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# Abnormalities of *in vitro* immunoglobulin production in apparently healthy haemophiliacs: relationship with alterations of T cell subsets and with HTLV-III seropositivity

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## SUMMARY

The pokeweed mitogen (PWM)-induced immunoglobulin (Ig) production by cultures of peripheral blood mononuclear cells (PBMC) was reduced in healthy haemophiliacs treated with commercial factor VIII (or IX) concentrate, whereas the spontaneous IgG synthesis *in vitro* was enhanced. PWM-induced Ig production was lower in those who had received greater amounts of concentrate, in those with inverted T4/T8 lymphocyte ratios and in those with antibody to HTLV-III. The spontaneous IgG production *in vitro* was higher in haemophiliacs who had received larger amounts of concentrate, in those with inverted T4/T8 ratio and in those with antibody anti-HTLV-III. However, some patients with normal T4/T8 ratio and some with HTLV-III antibody also had raised spontaneous IgG production.

**Keywords** haemophilia HTLV-III virus T-cell subsets Ig synthesis *in vitro*

## INTRODUCTION

Patients with haemophilia receiving lyophilized factor VIII concentrate are at increased risk from acquired immunodeficiency syndrome (AIDS) and the AIDS-related complex (ARC) (Elliott *et al.*, 1983; Poon *et al.*, 1983). Many healthy haemophiliacs also have alterations in T-lymphocyte subsets similar to those found in the prodromal stage of AIDS (Lechner *et al.*, 1983; Lederman *et al.*, 1983; Menitove *et al.*, 1983; Mannucci *et al.*, 1984) and European haemophiliacs treated with factor VIII concentrate imported from the USA may have antibody to HTLV-III (Melbye *et al.*, 1984; Rossi *et al.*, 1984).

Patients with AIDS have abnormalities of B cell function (Lane *et al.*, 1983; Amman *et al.*, 1984; Pahwa *et al.*, 1984) but the mechanism remains unknown. To this effect we have studied spontaneous and PWM-stimulated IgG and IgM synthesis by peripheral blood lymphocytes (PBL) from haemophiliacs treated with commercial factor VIII or IX concentrate.

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## MATERIALS AND METHODS

*Patients.* Seventy-five consecutive haemophiliacs (62 with haemophilia A, 13 with haemophilia B) were studied at the Haemophilia Centre of Florence. Information on replacement therapy between 1981 and 1984 was obtained from hospital or patient records; commercial concentrates manufactured from large plasma pools in the USA were used by all. Patients were examined at the time of blood taking; no abnormalities were detected. None were known to be homosexuals or drug abusers. Volunteer healthy members of the laboratory staff, their parents and children were also studied as controls (total 31).

*Lymphocyte separation and characterization.* Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by centrifugation over Ficoll-Hypaque. T-cell subpopulations were counted by indirect immunofluorescence using three monoclonal antibodies (OKT3, OKT4 & OKT8; Ortho Pharmaceutical, Raritan, NJ, USA), followed by fluorescein-labelled affinity-purified $F(ab')_2$ fragments of anti-mouse Ig rabbit antibodies (Romagnani *et al.*, 1983).

*In vitro Ig production. In vitro* synthesis of IgM and IgG by PBMC was measured as described (Romagnani *et al.*, 1983a). Briefly, $1 \times 10^6$ PBMC were cultured for 7 days in 1 ml of 10% fetal calf serum-containing RPMI 1640 medium (GIBCO, Grand Island, NY, USA) with or without PWM (GIBCO) at the final concentration of 5 $\mu$l/ml. IgM and IgG in the supernatant was determined by solid phase radioimmunoassays. Parallel cultures containing cycloheximide, 100 $\mu$g/ml, were always performed. Synthesis of IgM and IgG was calculated by subtracting from values from unstimulated or PWM-stimulated cultures values of equivalent cultures containing cycloheximide.

*Antibody to HTLV-III.* Serum antibody to HTLV-III structural proteins was assayed either by indirect immunofluorescence on HTLV-III-infected HT cell line (Rossi *et al.*, 1984; Sirianni *et al.*, 1985; Aiuti *et al.*, 1985) or by two different commercial immunoenzymatic assays (Abbott HTLV III EIA, Abbott Laboratories, North Chicago, USA; HTLV-III Bio-Enzabead, Litton Bionectics, Charleston, USA).

## RESULTS

*Abnormalities of spontaneous and PWM-induced Ig production in haemophiliacs*

Spontaneous IgM synthesis did not significantly differ between 75 haemophiliacs and 31 controls, whereas PWM-induced IgM synthesis from patient PBMC was significantly reduced ($P < 0.05$) (Table 1). Spontaneous production of IgG was significantly greater in PBMC cultures from haemophiliacs than from controls ($P < 0.002$). IgG concentrations in supernatants from patient's cultures stimulated with PWM did not differ from those of controls; spontaneous IgG synthesis in patient cultures was greater, and the increased IgG production following PWM-stimulation was less in the haemophiliac group than in controls.

*Relationship between abnormalities of Ig production and changes in the proportion of T-cell subsets.*

The mean absolute value of $T4^+$ cells in haemophiliacs was $1,055 \pm 47/mm^3$ of blood, whereas the mean value of $T8^+$ cells was $792 \pm 50/mm^3$. Twenty-three haemophiliacs, but none of controls, had a T4/T8 ratio $< 1$. PWM-induced IgM production in haemophiliacs with inverted T4/T8 ratio was significantly lower than in the other patients ($P < 0.05$) and controls ($P < 0.002$) (Table 2). The spontaneous IgG synthesis was significantly greater in haemophiliacs with T4/T8 ratio $< 1$ in comparison with patients showing T4/T8 ratio $> 1$ ($P < 0.02$); in both groups of patients it was significantly higher than in controls ($P < 0.0005$ & $P < 0.02$, respectively). IgG production in PWM-stimulated cultures of the three groups did not differ significantly.

*Relationship between abnormalities of Ig production and total dose concentrate*

Haemophiliacs treated with < 50,000 iu of factor VIII (or IX) concentrate in the previous 4 years did not differ significantly from controls in spontaneous IgM and PWM-induced IgM and IgG production. However, spontaneous IgG production by PBMC of patients treated in the previous 4

**Table 1.** Concentration of IgM and IgG in PBMC cultures of haemophiliacs and controls

| Subjects | Number | IgM (ng/ml) | | IgG (ng/ml) | |
|---|---|---|---|---|---|
| | | Unstimulated | PWM-stimulated | Unstimulated | PWM-stimulated |
| Hemophiliacs | 75 | 322±83* | 2,763±371 | 616±60 | 2,277±299 |
| Controls | 31 | 319±124 | 3,991±517 | 320±65 | 2,474±367 |

* Mean values ± s.e.

**Table 2.** Concentration of IgG and IgM in culture supernatants of haemophiliacs with T4/T8 cell ratios above or below 1

| Subjects | Number | IgM (ng/ml) | | IgG (ng/ml) | |
|---|---|---|---|---|---|
| | | Unstimulated | PWM-stimulated | Unstimulated | PWM-stimulated |
| Haemophiliacs | | | | | |
| T4/T8 ratio < 1 | 23 | 258±86 | 1,870±459 | 792±106 | 2,044±622 |
| T4/T8 ratio > 1 | 51 | 347±115 | 3,182±497 | 525±71 | 2,307±338 |
| Controls | 31 | 319±124 | 3,991±517 | 320±65 | 2,474±367 |

**Table 3.** Relationship between *in vitro* Ig production and total dose of concentrate consumed

| Subjects | Number | IgM (ng/ml) | | IgG (ng/ml) | |
|---|---|---|---|---|---|
| | | Unstimulated | PWM-stimulated | Unstimulated | PWM-stimulated |
| Haemophiliacs | | | | | |
| > 50,000 iu | 32 | 247±92 | 1,766±413 | 719±104 | 1,232±247 |
| < 5,000 iu | 36 | 443±162 | 3,848±630 | 480±67 | 3,077±455 |
| Controls | 31 | 319±124 | 3,991±517 | 320±65 | 2,474±367 |

**Table 4.** Relationship between abnormalities of *in vitro* Ig production and presence in the serum of antibody to HTLV-III

| Subjects | Number | IgM (ng/ml) | | IgG (ng/ml) | |
|---|---|---|---|---|---|
| | | Unstimulated | PWM-stimulated | Unstimulated | PWM-stimulated |
| Haemophiliacs | | | | | |
| HTLV-III-seropositive* | 28 | 207±164 | 999±279 | 780±108 | 1,273±331 |
| HTLV-III-seronegative | 46 | 301±67 | 3,791±520 | 475±56 | 2,872±415 |
| Controls† | 31 | 319±124 | 3,991±517 | 320±65 | 2,474±367 |

* Serum antibody to HTLV-III was detected by two different immunoenzymatic assays, as described in Materials and Methods.

† All controls were seronegative.

years with > 50,000 iu was significantly higher than in patients treated with less than 50,000 iu ($P < 0{\cdot}05$) and in controls ($P < 0{\cdot}002$) PWM-induced IgM and IgG synthesis was lower than in controls ($P < 0{\cdot}002$ & $P < 0{\cdot}0005$) and lower than in patients treated with < 50,000 iu ($P < 0{\cdot}005$ & $P < 0{\cdot}002$) (Table 3). Seven patients were omitted as the dose was uncertain.

*Relationship between abnormalities of Ig production and HTLV-III antibody*

Fifteen of the 70 patients tested by indirect immunofluorescence had serum antibody to HTLV-III. When antibody to HTLV-III was sought using two different immunoenzymatic assays it was found in the serum of a higher number of patients (28 out of 74), but in none of controls (Table 4). Those with HTLV-III antibody had lower PWM-induced IgM synthesis ($P < 0{\cdot}0005$). PWM-induced IgG synthesis was also lower in HTLV-III seropositive than in controls ($P < 0{\cdot}01$) or seronegative patients ($P < 0{\cdot}002$), whereas spontaneous IgG synthesis in seropositive and seronegative patients was higher than in controls ($P < 0{\cdot}0005$ & $P < 0{\cdot}05$, respectively). However, seropositive patients had higher spontaneous IgG synthesis than seronegative and the difference was statistically significant ($P < 0{\cdot}025$).

## DISCUSSION

Lymphocytes from symptom-free Italian haemophiliacs treated with commercial concentrate imported from the USA had greater spontaneous IgG production and less PWM-stimulated IgM production than controls.The amount of IgG produced *in vitro* by patient B cells following PWM-stimulation was lower than that released by PWM-stimulated cells from control individuals. Similar alterations have been described in patients with AIDS or LAS (Lane *et al.*, 1983) and, more recently, in asymptomatic homosexuals at risk for AIDS (Pahwa *et al.*, 1984) and are thought to result from polyclonal B cell activation *in vivo* (Lane *et al.*, 1983; Pahwa *et al.*, 1984).

The abnormalities were related to the amount of concentrate consumed in the last 4 years, suggesting that these abnormalities were not an inherent feature of haemophilia, but were a consequence of substitution therapy. The decrease of PWM-induced IgM synthesis was significantly related to the T4/T8 lymphocyte ratio. Patients with T4/T8 ratio > 1 showed PWM-induced IgM (& IgG) production comparable with that of controls, whereas in patients with inverted T4/T8 ratio PWM-induced IgM production was consistently depressed. Patients with inverted T4/T8 ratio showed a significantly higher spontaneous IgG synthesis than those without. However, the amount of IgG spontaneously synthesized by haemophiliacs with normal T4/T8 ratio > 1 was significantly higher than in controls.

PWM-induced Ig production strictly depends on the regulatory activity of helper and suppressor T lymphocytes; so the reduction of PWM-induced Ig production in asymptomatic haemophiliacs may reflect abnormal proportions of immunoregulatory T cells.

PWM-induced Ig production in HTLV-III seronegative patients was not significantly different from controls, whereas PWM-stimulated production of both IgM and IgG was low in HTLV-III-seropositive patients. Since HTLV-III retrovirus infects and destroys the $T4^+$ subset and can induce an increase of the $T8^+$ subset (Sarin & Gallo, 1984), both the alteration of T4/T8 ratio and the reduction of Ig synthesis in cultures stimulated with a T-cell dependent B-cell activator may be the result of HTLV-III-induced imbalance between $T4^+$ ('helper/inducer') and $T8^+$ ('cytotoxic/suppressor') lymphocytes.

A statistically significant difference in spontaneous IgG synthesis between seronegative and seropositive patients was also found, suggesting that the increase in spontaneous IgG production does at least in part reflect HTLV-III retrovirus infection. However, seronegative patients showed significantly higher spontaneous IgG production than controls. This may suggest a second abnormality in B cell function independent from HTLV-III infection, unless some HTLV-III-infected individuals did not make antibody to HTLV-III; HTLV-III has been isolated from symptom-free seronegative persons (Salahuddin *et al.*, 1984). Thus, the possibility that B cells from haemophiliacs may already be polyclonally activated *in vivo* before infection of T cells by HTLV-III retrovirus cannot be excluded.

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