.

lymphocyte response while plasma from the patients with metastatic adenocarcinoma, granulomatous hepatitis, uraemia and allergic rhinitis exhibited a blocking effect.

Significant negative correlations were found between the percent of PHA stimulation in patients' plasma and levels of γG, γA, γM, the sum of γG, γA, and γM, and the levels of the sum of β- and γ-globulins, but not with levels of albumin, α_1-, α_2- or the sum of α_1- and α_2-globulins (Table 1).

TABLE 1. Serum protein and immunoglobulin levels

Group	Name	Serum protein (g %)	Serum protein electrophoresis (g%)					Plasma immunoglobulins (mg%)		
			Albumin	α_1	α_2	β	γ	γG	γA	γM
	Normal range	6·8–8·0	3·5–5·3	0·16–0·37	0·45–1·10	0·62–1·20	0·60–1·70	600–1200	288 ± 121	80 ± 29
I	Lo							900	240	140
	Va							1050	250	240
	Ke							600	370	180
	Rei							700	220	105
	Ce							900	370	135
	Fr							900	68	73
	Ra							900	320	96
	Po							700	390	127
	Ma									
	Raj									
II	Su	6·5	2·79	0·12	0·46	0·70	2·44	2200	1000	380
	Mo	6·3	2·44	0·25	0·43	0·49	2·69	1650	660	240
	Mi	7·2	2·10	0·36	0·60		4·14*	2100	840	340
	Ad	7·1	2·66	0·45	0·99	1·09	1·92	1200	590	280
	Fa	6·9	2·62	0·36	0·59	1·01	2·32	1400	940	170
	Sh	6·9	1·77	0·21	0·52		4·39*	1850	1100	510
	Ja	6·8	2·94	0·20	0·46	0·78	2·42	2400	840	130
	Ri	6·8	2·69	0·40	0·86	1·48	1·37	800	240	150
	An	7·8	2·82	0·27	0·59	0·98	3·41			

TABLE 1 (*cont.*)

III	Mir							900	240	140
	Ch							700	100	96
	Mei	7·6	4·79	0·23	0·62	0·84	1·12	700	135	310
	Hs	7·8	5·09	0·20	0·41	0·76	1·33	1050	330	160
	Ya							800	280	140
IV	Ko	6·0	3·78	0·24	0·36	0·72	0·90	700	135	165
	Ba	7·2	4·01	0·30	0·70	0·90	1·30			
	Cu	6·9	3·63	0·26	0·72	0·99	1·30	800	220	46
	Vi	7·4	4·22	0·19	0·84	1·08	1·08	800	100	115
	McK	7·2	3·13	0·44	0·83	1·13	1·66	1100	300	180
	Li	8·6	4·13	0·34	1·01	1·01	2·09	980	290	190
	Wi	7·2	3·84	0·29	0·87	0·62	1·57	1050	210	140

* The sum of β-and γ-globulins.

Direct cell counts performed at the end of the culture period revealed a mean cell count of $0{\cdot}39 \times 10^6$ per tube with SD of $0{\cdot}07 \times 10^6$ in seven cirrhotic sera, two calf sera, and five normal plasmas with no difference between the various types of sera.

Plasma blocking effects persisted throughout 5 days of culture period (Fig. 4) When the dose of PHA was increased, the inhibitory effect disappeared (Fig. 5). A dose of 0·02 ml of 1 : 8 dilution of PHA produced 12·4%, 8·0%, 0·8%, and 2·0% blast transformations for normal plasma, calf serum, cirrhotic plasma and allergic plasma, respectively. A dose of

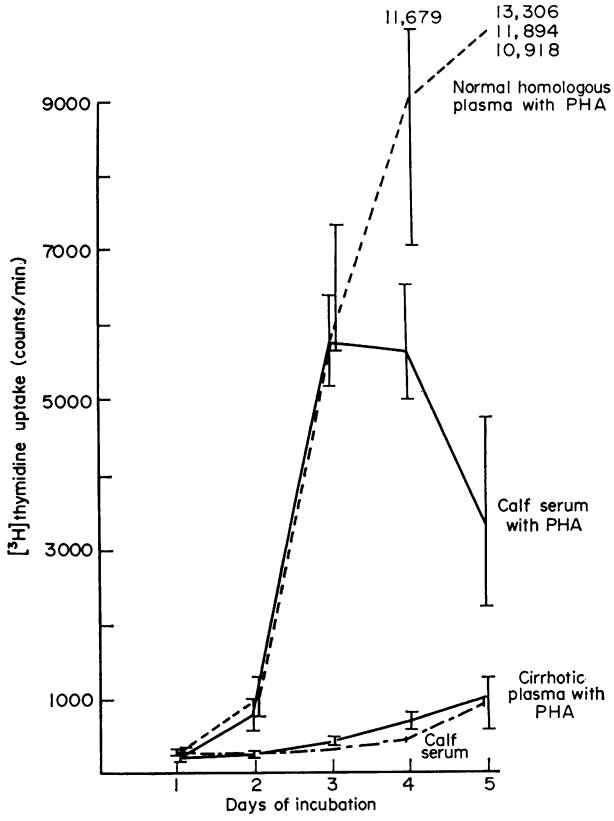

FIG. 4. Culture duration curve of lymphocytes in normal homologous plasma, calf serum and cirrhotic plasma with PHA.

0·04 ml of PHA produced 26·2%, 19·5%, 12·2% and 23·3% blast transformation for normal plasma, calf serum, cirrhotic plasma and allergic plasma, respectively.

Using PWM a cirrhotic plasma gave a SI of 1·30 while calf serum, a normal plasma and an allergic plasma produced a SI of 2·18 to 2·40. When PWM produced higher stimulation, SI of 8–11, no difference was noted. In cultures with Candida antigen stimulation, two cirrhotic plasmas gave a SI of 2·3 and 2·4, while two normal plasmas gave SI of 6·8 and 8·3. In mixed lymphocyte reaction, no differences were noted between two normal, three cirrhotic, and three allergic plasma.

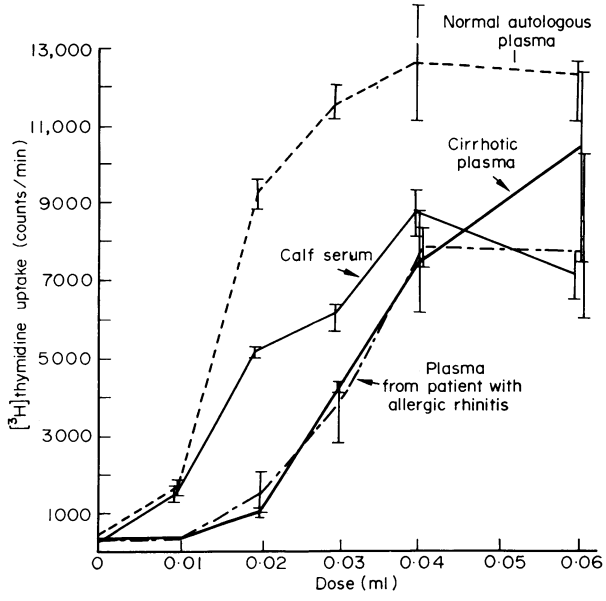

FIG. 5. Dose response curve of lymphocytes to PHA (1 : 8 dilutions) cultured in normal autologous plasma, calf serum, cirrhotic plasma and allergic plasma.

DISCUSSION

Serum or plasma factors which block lymphocyte response to PHA have been reported in uraemia (Silk, 1967a), cancer (Silk, 1967b), Hodgkin's disease (Trubowitz, Masek & Del Rosario, 1966), active tuberculosis (Heilman & McFarland, 1966), idiopathic steatorrhea (Winter *et al.*, 1967; Blecher *et al.*, 1969), cirrhosis (Winter *et al.*, 1967), syphilis (Levene *et al.*, 1969), multiple sclerosis (Knowles *et al.*, 1968), ataxia telangiectasia (McFarlin & Oppenheim, 1969), normal sera (Cooperband *et al.*, 1967), and rag-weed allergy (Richter & Naspitz, 1968). Similar effects have resulted from perphenazine (Hughes & Field, 1969), chloroquine (Hurvitz & Hirschhorn, 1965) glucocorticoids (Nowell, 1961), actinomycin D (Hirschhorn *et al.*, 1963), chlorpromazine (Pisciotta, Westring & De Prey, 1967), chloramphenicol (Pisciotta & De Prey, 1967; McIntyre & Ebaugh, 1962), ouabain (Quastel & Kaplan, 1968) and *N*-acetyl-D-galactosamine (Borberg *et al.*, 1968; Fisher & Mueller, 1969).

Theoretically, PHA stimulation may be blocked by a factor which inhibits reaction sites on the lymphocyte or inactivates the mitogenic property of the PHA. There is evidence that blocking factors occur in plasma and serum: (a) 'protein J' in extract of Jack bean extracts will react with α_1-, α_2-, β-globulin and probably γ-globulin, but not with albumin (Nakamura, Tanaka & Murakawa, 1960); (b) PHA precipitates with twenty different human sera using Ouchterlony agar diffusion plates (Beckman 1962); (c) precipitin bands are formed between PHA and Cohn's human serum fractions II, III, IV, VI, human albumin, agammaglobulinaemic serum and pure bovine serum albumin and precipitating and haemagglutinating but not mitogenic properties of PHA can be abolished by absorbing the PHA with human RBC for 30 min or by heat inactivation at 85°C for 5 min (Holland & Holland, 1965); (d) PHA-P precipitates with γM pathological macroglobulins and γA globulins from myeloma patients in gel diffusion (Morse, 1968).

Whether precipitation is responsible for inactivation of the mitogenic effect of PHA in our study is not known. The degree of lymphocyte transformation is dependent on the PHA : serum ratio; however, there is no significant difference in PHA requirement when the macroglobulin fraction is added, or lymphocytes are cultured in sera rich or poor in macroglobulin concentration (Forsdyke, 1966; 1967). An antiPHA antiserum has been prepared in rabbits by injecting purified PHA in Freund's adjuvant. This antiPHA serum inactivates the mitogenic effect of PHA (Simons, Fowler & Fitzgerald, 1968). Recently it has been shown that α-globulin separated from normal human sera has an immunosuppressive effect (Cooperband *et al.*, 1968, 1969). It blocks the lymphocyte response to PHA and this effect can be overcome by increasing the dose of PHA. This effect is not noted if the α-globulin is added to the culture 24 hr after the addition of PHA. It was also shown to interfere with the migration inhibition produced by sensitized guinea-pig lymphocytes by specific antigen. Therefore, this α-globulin was probably blocking the recognition site of lymphocytes competitively and is postulated to be the humoral factor regulating immune reactivity and prevents lymphoid cell proliferation.

It was shown in this study that cirrhotic plasma blocks the lymphocyte response to PHA. Since this effect could be partially abolished by heating, this is most likely due to the presence of unstable factor(s) in the cirrhotic plasma. The mechanism of this effect is still not clear. This effect is negatively correlated with the γ-globulin level, which in turn is related to the severity of the disease (Wilson, Onstad & Williams, 1969). This may result from toxic effects of unmetabolized products, abnormal protein produced by the diseased liver, the increase in immunoregulatory humoral factor in response to certain stimuli such as hypergammaglobulinaemia, or inactivation of PHA by antiPHA proteins in the cirrhotic plasma. Efforts to clarify the mechanism using other stimulants gave inconclusive results.

Lymphocytes from patients with primary biliary cirrhosis exhibit subnormal PHA stimulation in autologous plasma (Fox *et al.*, 1969). These patients have a high incidence of negative Mantoux test and decreased DNCB sensitization, suggesting the presence of impaired delayed hypersensitivity in these patients. The depressed lymphocyte response was not found in four patients at early stages of biliary cirrhosis suggesting that the impairment of the delayed hypersensitivity is the result rather than the cause of the disease. This suggestion is interesting although it is not known whether the depressed lymphocyte response to PHA was the result of defective lymphocytes or the presence of certain plasma factors.

Lymphocytes from patients with alcoholic cirrhosis behaved normally in the absence of their sera. Similar findings have been reported by others (Tobias, Safran & Schaffner, 1967). In contrast it has been found that lymphocytes from patients with acute infectious hepatitis and aggressive chronic hepatitis exhibit decreased response to PHA (Rössler, Havemann & Dölle, 1969; Willems, Melnick & Rawls, 1969).

The concept that an increase in an immunoregulatory humoral factor may occur is suggested by the finding of plasma inhibitory factors in four patients with allergic rhinitis.

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ABBREVIATIONS

H^3T	tritiated thymidine
PCA	perchloric acid
PWM	poke weed mitogen
SI	stimulation index