

Gamma-globulin Inhibits Superantigen-induced Lymphocyte Proliferation and Cytokine Production

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ABSTRACT

Background: High-dose pooled human immunoglobulin (PHIG) treatment is sometimes effective in superantigen related inflammatory diseases, such as toxic shock syndrome and Kawasaki disease. Neutralizing antibody to superantigen might provide protection, but antigen independent immune regulation of PHIG is also a proposed mechanism.

Methods: Staphylococcal enterotoxin B (SEB)-specific IgG antibody in PHIG products (Venoglobulin IH[®]) was detected by ELISA. The suppressive effect of PHIG or its fragments on proliferation and cytokine (IL-4 and IFN- γ) production from SEB-stimulated peripheral blood mononuclear cells was examined.

Results: SEB-specific IgG was detected in PHIG products. PHIG (6.25–25 mg/ml) suppressed SEB-induced proliferation and cytokine production in a dose-dependent manner. Fab and F (ab')₂ fractions of PHIG also suppressed the responses, but depletion of SEB-specific antibody from PHIG did not affect the inhibitory effects. The Fc fragment of PHIG also showed partial, but significant suppression.

Conclusions: These data suggested the possibility that PHIG suppressed SEB-induced proliferation and cytokine production by some mechanisms independent of the presence of neutralizing antibody.

KEY WORDS

neutralizing antibody, pooled human immunoglobulin, Staphylococcal enterotoxin B, superantigen

INTRODUCTION

Superantigens are exotoxins released from several kinds of bacteria or viruses. They stimulate as much as 10–20% of T lymphocytes bearing specific V β regions of the T-cell receptor and contribute to many kinds of inflammatory diseases, including toxic shock syndromes,¹ enterocolitis, neonatal toxic shock syndrome-like exanthematous disease (NTED),² and possibly Kawasaki disease.³ Exposure to superantigens produced by *Staphylococcus aureus* on skin lesions affects the severity of atopic dermatitis.⁴

Corticosteroid is widely used in the treatment of inflammatory diseases. However, corticosteroid does not suppress superantigen-induced lymphocyte proliferative responses.⁵ Resistance to superantigen-induced T cell responses may be due to inactivation of glucocorticoid receptors or modification of the T cell receptor signaling pathway, which leads to gluco-

corticosteroid receptor α phosphorylation and inhibition of the nuclear translocation of the receptor.^{6,7}

Calcineurin inhibitors such as FK506 effectively inhibit superantigen-induced responses. However, treatment of the superantigen-mediated diseases with these inhibitors is still challenging.⁸

High-dose pooled human immunoglobulin (PHIG) therapy was of therapeutic use in some of the superantigen related diseases. Specific antibodies to superantigens can be detected in the sera from healthy donors or PHIG products.¹ Toxic shock syndrome toxin (TSST-1) specific IgG antibody in cord blood appears to protect babies from NTED,⁹ suggesting that neutralizing antibodies against superantigen can be protective.¹⁰ On the other hand, several reports have suggested the mechanisms of PHIG therapy to be free from the presence of neutralizing antibody.¹¹

In this paper, we investigated the suppressive effects of PHIG on the SEB-induced lymphocyte activ-

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ity and aimed to reveal antigen-specific and non-specific mechanisms of the suppressive effect.¹

METHODS

MEASUREMENT OF SEB-SPECIFIC IGG ANTIBODY

SEB-specific IgG antibody was measured in PHIG (Venoglobulin IH[®], Mitsubishi Pharma Corporation, Osaka, Japan) using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated for 24 hours with 5 µg/ml (0.5 µg per well) of SEB dissolved in coating buffer (BD Pharmingen, San Diego, CA, USA). After blocking nonspecific reactivity with 2% human albumin (Mitsubishi Pharma), plates were incubated with 100 µl of PHIG (50–0.0008 mg/ml) for 2 hours. Alkaline phosphatase (ALP)-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was incubated for 1 hour and developed with P-NPP (KPL, Inc. Gaithersburg, MD, USA). Bound IgG was detected by absorbance at 450 nm using a plate reader (BIO-RAD, Tokyo, Japan).

CELL CULTURE

Peripheral blood mononuclear cells (PBMC) from healthy adult donors were isolated by Ficoll-Hypaque (Pharmacia Co. Uppsala, Sweden) gradient centrifugation and suspended in RPMI 1640 (Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal bovine serum (Life Technologies Limited, Auckland, New Zealand), 100 U/ml of penicillin G sodium (Sigma Chemical Co.) and 100 U/ml of streptomycin (Sigma Chemical Co.). Cells (1×10^5 per well) were plated in 96-well round-bottom culture plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and stimulated with 100 ng/ml of SEB (Toxin Technology, Sarasota, FL, USA) or 10 µg/ml of concanavalin A (ConA) (Sigma Chemical Co.).

Dexamethasone (10^{-10} to 10^{-6} mol/l, Sigma Chemical Co.), PHIG (6.25–25 mg/ml) or fractions of PHIG were added at the beginning of the culture. Mixtures of PHIG and human albumin at different ratios were applied to the culture to avoid the effect of dilution of the culture medium.

Cultures were incubated for 72 hours at 37°C in a humidified, 5% carbon dioxide-containing incubator. To detect proliferative responses, [³H]-thymidine (0.25 µCi/well, Amersham Pharmacia, Buckinghamshire, UK) was added for the final 16 hours, and radioactivity was determined by liquid scintillation counting (Beckman Coulter, Inc., Fullerton, CA, USA).

CYTOKINE ELISA

Cytokine levels in the culture supernatant were determined by sandwich ELISA, using rat monoclonal antibodies specific for human IFN-γ or human IL-4 (BD OptEIA ELISA Set, BD Pharmingen) according to the

manufacturer's protocol. Samples were diluted $\times 100$ for assay of IFN-γ and the sensitivity was 300 pg/ml. They were undiluted for assay of IL-4, and the sensitivity was 7.8 pg/ml.

DEPLETION OF SEB-SPECIFIC NEUTRALIZING ANTIBODY FROM PHIG

To obtain PHIG lacking SEB-specific antibody, PHIG was dialyzed in PBS and applied to an SEB-conjugated affinity column. The affinity column contained SEB coupled to sodium cyanobromide-activated formyl-cellulofine beads.

The flow-through fractions were pooled and concentrated to a protein concentration of 50 mg/ml in PBS, and designated "anti-SEB-depleted PHIG." Preparation of mock-treated PHIG followed the same procedure but substituted a column containing the same beads without SEB. Depletion of SEB-specific antibody was examined using ELISA. Depletion of SEB-specific antibody was, however, incomplete. Compared with the native PHIG, 8-fold less SEB-specific antibody was detected in the anti-SEB-depleted PHIG.

PREPARATION OF PHIG FRAGMENTS

For further experiments, three fragments of IgG (Fc, Fab, and F (ab')₂) were prepared. Fab fragment is a heterodimer of the variable (V) domain and part of the constant (C) domain from heavy (V_H, C_H) and light (V_L, C_L) chains of immunoglobulin. Fc fragment is a homodimer of the C_γ1 and C_γ2 domains of IgG heavy chain. Fab and Fc fragments of PHIG were obtained by papain digestion. The digested fragments were applied to a protein G column. Fab fragments were obtained in the flow-through fractions, and Fc fragments were eluted from the column.

F (ab')₂ fragment is a covalently conjugated pair of Fab fragments. To obtain F (ab')₂ fragments, PHIG was digested by pepsin and applied to a protein A column. The low-molecular weight contaminants were removed by filtration (50,000 Da cutoff). The concentrations of each fraction were adjusted to obtain molar concentrations identical to those in the original PHIG.

The specificity and purity of the Fab, Fc, and F (ab')₂ fragments were confirmed using SDS-polyacrylamide gel electrophoresis in reducing and non-reducing conditions, cellulose acetate electrophoresis, and gel-filtration analysis. Commercially available Fab, Fc, and F (ab')₂ fragments (Capell Laboratories, West Chester, PA, USA) were used as controls.

RESULTS

SEB-SPECIFIC IGG ANTIBODY IN PHIG

SEB-specific IgG antibody was detected by ELISA in PHIG (Fig. 1). Specificity of the ELISA was examined by pre-incubating PHIG (50 µg/ml) with serial con-

centrations of SEB (1 ng/ml–10 µg/ml). Dose-dependent inhibition was observed, and the ELISA titer was completely abolished (reduced to the background level) by adding 10 µg/ml of SEB (Fig. 2).

PHIG SUPPRESSED SEB-INDUCED PROLIFERATION AND CYTOKINE PRODUCTION

SEB (100 ng/ml) and ConA (10 µg/ml) induced similar levels of proliferative responses to PBMC from healthy donors. But SEB induced significantly more

IFN-γ and less IL-4 compared with ConA stimulation (Fig. 3). Dexamethasone did not suppress SEB-induced proliferative responses (data not shown).

PHIG significantly and dose-dependently suppressed SEB-induced proliferation, IFN-γ and IL-4 production. Almost complete suppression of IL-4 was obtained at the lower concentration (6.25 mg/ml) of PHIG, but proliferation and IFN-γ was also sup-

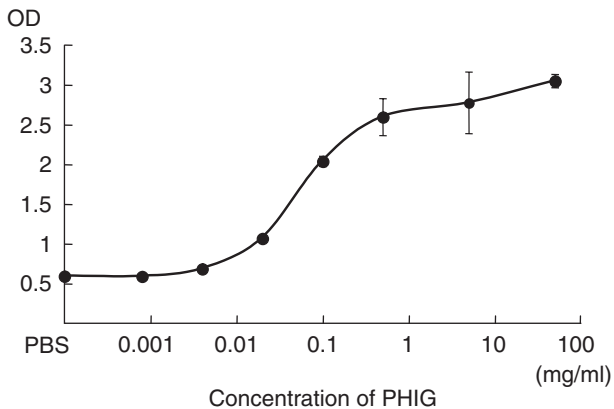


Fig. 1 Detection of SEB-specific IgG antibody in PHIG. SEB-specific IgG antibody was detected by ELISA. Although relatively high background using PBS was seen, serial dilution (50–0.0008 mg/ml) of PHIG resulted in concomitant decrease of OD titer, suggesting the specificity of the ELISA.

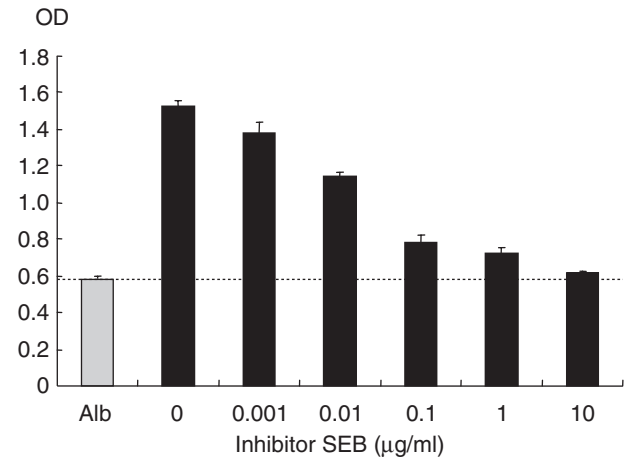


Fig. 2 Specificity of the ELISA was confirmed using an inhibition ELISA. PHIG (50 µg/ml) was pre-incubated with serial concentrations of SEB before ELISA detection. Dose-dependent inhibition was observed, and SEB (10 µg/ml) completely blocked the detection.

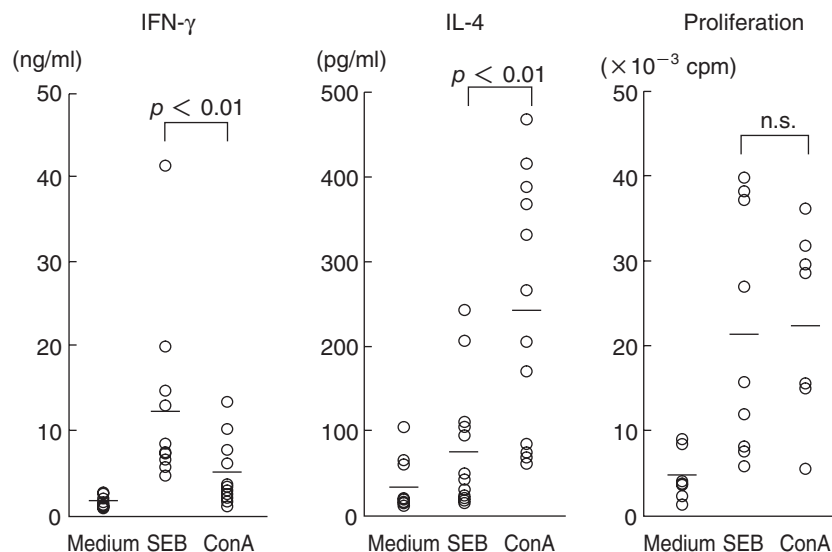


Fig. 3 Proliferation and cytokine production of PBMC stimulated with SEB and ConA. PBMC (1×10^5) from healthy adult donors were stimulated with SEB (100 ng/ml) or ConA (10 µg/ml) for 3 days. Either stimulant induced identical proliferative responses, but SEB induced IFN-γ dominant cytokine synthesis. Statistical difference was calculated by the Mann-Whitney *U* test.

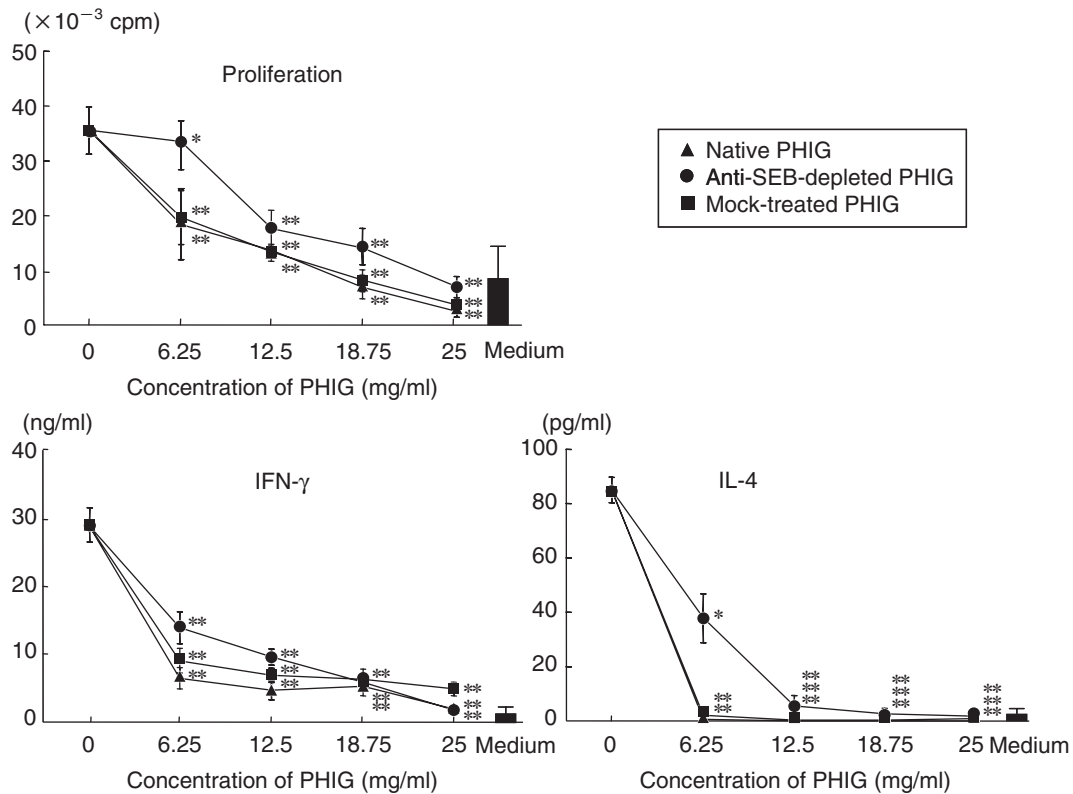


Fig. 4 Suppression of SEB-induced proliferation and cytokine production by PHIG. PHIG suppressed SEB-induced proliferation and cytokine production in a dose-dependent manner (triangle). Anti-SEB-depleted PHIG (circles) also showed significant suppressive effects. The suppression was not statistically different from that of mock-treated PHIG (squares) at any concentrations. * $p < 0.05$, ** $p < 0.01$ compared with the responses without PHIG by paired t -test. Data are expressed as mean $\pm$ SD of 3 independent triplicate cultures.

pressed completely at the highest concentration (25 mg/ml) (Fig. 4, native PHIG). However, PHIG did not suppress the ConA-induced responses at all (data not shown), suggesting the suppression was not due to a toxic effect of PHIG.

EFFECT OF REMOVING SEB-SPECIFIC ANTI-BODY FROM PHIG

The role of neutralizing antibody in the inhibition of SEB-induced responses was examined using anti-SEB-depleted PHIG. As is shown in Figure 4, anti-SEB-depleted PHIG also showed a suppressive effect as well as the native PHIG. In the lower concentration (6.25 mg/ml), the suppression seemed to be less than that of mock-treated PHIG, but the difference was not statistically significant.

EFFECTS OF PHIG FRAGMENTS ON THE SEB-INDUCED RESPONSES

Fc, Fab, and F (ab')₂ fragments of PHIG were prepared to reveal the mechanism of PHIG's inhibitory effect on SEB-induced responses (Fig. 5). Both Fab and F (ab')₂ fragments also inhibited proliferation

and cytokine production at the concentrations comparable to those of native PHIG. Similar to the native PHIG, complete suppression of IL-4 was observed at low concentrations of Fab and F (ab')₂ fragments, but IFN- γ was also suppressed completely at the highest concentration.

Fc fragment also suppressed SEB-induced proliferation and cytokine production in a dose-dependent manner. IL-4 production was significantly suppressed at as low as 2 mg/ml of Fc fragment (corresponding to 6.25 mg/ml of PHIG), but the suppression was not complete at the highest concentration. Significant suppression of IFN- γ production and proliferation was also observed at 6 mg/ml (corresponding to 18.75 mg/ml of PHIG) or higher concentrations of Fc fraction.

DISCUSSION

High-dose PHIG treatment has been reported to be effective in superantigen-related inflammatory diseases.¹ Several reports, including the present paper, have shown the presence of superantigen-specific antibody in PHIG preparations. Superantigen-specific

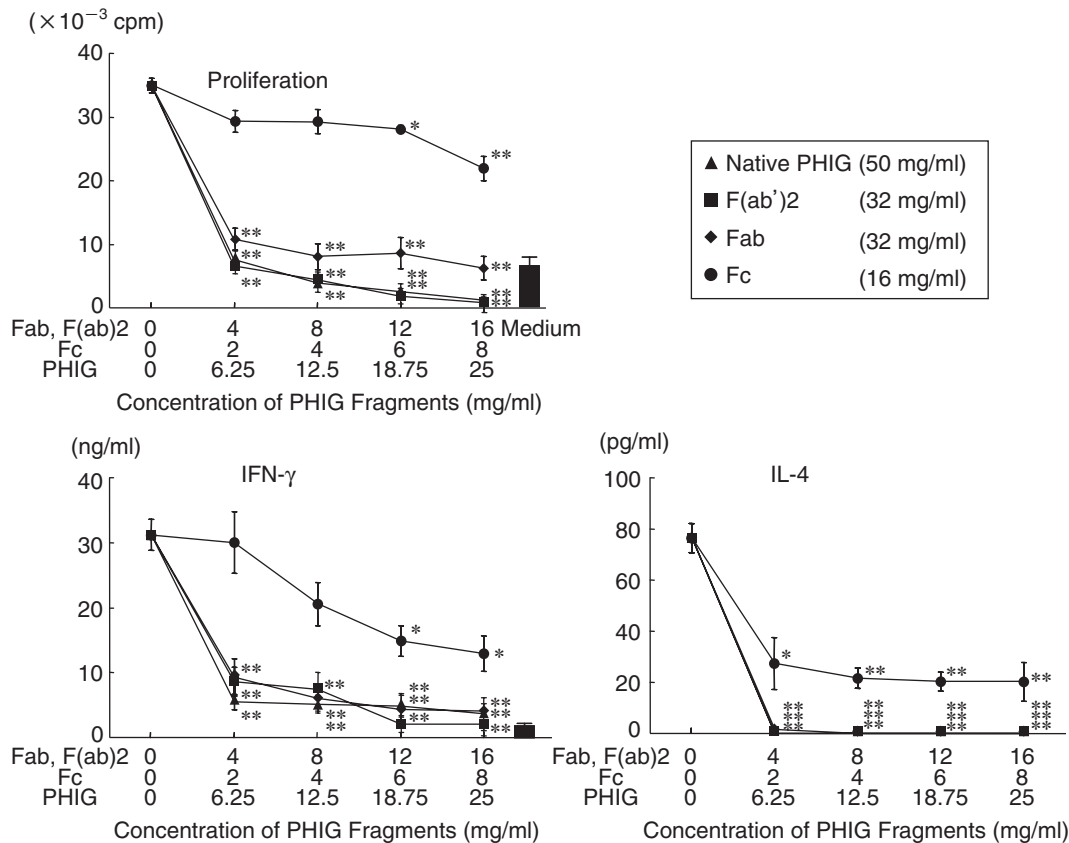


Fig. 5 Suppression of SEB-induced proliferation and cytokine production by PHIG fragments. Fab (diamonds) and F(ab')₂ (squares) fragments suppressed the responses as effectively as native PHIG (triangles). Fc fragment (circles) also partially and significantly suppressed IL-4, and less effectively, IFN-γ and proliferation. * $p < 0.05$, ** $p < 0.01$ compared with the responses without PHIG by paired t -test. Data are expressed as mean $\pm$ SD of 3 independent triplicate cultures.

neutralizing antibody may partially explain the mechanism of high-dose PHIG therapy.¹ The protective effect of maternal TSST-1 specific antibody in NTED has also been suggested.⁹

However, several reports have suggested that the effect of high-dose PHIG is not due to a neutralizing activity alone. Takata¹¹ reported that PHIG decreased superantigen-induced IL-12 and IFN-γ production, but the effect was independent of the neutralizing activity of IgG. Aukrust¹² reported that patients with primary hypogammaglobulinemia showed elevated levels of IL-1, and administration of PHIG down-regulated the IL-1 cytokine network *in vivo*. Campbell¹³ also reported that 6 mg/ml of PHIG inhibited proliferation and IL-4 secretion but not IFN-γ secretion of SEB-stimulated PBMC. The inhibition was unrelated to the presence of anti-SEB IgG, because PHIG depletion of SEB-IgG showed similar inhibition of proliferation and IL-4 secretion.

We have shown that PHIG suppressed proliferation and cytokine production of SEB-stimulated PBMC (Fig. 4). Consistent with Campbell's report¹³, IL-4 production was susceptible to the suppression at the

low concentration of PHIG (6.25 mg/ml), but higher concentrations (25 mg/ml) of PHIG almost completely suppressed IFN-γ production as well. The concentrations of PHIG used in the experiment were comparable to serum IgG levels in healthy adults (5–15 mg/ml) and levels after high-dose PHIG therapy (20–30 mg/ml).

Removal of SEB-specific antibody from PHIG resulted in a small, statistically insignificant decrease in the suppressive effect at the low concentration (6.25 mg/ml). At the higher concentrations of anti-SEB-depleted PHIG, level of suppression was similar to that of native PHIG. Although depletion of SEB-specific antibody was not complete (only 8-fold less activity), it was concluded that the role of neutralizing activity in the PHIG was limited.

For further analysis of the suppressive mechanisms, we have examined the effects of Fab and Fc fractions of IgG. Purified Fab and F(ab')₂ fragments of PHIG suppressed the responses as effectively as native PHIG, and complete suppression was obtained at the highest concentration. These findings might suggest that these fragments, containing SEB-

specific binding activity, directly blocked the binding of SEB to T cell receptors or MHC Class II molecules. But the role of SEB-specific antibody remains unknown, because these fragments contain variable regions of IgG antibodies to any other components, or the role of trace amounts of contaminating factors could not be ruled out.

Interestingly, high concentration of Fc fragment, which is completely free from neutralizing activity, partially but significantly suppressed IL-4 production and, less markedly, proliferation and IFN- γ production induced by SEB. Different susceptibility to the suppression might suggest the presence of a specific mechanism to regulate the response to superantigens. Suppression by Fc fragment was not thought to be due to direct inhibition of T cell function, because PHIG did not suppress ConA-induced proliferation.

In several recent reports, Fc γ RIIb was shown to induce signal inhibition to B cells or antigen presenting cells (APC).¹⁴ Generally, the induction involves cross-linking of Fc γ RIIb and other Fc γ Rs, as well as Fc ϵ RI¹⁵ via antigen-antibody complex. Our findings might suggest the possibility that binding of Fc components to Fc γ RIIb itself partially induce inhibitory signals to APC, apart from antigen-specific binding of the antibody. Further studies may be needed to reveal the mechanism of regulation of immune systems by immunoglobulin itself. Highly purified or recombinant immunoglobulin fragments might prove useful for treatment or prophylaxis of superantigen-induced diseases.

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