

Intravenous immunoglobulin inhibits IgE production in human B lymphocytes

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Background: Intravenous immunoglobulin (IVIG) is commonly used as both an immune-enhancing and immune-modulating agent. Treatment with high doses of IVIG diminishes IgE secretion in patients with severe steroid-dependent asthma.

Objective: We studied the action of IVIG on IgE production in highly purified B lymphocytes stimulated without additional T cells to determine the action of IVIG on B lymphocytes.

Methods: Human B cells were purified from tonsils, and T lymphocytes were removed by E-rosetting. B cells were cultured with IL-4 (400 U/mL) and anti-CD40 antibodies (1 µg/mL), with or without additional IVIG. Cell proliferation was determined by [³H]-thymidine uptake, and supernatant IgE was determined by ELISA. Cell cycle analysis was performed by flow cytometry, and IgE transcripts were measured by in situ hybridization.

Results: IVIG (5 mg/mL) decreased B-cell proliferation in IL-4/anti-CD40-stimulated B cells by an average of 74% (±6%). Addition of IVIG up to 48 hours after initiation of cell culture led to significant diminution of cell proliferation at 96 to 120 hours. This effect was dose dependent, with 10 mg/mL being the most effective and doses under 0.1 mg/mL having minimal effect. IVIG diminished the number of stimulated cells progressing in the cell cycle by 30%, and there was no difference in cell viability between IVIG-treated and IVIG-untreated cells. The production of IgE in culture by anti-CD40/IL-4-stimulated B lymphocytes was curtailed by greater than 80% after addition of 5 mg/mL IVIG. This was associated with a decrease in IgE (ε) transcripts in IVIG-treated cultures.

Conclusion: These data indicate that diminution of IgE production in anti-CD40/IL-4-stimulated B cells by IVIG is due to inhibition of early events related to proliferation and progression in the cell cycle. (*J Allergy Clin Immunol* 1998;102:421-7.)

Key words: Intravenous Ig, IgE, B lymphocytes, cell cycle

Intravenous immunoglobulin (IVIG) is an important therapeutic modality in immune deficiency and autoimmune disease. Because it contains antibodies pooled

Abbreviations used

αCD40:	Anti-CD40 antibodies
IVIG:	Intravenous immunoglobulin
PDB:	Phorbol 12,13 dibutyrate
PWM:	Pokeweed mitogen

from a large number of donors, it confers protection from common pathogens to individuals with antibody deficiencies. It is also used extensively in conditions such as immune thrombocytopenic purpura, Kawasaki syndrome, the Guillaine-Barré syndrome, and myasthenia gravis, diseases featuring autoantibody production, B-lymphocyte activation, or both.¹ However, it is unclear what the exact mechanism of action of IVIG is in many of these conditions. Indeed, there may be several plausible mechanisms, including blockade of Fc receptors,² neutralization of offending pathogens or superantigens,³ provision of antiidiotypic antibodies,³ and immune regulation with anticytokine antibodies and other regulatory molecules.⁴ It is certain that discerning the mode of immune modulation by IVIG will help better focus its use in immune-mediated diseases.

Diseases such as asthma and severe atopic dermatitis are common conditions for which corticosteroid therapy is the main method of modulating the immune system. A common feature of these atopic diseases is the overproduction of IgE, both total⁵ and allergen-specific IgE.⁶ IVIG has been used in severe steroid-dependent asthma in an attempt to diminish the debilitating side effects of chronic steroid therapy.^{7,8} In an open-label study, Mazer and Gelfand⁷ treated 8 pediatric patients with high-dose IVIG therapy (2 g/kg/mo) for 6 months, monitoring their symptom scores, pulmonary function, and steroid requirements. IVIG therapy was associated with significant improvements in symptom scores and improved asthma control. The average steroid dose was decreased by 66% over the trial. Similar results in a cohort of adult asthmatic patients was reported by Gelfand et al.⁹ Importantly, serum IgE levels fell an average of 50% in all individuals, and specific IgE also greatly decreased as evidenced by a decrease in skin test reactivity to a panel of antigens.⁷

In this study we examined whether the action of IVIG on IgE production in steroid-dependent asthmatic subjects could be duplicated in vitro. Although there is evidence for a direct action of IVIG on T lymphocytes^{10,11}

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Supported by the Bayer-Canadian Red Cross Research and Development fund and Fonds de la Recherche en Santé du Québec.

Received for publication Oct 31, 1997; revised May 12, 1998; accepted for publication May 12, 1998.

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0091-6749/98 \$5.00 + 0 1/1/91750

and on T-cell dependent B-cell activation,¹²⁻¹⁴ in this study we used a T-cell independent system to better address the question of the direct action of IVIG on B-cell function. Specifically, we investigated the action of IVIG on early events in B-cell maturation, including apoptosis, cell cycle analysis, and cell proliferation. We also sought to determine if IVIG had any effect on the genetic program of isotype switching from IgM to IgE mRNA. Our data suggest that IVIG diminishes IgE production in anti-CD40/IL-4-stimulated B cells and that this is likely caused by inhibition of events related to proliferation and progression in the cell cycle.

METHODS

Cell culture materials

Recombinant IL-4 was purchased from R and D Systems (Minneapolis, Minn). Anti-CD40 antibodies (α CD40) were purified from cell culture supernatants harvested from the G28.5 B-lymphoblastoid cell line (American Type Culture Collection, Rockville, Md).¹⁵ Phorbol-12,13-dibutyrate (PDB) and ionomycin were purchased from Calbiochem (La Jolla, Calif). IVIG for human use was provided courtesy of Bayer Inc (Etobicoke, Ontario). It was used as supplied diluted in complete medium or as a pH 7-adjusted solution. To adjust the pH from 4 to 7, IVIG was slowly titrated with NaOH as suggested by the manufacturer. The pH-adjusted solution was stable for up to 10 days at 4°C.

B-cell preparation

Human B lymphocytes were isolated from tonsils surgically removed for a variety of indications. Mononuclear cells were purified by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. B lymphocytes were separated from T cells by E-rosetting with neuraminidase-treated (Calbiochem, La Jolla, Calif) sheep red blood cells. Monocytes were adherence depleted on plastic petri dishes. B-lymphocyte purity was routinely demonstrated to be 98% or greater by flow cytometry (CD19+), with less than 2% CD3+ (T lymphocytes) and less than 1% CD14 (monocytes). Informed consent was routinely obtained for use of discarded surgical tissue.

Cell culture

B cells were resuspended in complete medium consisting of RPMI 1640, supplemented with 10% FCS, 10 mg/mL L-glutamine, 500 U/mL penicillin, and 500 μ g/mL streptomycin. The cells were plated at a concentration of 10^6 per milliliter and were cultured with α CD40 (1 μ g/mL) and recombinant IL-4 (400 U/mL) in 24-well plates. In some experiments PDB (10^{-8} mol/L) and ionomycin (0.5 μ mol/L) were added in place of α CD40/IL-4. IVIG or an equal volume of control (albumin- or FCS-containing medium) was added as indicated at appropriate doses and times. Neither control adversely affected IgE production. At the end of culture, cell counts were performed with a CBC-5 benchtop automatic cell counter (Coulter Electronics, Hialeah, Fla).

Cell cycle analysis

B cells were cultured with either α CD40 plus IL-4 or PDB and ionomycin in 24-well tissue culture plates. After cell culture for the indicated times, the cells were washed twice with PBS by centrifugation at 1200 rpm for 10 minutes, resuspended in 250 μ L FCS and 250 μ L RPMI 1640, and fixed in 1.5 mL 70% ethanol at 4°C for a minimum of 30 minutes. After fixation, the cells were again washed twice with PBS by centrifugation at 4°C and 2400 rpm for 5 minutes and then resuspended in 1 mL PBS with 0.05 mg/mL RNase A (50 U/mg) (Boehringer Mannheim, Laval, Quebec) and incubated for

30 minutes at 37°C. After this, 50 μ g/mL propidium iodide was added, and the cells were analyzed by flow cytometry with Cell Fit Software (Becton Dickinson, La Jolla, Calif).

³[H]-thymidine incorporation

B lymphocytes were cultured in 96-well plates for a maximum of 120 hours after the addition of the indicated mitogens with or without IVIG. At 90 to 114 hours, 1 μ Ci/well ³[H]-thymidine (ICN, Mississauga Ontario) was added, and the cells were incubated for an additional 6 hours. Cells were harvested by water lysis (PHD Cell Harvester, Cambridge, Mass), and ³[H]-thymidine incorporation was measured by liquid scintillation counting (Wallac LKB, Chicago, Ill).

IgE production

B cells were cultured with α CD40 (1 μ g/mL), and recombinant IL-4 (400 U/mL) in 24-well plates for 14 days with IVIG added at the indicated doses. Supernatants were harvested, and IgE was measured by ELISA (Kallestad; Sanofi Diagnostics, Chaska, Minn) according to the manufacturer's instructions.

In situ hybridization for IgE transcripts

B cells were cultured with α CD40 (1 μ g/mL) and recombinant IL-4 (400 U/mL) in 24-well plates for 5 days, with IVIG added at the indicated doses. The cells were harvested and washed and then applied to polylysine-coated slides, and in situ hybridization with 35S-labeled complementary RNA probes coding for C ϵ RNA was performed. Permeabilization of the cell membranes was achieved through incubation with Triton X-100 and proteinase K solution (1 mg/mL) in 0.1 mol/L Tris containing 50 mmol/L EDTA for 20 minutes at 37°C. Slides were then immersed in 0.1 mol/L triethanolamine and 0.5% acetic anhydride for 10 minutes and then in N-ethylaldehyde (1.25 mg/mL) and iodoacetamide (1.85 mg/mL) for 20 minutes at 37°C to prevent the nonspecific binding of the cRNA probes. Prehybridization of the samples was completed with 50% formamide in 2 $\times$ standard saline citrate for 15 minutes at 37°C. Cytospins were incubated overnight with a hybridization mixture containing the C ϵ probe (0.75×10^6 cpm/slide). Posthybridization involved high stringency washing of the samples in decreasing concentrations of standard saline citrate at 42°C. To remove any unbound RNA probe, samples were washed with an RNase solution (20 mg/mL) for 20 minutes at 42°C. The samples were then dehydrated with increasing concentrations of ethanol and left to air dry. After this, cytopins were dipped in Amersham LM-2 emulsion and exposed for a period of 9 days. The autoradiographs were developed in Kodak D-19 developer, fixed, and counterstained in Mayer's haematoxylin for 60 seconds. The samples were mounted with a coverslip and examined under a graduated microscope for positive signals.

Statistical analysis

All statistical analyses were performed with Instat (Graphpad software, San Diego, Calif). All conditions were performed in duplicate, and each experiment was performed multiple times as indicated. Means of replicate experiments were compared with control means with Student *t* test.

RESULTS

IVIG diminishes IgE secretion in α CD40/IL-4-stimulated B lymphocytes

To investigate the action of IVIG on IgE production, we isolated B lymphocytes to study in vitro IgE production without the addition of T cells. The need for T lymphocytes was obviated by using exogenous recombinant

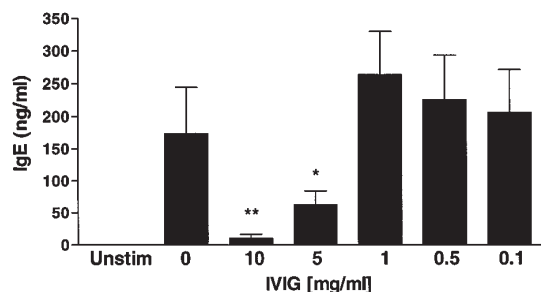


FIG 1. IVIG inhibits production of IgE. Highly purified B lymphocytes were cultured with α CD40 (1 μ g/mL) and IL-4 (400 U/mL) for 14 days. IVIG was added at initiation of culture period at indicated doses. After 14 days, supernatants were harvested and IgE production was measured by specific ELISA. IVIG (5 to 10 mg/mL) significantly decreased the production of IgE. *Unstim*, Cells cultured in absence of α CD40, IL-4, and IVIG. Histograms indicate means $\pm$ SEM. $n = 5$; ** $P < .005$; * $P < .05$.

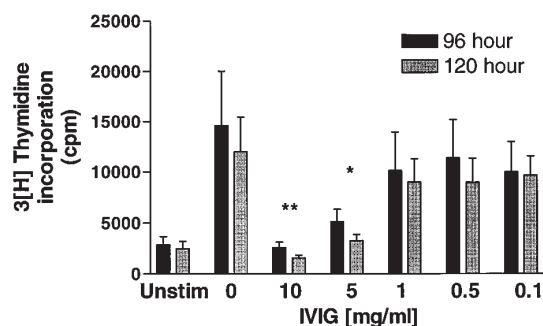


FIG 2. IVIG inhibits proliferation induced by α CD40/IL-4. Highly purified B lymphocytes were cultured with α CD40 (1 μ g/mL) and IL-4 (400 U/mL) for 96 to 120 hours. IVIG was added at initiation of culture period at indicated doses. Before harvesting, ^{3} H-thymidine (1 μ Ci/well) was added, and cultures were incubated for an additional 6 hours. IVIG (5 to 10 mg/mL) markedly decreased cell proliferation at peak of ^{3} H-thymidine incorporation, between 96 and 120 hours. *Unstim*, Cells cultured in the absence of α CD40, IL-4, and IVIG. Histogram values indicate means $\pm$ SEM. $n = 4$; ** $P < .005$; * $P < .05$.

IL-4 and α CD40. Fig 1 demonstrates the effect of IVIG, added at the initiation of culture, on IgE synthesis by α CD40/IL-4-stimulated B cells. High-dose IVIG (5 to 10 mg/mL) significantly inhibited the production of IgE. This dose range achieves concentrations equivalent to that achieved in the serum of patients treated for autoimmune disease by using high doses of IVIG (eg, 2 g/kg).¹² Lower doses of IVIG did not significantly diminish IgE production (Fig 1). The maximal inhibition of IgE production was achieved when IVIG (5 mg/mL) was added up to 48 hours after the initiation of culture (data not shown).

The preparation of IVIG that we used (Human Immunoglobulin for Intravenous Use; Bayer, Etobicoke, Ontario) is prepared and supplied at pH 4 to maintain solubility and stability. To determine whether the effect of IVIG was simply a toxic effect of the pH, we adjusted the solution to pH

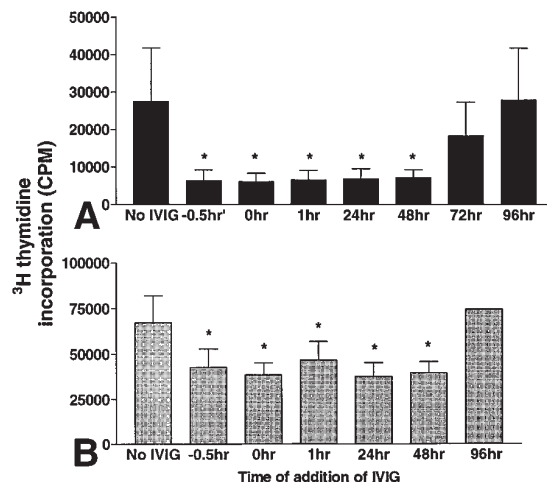


FIG 3. IVIG inhibits proliferation induced by α CD40/IL-4. Highly purified B lymphocytes were cultured with α CD40 (1 μ g/mL) and IL-4 (400 U/mL) (A) or PDB (10⁻⁸ mol/L) and ionomycin (0.5 μ mol/L) (B) for 96 hours. IVIG (5 mg/mL) was added at indicated times. Before harvesting, ^{3} H-thymidine (1 μ Ci/well) was added, and cultures were incubated for an additional 6 hours. IVIG (5 mg/mL) significantly decreased ^{3} H-thymidine incorporation if added up to 48 hours after mitogenic stimulation but not if added at 72 hours (A) or 96 hours (A and B). *No IVIG*, Cells cultured in presence of α CD40 and IL-4 with no added IVIG. Histogram values indicate means $\pm$ SEM. $n = 4$; ** $P < .005$; * $P < .05$.

7. The effect on IgE production of the pH-adjusted IVIG was identical to the pH 4 solution (data not shown). However, if the IVIG was stored at pH 7 for 14 days or more, the preparation became ineffective (data not shown).

To ensure that the effect of IVIG was not simply a provision of blocking antibody against supernatant IgE, IgE-standard sera were incubated with 10 mg/mL IVIG and then plated on the ELISA plates. There was no significant difference between the results of the IgE determination for standard sera in the presence or absence of IVIG.

IVIG inhibits B-cell proliferation

α CD40 and IL-4 provide initial mitogenic signals to B lymphocytes causing them to multiply before their maturation into Ig-secreting cells. To assess whether the reduction in IgE production by IVIG was caused by inhibition of these early cell-proliferation events, we examined B-cell replication and de novo DNA synthesis by measuring ^{3} H-thymidine incorporation after a 96- to 120-hour culture period. IVIG (5 mg/mL) decreased ^{3} H-thymidine incorporation into B cells after IL-4/ α CD40 stimulation by an average of 74% ($\pm$ 6%) (Fig 2). This effect was dose dependent, with 10 mg/mL being the most effective and doses under 0.1 mg/mL having minimal effect. Addition of 5 mg/mL IVIG up to 48 hours after initiation of cell culture, but not after 72 or 96 hours, led to significant diminution of cell proliferation at 96 to 120 hours compared with cells that were not treated with IVIG (Fig 3, A). The effect of IVIG on cell

TABLE I. Percentage of apoptotic cells and cells in each phase of cell cycle (% means $\pm$ SD)

Stimulus	Apoptosis	G0-G1	S	G2-M
Control	14.8 $\pm$ 8.1	74 $\pm$ 6.0	8.39 $\pm$ 3.9	2.31 $\pm$ 1.0
IVIG	12.4 $\pm$ 9.4	76 $\pm$ 6.6	9.65 $\pm$ 4.1	2.2 $\pm$ 0.9
CD40/IL-4	15 $\pm$ 6.5	69.6 $\pm$ 4.5	10.9 $\pm$ 2.7	2.12 $\pm$ 1.3
CD40/IL-4/IVIG	12.5 $\pm$ 5.5	70.2 $\pm$ 6.5	11.23 $\pm$ 3.1	2.53 $\pm$ 1.6
PDB/Ionomycin	19.2 $\pm$ 8.3*	52.5 $\pm$ 3.9*	19.9 $\pm$ 4.7*	3.21 $\pm$ 2.5
PBD/Ionomycin/IVIG	15.6 $\pm$ 6.3†	59 $\pm$ 6.8†	16.4 $\pm$ 5.3†	2.46 $\pm$ 1.7

B cells were cultured with α CD40/IL-4 as above or PDB (10^{-8} mol/L) and ionomycin ($0.5 \mu\text{mol/L}$) for 72 hours, and IVIG (5 mg/mL) was added at initiation of culture. Cell cycle analysis and apoptosis were detected by permeabilizing cells and staining with propidium iodide and then analyzing by flow cytometry. Data presented are cell numbers $\pm$ SEM in each population. Little change in cell cycle is seen with α CD40/IL-4 stimulation in spite of consistent increases in ^3H -thymidine incorporation. IVIG (5 mg/mL), if added at initiation of culture, inhibited progression of cells into G2-M phase and decreased number of apoptotic cells that were detected in the PDB/ionomycin-stimulated cultures. $n = 3$.

* $P < .01$ compared with unstimulated controls.

† $P < .05$ compared with PDB/ionomycin.

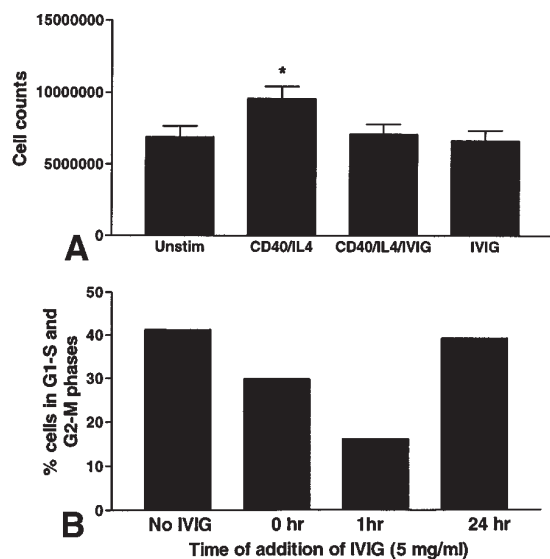


FIG 4. Effect of IVIG on cell viability. **A**, Tonsillar B cells were incubated with α CD40 (1 $\mu\text{g/mL}$) and IL-4 (400 U/mL) as indicated, and cell counts were performed at termination of 14-day culture period with benchtop Coulter Counter (CBC-5). Mean cell counts $\pm$ SEM are indicated in panel. There was increased number of cells in α CD40/IL-4-stimulated cultures compared with both control and α CD40/IL-4/IVIG. This correlated with hemocytometer counts and trypan blue exclusion (data not shown). *Unstim*, Cells cultured in absence of α CD40, IL-4, and IVIG. * $P < .05$; $n = 5$. **B**, Assessment of cell cycle was performed on cells cultured with PDB (10^{-8} mol/L) and ionomycin ($0.5 \mu\text{mol/L}$) for 72 hours. IVIG (5 mg/mL), if added at initiation of culture (0 to 1 hours) but not after 24 hours, inhibited progression of cells into G2-M phase. There was concomitant increase in cells in G0-G1 (resting) phase. This is a single representative experiment; remaining data are summarized in Table I. *No IVIG*, Cells cultured in presence of α CD40 and IL-4 without additional IVIG.

proliferation was not specific for α CD40 and IL-4. IVIG also reduced ^3H -thymidine incorporation when B cells were induced to proliferate by the combination of the potent pharmacologic stimuli PDB and the calcium ionophore ionomycin (Fig 3, B).

IVIG does not induce apoptosis in B lymphocytes

The decrease in cell proliferation and subsequent decrease in IgE production could be explained by toxicity or an increase in programmed cell death induced by IVIG. Indeed, other investigators have found that similar doses of other IVIG preparations have caused necrotic cell death and perhaps apoptosis and as such dialyze their IVIG.¹⁶ We did not find any such toxicity, even with doses as high as 10 mg/mL. At 24 to 72 hours, there was no increase in cell necrosis as demonstrated by trypan blue exclusion in IVIG-treated cultures (data not shown). We performed cell counts after 14 days of cell culture and found no significant difference in cell viability between control cells, cells treated with IVIG alone, and α CD40/IL-4/IVIG-treated cells in terms of morphology, trypan blue uptake, or cell counts at termination of culture (Fig 4, A).

Alternatively, IVIG could act by inducing apoptosis. To assess this, B cells were stained with propidium iodide 96 hours after initiation of culture and assessed by flow cytometry according to the method of Darzynkiewicz et al.¹⁷ We have shown that this method correlates well with more direct measures of apoptosis, such as gel electrophoresis and fluorescent labeling of 180 to 200 bp fragments (TUNEL Assay).¹⁸

Table I shows the results of 4 experiments assessing cell cycle and programmed cell death in purified B cells. Treatment of the cells with IVIG did not increase the percentage of apoptotic cells over control values. In spite of clear differences in ^3H -thymidine uptake, α CD40/IL-4 did not induce large changes in the cell cycle at 48 hours in our study. However, PDB/ionomycin stimulation led to 2 clear patterns; a group of cells were driven to apoptosis ($19\% \pm 8.3\%$), and numbers of cells in S+G2-M (proliferating) phases of the cell cycle were increased (Fig 4, B and Table I). Addition of IVIG also inhibited the induction of apoptosis by PDB/ionomycin (Table I). Furthermore, less cells were found in the mitotic phases of the cell cycle (Table I). These data suggest that IVIG has an effect on early signals initiated by receptor-ligand interaction or pharmacologic stimuli. These include signals involved in initiating proliferation or programmed cell death.

Effect of IVIG on IgE transcripts

The stimulation of B cells by α CD40/IL-4 leads to switching of the germline IgM (μ) transcripts to the ϵ gene that codes for the constant region of IgE. We therefore examined whether IVIG decreased the number of cells that express the C ϵ gene after incubation with α CD40/IL-4. In control cells and in IVIG-treated cells, few B cells express transcripts for C ϵ (Table II). As expected, a greater number of B cells that were exposed to α CD40/IL-4 were positive for the C ϵ transcript, and preincubation with IVIG markedly diminished the number of cells that were positive for C ϵ (Table II, Fig 5).

DISCUSSION

In these studies we have demonstrated that IVIG can diminish the synthesis of IgE in vitro. Using highly purified B cells stimulated to produce IgE in the absence of T cells, we have demonstrated conclusively that IVIG acts directly on B lymphocytes. IVIG decreased α CD40/IL-4-stimulated B-cell proliferation and blocked the early events induced by PDB/ionomycin that induced progression in the cell cycle. We have also shown that IVIG reduced the generation of C ϵ transcripts, a key feature of the genetic switch of immunoglobulin genes from IgM to IgE.

There is compelling evidence that IVIG contains molecules that modulate immune function. Its effectiveness in autoimmune disease and its rapid action in inflammatory conditions, such as Kawasaki syndrome, argue that administration of pooled human antibodies may mimic normal mechanisms of immune regulation. Experience with diseases, such as immune thrombocytopenic purpura,¹⁹ Kawasaki syndrome,²⁰ and Guillain-Barré syndrome,²¹ has clearly demonstrated the therapeutic efficacy of IVIG. Its mechanism of action in these diseases is unclear, but Fc receptor blockade,¹⁹ provision of toxin-neutralizing antibodies,²² anticytokine antibodies,⁴ or regulation of idiotypic networks¹⁹ have all been proposed.

The in vivo effects of IVIG on IgE production have been previously shown in several studies. Durandy et al²³ treated a cohort of children with recurrent infections with low doses of intramuscular Ig (80 mg/kg). They noted that several allergic children in the group with high serum IgE concentrations normalized their serum IgE levels after 3 to 4 months of Ig therapy. In a trial of IVIG for severe steroid-dependent asthma, all patients had an average 66% decrease in their serum IgE, and 7 of 8 had a 1 to 2 dilution improvement in end point titration of their skin tests. The effect of IVIG on specific IgE production was further emphasized by the actual extinction of skin test sensitivity to several antigens in 4 patients.⁷ Additionally, in patients with hyper-IgE syndrome, Kimata¹⁴ determined that there was a reduction in both their in vivo and in vitro IgE production after IVIG therapy.

Our findings confirm the validity of these observations. Five to ten milligrams per milliliter IVIG, a dose achieved in high-dose infusion regimens, decreased the production of IgE by 71% to 88% in B cells cultured with α CD40 and IL-4 (Fig 1). Our studies have shown this to

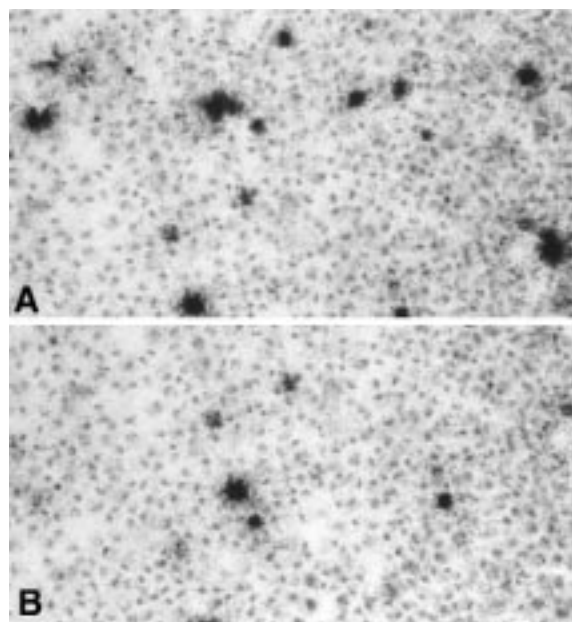


FIG 5. Photomicrographs of in situ hybridization. Positive signal for C ϵ mRNA was identified as dense collections of silver granules overlying cells. **A**, C ϵ expression in purified B cells incubated with α CD40 (1 μ g/mL) and IL-4 (400 U/mL). **B**, B cells cultured with α CD40, IL-4, and IVIG (5 mg/mL).

TABLE II. Effect of IVIG on IgE transcripts

Condition	C ϵ
Control	$\pm$
IVIG	$\pm$
α CD40/IL-4	++
α CD40/IL-4 plus IVIG	+

Purified B lymphocytes (1×10^6) were incubated with α CD40 and 400 U/mL IL-4 with or without 5 mg/mL IVIG as indicated. After 5 days, cells were harvested, applied to polylysine-coated slides by cytopspin, and probed for presence of C ϵ transcripts as detailed in Methods section. After a 9-day exposure, the slides were counted by microscopy for cells that have a positive signal, as seen in Fig 5.

$\pm$, Indeterminant signal; +, few cells with C ϵ transcripts; ++, moderate presence of C ϵ transcripts.

be due to an effect on the events that occur early in B-cell activation. IVIG-treated B cells proliferated much more poorly than untreated cells, and proliferation was inhibited if IVIG was added to culture up to 48 hours after the addition of mitogens. Importantly, there was no increase in necrotic cell death or apoptosis compared with control cells. The action on cell proliferation was demonstrated both for receptor-ligand signaling (CD40 plus IL-4) and pharmacologic signaling (PDB plus ionomycin). IVIG's mechanism of action therefore must include pathways that interrupt signal transduction in activated cells without inducing an apoptotic signal.

Previous in vitro studies on IVIG and B lymphocytes have used B cells in culture with T lymphocytes. IVIG has established effects on T-lymphocyte proliferation¹¹ and cytokine production.^{10,16} These would influence the

signals that B cells require for Ig production. Additionally, a common cell activator used in these studies, pokeweed mitogen (PWM), depends on T cells to transmit cytokines, such as IFN- γ , to B cells for in vitro Ig production.²⁴ Durandy et al²³ studied children with atopic dermatitis treated with low doses of intramuscular Ig. They cultured B and T lymphocytes from their patients with PWM before and after intramuscular Ig treatment. The patients showed decreased Ig-secreting cells while receiving therapy compared with normal control subjects or with the patients' own cells once therapy was stopped. Tsubakio et al,²⁵ also using PWM-cultured lymphocytes, documented decreased IgG, IgA, and IgM secretion in vitro after much higher dose IVIG therapy (2 g/kg) for immune thrombocytopenic purpura. In a series of studies Stohl^{26,27} attempted to discern the mechanism of IVIG's effect on Ig-secreting cells by adding it directly to PWM-stimulated mixed lymphocyte cultures. IVIG decreased the number of Ig-secreting cells detected by plaque-forming cell assays. More recently, Stohl and Elliot¹² used the T-cell dependent *Staphylococcus* toxins as superantigens to stimulate B cells and demonstrated that IVIG inhibited B-cell maturation as measured by enumeration of Ig-secreting plaque-forming cells. Kondo et al¹³ used various IVIG preparations in a T- and B-lymphocyte culture system. They also demonstrated a dose-dependent decrease in Ig-secreting cells and in cell proliferation assessed by ³[H]-thymidine incorporation.

Our data agree with other in vitro studies on IgE production performed by Kimata¹⁴ and Haas and Schlaak,²⁸ who demonstrated a decrease in IgE production by human IgG. Both of these previous studies were performed with mixed lymphocyte populations, and Haas and Schlaak used human serum from single donors in their studies.²⁸ Our work furthers these observations by examining B cells alone and determining that the action of commercially available IVIG is primarily on early activation events that regulate proliferation and cell cycle.

How may IVIG act on B cells to block their proliferation, genetic switch, and subsequent production of IgE? Anticytokine antibodies have been found among the repertoire of IVIG,²⁹ and therefore IVIG may block the action of the cytokine IL-4. Additionally, the preparations may contain neutralizing antibodies or antiidiotype antibodies to α CD40 antibodies or bind directly to the CD40 molecule on B cells to block the action of the mitogen.^{30,31} The effect of IVIG up to 48 hours after addition of α CD40 or cytokines argues against this because neutralization after receptor-mediated signal transduction has begun may not be effective. This will be tested in future studies.

B cells express a particular low-affinity Fc γ receptor subtype known as Fc γ RIIb1.³² Although Fc γ receptors generally have intracellular signaling chains that promote cellular activation events, such as phagocytosis and cytotoxicity,³² the B-cell receptor subtype has unique properties. Fc γ RIIb1 has a 13 amino acid chain in its cytoplasmic domain that is known as the immunoreceptor tyrosine-based inhibitory motif. In

contrast to other Fc receptors, activation of Fc γ RIIb1 sends inhibitory signals, decreasing B cell proliferation and Ig synthesis.^{31,32} D'Ambrosio et al³³ demonstrated that ligation of Fc γ on B cells leads to recruitment of a phosphatase known as SHP-1 (or protein tyrosine phosphatase 1C). Phosphatases are recognized to play important roles in regulating T- and B-cell responses³³; SHP-1 may be responsible for decreasing phosphorylation of PLC γ ^{34,35} and other inhibitory events. The consequences of Fc γ RIIb1 ligation on B cells in vitro include attenuated production of inositol phosphates, decreased cell proliferation, and decreased antibody synthesis.³² Therefore the Fc portions of the Ig molecules provided by IVIG may interact with B-cell Fc γ RIIb1 receptors and contribute inhibitory signals without inducing apoptosis. In previous studies investigators have found evidence suggesting that the Fc portion plays a role in regulating both T-cell¹⁶ and B-cell responses,³⁶ although work by Klaesson et al³⁷ questions a role for the Fc fragment in their experiments on Ig production by B cells. It is crucial to determine the elements of the IgG molecule in IVIG that inhibit the production of IgE.

The regulatory events leading to the cessation of B-cell antibody production once an immune response is initiated are poorly understood. Even more poorly understood are the signals that can interrupt a maladaptive immune response such as the production of autoantibodies. We have shown that high concentrations of antibodies can inhibit B-lymphocyte proliferation, the genetic switch of the cells from IgM to IgE, and the production of IgE. Future experiments will focus on which portion of the antibody molecule is important in this inhibitory activity. Should the F ab'2 molecule be implicated, then investigation will focus on the action of IVIG on cytokines or as an antiidiotype antibody. Should the Fc portion be the crucial part, this would suggest interaction with Fc γ RIIb1 receptors. Observations on the mechanism of action of IVIG should provide future crucial data on the natural regulation of immunoglobulin production by B lymphocytes.

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