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Varying Effects of Intravenous Immunoglobulin on Mononuclear Cell Proliferation In Vitro

Intravenous immunoglobulin (IVIG) is being increasingly used to treat numerous immune-mediated diseases. However, there is a paucity of knowledge on the specific mode of action of IVIG in vivo. In this study, the in vitro effects of IVIG on peripheral blood mononuclear cell (PBMC) proliferation using phytohemagglutinin (PHA), anti-CD3 monoclonal antibody (MAb), phorbol myristate acetate (PMA), or purified protein derivatives (PPD) have been analyzed. The PBMCs were obtained from more than 10 individual donors. In all cases, IVIG almost completely inhibited PBMC proliferation at concentration above 20 mg/mL except when used in conjunction with PMA. PHA-induced proliferation of PBMCs at concentrations ranging from 1 to 15 mg/mL did not show significant differences. Anti-CD3 MAb-induced proliferation showed dose-dependent inhibition at concentrations ranging from 1 to 10 mg/mL. Interestingly, PMA-induced proliferation of PBMCs showed a dose-dependent increase at the same concentration range. PPD-induced proliferation of PBMC at concentrations ranging from 1 to 10 mg/mL did not show any statistically significant differences. These results suggest that high dose IVIG may be necessary to immune modulation in vivo and IVIG has various effects on PBMCs proliferation in limited concentration in vitro.

Key Words : *Immunoglobulins, Intravenous; Proliferating Cell ; Nuclear Antigen*

Kyung-Yil Lee, Dae-Kyun Koh, Joon-Sung Lee, Kyung-Tae Whang

Department of Pediatrics, College of Medicine, The Catholic University of Korea, Seoul, Korea

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Address for correspondence
Dae-Kyun Koh, M.D.
Department of Pediatrics, St. Vincent's Hospital
The Catholic University of Korea, 93 Chi-dong, Paldal-gu, Suwon 442-060, Korea
Tel : +82.31-249-7320, Fax : +82.31-257-9111
E-mail : kdk7016@hanmail.net

INTRODUCTION

Intravenous immunoglobulin (IVIG) is an established replacement therapy for primary immunodeficiency (1). However, the mechanism of immunoregulatory action is poorly understood, even though it is used as an immunomodulatory treatment for many immune-mediated diseases including Kawasaki disease, and immune thrombocytopenic purpura (ITP) (2, 3). Several studies have presented evidence for IVIG as a modulater of cell mediated immune responses both in vivo and in vitro. IVIG prolongs the Fc receptor-mediated clearance of IgG coated cells and decreases antibody-dependent cytotoxicity (3-5). It also solubilizes formed tissue immune complexes and inhibits autoantibody production (6, 7). Furthermore it inhibits the production of certain cytokines (8, 9), decreases T-cell dependent polyclonal immunoglobulin production by B cells (10-12) and neutralizes pathogenic antibodies or toxins (13, 14).

Research into the effects of IVIG have shown that in a dose-dependent fashion, it can inhibit lymphocyte proliferation in vitro to phytohemagglutinin (PHA) (15-17), concanavalin A, pokeweed mitogen, and *Staphylococcus aureus* protein A (17), phorbol myristate acetate (PMA) with ionomycin (18), anti-CD3 monoclonal antibody (MAb) (9, 18) and in the mixed lymphocyte reaction (16, 17). However, low concentrations of IVIG which are enough to suppress the production of immunoglobulins by B cells do not effect T cell proliferation (19).

In this study, the in vitro effects of IVIG on PBMC activation and proliferation using different T-cell stimulants have been analyzed. PBMCs were activated in an antigen nonspecific cell surface receptor-mediated manner using PHA or anti-CD3 MAb, and in a direct activation manner that bypasses the cell surface receptor using PMA, and in an antigen-specific manner using purified protein derivatives of *Mycobacterium* (PPD). The main focus of this investigation was the effects of IVIG concentration on cellular proliferation in vitro. The data provides evidence that in limited IVIG concentrations in vitro, there may be various mechanisms responsible for modulating PBMCs.

MATERIALS AND METHODS

Cell preparation

Human PBMCs were isolated from healthy donors who tested positive for Mantoux by Ficoll-Hypaque gradient centrifugation (Phamacia Biotech, Uppsala, Sweden). The cells were used immediately after isolation. Freeze-dried

IVIG for clinical use, derived from a large pool of donor plasma (>10,000), was obtained from the Green Cross Co. (IV globulin, Korea). The final concentrations of PHA (Sigma, St. Louis, U.S.A.), anti-CD3 MAb (Sigma, St. Louis, U.S.A.), PMA (Sigma, St. Louis, U.S.A.) and PPD (lysate of H37Rv strain, gift from Dr. Paik, Dept. of Microbiology, Chungnam National University, Korea) used were 6.25 μg/mL, 50 ng/mL, 100 ng/mL, and 2.5 μg/mL, respectively. The IVIG for clinical use was dissolved in culture medium, and human albumin (20%, Green Cross Co. Korea) and glucose (50%, JoongWae Co, Korea) were used as controls.

Cell cultures

PBMCs were incubated in 96-well flat bottom tissue culture plates at 1×10^5 cells and 200 μL per well. The culture medium consisted of RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), 2-mercaptoethanol (5 mM), HEPES buffer (15 mM), sodium bicarbonate (1 mM), and fetal bovine serum (10%). The following mitogens added to the plates: PHA (6.5 μg/mL), anti-CD3 MAb (50 ng/mL), PMA (100 ng/mL), or PPD (2.5 μg/mL) with graded concentrations of IVIG (0-50 mg/mL). The final volume of each well was adjusted to 250 μL with the culture media. Culture plates were kept in a humidified, 37℃ incubator with 5% CO_2 for 4 days. After 3 days, 1 μCi ^{3}H-thymidine was added and the cells were harvested 20 hr later. ^{3}H-thymidine incorporation was measured with a Packard beta-counter (Matrix 9600, Meriden, CT, U.S.A.). Experimental counts (cpm) were calculated by subtracting the average back ground from the mean of duplicate experimental values. Cell viability, assessed by trypan blue staining, was unaffected by IVIG supplementation in any of the cultures.

Statistical methods

The data was presented using the mean and standard deviations. The ratio of proliferation was calculated within individual experiments. The ratio from the control value (without IVIG) was set to 1.0 and compared to the experimental results. The differences were computed for their statistical significance using the t-test for paired data within the range of IVIG concentrations 0 mg/mL to 30 mg/mL. A $p<0.05$ was considered statistically significant (SPSS version 8.0).

RESULTS

From around 10 individual donors, nearly complete inhibition of various stimuli-induced PBMC proliferation except PMA was observed at IVIG concentration above 20 mg/mL. At a final IVIG concentration of 30 mg/mL or more almost 100% inhibition was observed in all experiments. In these experiments, the background cpm (without stimulants) was <30. In each experiment, the mean cpm of the graded IVIG concentrations was calculated with the mean cpm without IVIG (0 mg/mL) set at 1.0. The proliferation ratio is the ratio between the mean cpm (0 mg/mL IVIG) and the graded IVIG concentrations. In control study, various concentrations ranging 1-30 mg/mL of albumin did not show any inhibitions irrespective of stimulants, however, glucose showed dose-dependent inhibition at the same range of concentration in all stimulants (Fig. 1) Glucose concentration above 20 mg/dL showed almost complete inhibition.

PHA-induced proliferation of PBMC at concentrations ranging from 1 to 15 mg/mL did not show any statistically significant differences. The mean cpm without IVIG (0 mg/

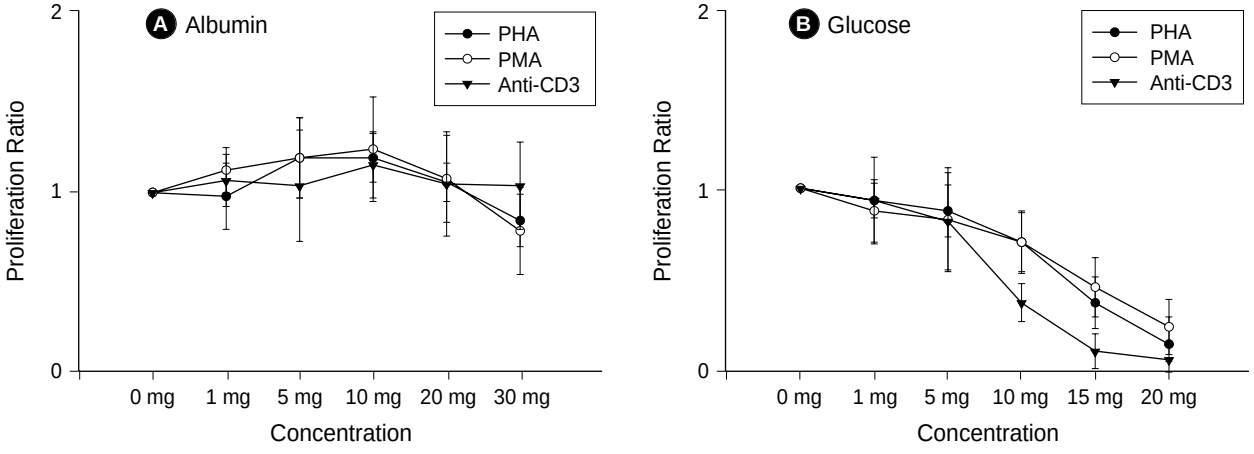

Fig. 1. Influences of albumin and glucose as controls on peripheral mononuclear cell proliferation induced by various stimulants. Albumin (n=6) did not affect on cell proliferation, but glucose (n=6) inhibited cell proliferation in dose-dependent fashion. Proliferation ratio means the ratio between cpm of 0 mg/mL of albumin or glucose and that of graded concentrations of albumin or glucose (ranging from 1 mg/mL to 20-30 mg/mL).

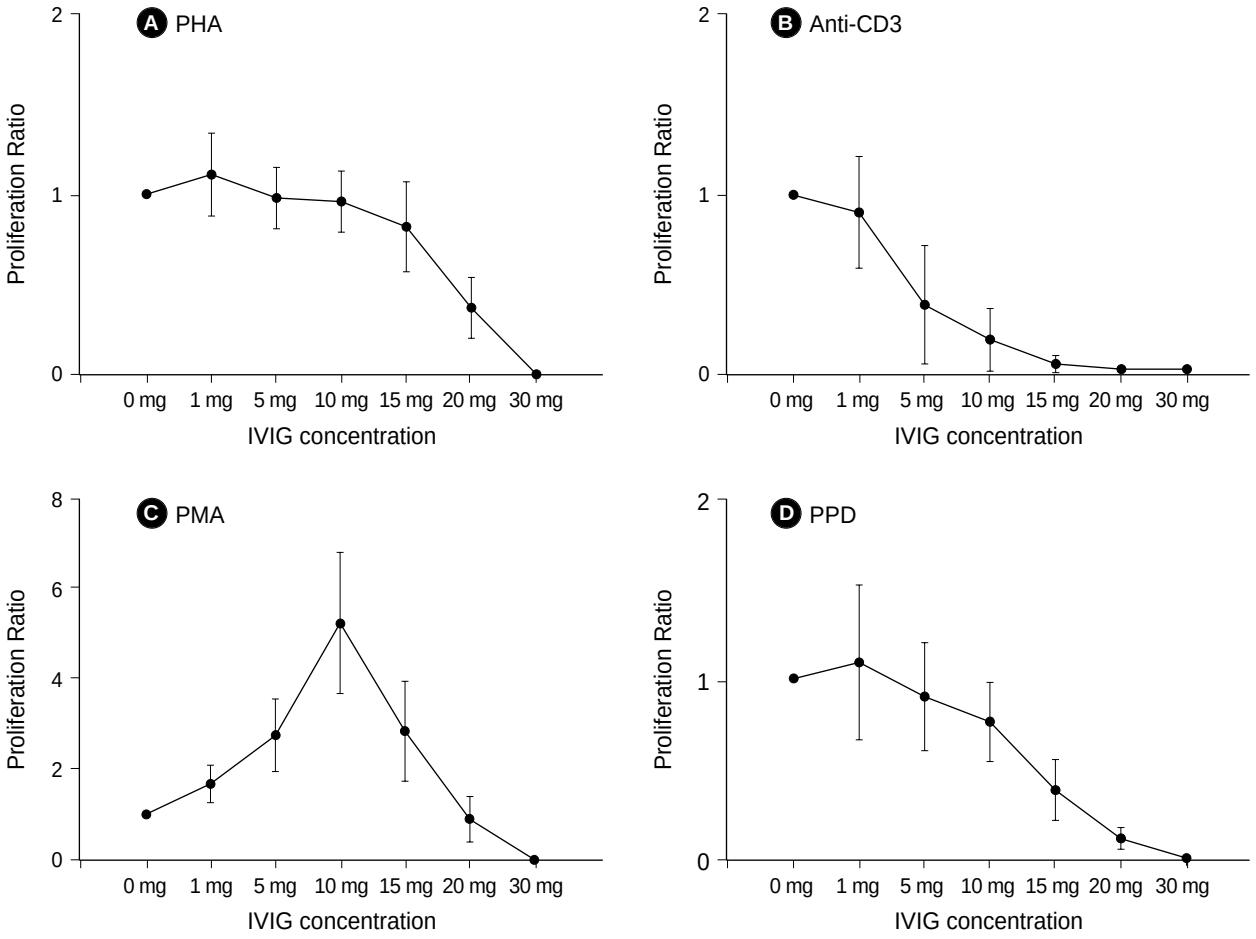

Fig. 2. Effects of IVIG on peripheral blood mononuclear cell proliferation induced by various stimulants. Nearly complete inhibition except PMA by IVIG at concentration above 20 mg/mL was observed. At a final concentration of 30 mg/mL or more of IVIG, almost 100% inhibition was observed in all experiments. (A) PHA (6.25 μg/mL)-stimulated proliferations (n=12) of ranging from 1 mg/mL to 15 mg/mLof IVIG were not statistically significant. Mean cpm without IVIG (0 mg/mL) was 7,142±2,713 and set 1.0. (B) In anti-CD3 monoclonal antibody (50 ng/mL) stimulaton (n=10), proliferation ratios of ranging from 1 mg/mL to 10 mg/mL of IVIG show significant dose dependent decrease (mean cpm without IVIG was 3,261±1.664). (C) Proliferation ratios of PMA (10 ng/mL, n=10) show significant dose-dependent increase of ranging from 1 mg/mL to 10 mg/mL (mean cpm without IVIG, 1,351±422). (D) PPD-stimulated proliferations (2.5 μg/mL, n=8) show no significant inhibition between from 1 mg/mL to 10 mg/mL of IVIG concentration (mean cpm without IVIG, 272±219). In all experiments, background cpm (without stimulant) was below 30.
Abbreviations; n, numbers of materials; PHA, phytohemagglutinin; anti-CD3, anti-CD3 monoclonal antibody; PMA, phobol myristate acetate; PPD, purified protein derivatives.

mL) was 7,142±2,723 (Fig. 2A). Anti-CD3 MAb-induced proliferation at concentrations ranging from 1 to 10 mg/mL showed dose-dependent inhibition (Fig. 2B). Anti-CD3 MAb induced proliferation was inhibited quite efficiently by IVIG, and at 5 mg/mL the proliferation ratio was 0.34. However, interestingly PMA-induced the proliferation of PBMC at concentrations ranging from 1 to 10 mg/mL showed dose-dependent increase. PMA induced the active proliferation of PMNCs, and the proliferation ratios at concentrations of 5, 10, 15 and 20 mg/mL were 2.7±1.0, 5.2±1.9, 2.8±1.3, and 0.9±0.6, respectively (Fig. 2C). PPD-induced proliferation of PBMC showed a dose-dependent pattern, however at concentrations ranging from 1 to 10 mg/mL did not show any statistical differences (Fig. 2D).

DISCUSSION

In this investigation, it was found that in all stimulants, IVIG (>20 mg/mL) have an inhibitory effect on PBMC proliferation, as measured by ^{3}H-thymidine. High-dose induced inhibition was observed regardless of how the cell was activated, i.e., by antigen specific stimulation (PPD) for sensitized T cell, via T-cell receptor (anti-CD3 MAb), via antigen-nonspecific lectin stimulation (PHA), or direct stimulation without receptors (PMA).

However, at IVIG concentrations ranging from 1 mg/mL to 10 or 15 mg/mL, PHA- and PPD-induced proliferation showed no significant differences (Fig. 2A, D). Anti-CD3 MAb-induced proliferation was inhibited in a dose-dependent manner at concentrations between 1 mg/mL and 10 mg/mL (Fig. 2B). However, PMA-induced proliferation showed dose-dependent increases at the same range. These results suggest that IVIG has varying effects on cell activation and proliferation in vitro and modulates cell proliferation in limited concentrations in vitro.

Other studies have shown that high IVIG concentrations inhibit lymphocyte proliferation in the presence of various stimulants irrespective of the cell types including several malignant cell lines (16), and of the IVIG preparations (whole IgG, $F(ab')_2$ fragment (17, 20), Fc fragments (15, 20), or repurified IVIG (17), IgG from single or five donors (17)).

IVIG have shown diverse immunoregulatory actions. However, the inhibitory mechanism of cell proliferation is at present unknown. High-dose IVIG seems not to induce cell death (15-20), but arrests the cell cycle in G0/G1 stage (16). It is suggested that IVIG interacts with a number of surface antigens of T cells including the T-cell receptor (16, 17), the Fc receptor (15) and modulates IL-2 (18, 20). Recently it is reported that high dose IVIG induced apoptosis in vitro in leukemic cell lines and in CD40-activated normal tonsillar B cells via Fas apoptotic pathway (21). However, the role of anti-Fas antibodies in IVIG on apoptosis is controversial (22).

It is suggested that materials containing IVIG as stabilizers (glucose, albumin, sucrose, maltose, etc), different culture conditions or IVIG preparations used in the experiments may lead to varying results (17, 23). The preparation used in this study contains glucose (5%) and albumin (20%) as stabilizer. We used commercially available human serum albumin (HAS, 20%) and glucose (50%) as controls. HSA and glucose, at concentrations corresponding to the dose present in IVIG preparation (0.2-4.0 mg/mL and 0.08-1.0 mg/mL, respectively) and high-dose HAS (5-30 mg/mL), had no significant effect on PBMC proliferation that was induced by any of the stimulants. On the other hand, high-dose glucose inhibits PBMC proliferation in dose-dependent fashion (Fig. 1).

Therefore one possible explanation can be proposed that high doses of IVIG may affect the experimental culture environment regardless of any type of stimuli like a glucose in control study, however, varying effects of IVIG in limited concentrations can rule out this possibility.

Although several studies have shown that low IVIG doses (below 10 mg/mL) can inhibit lymphocyte proliferation, inhibition of proliferation induced by various stimuli at higher IVIG concentration in vitro may be inferred that immune modulation of IVIG in vivo requires a high concentration.

It has been suggested that IVIG concentrations in vitro ranging from 20 mg/mL to 40 mg/mL may correspond to serum levels observed when IVIG is given in clinical doses (15, 16, 21). In our clinical study of twelve children with Kawasaki disease, it was found that after administration of IVIG of 2 g/kg for 12 hr, the serum IgG levels showed a concentration of $3,091 \pm 270$ mg/dL (30.9 mg/mL) after 2 hr, $2,723 \pm 259$ mg/dL after 24 hr, $2,470 \pm 311$ mg/dL after 7 days, and 908 ± 218 mg/dL before IVIG infusion, respectively. This finding show that a high IVIG dose can maintain the serum level of IgG above 20 mg/mL for longer than 7 days. Thus, although the inhibitory effects of IVIG in vitro requires supraphysiologic levels (>20 mg/mL) of IgG, this range of level may be easily reached in vivo by using standard treatment schemes with a IVIG of high dose for autoimmune disease. If the inhibition of proliferation to various stimulants inferred from immune modulatory effect of IVIG, IVIG concentrations above 20 mg/mL are necessary to work and maintain an immune modulation in vivo. In Kawasaki disease or ITP, a high single dose of IVIG (2.0 g/kg) or divided low doses of IVIG (500 mg/kg/day for 4 days) did not have any apparent therapeutic results (24). This finding suggests that a higher serum concentration of IVIG is required in order to obtain the immunoregulatory effects regardless of therapeutic schemes. Also we found that high-dose IVIG treatment in patients with Kawasaki disease induced the changes of the various protein and lipid profiles including albumin and apolipoprotein A-I within two hours after IVIG infusion. These findings make us to propose a hypothesis that immunoregulatory action of IVIG results from the changes on systemic protein metabolism or the acceleration of protein catabolism (unpublished observation). The results of our in vitro study can partly explain the hypothesis derived from our in vivo observation. If IVIG modulate proteins in vitro system in dose-dependent fashion, IVIG inhibit the cell proliferation induced by stimulants which are derived from proteins (anti-CD3, PPD, and PHA) more effectively than by phobol ester (PMA). In case of PHA, mitogenic activity of PHA may have a wide range of its concentration to induce peak proliferation. Furthermore, high concentrations of IVIG may affect the proteins which are essential to cell proliferation including cytokines synthesized from the cells with stimulants.

Our data showed that IVIG in a limited concentration (1-15 mg/mL, maybe within physiologic level) has varying effects on PBMCs in conjunction with other stimuli. Although most of studies have reported that IVIG inhibits cell proliferation in a dose-dependent fashion, some similar findings to our results could be found; repurified IVIG via DEAE-Sephadex adsorption did not inhibit PBMC proliferation to PHA at a concentration of 15 mg/mL (23), the proliferative response to PMA/ionomycin was not inhibited over concentrations ranging from 1 to 10 mg/mL (9).

To what extent the various effects that IVIG has on cell proliferation contribute to the clinically beneficial effect that

is observed in many autoimmune diseases, is not known. However, it is possible that several immunoregulatory effects of IVIG on immune cells affect patients in different immunologic disease states.

In conclusion, our results suggest that IVIG in higher concentrations inhibits and IVIG in limited concentrations modulates the response of PBMCs to various stimuli in vitro. Further investigation is needed in order to clarify whether these mechanisms also work in vivo.

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