Mitogen responsiveness in viral hepatitis and chronic active hepatitis: the role of reversible suppressive influences

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SUMMARY Depressed phytohaemagglutinin and concanavalin-A responsiveness was found in patients with acute viral hepatitis (VH) when a suboptimal mitogen stimulus was used. Normal responsiveness was observed with optimal mitogen stimulation. These findings were independent of extrinsic serum inhibitors. When viral hepatitis lymphocytes were preincubated before mitogen addition an enhanced responsiveness similar to the control group occurred. These *in vitro* findings are in favour of a primary defect in lymphoproliferation in viral hepatitis and do not suggest the presence of reversible suppressive influences such as an excess of short-lived suppressor cells or the presence of cell bound inhibitors. In chronic active hepatitis (CAH) lymphoproliferation induced by immediate mitogen stimulation was similar to control studies. However when CAH cells were preincubated before mitogen addition, enhanced responsiveness significantly greater than in controls occurred. It is suggested that suppressive influences are present in CAH and that their effect can be reversed by cellular preincubation.

In acute viral hepatitis mitogen induced lymphoproliferation is frequently depressed,¹⁻³ but the cause of this depression is disputed. In some studies hyporesponsiveness was found in lymphocyte cultures performed in the absence of autologous serum¹³ and an intrinsic lymphocyte defect was implicated. Others have reported normal responsiveness in similar cultures⁴⁻⁵ and claimed that the depressed lymphoproliferation in viral hepatitis is due to the serum inhibitory factors which have been described in this disease.³⁻⁶ Mitogen studies in chronic active hepatitis (CAH) have also produced conflicting findings: both normal^{4-5, 7-9} and depressed responsiveness¹⁰⁻¹² have been reported. Serum factors which depress mitogen stimulation have also been found in CAH.⁴⁻⁵

In other disease states, the adsorption of inhibitory factors to mononuclear cell surfaces has been described and this was additional to the presence of similar unbound factors in the serum.¹³⁻¹⁵ In these reports, an enhanced lymphoproliferative capacity was observed after the incubation of these cells at 37°C. In viral hepatitis and CAH, improved

sheep erythrocyte rosetting has been described after similar incubation¹⁶ but the effect on mitogen responsiveness has not previously been investigated.

In this study of mitogen induced lymphoproliferation in viral hepatitis and CAH, the influence of unbound serum inhibitory factors was avoided by culturing mononuclear cells washed free of autologous serum. Additional cultures were performed using cells which were preincubated before mitogen stimulation in the expectation that this manoeuvre would abrogate the effect of cell-bound inhibitors or other possible suppressive influences.¹⁷ Three mitogens were used, phytohaemagglutinin (PHA), concanavalin-A (Con-A), and pokeweed mitogen (PWM.) A range of concentrations was used, as it has been reported that hyporesponsiveness may be best demonstrated with a suboptimal mitogen stimulus.^{13, 18-20}

When suboptimally stimulated, the viral hepatitis patients were found to be significantly hyporesponsive to PHA and Con-A. The lymphoproliferation observed in the CAH group did not differ significantly from the controls studied. In the experiments in which cells were preincubated before mitogen stimulation, the control and both patient groups demonstrated significantly enhanced proliferation. The degree of enhancement found in the viral

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hepatitis patients was no different from the controls, whereas the CAH patients developed significantly greater enhancement.

Methods

PATIENTS

Thirty patients were studied. Nineteen had acute viral hepatitis, 15 of whom were studied within five weeks of the onset of their symptoms. Three patients were seropositive for the hepatitis-B surface antigen (HBsAg) as measured by reverse passive haemagglutination (Wellcome Reagents). The mean age was 23 years (range 5–60 years).

Eleven patients with chronic active hepatitis (eight females) were studied. The diagnosis was based on established histological criteria²¹ in conjunction with typical clinical and laboratory parameters²² in all patients. One patient was persistently positive for HBsAg. Seven were being treated with steroids at the time of study (prednisolone 7.5–45 mg/day). The mean age was 42 years (range 17–62 years).

CONTROLS

The control group consisted of 32 normal subjects, mean age 40 years (range 18–71 years). In individual studies, sex and age-matched controls were used.

LYMPHOCYTE CULTURES

Methods similar to those previously described were used.²³ Mononuclear cells were obtained from heparinised blood by plasma gel (Laboratoire Roger Bellon) sedimentation and density gradient separation using Ficoll-Hypaque.²⁴ Cells were then washed twice with 25 ml RPMI 1640 (Gibco, Biocult) and resuspended in RPMI supplemented with 10% fetal calf serum and 100 U/ml penicillin-streptomycin solution (Gibco, Biocult).

10^5 cells in 0.2 ml of culture medium were then added to the wells of a microculture plate (Sterilin microtitre F) and incubated at 37°C in a humidified 5% CO₂/air atmosphere. All cultures were performed in triplicate and cells were incubated in the presence or absence of mitogen. Three mitogens were used: phytohaemagglutinin (PHA-P, Bacto Difco) with a concentration range of 1.6 to 100 µg/ml; concanavalin-A (Con-A, Pharmacia) with a concentration range of 1 to 25 µg/ml; and pokeweed mitogen (PWM, Grand Island Biological Co.) with a concentration range of 0.1 to 1000 µg/ml. The PHA and Con-A cultures were terminated after 48 hours of incubation and PWM cultures after 120 hours.

Four hours before the end of the incubation period, 0.3 µCi of (³H) thymidine (sp.act.2.0 mCi/mM; Radiochemicals) was added to each culture

well. The contents of each well were then harvested onto glass fibre discs (Whatman), washed in 10% trichloroacetic acid, and dehydrated with methanol. The incorporation of radioactive thymidine in the dried discs was measured by liquid scintillation counting (Tricarb, Packard).

PRE-MITOGEN INCUBATION STUDIES

In a further series of experiments cells were cultured for 24 hours before Con-A stimulation. These cultures (t_{24}) were compared with cultures to which Con-A was added at the commencement (t_0). In each instance the culture was terminated 48 hours after the addition of mitogen. The t_{24} counts per minute (ct/min) were expressed as a ratio of the t_0 ct/min.

ANALYSIS OF DATA

Student's *t* test for paired and non-paired data were used to analyse the statistical significance of the results.

Results

STUDIES IN VIRAL HEPATITIS PATIENTS

Ten viral hepatitis patients were studied using PHA, Con-A, and PWM as mitogen stimuli in lymphocyte cultures. Their responses were compared with those of 41 control subjects.

PHA

Five concentrations of PHA were used (Fig. 1). At low PHA concentrations (3.1 and 6.3 µg/ml)

Fig. 1 Lymphocyte transformation after PHA stimulation in 41 control studies (●---●) and in 10 viral hepatitis patients (○---○). Points represent means and bars show standard errors.

Fig. 2 Scatter diagram representation of lymphocyte transformation after PHA stimulation in controls (●) and in viral hepatitis patients (○). A suboptimal (3.1 µg/ml) and an optimal (25 µg/ml) concentration of PHA are shown.

highly significant hyporesponsiveness was observed in the viral hepatitis patients ($P < 0.001$). At higher concentrations (25 and 50 µg/ml) the difference was no longer significant ($P < 0.05$ and $P < 0.2$ respectively). Figure 2 shows the results of the PHA concentrations 3.1 and 25 µg/ml as a scatter diagram. When the low PHA concentration (3.1 µg/ml) was used, the viral hepatitis patients showed only a minor proliferative response, whereas at an optimal concentration (25 µg/ml), the majority demonstrated normal isotope incorporation.

Con-A

The viral hepatitis patients were also hyporesponsive to Con-A stimulation (Fig. 3). The lowest Con-A concentration (2 µg/ml) resulted in minimal proliferation in the control and patient groups. At the higher concentrations (5.8 and 10 µg/ml) the viral hepatitis patients responsiveness was significantly depressed ($P < 0.025$; $P < 0.005$; $P < 0.025$ respectively).

PWM

In the PWM stimulation experiments, the lymphocyte cultures were incubated for six days and under these culture conditions, the viral hepatitis patients' responses did not differ from the controls (Fig. 4).

STUDIES IN CHRONIC ACTIVE HEPATITIS PATIENTS

Eleven patients with CAH were studied. Although considerable variation in lymphoproliferation was observed, the mean responsiveness of the CAH

Fig. 3 Lymphocyte transformation after Con-A stimulation in 10 control studies (●---●) and in 10 viral hepatitis patients (○---○). Points represent means and bars show standard errors.

patients to PHA (Table 1), Con-A (Table 2), and PWM (results not shown) did not differ significantly from the controls. Three of the CAH group were young HBsAg negative females, with multiple autoantibodies, raised gamma globulin, typical liver histology, and clinically aggressive disease requiring immunosuppressive therapy: their responses were very similar to young healthy controls. A fourth female CAH patient with clinically inactive disease was studied serially during a six week period (Fig. 5). The initial culture resulted in supranormal lymphocyte proliferation. Three weeks later this enhanced responsiveness was less apparent but,

Fig. 4 Lymphocyte transformation after PWM stimulation in 10 control studies (●---●) and in 10 viral hepatitis patients (○---○). Points represent means and bars show standard errors.

Table 1 PHA responsiveness in control subjects and patients with chronic active hepatitis

PHA ($\mu\text{g/ml}$)	Controls		Chronic active hepatitis		Significance†
	No.	CPM $\pm$ SEM*†	No.	CPM $\pm$ SEM	
0	41	259 $\pm$ 17	12	285 $\pm$ 38	} NS
1.6	24	1319 $\pm$ 213	11	903 $\pm$ 277	
3.1	31	3352 $\pm$ 440	10	2375 $\pm$ 719	
6.3	40	5758 $\pm$ 514	12	5849 $\pm$ 1510	
12.5	38	6697 $\pm$ 587	8	6342 $\pm$ 2182	
25.0	26	6896 $\pm$ 750	6	7994 $\pm$ 3426	
100.0	28	4006 $\pm$ 494	8	4948 $\pm$ 1679	

*Mean (^3H) thymidine incorporation in counts per minute.

†Standard error of the mean.

‡Analysed by Student's *t* test for non-paired data.

NS: not significant.

Table 2 Con-A responsiveness in control subjects and patients with chronic active hepatitis

Con-A ($\mu\text{g/ml}$)	Controls		Chronic active hepatitis		Significance†
	No.	CPM $\pm$ SEM*†	No.	CPM $\pm$ SEM	
0	40	260 $\pm$ 18	13	277 $\pm$ 36	} NS
2	40	589 $\pm$ 73	13	674 $\pm$ 221	
5	40	2352 $\pm$ 242	13	2831 $\pm$ 1194	
8	22	4026 $\pm$ 511	5	4916 $\pm$ 2920	
10	40	3888 $\pm$ 371	13	4677 $\pm$ 1464	
25	18	2069 $\pm$ 278	7	3886 $\pm$ 973	

*Mean (^3H) thymidine incorporation in counts per minute.

†Standard error of the mean.

‡Analysed by Student's *t* test.

NS: not significant.

when a third culture was performed after a further three week interval, the patient demonstrated minimal lymphoproliferation only. This finding could not be explained by extrinsic factors such as loss of mitogen potency. The results of seven studies of a male control subject conducted over a six month period are also illustrated for comparison.

Fig. 5 Serial PHA induced lymphocyte transformation studies in patient SC with chronic active hepatitis. The mean response of three sequential studies are represented by Δ , $\circ$, and $\square$ respectively. Seven repeated cultures in a male control subject are shown ($\blacksquare$) and bars indicate standard errors.

PRE-MITOGEN INCUBATION STUDIES

The effect of a 24 hour incubation of cells before Con-A stimulation was examined (Table 3). In normal controls t_{24}/t_0 ratios of 5.1 (Con-A 1 $\mu\text{g/ml}$), 2.5 (Con-A 2 $\mu\text{g/ml}$), 2.2 (Con-A 5 $\mu\text{g/ml}$), and 1.3 (Con-A 10 $\mu\text{g/ml}$) were found. This represented a significantly increased response at all four Con-A concentrations: 1, 2, 5 $\mu\text{g/ml}$ ($P < 0.001$) and 10 $\mu\text{g/ml}$ ($P < 0.005$). The CAH patients studied also demonstrated enhanced responses but their ratios of enhancement (t_{24}/t_0) were greater than the controls at all Con-A concentrations and this was significant with Con-A 5 and 10 $\mu\text{g/ml}$ ($P < 0.005$). Moreover, the t_{24}/t_0 ratios of the total 23 CAH patient experiments were significantly greater ($P < 0.025$) than the 110 control experiments.

In similar experiments the proliferative responses of 11 VH patients were investigated after a 24 hour incubation (Table 4). This resulted in significantly improved responses but this was not different from the control group.

Discussion

In this study, viral hepatitis patients demonstrated significant hyporesponsiveness to PHA and Con-A stimulation and this finding could not be explained by serum inhibitory factors, as these experiments

Table 3 Con-A addition at start of culture (t_0) or after 24 hours (t_{24}): control subjects compared with chronic active hepatitis patients

Con-A ($\mu\text{g/ml}$)	Controls			Chronic active hepatitis			Significance†
	t_0^*	t_{24}	$t_{24}/t_0^\dagger$	t_0	t_{24}	t_{24}/t_0	
0	186 $\pm$ 17	190 $\pm$ 12	1.2 $\pm$ 0.1	213 $\pm$ 34	255 $\pm$ 44	1.3 $\pm$ 0.1	P > 0.4
1	241 $\pm$ 25	977 $\pm$ 253	5.1 $\pm$ 1.3	252 $\pm$ 53	1410 $\pm$ 616	7.7 $\pm$ 4.9	P > 0.4
2	726 $\pm$ 96	2205 $\pm$ 300	2.5 $\pm$ 0.4	436 $\pm$ 126	1924 $\pm$ 610	6.4 $\pm$ 2.9	P > 0.05
5	2333 $\pm$ 254	4682 $\pm$ 491	2.2 $\pm$ 0.2	1403 $\pm$ 616	4359 $\pm$ 1317	6.2 $\pm$ 2.7	P < 0.005
10	4388 $\pm$ 542	5955 $\pm$ 844	1.3 $\pm$ 0.1	3640 $\pm$ 1115	7183 $\pm$ 1813	2.4 $\pm$ 0.5	P < 0.005

*Mean (^3H) thymidine incorporation in ct/min $\pm$ SEM in t_0 and t_{24} cultures.†The t_{24} ct/min was expressed as a ratio of the t_0 ct/min.‡The patients' t_{24}/t_0 ratios were compared with the control values and the data analysed by non-paired Student's t test.Table 4 Con-A addition at start of culture (t_0) or after 24 hours (t_{24}): control subjects compared with viral hepatitis patients

Con-A	Controls		Viral hepatitis		Significance†
	No.	t_{24}/t_0^*	No.	t_{24}/t_0	
0	30	1.2 $\pm$ 0.1	11	0.9 $\pm$ 0.1	} NS
2	30	2.5 $\pm$ 0.4	11	2.3 $\pm$ 0.3	
5	23	2.2 $\pm$ 0.2	11	1.8 $\pm$ 0.2	
10	17	1.3 $\pm$ 0.1	11	1.3 $\pm$ 0.1	

*The t_{24} ct/min was expressed as ratio of the t_0 ct/min.†The patients' t_{24}/t_0 ratios were compared with the control values and the data analysed by non-paired Student's t test. NS: not significant.

were conducted in the absence of autologous serum. This impaired proliferative ability was most apparent when suboptimal concentrations of mitogen were used. Similar findings with suboptimal stimulation have been reported in other diseases including systemic lupus erythematosus (SLE),^{13,18} primary biliary cirrhosis,¹⁹ and ataxia telangiectasia.²⁰ It would appear that more subtle defects in blastogenic capabilities can be detected by low concentrations of mitogen, whereas higher mitogen concentrations can overcome the inhibitory or deficiency factors involved. The proliferative response induced by PWM showed no difference between the viral hepatitis and control groups. This finding may be explained by the prolongation of PWM stimulated cultures to six days. Willems *et al.*¹ also described VH hyporesponsiveness to PHA which was evident in three day cultures but not seen in six day cultures. The normal response induced by PWM might also be due to the different stimulatory capacity of this mitogen²⁵, but this possibility was not analysed in these data.

The finding of mitogen hyporesponsiveness in viral hepatitis is in agreement with earlier reports.^{1,3} The normal response reported by Wands *et al.*⁴ may be explained by their failure to use a suboptimal mitogen stimulus. The duration of this hyporesponsiveness is not known but it may last as long as 10 weeks.³ The three patients studied serially in these experiments all showed a definite improvement in proliferative capacity, indicating a return to normal reactivity.

In cultures performed in the absence of autologous serum, the CAH patients' lymphocyte response to PHA, Con-A, and PWM was similar to that observed in the controls. This finding is in agreement with the reports of some authors;^{4,7-9} but in conflict with others.¹⁰⁻¹² However, strict comparisons are difficult. In most of these studies the majority of the patients were HBsAg positive^{4,7,8,11} and, in some, autologous serum was present in the culture system.^{7,11} HBsAg positive CAH is now considered to be a disease entity distinct from HBsAg negative CAH^{26,27} and the heterogeneity of this latter group has also been recognised.²⁸ All but one of the 11 patients in this study were HBsAg negative and when analysed separately their responses were still not significantly different from the control group.

Within the CAH group, considerable variation in mitogen responsiveness was observed, similar to the variation seen in the control subjects. However, in addition, the results of one patient studied serially suggests that exaggerated changes in lymphoproliferation may occur in CAH. Some fluctuation in responsiveness can be expected in serial lymphocyte blastogenesis studies.^{29,30} Nonetheless, changes observed in this patient seemed in excess of this and were certainly greater than that of a normal subject also serially studied. During the six week study period, the patient's liver disease remained clinically stable. This variation may represent dynamic events in lymphocyte populations in CAH, and serial variations in lymphocytotoxic activity and T cell numbers have also been described in this disease.³¹

Seven of the CAH patients were being treated with prednisolone but this did not seem to inhibit their proliferative capacity. Other studies have reported similar findings.^{4,11} On the contrary, it has been suggested that mitogen responsiveness in CAH improved after immunosuppressive therapy.¹¹ Although the effect of bolus administration of corticosteroids to normal subjects has been reported to depress immunological function^{32,33}, the result of chronic administration of these drugs is largely unknown and needs to be assessed.³⁴

It has been reported that preincubation of mononuclear cells may abrogate the effect of cell bound inhibitors.^{13,16} In studies of patients with VH and CAH¹⁶ and Hodgkin's disease¹⁵ depressed T-lymphocyte rosette formation had reverted to normal when tested after preincubation. Likewise, this procedure resulted in enhanced lymphoproliferation in SLE^{13,14} and Hodgkins' disease.¹⁵

In the experiments described in this paper the viral hepatitis patients demonstrated improved mitogen responsiveness after preincubation which was similar to the control group. This finding of only normal enhancement suggests that the factors involved in mitogen hyporesponsiveness in viral hepatitis are not reversible *in vitro* and this would argue against a role for short-lived suppressor cells¹⁷ or elutable cell bound inhibitors.¹⁴ However, the CAH patients demonstrated an enhancement significantly greater than the control subjects. This exaggerated enhancement may follow the neutralisation of serum inhibitor factors as described by Wernet *et al.*¹⁴ or occur because the inhibitor had a short biological half life, as is the case with the rosette inhibitory factor described by Chisari and Edgington.¹⁶

The enhanced mitogen responsiveness observed in normal subjects after preincubation of their cells has been previously reported.^{17,23,35} It has been proposed that this is due to the loss of a suppressor cell subpopulation,¹⁷ similar to that described in the mouse by Dutton.³⁶ Interpreted in this manner, it is possible that excessive suppressor cell activity is present in CAH and, when this influence is diminished by incubation of these cells, an exaggerated enhancement phenomenon results. This concept is supported by the recent finding of increased numbers of T gamma cells in CAH.³⁷ T gamma cells, which contain receptor sites for the Fc portion of IgG are considered to include the suppressor T cell population.³⁸

These two explanations of exaggerated responsiveness in CAH could be unified if it were shown that the mitogen inhibitory factors described in CAH were the products of suppressor cells. Although there is no firm information about the source of

origin or chemical nature of mitogen inhibitors in VH or CAH,³⁻⁵ it has been suggested that suppressor cells and their products play a role in the immuno-suppression observed in virus diseases.^{39,40}

These results confirm the presence of mitogen hyporesponsiveness in viral hepatitis which is independent of extrinsic serum influences and appears to be due to a primary defect in lymphoproliferation. Such a finding is in keeping with depressed cell mediated immunity which has been observed in many other virus diseases.⁴⁰ Although the CAH patients responded normally to direct mitogen stimulation, the enhanced responsiveness after cellular preincubation suggested that inhibitory influences were also present in these patients. It is not known whether these inhibitors were derived from suppressor cells or if they are contained in previously described inhibitor factors.

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