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The suppressive effect of gammaglobulin preparations on *in vitro* pokeweed mitogen-induced immunoglobulin production

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SUMMARY

The effect of the supplementation with several gammaglobulin (GG) preparations on the *in vitro* immunoglobulin synthesis of peripheral blood mononuclear cells (PBMC) from normal subjects stimulated with pokeweed mitogen (PWM) was studied. Among the GG preparations used in this study, immune serum globulin (ISG) demonstrated the most suppressive effect, and S-sulfonation and polyethylene glycol (PEG)-treated preparations also had a suppressive effect. However, the preparation of pepsin degradation had no suppressive effect. And because IgG $F(ab')_2$ fragments also failed to induce the suppressive effect, it was considered to be triggered by the attachment of the Fc portion of GG to the corresponding membrane receptor.

To determine the cellular targets, PBMC were fractionated into E-rosetting cells (T cells) and non E-rosetting cells (B cells). The suppressive effect was induced by pre-incubation of either T cells or B cells with the GG preparations for 1 h, at 37°C in PWM-induced immunoglobulin (Ig) production. The failure of T cells pretreated with OKT8 monoclonal antibody and complement to induce the suppressive effect suggested that T8 positive T cells are one of the effector cells involved. The activation step of the suppressive effect was prostaglandin E_2-independent, and as effector cells contain an Fc receptor which is sensitive to pronase, it was suggested that monocytes were not involved in this activation process. Our observations further suggested that the Ig effects of GG therapy are not limited to antibody transfer, since GG preparations also suppress directly the differentiation of B cells and induce suppressor T cells in *in vitro* immunoglobulin production stimulated with PWM.

Keywords gammaglobulin preparations immunoglobulin synthesis pokeweed mitogen

INTRODUCTION

Gammaglobulin (GG) preparations have been used as a replacement therapy for primary immunoglobulin deficiency diseases. In recent years, the so-called intact type of GG preparations for intravenous use have been commercially available, and their ability to maintain safely a higher concentration of serum immunoglobulin (Ig) levels has been recognized (Morell *et al.*, 1980). With high dose therapy of intravenous GG preparations, it is possible to increase the trough level of serum IgG concentrations and arrive at a satisfactory clinical course in patients with severe humoral immunodeficiency (Schiff *et al.*, 1983; Sorensen & Polmar, 1984).

Many investigators have reported that passively administered Ig reduces the level of the

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humoral response, and antibody or antigen–antibody complexes suppress the antibody response *in vivo* and *in vitro* (Uhr & Moller, 1968; Hoffmann & Kappler, 1978; Milne, Chalon & Vaerman, 1980; Nicholson & McDougal, 1981). It has also been reported that immune serum globulin (ISG) by muscular injection induces an inhibition of proliferation of T and B lymphocytes and maturation of B lymphocytes (Durandy, Fischer & Griscelli, 1981). We have investigated the influence of several GG preparations on *in vitro* lymphocyte transformation and Ig production induced by polyclonal stimulation.

MATERIALS AND METHODS

Lymphocyte preparation and isolation of human T and B cells. Fresh peripheral blood mononuclear cells (PBMC) were isolated from consenting healthy human volunteers by Ficoll-Hypaque density gradient centrifugation. Enriched populations of T cells were then isolated by the method of rosette formation with neuraminidase-treated sheep erythrocytes and density gradient centrifugation. A highly enriched population of B cells was isolated from the non-rosette forming cells by the second rosette formation. In order to isolate T cell sub-populations enriched in either T4 positive cells or T8 positive cells, 5×10^6 of T cells were incubated with 1 ml of OKT4 or OKT8 monoclonal antiserum (Orthoclone, Raritan, NJ, USA) diluted to 1/15 in RPMI-1640 media for 45 min at room temperature. After incubation, fresh rabbit serum was added at a final dilution of 1/10, and incubation was further carried out for 30 min at 37°C in a humid atmosphere. Analysis of the resulting populations showed that the OKT4-treated population contained $< 20\%$ T4 positive cells and that the OKT8-treated population contained $> 90\%$ T4 positive cells and $< 10\%$ T8 positive cells. In view of this result, we decided to use only the population of T cells remaining after treatment with OKT8 plus complement in this study.

Culture conditions. Cells were cultured in round bottom culture tubes (Falcon CA, USA) at a final cell concentration of 5×10^5/ml/tube in RPMI-1640 supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin and 10 μl/ml of pokeweed mitogen (PWM) (GIBCO, Grand Island, NY, USA). The culture tubes were incubated at 37°C in a humid atmosphere containing 5% CO_2, 95% air. On the sixth day of the culture, the cells were resuspended at a concentration of 5×10^5 cells/ml in fresh minimal essential medium (MEM) without L-leucine (GIBCO, Grand Island, NY, USA) containing 10% dialysed FCS and 10 μCi/ml, L-4-5 ^{3}H-leucine (Amersham International Ltd., Amersham, UK). After incubation for 24 h, the supernatants were harvested by centrifugation at 400 ***g*** for 20 min.

B cells ($2{\cdot}5 \times 10^5$) and T cells (5×10^5) were incubated under the same condition. Graded numbers (see Results) of fractionated T cells or the T8 negative T cell subsets were added to the culture of fresh PBMC.

PBMC were also cultured in a round-bottomed tissue culture microplate (Costar, Cambridge, MA, USA) in triplicate with phytohaemagglutinin (PHA) (Difco, Detroit, MI, USA), concanavalin-A (Con A) (Calbiochem, San Diego, CA, USA) and PWM. The culture was performed in a final volume of 0·2 ml in RPMI-1640 with FCS, 10% AB serum or 10% FCS plus the GG preparations (1 mg/ml). Incorporation of ^{3}H-thymidine was determined after the addition of 1 μCi of ^{3}H-thymidine (New England Nuclear, Boston, MA, USA) during the last 16 h of a 3 day culture.

Solid phase radioimmunoassay for the quantification of immunoglobulin production. Immunoglobulin purified chromatographically was absorbed onto polystyrene balls (diameter 6·4 mm; Precision Plastic Ball Co., Chicago, IL, USA) in the following way. The balls were submerged in anti-IgG, anti-IgA, anti-IgM (Tago Inc., Burlingame, CA, USA) diluted to 100 μg/ml in carbonate-bicarbonated buffer (pH 8·5) and stored at 4°C overnight. The Ig coated balls were then put into 100 μl of culture supernatant containing an equal volume of phosphate-buffered saline (PBS) (pH 7·4, with 10% BSA). After incubation for 1 at 37°C, the balls were washed twice and placed in a scintillation vial for counting by a liquid scintillation counter. Data are presented as the per cent suppression of Ig production according to the following formula:

$$\text{Suppression (\%)} = \left[1 - \frac{\text{Ig production in the presence of or pretreatment with GG preparations}}{\text{Ig production in the absence of GG preparations}}\right] \times 100$$

Gammaglobulin preparations. Four different types of gammaglobulin preparations, standard immune serum globulin (ISG, cold ethanol fractionation) (Green Cross Co., Japan), pepsin degraded preparation (Gamma-Venin, Behring-Werke, FRG), PEG-treated preparation (Venoglobulin, Green Cross Co., Japan) and S-sulfonated preparation (Venilon, Teijin Inst., Japan) were examined. Purified human IgG $F(ab')_2$ (Cappel Lab. Inc., PA, USA) and intact IgG (Cappel Lab. Inc., PA, USA) were also used.

Treatment with pronase. T cells were treated with pronase (Calbiochem, San Diego, CA, USA) as previously described (Colombatti, Heumann & Moretta, 1981). The cells were incubated with pronase (4 mg/ml in RPMI-1640) for 30 min at 37°C.

RESULTS

Effect of GG preparations on PWM-induced immunoglobulin production

We examined the effect of GG on the Ig production of PWM-stimulated whole PBMC. Treatment of PBMC with GG preparations induced depression of Ig production in a dose-dependent manner (Fig. 1). Among the GG preparations, ISG (0·01–1·00 mg/ml) demonstrated the most suppressive effect on IgG, A and M production ($P < 0·01$), and S-sulfonation (0·05–1·00 mg/ml) and PEG treatment (0·5–1·0 mg/ml) also showed significant suppressive effects on IgG and IgA production ($P < 0·05$). Only a high concentration (1 mg/ml) of PEG treatment and S-sulfonation had a suppressive effect on IgM production ($P < 0·05$). However, in contrast to the effects induced by ISG or the intact GG preparations, the pepsin degradation preparation had no suppressive effect on any immunoglobulin isotypes at graded doses.

To clarify the dependence of the Fc portion of Ig on the suppressive effect, IgG $F(ab')_2$ fragments or intact human IgG were added to the culture (Fig. 2). A marked depression of Ig production was observed with intact IgG treatment, whereas $F(ab')_2$ fragments showed no depression of immunoglobulin production.

Effect of GG preparations on mitogen-induced proliferations of PBMC

PBMC cultured in the presence of a higher concentration of the GG preparations (1 mg/ml) used for analysis of the effect on Ig production were able to proliferate under stimulation of PHA, Con A or PWM, the same as the control culture (Table 1).

Target cells of the suppressive effect on immunoglobulin production

To determine whether the modulation of Ig production is due to the effect on regulatory T cells or B cells, we examined the effect on pre-incubated T cells or B cells with the GG preparations in a PWM-stimulated culture. B cells were preincubated with the GG preparations at 37°C for 1 h, and after washing, were incubated with fresh autologous T cells. B cells could not differentiate into Ig production ($P < 0·001$). T cells also were preincubated with the GG preparations and incubated

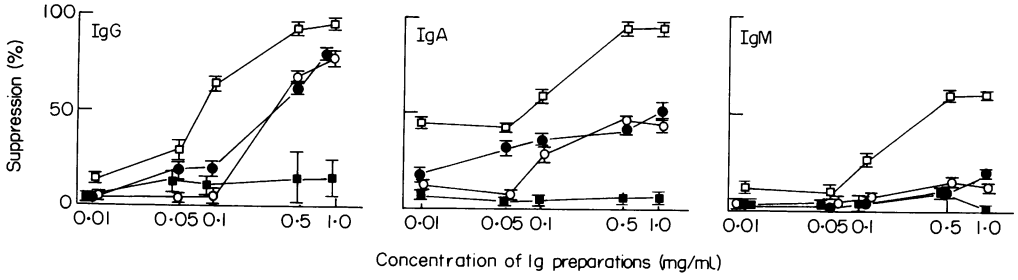

Fig. 1. Effect of GG preparations on Ig production by PBMC stimulated with PWM. PBMC (5×10^5) were cultured with graded doses of ISG (□), S-sulfonation (●), PEG treatment (○) and pepsin degradation (■) in addition to PWM. After 6 days, the culture supernatants were harvested and assayed by solid phase radioimmunoassay. Values represent mean ± s.d. of triplicate cultures.

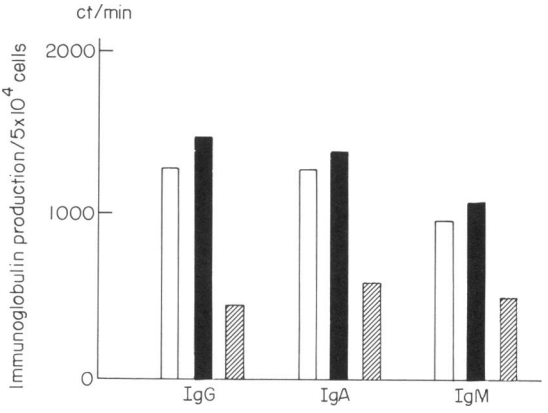

Fig. 2. Effect of Ig fragments on IgG production by PBMC stimulated with PWM. PBMC (5×10^5) were cultured with a supplement of: (□) medium; 1 mg/ml $F(ab')_2$; (▨) 1 mg/ml 7S intact IgG. PWM was added to all cultures. After 6 days the culture supernatants were harvested and assayed by solid phase radioimmunoassay. Mean ct/min of triplicate cultures are shown.

Table 1. Effect of ISG on mitogen-induced lymphocyte proliferation

	^{3}H-thymidine uptake (ct/min $\times 10^{-3}$)		
Cell culture	PHA	Con A	PWM
10% FCS	127·5±18·1	119·9±16·2	48·3±8·9
10% FCS+ISG	137·7±12·0	116·8± 8·8	39·4±8·2
10% AB serum	155·4± 8·2	117·7±12·5	51·4±2·0

PBMC were cultured at 1×10^5/ well with supplementation of 10% FCS or 10% human AB serum or 10% FCS+ISG (1 mg/ml) in the presence of mitogen. Incorporation of ^{3}H-thymidine was determined after the addition of 0·4 μCi of ^{3}H-thymidine.

* Mean ± s.e. of triplicate cultures.

with fresh autologous B cells. T cells were not able to help B cells to differentiate into Ig productions ($P < 0{\cdot}001$) (Fig. 3).

To clarify the phenotype of suppressor T cells, T cells were treated with OKT8 monoclonal antibody and complement before preincubation with the GG preparations. As shown in Fig. 4a, when increasing numbers of pre-incubated T cells which had been pretreated with OKT8 and complement were added to a constant number of PBMC, the suppressor activity of T cells was abolished to the same level as fresh T cells. But the suppression of IgG production was profoundly observed at even small numbers of T cells which were pre-incubated with the GG preparations. To determine the effect of the Fc receptor on T cells on the induction of suppressor activity, T cells were treated with pronase and then preincubated with the GG preparations. As shown in Fig. 4b, the suppressor activity of the pronase-pretreated T cells was the same as the T cells non-treated with the GG preparations.

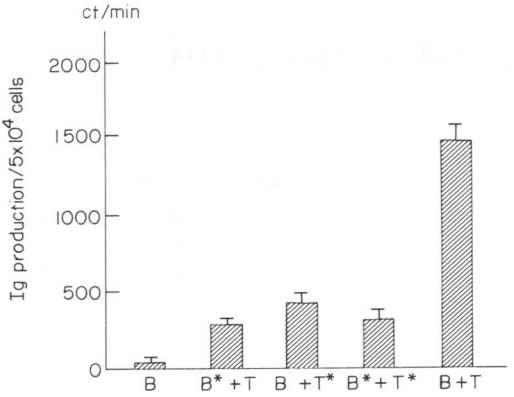

Fig. 3. B and T cells were preincubated with 1 mg/ml S-sulfonated preparation at 37°C for 1 h, and after washing each population was cultured together. $2{\cdot}5 \times 10^5$ pre-incubated or non-pre-incubated B cells were cultured alone or with 5×10^5 autologous preincubated or non-preincubated T cells, and after 6 days, the culture supernatants were analysed. Values represent mean ± s.d. of triplicate cultures.

* Preincubation.

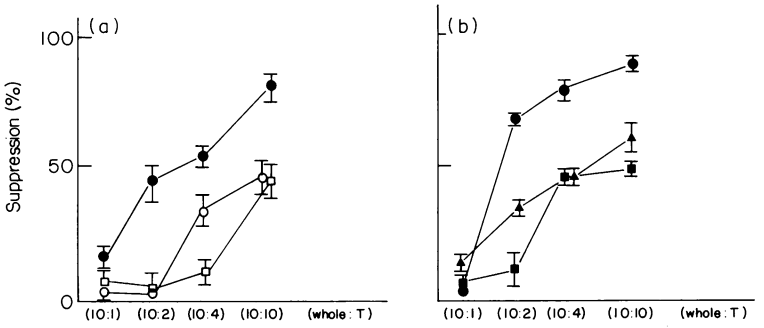

Fig. 4. (a) Effect of treatment with OKT8 monoclonal antibody on the suppressive activity of the T cells preincubated with ISC. After treatment with OKT8 and rabbit complement, T cells were preincubated with 1 mg/ml ISG. The various cell numbers of the T cells were added to 5×10^5 fresh autologous PBMC. (●) ISG pretreated; (○) OKT8 and ISG pretreated; (□) non-treated. (b) Effect of treatment with pronase on the suppressor activity of the T cells preincubated ISG. After treatment with pronase (4 mg/ml, at 37°C, for 30 min), the T cells were preincubated with 1 mg/ml ISG. (●) ISG pretreated; (■) pronase and then ISG pretreated; (▲) non-treated. Values represent mean ± s.d. of triplicate cultures.

DISCUSSION

The present study investigated the effect of GG preparations such as immune serum globulin (ISG), S-sulfonated, PEG treatment and pepsin degradation on *in vitro* proliferation and Ig production of PBMC. The results of our experiments revealed that small amounts of the GG preparations such as S-sulfonation and PEG treatment preparations had no effect on the proliferation of PBMC stimulated with PHA, Con A and PWM, but induced a severe suppression of immunoglobulin production stimulated with PWM. As neither pepsin degradation nor $F(ab')_2$ are capable of eliciting suppression of Ig production, it is possible that the GG preparations suppressed Ig production by interacting with Fc receptors of the cells needed for Ig production in response to PWM. The induction of proliferation and differentiation of B cells by PWM is a complex

phenomenon that includes activation of helper and suppressor T cells and monocytes. Since some of these cells could be affected by binding GG preparations to their Fc receptors, in this study T and B cells were individually preincubated with the GG preparations, and then were recombined with their complementary population for Ig production. These studies showed that GG preparations affected both B and T cells. The pre-incubated T cells were not able to help the differentiation of fresh B cells. The graded numbers of T cells pre-incubated with GG preparations suppressed Ig production of fresh PBMC. These results suggested that the dysfunction of the helper T cells was due to the activation of suppressor T cells. We next examined T cells treated with OKT8 monoclonal antibody and complement to investigate the phenotype of the suppressor T cells. It was suggested that $T8^+$ T cells were involved in the induction of the suppressive effect. However, it was not possible to determine whether or not $T4^+$ T cells are required to generate the suppressive effect because the separated $T4^+$ T cell population was contaminated with $T8^+$ T cells.

It has been reported that monocytes exert a non-specific suppression on Ig production through activating short lived suppressor T cells by secreting of prostaglandin E_2 (Fischer, Durandy & Griscelli, 1981). We examined the effect of indomethacin on a culture treated with the GG preparations, but indomethacin did not affect suppressor T cell activity (Table 2). The Fc receptor of T cells and B cells is sensitive to pronase, but monocytes are resistant (Colombatti *et al.*, 1981). Our pronase treatment of T cells reduced suppressor activity to the same levels as fresh T cells. Taken together, these observations suggest that monocytes and prostaglandin E_2 were not involved in the induction of suppressor T cells in our system.

Among the GG preparations, ISG had a more severe suppressive effect on Ig production. This finding cannot be explained by the different concentrations of aggregates in the GG preparations. It has been reported that ISG contains 30% of aggregates, PEG treatment, 4% and S-sulfonation, 15% (Romer *et al.*, 1982). But the different amounts of aggregates in these preparations did not correspond to differences in suppressive effect. PEG treatment and S-sulfonation treatment in a 62°C water bath for 20 min increased the anticomplementary activity significantly, whereas the suppressive effect of each preparation did not change after the treatment.

It has been reported that aggregated IgG or insoluble immune complexes suppress both mitogen-induced proliferation and Ig production of PBMC (Moretta *et al.*, 1977; Samarut, Cordier & Revillard, 1979). In our results, the GG preparations did not suppress proliferation of PBMC stimulated with PHA, Con A or PWM. Thus our present studies suggest that the mechanism of the suppression induced by the GG preparations on Ig production stimulated by PWM is different from that observed for aggregated IgG molecule and the suppressive effect seems to be dependent principally on the Fc of the intact monomelic IgG molecule.

We also showed that B cells pre-incubated with the GG preparations did not differentiate into immunoglobulin producing cells with the combination of fresh T cells under stimulation of PWM.

Table 2. Effect of indomethacin on the suppressive activity of T cells preincubated with ISG

	Suppression (%)	
Cell culture	without indomethacin	with indomethacin
PBL + 5×10^4 T cell	8·6 ± 2·6	10·0 ± 5·7
PBL + 1×10^5 T cell	41·5 ± 5·2	74·9 ± 4·7
PBL + 2×10^5 T cell	87·3 ± 3·6	72·7 ± 7·3
PBL + 5×10^5 T cell	89·5 ± 1·8	85·1 ± 2·2

PBL (5×10^5) were cultured with various numbers of autologous T cells which had been preincubated with 1 mg/ml ISG. The cultures were performed with or without supplementation of indomethacin (0·1 μg/ml). After 6 days, the culture supernatants were harvested and assayed on IgG production. Values represent mean ± s.d. of triplicate cultures.

Epstein–Barr virus-induced Ig production, which is T cell-independent, was also suppressed without affecting proliferation by the GG preparations (data not shown). To determine the stage at which the GG preparations exerted their effects in the culture, the GG preparations were added to the culture at various times after the start of the culture. The suppressive effect was observed up to day 4 of culture (data not shown). The preparations seemed to have a direct affect on the differentiation stage of B cells by binding to the Fc receptor.

In the present study we observed suppression of polyclonal Ig production in an *in vitro* culture system by the GG preparations used. Further experiments are needed to define whether high dose therapy with the GG preparations affects antibody production *in vivo*.

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