

Suppression of IL-4- and CD40-induced B-lymphocyte activation by intravenous immunoglobulin is not mediated through the inhibitory IgG receptor Fc γ RIIb

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Background: Intravenous immunoglobulin (IVIG) has been used extensively in the treatment of autoimmune and allergic diseases, but the precise mechanism behind its efficacy remains unclear. Ligation of the low-affinity IgG Fc receptor Fc γ RIIb can inhibit B-lymphocyte activation. Our laboratory has shown that IVIG suppresses proliferation and IgE production by human B cells stimulated with IL-4 and anti-CD40 antibodies. **Objective:** We sought to determine whether the regulatory action of IVIG is mediated through binding Fc γ RIIb, phosphorylation of the receptor, and induction of phosphatases, including SH2-containing inositol-5'-phosphatase.

Methods: All experiments were performed on human tonsillar B cells. Phenotyping was performed by means of flow cytometry. Cells were cultured with IL-4 and anti-CD40 antibodies with or without IVIG (10 mg/mL), and Fc γ RIIb receptor activation and phosphorylation were measured by means of Western blot analysis.

Results: Fc γ RIIb was the predominant isoform of Fc γ receptor expressed on tonsillar B cells, and preincubation with IVIG failed to block binding of Fc γ RIIb antibody. Anti-Fc γ RIIb antibodies did not reverse inhibition of B-cell proliferation or IgE production by IVIG. Treatment of stimulated B lymphocytes with IVIG for 1 to 60 minutes did not change the global protein tyrosine phosphorylation pattern, except for tyrosine phosphorylation of an unidentified 30-kd protein. We directly examined tyrosine phosphorylation of Fc γ RIIb and its downstream-associated phosphatase, SH2-containing inositol-5'-phosphatase. Both remained unchanged after IVIG treatment, as did other related phosphatases.

Conclusion: These data argue against the involvement of Fc γ RIIb in the inhibition of CD40/IL-4-induced B-cell activation by IVIG. (*J Allergy Clin Immunol* 2002;110:480-3.)

Key words: Antibodies, B lymphocytes, Fc receptors, protein kinases-phosphatases, cellular proliferation, intravenous immunoglobulin, IgE

Abbreviations used

Fc γ R: Fc receptor γ
ITAM: Immune receptor tyrosine-based activation motif
ITIM: Immune receptor tyrosine-based inhibition motif
IVIG: Intravenous immunoglobulin
SHIP: SH2-containing inositol-5'-phosphatase

Intravenous immunoglobulin (IVIG) is an important and frequently used immunoregulatory agent, although its mechanism of action is unclear. Potentially, the Fc portion of IVIG might interact directly with Fc γ receptors. Fc γ RIIb is the primary isoform of Fc γ receptor expressed on human B lymphocytes.¹ It is a member of a family of inhibitory immune receptors, including PIR-B on B lymphocytes and Ly49, KIR, and CD94 on natural killer cells.² These receptors all contain cytoplasmic immune receptor tyrosine-based inhibition motifs (ITIMs).³ Coligation of Fc γ RIIb with immune receptor tyrosine-based activation motif (ITAM)-containing receptors, such as the B-cell receptor, induces phosphorylation of tyrosine residues within the ITIM, which provides a docking site for the cytoplasmic SH-2 domain-containing protein tyrosine phosphatase SHP-1^{4,5} and SHP-2,⁶ as well as the SH2-containing inositol-5'-phosphatase 1⁷ (SHIP-1) and SHIP-2.⁸ These phosphatases dephosphorylate the intracellular substrates required for B-cell activation. This provides a potential explanation for the immune regulatory effects of IVIG by interacting directly with Fc γ RIIb and phosphorylating phosphatases. Because ligation of Fc γ RIIb requires aggregates of IgG, such as antigen-bearing immune complexes, it is unclear whether monomeric IgG in IVIG can induce negative signals in B lymphocytes through Fc γ R. Since we have shown that IVIG inhibits IgE production *in vitro*, we investigated the contribution of Fc γ RIIb ligation by IVIG to the regulation of IgE synthesis by examining Fc γ RIIb staining and phosphorylation events. Our data suggest that the inhibition of CD40/IL-4-triggered B-cell activation by IVIG is not likely mediated through Fc γ RIIb.

METHODS

Antibodies and reagents

The following reagents were used: IVIG (Human Immune Globulin Intravenous, 50 mg/mL in 10% maltose, pH 4.2; Bayer Inc,

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Toronto, Ontario, Canada); recombinant human IL-4 (R&D Systems, Minneapolis, Minn); mouse anti-human CD40 antibody (α CD40) purified from culture supernatant harvested from the G28.5 hybridoma cell line (ATCC, Rockville, Md); mouse anti-human CD32 antibody C1KM5 (Caltag Laboratories, Burlingame, Calif); rabbit polyclonal anti-SHP2 and anti-SHIP antibodies (Upstate Biotechnology Inc, Lake Placid, NY); the horseradish peroxidase-conjugated antiphosphotyrosine antibody RC20H (Transduction Laboratories, Lexington, Ky); and horseradish peroxidase-conjugated goat anti-mouse IgG (H+L; Zymed Inc, San Francisco, Calif). Rabbit polyclonal anti-SHP1 antibody was a gift from Dr Katherine Siminovitch (University of Toronto, Ontario, Canada), and the mouse anti-human Fc γ RIIb antibodies KB61 and FUN-2 were gifts from Dr Karen Pulford (John Radcliffe Hospital, Oxford, United Kingdom) and Dr Yoshiro Nozawa (Fukushima Medical University, Fukushima, Japan), respectively.

B-lymphocyte isolation and culture

Human B lymphocytes were isolated from tonsils, as previously described.⁹ Cell proliferation was measured by means of tritiated thymidine incorporation. IgE was assayed by means of ELISA (Kallistad Total IgE microplate kit; Sanofi Diagnostics, Chaska, Minn), according to the manufacturer's instructions, by using supernatants from B lymphocytes (10^6 /mL) cultured with α CD40 and IL-4 for 14 days. Analysis of cell-surface receptor expression was performed according to standard protocols on a FACSCalibur flow cytometer and analyzed with CellQuest software (Becton Dickinson, La Jolla, Calif).

Cell lysis, immunoprecipitation, and immunoblotting

Tonsillar B cells were incubated at 37°C with α CD40/IL-4 with or without IVIG (10 mg/mL), as indicated. The reaction was stopped with ice-cold PBS containing 1 mmol/L sodium orthovanadate. After washing, the cells were incubated for 1 hour at 4°C in lysis buffer and then clarified by means of centrifugation at 14,000 rpm for 10 minutes. Extracts were precleared with 20 μ L of *Staphylococcus aureus* protein A or protein G sepharose beads (Amersham-Pharmacia Biotech, Buckinghamshire, United Kingdom) for 30 minutes at 4°C and then incubated with optimal amounts of antibody for 1 to 2 hours at 4°C. Immune complexes were collected with 20 μ L of protein A/G sepharose for an additional hour. Immunoprecipitates were washed 3 times in lysis buffer containing 0.5% Triton X-100, solubilized in boiling Laemmli sample buffer, subjected to 8% to 10% SDS-PAGE, and transferred to nitrocellulose. After blocking, membranes were incubated with appropriate antibodies and developed with Enhanced Chemiluminescent Reagent (ECL; Amersham-Pharmacia). For reprobing, membranes were stripped at 55°C for 30 minutes with stripping buffer containing 100 mmol/L β -mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris (pH 6.7) and reblotted with indicated antibodies.

RESULTS AND DISCUSSION

The majority ($85\% \pm 7.24\%$) of human tonsillar B cells expressed Fc γ RIIb, as determined by using the Fc γ RIIb-specific antibodies KB61¹⁰ and FUN-2¹¹ (Fig 1, B-E). There were 2 subpopulations of Fc γ RIIb⁺ cells, with $57.07\% \pm 5.83\%$ displaying brighter fluorescence (Fig 1, D). In contrast, C1KM5, an Fc γ RIIa-specific antibody,¹⁰ reacted minimally with tonsillar B cells (Fig 1, A). Preincubation with 10 mg/mL IVIG for 30 minutes before staining with KB61 or FUN-2 did not alter binding of anti-Fc γ RIIb antibodies to B cells (Fig 1, D and E).

Thus IVIG was unable to block Fc γ RIIb staining at the doses we used to inhibit IgE production.^{9,12} IVIG did not have a direct steric hindrance effect on Fc γ RIIb binding, and binding of the specific antibodies was stronger than the affinity that IVIG has for the Fc γ R.

Because IVIG diminishes B-cell proliferation and IgE production after addition of α CD40 and IL-4,⁹ we investigated whether this inhibition could be abrogated by antibodies against Fc γ RII. Human tonsillar B cells were preincubated with KB61 (10 μ g/mL) for 2 hours at 37°C before addition of IVIG (10 mg/mL) with or without anti-CD40 (1 μ g/mL) and IL-4 (400 U/mL). KB61 treatment alone did not inhibit B-cell proliferation, nor did it reverse the $63\% \pm 10\%$ inhibition of cell proliferation caused by IVIG. Similarly, suppression of CD40/IL-4-induced IgE production by IVIG (42 ± 16 vs 14 ± 6 ng/mL) was not blocked by pretreatment with KB61 (6 ± 6 ng/mL) or by antibodies against Fc γ RIIa ($n = 3$).

Regulation of inflammation by IVIG might be achieved by means of direct inhibition of B-lymphocyte proliferation by ligation of Fc γ R receptors on B lymphocytes, with subsequent activation of ITIMs and distal protein tyrosine or inositol phosphatases.^{13,14} Several models implicate the Fc segment of IVIG as the key effector of immune regulation. For example, using Fc γ RIIb knockout mice, Samuelsson et al¹⁵ demonstrated that the presence of Fc γ RIIb receptors on macrophages was essential for IVIG to rescue platelets in their model of immune thrombocytopenic purpura. They hypothesized that negative signaling through Fc γ RIIb could explain the action of IVIG. To determine whether inhibition by means of Fc γ RIIb is achieved through tyrosine phosphorylation of the receptor by IVIG, leading to association and activation of protein or inositol phosphatases, we evaluated protein phosphorylation events in tonsillar B cells after incubation with IVIG, α CD40/IL-4, or both. As shown in Fig 2, IVIG did not alter the pattern of protein tyrosine phosphorylation 2 to 60 minutes after anti-CD40/IL-4 treatment. IL-4 stimulation enhanced the tyrosine phosphorylation of a protein at approximately 100 to 110 kd, which was not modified by coincubation with IVIG (Fig 2). IVIG consistently induced tyrosine phosphorylation of an approximately 30-kd protein (Fig 2), which we have not yet characterized.

Neither IVIG nor α CD40/IL-4 treatment caused significant alteration in the basal level of Fc γ RIIb tyrosine phosphorylation after incubation times ranging from 1 minute to 1 hour. Similar results were obtained for SHIP. Coimmunoprecipitation revealed that incubation with IVIG did not induce association of SHIP, SHP-1, or SHP-2 with Fc γ RIIb (data not shown), which indicates that these molecules were not effectively recruited after addition of monomeric IVIG to B lymphocytes.

Phosphorylation of the tyrosyl residues within the Fc γ RIIb ITIM motif creates docking sites for the SH2-containing phosphatases SHIP1, SHIP2, SHP-1, and SHP-2, among which SHIP-1 has been shown to be the major mediator of Fc γ RIIb inhibitory effects in B cells.^{5,16} Enhanced tyrosine phosphorylation of SHIP is

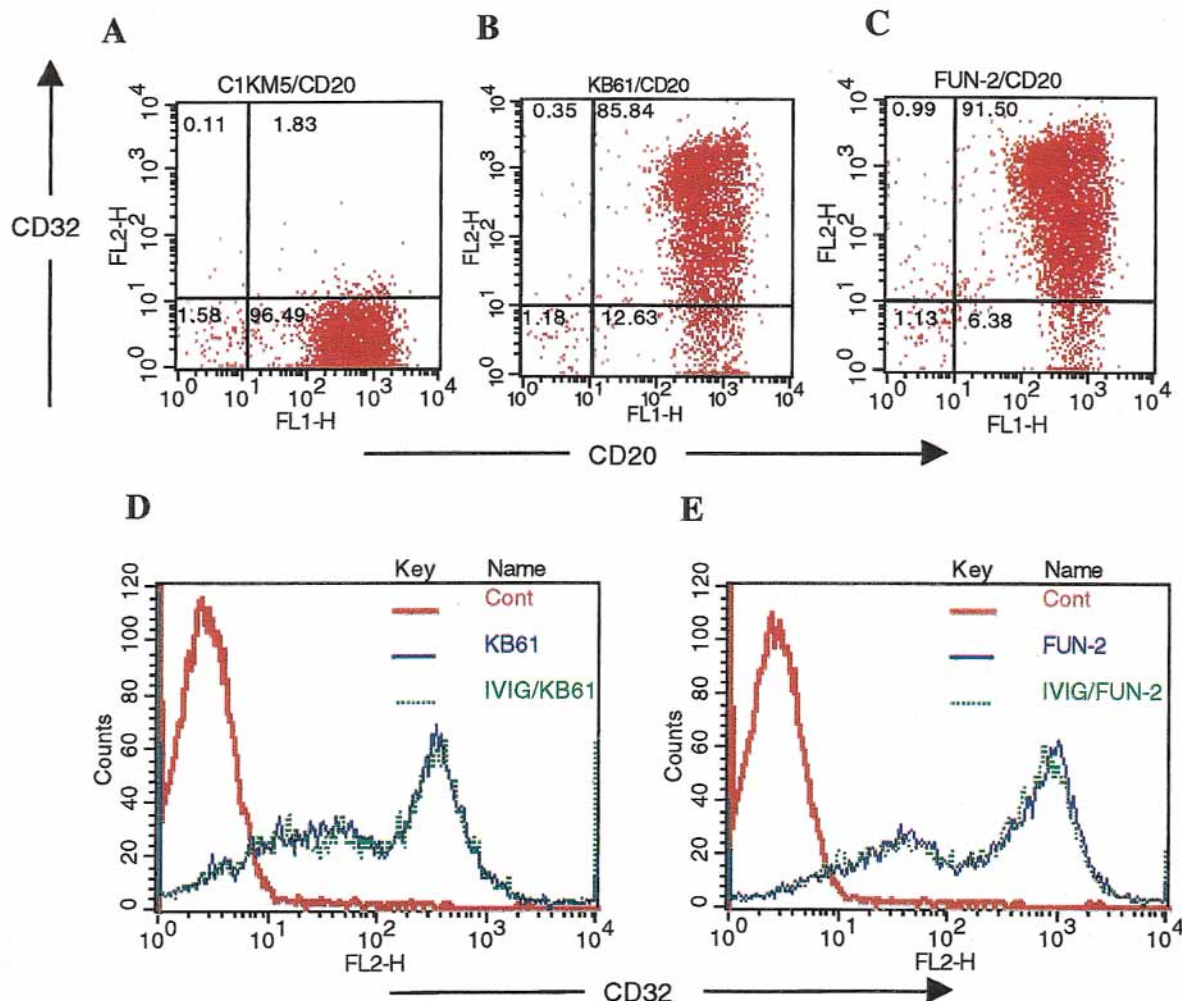


FIG 1. Expression of CD32 on human B lymphocytes. Freshly isolated human tonsillar B cells were stained with FITC-conjugated anti-CD20 antibodies (FL1) and various anti-CD32 antibodies (FL2): **A**, C1KM5; **B**, KB61; and **C**, FUN-2. In **D** and **E** the cells were preincubated with 10 mg/mL IVIG at 4°C for 30 minutes before staining with either KB61 (Fig 1, **D**) or FUN-2 (Fig 1, **E**). IVIG did not appear to inhibit staining by either FcγRIIb antibody. These are representative histograms from 4 identical experiments.

elicited by FcγRIIb coaggregation with the B-cell receptor.¹⁷ Accordingly, enhanced tyrosine phosphorylation of FcγRIIb and SHIP would support the hypothesis that these molecules are implicated in the suppression of B-cell activation by IVIG. However, no phosphorylation of FcγRIIb or phosphatases, such as SHIP, was induced by IVIG. FcγRIIb-mediated negative signaling through the ITIM is directed at regulating ITAM activation.¹⁸ To date, cross-talk between ITIM and other signaling pathways has not been observed.¹⁶ Phosphorylation of ITIM requires ligation of FcγRIIb and simultaneous ligation of an ITAM-containing receptor, such as the B-cell receptor, T-cell receptor, or FcεRI.¹⁶ In our experiments IgE production is induced by ligation of CD40 and the cytokine IL-4, neither of which are linked to an ITAM. Because ITAM activation is not achieved in our model of

IgE production, it is not surprising that we could not demonstrate FcγRIIb phosphorylation after the addition of IVIG to cultured human B lymphocytes.

Unfortunately, we cannot comment on whether immune complexes or aggregates of IVIG can signal through FcγRIIb in vivo. The data presented here and in our previous work imply the existence of a further level of regulation by IVIG, apart from possible interactions with Fc receptors. For example, IVIG consistently augmented the phosphorylation of a protein approximately 30 kD in size (Fig 2). This is the first demonstration in human cells that IVIG is capable of inducing phosphorylation of a protein in human B cells, and we are currently in the process of identifying this protein. We have found that the Fc portion was less potent than the F(ab')₂ fragment of IVIG in inhibiting activation of B lympho-

cytes.¹² We have also recently identified IVIG-responsive and IVIG-nonresponsive subsets of B lymphocytes (Janani et al, submitted for publication). The responsive subset, characterized by higher levels of surface IgD, appears to express less FcγRIIb than the more mature CD38⁺ cells (data not shown). Proper characterization will allow us to focus our efforts on key populations of B cells and better define the variable regions recognized by IVIG that play a role in inhibition of pathogenic antibody production.

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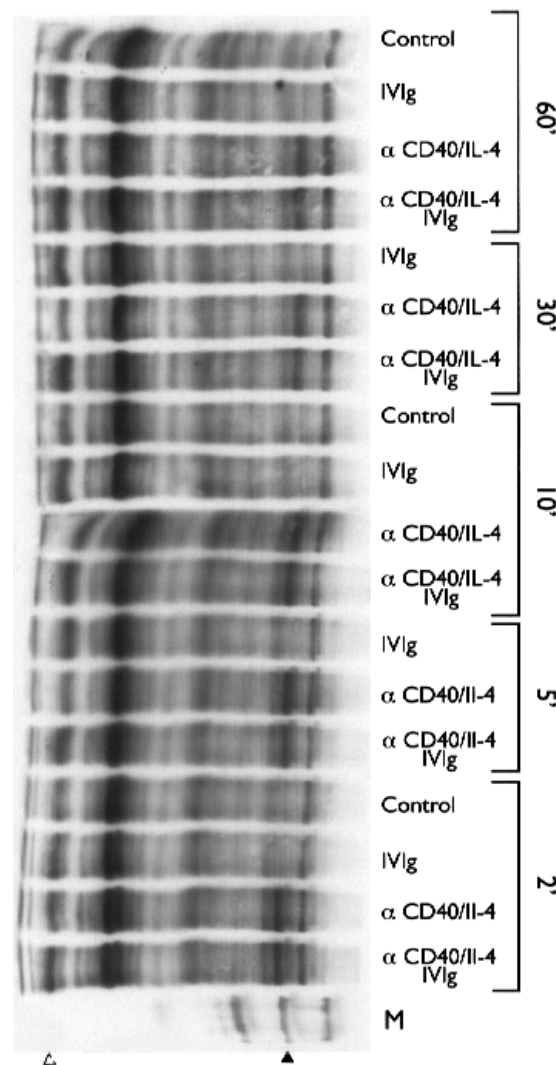


FIG 2. Tyrosine phosphorylation of proteins in human tonsillar B lymphocytes. After treatment with αCD40 (1 μg/mL)/IL-4 (400 U/mL) with or without IVIG (10 mg/mL) for 2 to 60 minutes as indicated, B cells were lysed, and cellular proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with RC20H antibody. Anti-CD40/IL-4 was responsible for consistent phosphorylation of several proteins, including a band of approximately 103 kD (solid arrow); this was not altered by the presence of IVIG. The presence of IVIG consistently led to the appearance of a phosphorylated band at 33 kD, beginning after 5 minutes of incubation (open arrow). One of 3 similar experiments is shown.

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