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Decreased suppressor T cell activity in patients with hepatic cirrhosis (HC)

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SUMMARY

Hypergammaglobulinaemia (HGG) is frequently found in patients with hepatic cirrhosis (HC). Using an assay system of *in vitro* PWM-stimulated immunoglobulin (Ig) production, the amounts of IgG, IgA, and IgM produced by peripheral blood lymphocytes (PBL) from 15 HBs Ag-negative patients with HC and from 16 age-matched healthy subjects were quantitated by radioimmunoassay. We found that PBL from patients with HC produced significantly greater amounts of IgG ($P < 0{\cdot}05$) but not IgA or IgM than did those from control subjects. This increased IgG production by PBL from patients with HC was attributed to enhanced T helper activity and not to enhanced B cell function. We also searched for defects in naturally occurring suppressor T cell activity which is sensitive to irradiation. Irradiation-induced enhancement for IgG production was significantly lower in patients with HC compared with age-matched control subjects ($P < 0{\cdot}01$). Similarly, we examined the effect of Con A-induced suppressor T cells on the *in vitro* PWM-stimulated IgG production by allogeneic PBL and observed the decrease of Con A-induced suppressor T cell activity in patients with HC ($P = 0{\cdot}01$). We conclude, therefore, that the increased serum levels of Ig, particularly IgG in patients with HC may result from in part on the basis of depressed ability of naturally occurring suppressor T cells or Con A-induced suppressor T cells to suppress Ig production.

INTRODUCTION

Polyclonal hypergammaglobulinaemia (HGG) is a well recognized manifestation of patients with chronic active hepatitis or hepatic cirrhosis (HC), and constitutes a characteristic feature of abnormal humoral immunity in chronic liver diseases.

Although several hypotheses are advocated to explain this phenomenon, (Doniach *et al.*, 1966; Triger, Alpm & Wright, 1972; Bjørneboe, Prytz & Ørskov, 1972; Triger & Wright, 1973), very little has been known about the mechanisms responsible for this HGG at the cellular level.

Hypergammaglobulinaemia in HC appears to be due to increased production rather than delayed catabolism (Havens *et al.*, 1954; Cohen, 1963). It has been well known that immunoglobulins (Ig) are produced by cells into which B cells have differentiated. This differentiation process is regulated by both helper and suppressor T cells, and also by macrophages. Therefore, it may be

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possible to assume that in HC, an enhanced B cell function and/or increased helper or decreased suppressor T cells activity may lead to HGG.

Recently, it has been accepted that pokeweed (PWM)-stimulated Ig production by peripheral blood lymphocytes (PBL) is highly reproducible and that this experimental system is a useful tool to study the pathogenesis of some immunological diseases. In addition, it has been known that suppressor T cells, when activated by concanavalin A (Con A), suppress Ig production by normal B cells (Haynes & Fauci, 1977, 1978a, 1978b) and that they are sensitive to irradiation (Siegal & Siegal, 1977).

The present study has been undertaken to elucidate the mechanism(s) for HGG in HC at the cullular level by using the assay system of PWM-stimulated Ig production. In addition, the activities of naturally occurring suppressor T cells that are sensitive to irradiation and of Con A-activated suppressor T cells in patients with HC were investigated.

MATERIALS AND METHODS

Patients. Fifteen HBs Ag-negative patients with hepatic cirrhosis (HC) (12 males and three females), ranging in age from 28 to 52 years (mean age, 41 years), were investigated. The diagnosis of HC was based on clinical, histological, and biochemical findings. One patient (male, 28 years old) with a history of having had heavy alcohol intake, had alcholic cirrhosis and others had undefined or cryptogenic cirrhosis. All patients had hypergammaglobulinaemia, ranging from 1·8 g to 3·8 g/dl (mean concentration, 2·6 g/dl). None of patients had received drugs apparently affecting the immune system during this study.

Sixteen nearly age-matched healthy subjects (12 males and four females) aged 29–47 years (mean age, 37 years) were used as controls in this study.

Preparation of peripheral blood lymphocytes (PBL) and subpopulations. Purified mononuclear cell suspensions were prepared from heparinized venous blood of patients and controls by Ficoll-Conray gradient centrifugation. To obtain populations of cell suspensions enriched for B cells or T cells, neuraminidase-treated sheep erythrocytes (En) rosettes were formed as previously described (Kishimoto *et al.*, 1978). Mononuclear cells binding En to their surface were then separated from cells not forming rosettes by centrifugation over Ficoll-Conray, cells remaining at the top of the Ficoll-Conray as well as cells sedimenting to the bottom of the Ficoll-Conray were removed separately. In the T cell enriched suspensions the pelleted cells were gently suspended in Eagle's MEM (Nissui Seiyaku Co. Ltd., Tokyo). Sheep erythrocytes were then lysed with Tris-buffered ammonium chloride. This isolation method yielded T cell enriched suspension (T cells) containing more than 95% T cells. Similarly, in several experiments in which the T cell depleted suspensions contained contaminating En rosette forming cells, those cells were rosetted again with En and depleted of En-rosette forming cells. This procedure yielded a population of B cell enriched suspensions (B cells) containing less than 5% En rosette forming cells. Cell viability was determined by the eosin Y dye exclusion methods.

Irradiation of lymphocyte subpopulation. T cells obtained by En-rosette formation at a concentration of 1×10^6 cells/ml in polyethylene tubes (2003, Falcon plastics, Oxnard, California), were exposed to γ-irradiation from a ^{60}Co source (RI-107-type, Toshiba, Tokyo) at a dose rate of 48 rads/min. The radiation doses employed for this study were 1000 and 3000 rad as previously described.

Con A-activation of T lymphocytes. T cells at a density of 1×10^6 cells/ml were cultured in a 5% CO_2 gas incubator at a concentration of 10 μg/ml Con A. After 2 days culture, cells were harvested and washed three times with MEM. Control cells were defined as cells that were not exposed to Con A on initiation of culture, but were cultured alone for 2 days.

Assay for in vitro *immunoglobulin production.* The quantitation of the Ig synthesized *in vitro* by lymphocytes was performed as previously described (Kishimoto *et al.*, 1978). Briefly, 2×10^5 cells in each cell of Microplate (Falcon Plastics) were cultured for 7 days in triplicate in 0·2 ml of the culture medium with PWM (10 μg/ml) (GIBCO, New York, USA) in a 5% CO_2 gas incubator. The culture medium used was RPMI 1640 (Nissui Seiyaku Co., Tokyo) containing 10% fetal calf serum (FCS),

100 units/ml of penicillin G, 100 μg/ml of streptomycin sulphate, and 5×10^{-5}M mercaptoethanol. The cultures were terminated by centrifugation and the culture medium was assayed for its ability to inhibit the binding of known quantities of radiolabelled IgG, IgA, or IgM to anti-IgG, -IgA, or -IgM antibody coupled to the well of a Microplate (Sanko Junyaku, Tokyo). By utilizing standard curves constructed from the inhibition of binding noted with known concentrations of unlabelled Ig; the amount of Ig present in the culture medium could be determined.

Determination of suppressor activity in vitro. In some experiments to determine naturally occurring suppressor activity that is susceptible to irradiation, 1×10^5 irradiated T cells were co-cultured with an equal number of B cells from a single donor in 0·2 ml of culture medium for 7 days, in triplicate, in the presence of PWM. In other experiments to clarify the effect of Con A-activated T cells, 2×10^5 PBL from each of two to three separate donors were co-cultured with 1×10^5 Con A-activated or control T cells from individuals of either patients or healthy subjects with PWM for 7 days. The amounts of the IgG, IgA, or IgM present in the supernatants of culture media were determined as described above. The amount of the Ig produced in the co-culture of PBL or B cells with Con A-activated, or irradiated T cells was expressed as a percentage of that in comparable cultures with control T cells, as determined by the following formula:

$$\% \text{ Ig production of control} = \frac{\text{Amount of Ig produced in the co-cultures of PBL or B cells + Con A-activated or irradiated T cells}}{\text{Amount of Ig produced in the co-cultures of PBL or B cells + control T cells}}.$$

Furthermore, to standardize results and permit evaluation of the degree of suppression, the following formula was used: %suppression = 100 − %Ig production of control.

Statistical methods. Results were compared by Wilcoxon's two-tailed paired *t*-test.

RESULTS

In vitro *PWM-stimulated Ig production by PBL*

Quantities of each of three classes of Ig produced by PBL from each of patients with HC or healthy subjects are shown in Fig. 1.

The geometric means of IgA and IgM produced by PBL from patients with HC were 1288 ng/ml and 1737 ng/ml, while those by PBL from healthy subjects were 468 ng/ml and 1603 ng/ml respectively. The mean amounts of IgA and IgM produced were not significantly different in both groups. By contrast, the geometric mean of IgG produced was 708 ng/ml in patients with HC and 331 ng/ml in controls IgG production was significantly increased in patients compared with that in healthy subjects ($P < 0{\cdot}05$).

B cell function in patients with HC and healthy subjects

To elucidate whether increased IgG production by PBL from patients with HC is due to an enhanced B cell function, 1×10^5 B cells prepared from each of patients or controls were co-cultured with equal numbers of T cells from a single donor in the presence of PWM for 7 days.

The geometric means of IgG, IgA and IgM produced in culture medium were 263 ng/ml, 209 ng/ml, and 366 ng/ml in patients and 372 ng/ml, 372 ng/ml, and 477 ng/ml in controls, respectively. When the amounts of IgG, IgA, and IgM produced in patients were compared with those in controls, no significant differences in IgG, IgA, and IgM production were observed between both groups (Fig. 2).

T helper activity in Ig production

To investigate T cell helper activity, 1×10^5 B cells isolated from a single donor were co-cultured with equal numbers of T cells prepared from each of patients or controls in the presence of PWM for 7 days.

The geometric means of IgA and IgM produced in the culture medium were 398 ng/ml, and 1285

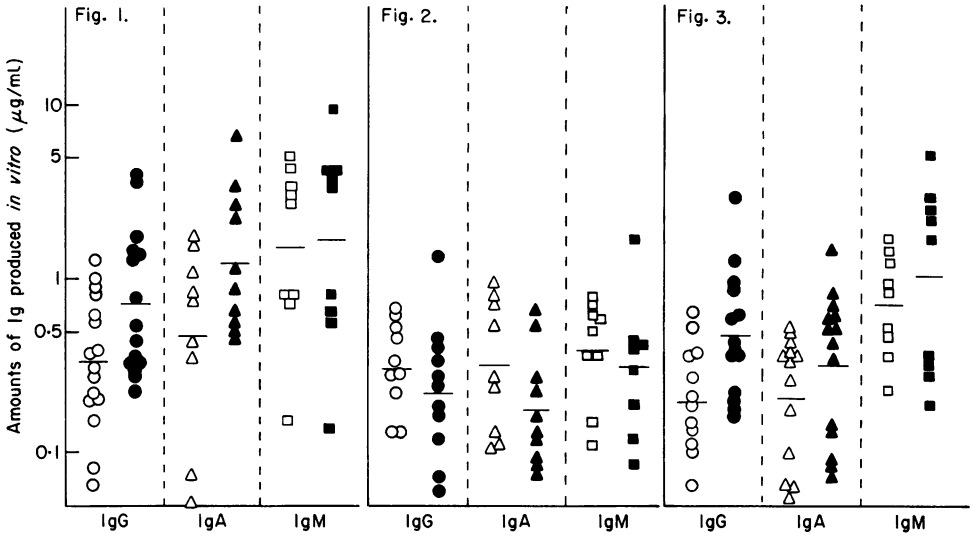

Fig. 1. *In vitro* PWM-stimulated Ig production by PBL. PBL 2×10^5, from patients with HC (●, ▲, ■) or healthy subjects (○, △, □) were cultured for 7 days in the presence of PWM (10 μg/ml). IgG, IgA, and IgM produced in the supernatants of the culture were quantitated by radioimmunoassay. The horizontal bars indicate the geometric means for each immunoglobulin class. (IgG, $P < 0{\cdot}05$; IgA, $P > 0{\cdot}05$; IgM, $P > 0{\cdot}05$).

Fig. 2. B cell function in Ig production. One hundred thousand B cells fractionated from each of patient with HC (●, ▲, ■) or healthy subjects (○, △, □) were co-cultured with equal number of T cells isolated from a single donor. After 7 days in culture with PWM, the amount of Ig produced in the culture supernatants was determined. No significant differences in IgG, IgA, and IgM production were observed between both groups.

Fig. 3. T helper activity for *in vitro* PWM-stimulated Ig production of B cells. B cells, 1×10^5, isolated from a single donor were cultured with T cells from each of patients with HC (●, ▲, ■) or healthy subjects (○, △, □) in the presence of PWM for 7 days. The amounts of Ig produced in culture were determined. (IgG, $P < 0{\cdot}01$; IgA, $P > 0{\cdot}05$; IgM, $P > 0{\cdot}05$).

ng/ml in the cultures containing T cells from patients, and were 251 ng/ml and 895 ng/ml in the cultures containing T cells from controls, respectively. In contrast, the geometric mean of IgG produced was 575 ng/ml in patients and 240 ng/ml in controls.

T cell helper activity in IgG production was significantly increased in patients when compared with that in controls, ($P < 0{\cdot}01$), whereas no significant differences in IgA and IgM production were apparent between both groups (Fig. 3). However, increased T cell helper activity in IgG production in patients with HC could also result from reduced suppressor T cell activity.

Effect of irradiated T cells on Ig production

The geometric mean of IgG produced in the co-culture with non-irradiated T cells, was 575 ng/ml in five patients with hepatic cirrhosis (HC) and 213 ng/ml in six healthy subjects. T helper activity in IgG production was significantly increased in patients compared with controls ($P < 0{\cdot}05$).

On the other hand, in co-cultures with T cells irradiated with 1000 rad, IgG production in healthy controls apparently increased by 210–640% compared to co-cultures with non-irradiated T cells. However, this enhancing effect on IgG production was smaller in patients (ranging from 110% to 200%). The irradiation-induced enhancement for IgG production was significantly lower in patients than in healthy subjects. ($P < 0{\cdot}01$) (Fig. 4). This enhancing effect on IgG production diminished in the co-cultures with T cell irradiated with 3000 rad in both groups.

Effect of Con A-activated T cells on the in vitro *PWM-stimulated IgG production by allogeneic PBL*

As shown in Fig. 5, Con A-activated T cells from all healthy subjects (100%), suppressed IgG

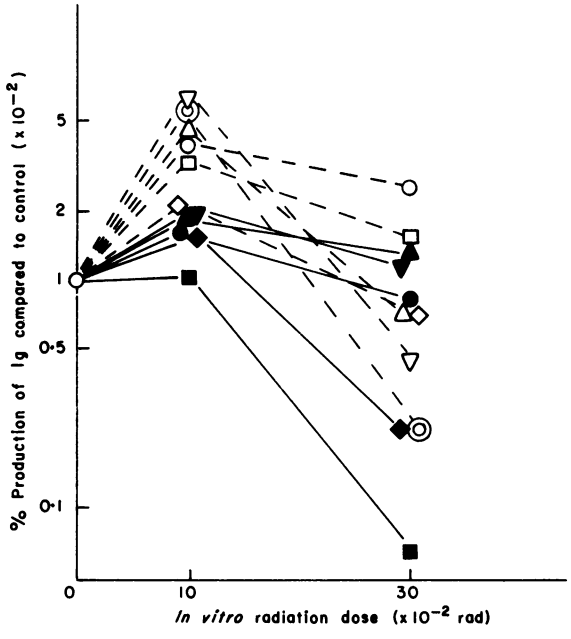

Fig. 4. Effect of irradiated T cells on the *in vitro* PWM-stimulated IgG production by B cells. T cells (1×10^5) from five patients with HC and six age-matched healthy subjects that had been irradiated were co-cultured with an equal number of B cells isolated from a single donor in the presence of PWM for 7 days. The amounts of IgG produced in culture with irradiated T cells are expressed as percentage production of those with non-irradiated T cells. Each symbol represents each individual. The open symbol and closed symbol indicate percentage IgG production from each of the healthy subjects and from each of patients with HC respectively.

production by PWM-stimulated PBL, although the magnitude of suppression ranged from 8 to 42%.

By contrast, Con A-activated T cells from only three out of seven patients with HC (43%) suppressed IgG production. Moreover in the remaining four patients, T cells, when pre-incubated with Con A for 2 days, mediated enhancement rather than suppression of IgG production. As illustrated at the right side in Fig. 5, the mean suppression mediated by Con A-activated T cells from seven controls was 27% for IgG production, and that from seven patients with HC was −4% for IgG production. The differences observed in the means are significant between the two groups ($P = 0{\cdot}01$).

DISCUSSION

HC is often accompanied by absolute increase in serum gammaglobulin concentration. All our patients used in this study had increased gammaglobulin levels of polyclonal peak type more than 1·8 g/dl. So far as we examined in the serum Ig, particularly IgG, and to a lesser extent, IgM and IgA had been elevated.

This elevated Ig in serum in HC is due to increased synthesis rather than decreased catabolism (Havens *et al.*, 1954; Cohen, 1963). Therefore, it may be that enhanced Ig production under these circumstances may occur elsewhere in the total body. In this regard, it has been reported that excess of serum gammaglobulin in human cirrhosis is produced at least in part by the lymph nodes and spleen (Glagov, Kent & Popper, 1959), in keeping with similar observations in ethiorine-induced rat cirrhosis (Kent *et al.*, 1957). Moreover, in some patients, Ig production in mononuclear cells of the

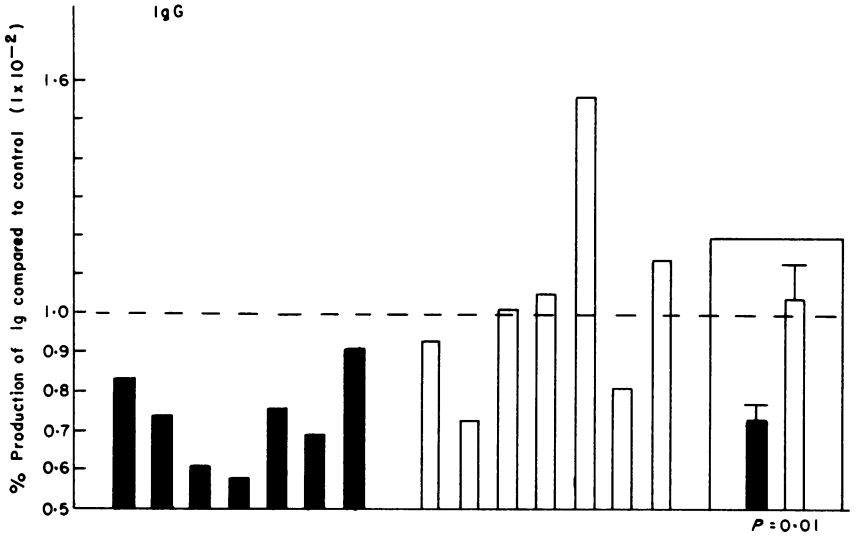

Fig. 5. Effect of Con A-activated T cells on the *in vitro* PWM-stimulated IgG production by allogeneic PBL. T cells (1×10^6) from seven healthy subjects (■) or seven patients with HC (□) were co-cultured with or without Con A (10 μg/ml). After 2 days in culture, T cells were harvested and washed three times and then T cells (1×10^5) were added to freshly prepared allogeneic PBL (2×10^5) from three separate donors and co-cultured for 7 days with PWM. IgG produced in culture was quantitated by radioimmunoassay. The amounts of IgG production are expressed as percentage production of those with T cells precultured without Con A.

liver may be a contributory factor in serum Ig levels (Hadziyannis *et al.*, 1969). However, the role of PBL in this process has not been explored.

Using the assay system of PWM-stimulated *in vitro* Ig production, the present study demonstrated that PBL from patients with HC produced significantly greater amounts of IgG but not IgA or IgM than did those from nearly age-matched healthy subjects. Although it is not clear to what extent Ig production by PBL reflects serum Ig levels, *in vitro* PWM-stimulated IgG production by PBL from patients with HC appears to be well correlated with serum IgG level. In addition our data suggested that this enhancement of IgG production by PBL in HC might result from the increased T helper activity due to depressed suppressor T cell activity and not from the enhanced B cell function in patients with HC.

In contrast, very recently, *in vitro* enhanced spontaneous IgG production by PBL from patients with chronic liver disease (CLD) as well as alcholic cirrhosis (AC) has been described (Mutchnick *et al.*, 1981). These studies suggests that spontaneous *in vitro* synthesis of IgG by B cells is enhanced in both CLD and AC and peripheral blood B cells may contribute to the elevated serum IgG seen *in vivo* in those patients. However, it is uncertain whether the results of these studies reflect significantly a true enhanced B cell function because the purified population of T and B cells have not been used to elucidate the mechanism resulting in increased Ig synthesis.

It is well known that T helper activity results from the change in balance between helper and suppressor T cell activity. Suppressor T cells in humans are known to be sensitive to irradiation whereas helper T cells are relatively radioresistant. (Siegal & Siegal, 1977; Haynes & Fauci, 1978a, 1978b). Previously, we have reported that the addition of irradiated T cells to B cells enhanced Ig production in culture with PWM beyond that obtained by the addition of non-irradiated T cells. (Kishimoto *et al.*, 1979).

In the present study, our data demonstrated that the irradiation-induced enhancement for IgG production was significantly lower in patients with HC compared with age-matched subjects. Our findings indicate that the function of naturally occuring suppressor T cells is decreased in many patients with HC.

Recently, human PBL activated by Con A have been shown to suppress the normal response to

mitogens, antigens and allogeneic cells (Shou, Schwartz & Good, 1976; Sakane & Green, 1977; Hallgren & Yunis, 1977). In addition our previous data (Kishimoto *et al.*, 1979) and those of others (Haynes & Fauci, 1977, 1978a, 1978b) have described that the activation by Con A of human PBL that, when co-cultured with fresh autologous or allogeneic cells, reproducibly suppressed the PWM-stimulated Ig production or anti-SRBC plaque forming cells (PFC) response. In the present study, the mean suppression on PWM-stimulated IgG production by Con A activated T cells were significantly lower in patients with HC than in nearly age-matched healthy subjects.

In both chronic active hepatitis (CAH) and primary biliary cirrhosis, loss of Con A induced suppressor cell activity has been demonstrated (Hodgson, Wands & Isselbacher, 1978; Dienstag, Weake & Wands, 1978; Woltjen & Zetterman, 1979). The most interesting findings observed in the present study are that T cells from four out of seven patients with HC, when pre-incubated with Con A for 2 days, exerted the enhancing effect rather than suppressive one on IgG production. Although the mechanism of this opposite phenomenon is entirely unknown, a recent report has indicated that Con A can activate both helper and suppressor T cells (Reinherz & Schlosman, 1979). Consequently, helper activity exceeds suppressor activity in patients with HC after Con A activation because the balance of T cell activity in patients with HC is in favour of helper rather than suppressor.

Patients with HC and CAH have been shown to have a decreased number of circulating T cells (Mcfarlane, Eddleston & Williams, 1977; Colombo, Vernace & Paronetto, 1977), and thus lack of Con A activated suppressor cells activity may be due to the absence of a Con A responsive T cell population. On the other hand, it has been shown that Con A activated suppressor cell are sensitive to mitomycin and require cell division to generate and to mediate suppression of PWM-stimulated PFC (Haynes & Fanci, 1978a, 1978b). Therefore, impaired Con A activated suppressor cell activity in patients with HC may be due to depressed proliferative response to Con A.

Taken together, the present study has shown that naturally occurring suppressor T cells, which are sensitive to irradiation, diminish in patients with HC. In addition, Con A activated suppressor T cells which are capable of suppressing Ig production by PWM-stimulated B cells, were confirmed to decrease in patients with HC. The loss or decreased suppressor T cell activity in these patients is probably a result of the established liver disease. The increased serum levels of Ig, particularly IgG in patients with HC may be explained in part on the basis of depressed ability of naturally occurring suppressor T cells and Con A-induced suppressor T cells to suppress Ig production.

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