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# *In vitro* synthesis of IgG by peripheral blood lymphocytes in chronic liver disease

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## SUMMARY

*In vitro* IgG synthesis by peripheral blood mononuclear cells (PBM) from patients with chronic liver disease (CLD) was studied. In addition, the effect of pokeweed mitogen (PWM), polyadenylic–polyuridylic acid complexes (poly A:U) and thymosin fraction 5 on IgG synthesis was determined. Unstimulated cultures of PBM from patients with chronic active hepatitis (CAH) and alcoholic cirrhosis (AC) synthesized significantly higher quantities of IgG than the controls. Moreover, there was a direct correlation between serum IgG concentrations and the quantity of newly synthesized IgG in these unstimulated cultures. PWM, poly A:U and thymosin each stimulated increased IgG synthesis in the controls. While neither poly A:U nor thymosin enhanced IgG synthesis in patients with CLD, PWM increased IgG synthesis in CAH but not AC. These results indicate that spontaneous *in vitro* B cell synthesis of IgG is enhanced in CLD and may reflect antigenic stimulation *in vivo*.

## INTRODUCTION

Hypergammaglobulinaemia is a frequent finding in chronic liver disease (CLD) for which the mechanism(s) remains unclear. It is generally agreed that the increased serum immunoglobulin (Ig) levels may involve all of the major Ig classes (Feizi, 1968; Deicher, Otto & Gleichmann, 1969; Wilson, Onstad & Williams, 1969; Ortmans, Wildhirt & Welcke, 1976; Van Epps *et al.*, 1976), and are a result of increased synthesis rather than decreased catabolism (Havens *et al.*, 1954; Ramsøe *et al.*, 1970; Jensen *et al.*, 1972). Among the suspected sites for the synthesis of the increased Ig observed in CLD are the spleen (Thomas, McSween & White, 1973; Prytz *et al.*, 1977), lymph nodes (Glagov, Kent & Popper, 1959) and bone marrow (Jarrold & Vilter, 1949; Glagov *et al.*, 1959; Zlotnick & Karshai, 1961; Prytz *et al.*, 1977), but apparently not the reticuloendothelial elements within the liver itself (Miller & Bale, 1954). Heretofore, the role of peripheral blood mononuclear cells (PBM) in this process has not been explored.

Accordingly, we measured *in vitro* IgG synthesis by peripheral blood B lymphocytes from patients with CLD, in the presence or absence of pokeweed mitogen (PWM), a potent mitogen for human T and B lymphocytes. In addition, we studied the effect on IgG synthesis of polyadenylic–polyuridylic acid complexes (poly A:U), a synthetic immunoadjuvant, and of the immunomodulator thymosin fraction 5, a polypeptide extract of the thymus gland.

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## MATERIALS AND METHODS

*Patient selection*

*Patients.* A total of 29 patients with CLD and proven hypergammaglobulinaemia were studied. The diagnosis was confirmed by biopsy in each case. Serum levels of IgM, IgG and IgA were determined by radial immunodiffusion (Behring Diagnostics, Somerville, New Jersey).

*Alcoholic cirrhosis (AC).* Ten patients (nine males, one female; aged 33–61), all with previous heavy alcohol intake, revealed histological evidence of micronodular cirrhosis. Five of these patients showed concomitant evidence of continuing liver cell necrosis, alcoholic hyaline and polymorphonuclear infiltration, indicative of alcoholic hepatitis.

*Chronic active hepatitis (CAH).* Nineteen patients were evaluated (CAH-T). All had CAH by clinical, biochemical, and histological criteria (Leevy, Popper & Sherlock, 1977). Five of these patients also had established cirrhosis. Nine patients (seven females, two males; aged 30–68) were untreated (CAH-N); of these two were $HBsAg^+$ and seven $HBsAg^-$. Ten patients (six males, four females; aged 22–68) were on steroid therapy (CAH-S) for at least 2 months prior to evaluation and of these, four were $HBsAg^+$ and six $HBsAg^-$.

*Controls.* Forty-three normal control subjects were selected from healthy adult volunteers (aged 21–55) who did not have a history of liver disease, immune disorders, or other chronic illness. This investigation was approved by the Committee for Clinical Research and Investigation Involving Human Beings of The University of Michigan Medical Center and all patients gave written consent.

*Isolation of PBM*

PBM were isolated from freshly drawn, heparinized (preservative-free heparin) blood by modification of the method of Böyum (1968). Blood was diluted 1:1 with normal saline (NS) and layered over one-third volume of Ficoll–Hypaque (Pharmacia, Piscataway, New Jersey) in conical-bottomed tubes. After centrifugation at 400 ***g*** for 40 min at room temperature, cells were harvested from the interface and washed three times with NS (cells isolated by this procedure were >98% mononuclear and >95% viable).

*Cell culture*

Cell suspensions were adjusted to contain $10^6$ mononuclear cells/ml in RPMI 1640 culture media with HEPES buffer, supplemented with penicillin, streptomycin, essential vitamins and glutamine (GIBCO, Grand Island, New York) plus 10% heat-inactivated fetal calf serum (Lot 22803; Reheis Chemical Co., Kankakee, Illinois). One-millilitre aliquots were cultured in loosely capped plastic tubes to which was added 0·1 ml of NS or 0·1 ml of one of the following modulating agents: PWM (GIBCO, Grand Island, New York), poly A:U (Miles Laboratories Inc., Elkhart, Indiana), or thymosin fraction 5 (generously provided by Professor A. L. Goldstein, George Washington University Medical Center, Washington, DC). PWM was used at dilutions of 1:100, 1:50 and 1:10; poly A:U at concentrations of 50, 100 and 200 μg/ml and thymosin at 50, 100 and 200 μg/ml. The peak IgG response to the variable concentrations of each agent was determined.

All cultures were prepared in duplicate and incubated for 7 days at 37°C in a humidified gas chamber (5% $CO_2$, 95% air). At the end of the incubation period, the culture tubes were centrifuged at 400 ***g*** for 15 min at 5°C to pellet cells, and the supernatant fluids aspirated and stored at −40°C until assayed.

*Assay of IgG*

Nanograms of IgG in culture supernatants were measured by tube-binding radioimmunoassay (Askenase & Leonard, 1970). This assay is based on competitive inhibition of the binding of radiolabelled IgG. Briefly, 10 × 75 mm siliconized glass culture tubes were coated with an appropriately diluted 0·7-ml aliquot of anti-human IgG (Miles Laboratories Inc., Elkhart, Indiana). Tubes were incubated overnight in humidified chambers, washed three times with phosphate-buffered saline containing 0·1% sodium azide (PBS), pH 7·2, then filled with 1% bovine serum albumin (BSA) to block other binding sites on the glass. The BSA was aspirated immediately prior to use.

For the assay, 0·2 ml of culture supernatant was diluted with 1·8 ml of 1% BSA containing

40–60,000 c.p.m./ml of $^{125}$I-human IgG with a specific activity of 49 $\mu$Ci/$\mu$g (IgG myeloma proteins were generously provided by Dr J. L. Claflin, University of Michigan, Ann Arbor, Michigan), labelled by chloramine T procedure (McConahey & Dixon, 1966). A volume of 0·9 ml from the resulting mixture was added to each of two culture tubes which were then incubated overnight at 37°C in humidified chambers. After incubation, the tubes were washed three times with PBS and bound $^{125}$I was quantitated in a Packard model 578 gamma counter. Standard curves were prepared with known amounts of IgG for each assay. It is linear within the range of 50–1,500 ng/ml and there is no cross-reactivity with IgM or with fetal calf serum. Results are expressed as ng IgG synthesized by $10^6$ PBM in 7 days. Student's independent *t*-test was used to compare study groups. Student's dependent *t*-test was used to compare IgG synthesis in the presence or absence of modulating agents within the same study group.

## RESULTS

The serum IgG levels were elevated in all groups of CLD patients (Table 1). The amount of IgG synthesized *in vitro* by unstimulated cultures was significantly higher in all patient groups as compared to controls. Poly A:U and thymosin stimulated increases in IgG synthesis by control B cells. In contrast, in the patient groups neither poly A:U nor thymosin were able to enhance immunoglobulin synthesis significantly above the already elevated baseline levels (Tables 1 and 2).

The addition of PWM caused a significant increase in IgG synthesis in controls and patient groups except AC. However, in the presence of this mitogen there were no significant differences in synthesis between control and patient groups (Table 1). That this was not due simply to a shift in the dose–response curve to PWM was verified by testing a number of patients and controls with PWM at varying dilutions. Significant differences were not observed in peak PWM response (data not shown).

## DISCUSSION

B cells from patients with CAH and AC spontaneously synthesized significantly greater quantities of IgG *in vitro* than the control group when cultured in media alone. Although PWM enhanced IgG synthesis in all groups, the degree of enhancement was less in the patient groups, and significantly so in the CAH-T ($P < 0{\cdot}02$).

**Table 1.** *In vitro* synthesis of IgG by PBM

| | | | ng IgG ($10^6$ cells)$^{-1}$ (7 days)$^{-1}$ (mean ± s.d.) | | |
|---|---|---|---|---|---|
| | | | | Modulating agents | |
| Subjects (*n*) | Age (mean ± s.e.m.) | Serum IgG (mean ± s.e.m.) (normal 92–207 i.u.) | Unstimulated NS | PWM | Poly A:U |
| CAH-T (19) | 46·2 ± 3·8 | 330 ± 26 | 872 ± 135 (19)* | 1,389 ± 192 (18)‡ | 804 ± 100 (18) |
| CAH-S (10) | 38·2 ± 5·1 | 304 ± 36 | 710 ± 129 (10)* | 1,210 ± 209 (10)§ | 734 ± 170 (9) |
| CAH-N (9) | 55·0 ± 4·2 | 358 ± 37 | 1,053 ± 241 (9)* | 1,613 ± 342 (8)¶ | 875 ± 112 (9) |
| AC (10) | 48·0 ± 3·1 | 359 ± 48 | 443 ± 74 (10)† | 706 ± 130 (10) | 510 ± 64 (10) |
| Controls (43) | 30·1 ± 1·2 | — | 276 ± 33 (43) | 1,126 ± 124 (39)** | 360 ± 35 (42)‡ |

Results in parentheses indicate number of subjects tested.
* $P < 0{\cdot}001$ control values, Student's independent *t*-test.
† $P < 0{\cdot}05$ control values, Student's independent *t*-test.
‡ $P < 0{\cdot}001$ NS baseline, Student's dependent *t*-test.
§ $P < 0{\cdot}005$ NS baseline, Student's dependent *t*-test.
¶ $P < 0{\cdot}05$ NS baseline, Student's dependent *t*-test.
** $P < 0{\cdot}0001$ NS baseline, Student's dependent *t*-test

**Table 2.** Influence of thymosin on *in vitro* synthesis of IgG

| | | ng IgG $(10^6$ cells$)^{-1}$ $(7$ days$)^{-1}$ (mean ± s.d.) | |
|---|---|---|---|
| Subjects | Number tested | NS | Thymosin |
| CAH-T | 13 | 1,001 ± 184 | 1,052 ± 183 |
| CAH-S | 8 | 729 ± 157 | 721 ± 138 |
| CAH-N | 5 | 1,435 ± 344 | 1,580 ± 308 |
| AC | 6 | 531 ± 106 | 610 ± 145 |
| Controls | 20 | 299 ± 49 | 403 ± 52* |

* $P < 0{\cdot}001$ NS baseline, Student's dependent *t*-test.

We have reported normal peripheral blood B cell numbers in alcoholic liver disease as well as other investigators (Thomas *et al.*, 1976; Van Epps *et al.*, 1976; Mutchnick & Goldstein, 1979). Several studies have described normal B cell proportions in CAH (Tolentino & Astaldi, 1975; Thomas *et al.*, 1976) while others report elevated counts in CAH patients without cirrhosis, and normal values in the presence of cirrhosis (Colombo, Vernace & Paronetto, 1977). Our data suggest that peripheral blood B cells of patients with CLD synthesize increased amounts of IgG *in vitro*. This increased rate of synthesis, as well as the relative unresponsiveness of patients' PBM to PWM, may be explained by prior activation *in vivo*. Additional evidence to support this conclusion is provided by using the Pearson product-moment correlation test to compare serum IgG levels of each patient to the concentration of IgG synthesized in unstimulated cultures. As shown in Table 3, there was a significant correlation observed between increasing serum levels of IgG and the quantity of newly synthesized IgG in unstimulated cultures of PBM from patients with CAH-N and AC. CAH-S patients failed to show this pattern, reflecting the *in vivo* inhibitory effect on IgG synthesis by steroids (Butler & Rossen, 1973).

That the data was not unduly influenced by serum IgG carry-over into the cultures was shown by preliminary kinetic studies. Experiments conducted on 12 control subjects did not demonstrate any measurable IgG in the supernatants prior to 72 hr in culture. The maximum rate of synthesis occurred from days 5 to 7. Neither poly A:U nor PWM shortened the 4-day latent phase. In addition, it is unlikely that cytophilic antibody would significantly alter the data as the presence of as little as 15 ng of IgG in the culture supernatant would require the shedding of $6 \times 10^7$ IgG molecules per cell into the culture, far in excess of the $1 \times 10^5$ molecules generally believed carried by such cells.

Previous studies of poly A:U have shown that this non-toxic, non-pyrogenic adjuvant can increase polyclonal IgG synthesis of human B cells in a system identical to that used in this study

**Table 3.** Relationship of serum IgG levels to IgG synthesized *in vitro*

| Group | No. | Correlation Coefficient (*r*) | Significance (*P*) |
|---|---|---|---|
| CAH-S | 10 | 0·1817 | n.s. |
| CAH-N | 9 | 0·6862 | < 0·05 |
| AC | 10 | 0·6717 | < 0·05 |
| CLD | 29 | 0·3687 | < 0·05 |

(Lederman, 1978). The precise mechanism for poly A:U enhancement of IgG synthesis is not yet known, but data in murine and human systems (Ishizuka, Braun & Matsumoto, 1971; Schmidtke & Johnson, 1971) suggest that poly A:U is non-mitogenic (Han & Johnson, 1975) and may activate helper T cells. In our studies, poly A:U did increase *in vitro* IgG synthesis in the controls but not in patients with CAH or AC. This suggests further that B cells of CLD patients were already highly stimulated *in vivo*.

Thymic gland extracts have been shown to exert an endocrine influence on the maturation and function of the immune system in a variety of animal and human models (Goldstein *et al.*, 1975). Thymosin fraction 5 is believed to hasten maturation of immature lymphocytes destined to become T cells and has been shown to influence the number and function of regulatory T cells *in vitro* (suppressor, helper) (Schulof & Goldstein, 1977). Thymosin is non-mitogenic and does not directly influence B cell or monocyte numbers or function (Horowitz & Goldstein, 1978). The addition of thymosin to PBM cultures significantly enhanced IgG synthesis in the controls but did not significantly alter IgG synthesis in patients with CLD. This, too, may be due to previous activation of B lymphocytes *in vivo* and/or to the presence of enhanced helper effect.

Alterations in immunoregulatory control have been described in patients with CAH and alcoholic liver disease (ALD). Decreased concanavalin A (Con A) induced suppressor cell activity (SCA) has been reported in CAH and ALD (Hodgson, Wands & Isselbacher, 1978; Kawanishi *et al.*, 1978; Schaffner, Mutchnick & Goldstein, 1979). It is not known if the results of these studies signify a true deficiency in suppressor cell numbers and/or function or reflect changes in helper cell effect which may mask SCA. We have previously shown that thymosin can increase Con A-induced SCA in patients with CAH, but not in normal controls (Schaffner *et al.*, 1979). Decreased T suppressor but not increased T helper effect on *in vitro* IgG synthesis has been described in primary biliary cirrhosis but not in AC or CAH when varying ratios of separated T and B cell fractions were co-cultured (James *et al.*, 1979).

Macrophages are required for optimal *in vitro* induction of antibody responses but it is not known if this reflects a specific helper function or a non-specific increase in viability (Dosch & Gelfand, 1977; Delfraissy *et al.*, 1978). All of the postulated effects of T cells may apply equally well to macrophage subpopulations. Antigens stimulating lymphocytes of patients with CLD *in vivo* might be carried over into culture medium, thus accounting for our results. Although the PBM were washed extensively prior to culture, this possibility cannot be discounted *a priori*.

Peripheral blood B cells may contribute to the elevated serum IgG seen *in vivo* in patients with CAH and AC. Use of purified populations of T and B cells as well as macrophages may help elucidate the mechanism resulting in increased Ig synthesis in these patients.

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