

Concurrent presence of agonistic and antagonistic anti-CD95 autoantibodies in intravenous Ig preparations

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Background: Although there have been several reports suggesting the presence of physiologic anti-CD95 (Fas, APO-1) autoantibodies in human intravenous Ig (IVIg) preparations, it is still unclear whether and under which conditions these autoantibodies block or stimulate the CD95 receptor.

Objective: We examined the effects of IVIg on CD95-mediated apoptosis in CD95-sensitive human blood neutrophils *in vitro*.

Methods: The presence of anti-CD95 antibodies was determined by competition assays with flow cytometry. Cell death and apoptosis were assessed by ethidium bromide uptake test and annexin V staining, respectively.

Results: Pretreatment of neutrophils with IVIg prevented binding of FITC-conjugated anti-CD95 mAb to the cell surface, suggesting that IVIg contains CD95 autoantibodies. By using low concentrations of IVIg (1 to 10 mg/mL), we observed a dose-dependent inhibition of anti-CD95 mAb (CH11)-mediated neutrophil apoptosis. Higher concentrations of IVIg (20 to 50 mg/mL), however, induced neutrophil death and apoptosis in a dose-dependent manner. This effect was partially blocked by soluble CD95 receptors (recombinant Fc-Fas) but not by an anti-CD95 blocking mAb, which was shown to recognize the CH11 epitope of CD95.

Conclusion: Both agonistic and antagonistic anti-CD95 antibodies are present in IVIg, and the effect on CD95 is dose-dependent. Our findings have potential implications for IVIg treatment, which is intended to target the CD95 receptor. (*J Allergy Clin Immunol* 2003;112:1185-90.)

Key words: Apoptosis, autoantibodies, Fas, IVIg, neutrophils

Intravenous Ig (IVIg) preparations are commonly used in the treatment of primary or secondary immunodeficiency disorders associated with hypogammaglobulinemia or agammaglobulinemia. IVIg application is believed to deliver missing antibodies against pathogens, and therefore this treatment is considered as substitution therapy or passive immunization against multiple infectious diseases. In contrast, the use of IVIg in diseases associated with hyperreactive conditions of the immune system is still in

Abbreviations used

IVIg: Intravenous Ig
TBS: Tris-buffered saline
TNFR: TNF receptor

discussion, although the efficacy of IVIg treatment has been demonstrated formally in several autoimmune diseases.¹ One of the reasons for this situation is that the mechanisms of how IVIg preparations suppress hypersensitivity reactions of the immune system are largely unclear.

In patients with toxic epidermal necrolysis, IVIg treatment dramatically improves the clinical situation.^{2,3} The increased keratinocyte apoptosis observed in this disease appears to be mediated by CD95 ligand/CD95 receptor molecular interactions, which can be blocked by antagonistic anti-CD95 autoantibodies present in IVIg preparations.²⁻⁴ In contrast, it was reported that IVIg contains anti-CD95 antibodies, which induce apoptosis in lymphocytes and monocytes in a caspase-dependent manner.^{5,6} Thus, although the presence of anti-CD95 autoantibodies has been demonstrated by several groups, it is not clear whether and under which conditions they contain agonistic or antagonistic anti-CD95 autoantibodies. Here we investigated the effect of IVIg on the regulation of apoptosis in human neutrophils, which have previously been demonstrated to be Fas-sensitive.⁷ The results of our study suggest that IVIg preparations contain both agonistic and antagonistic anti-CD95 autoantibodies and that they recognize different epitopes.

METHODS

Cell isolation

Neutrophils were isolated from peripheral blood of healthy donors by Ficoll-Hypaque centrifugation.^{8,9} The resulting cell populations contained greater than 95% neutrophils. Written consent was obtained from all donors, and the study was approved by the Medical Ethics Committee of the Canton Bern.

Reagents

Complete culture medium was RPMI 1640 (Life Technologies, Inc, Basel, Switzerland) supplemented with 2 mmol/L L-glutamine, 200 IU/mL penicillin/100 µg/mL streptomycin, and 10% FBS (all from Life Technologies). The agonistic anti-CD95 receptor mAb CH11¹⁰ and the fluorescein-conjugated anti-CD16 mAb were purchased from Beckman Coulter International SA (Nyon, Switzerland). The same anti-CD16 mAb was used in a nonconjugated form for immunofluorescence blocking experiments. The IVIg prepara-

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tion was Sandoglobulin (ZLB Bioplasma AG, Bern, Switzerland). Fluorescein-conjugated anti-CD95 mAb UB2 and the antagonistic anti-CD95 mAb ZB4 (inhibits apoptosis induced by CH11 at isomolar concentrations and targets the same epitope as CH11 corresponding to Lys126 to Cys135 of the extracellular domain of CD95)^{11,12} were from MBL Co, Ltd (Nagoya, Japan) and distributed by LabForce AG (Nunningen, Switzerland). The anti-CD95 mAb (clone 13) was purchased from Becton Dickinson Pharmingen (Basel, Switzerland), and anti- β -actin mAb was from Sigma (Buchs, Switzerland). Recombinant Fas-Fc protein, recombinant TNF-receptor 1 (TNFR1), and the pan-caspase inhibitor z-VAD-fmk were obtained from Alexis Corp (Läufelfingen, Switzerland). Human serum albumin (96%) was of low endotoxin content (Sigma). Unless stated otherwise, all other reagents were from Sigma.

Cell viability and apoptosis measurements

Neutrophils were cultured in complete culture medium in the presence and absence of the indicated concentrations of IVIg, CH11 (1 μ g/mL), ZB4 mAb (1 μ g/mL), or z-VAD-fmk (50 μ mol/L). When treated in combination, ZB4 mAb or z-VAD-fmk was given 1 hour before IVIg or CH11 addition, and IVIg was given 1 hour before CH11 addition without an intermediate washing step. Neutrophil death was assessed after 24 hours by uptake of 1 μ mol/L ethidium bromide and flow cytometric analysis (FACS Calibur; Becton Dickinson, Heidelberg, Germany).^{8,9} To determine whether cell death was apoptosis, redistribution of phosphatidylserine in the absence of propidium iodide was measured in 6-hour neutrophil cultures.¹³ Because neutrophils undergo spontaneous apoptosis, the difference between anti-CD95 mAb or IVIg-treated and untreated was defined as specific death and specific apoptosis, respectively. All experiments were controlled by using sucrose/human serum albumin and glucose/human serum albumin (1:1 in PBS) solutions at the appropriate concentrations that had no effect on neutrophil apoptosis.

Preincubation of IVIg

The indicated concentrations of IVIg were incubated on ice for 30 minutes with recombinant Fas-Fc (5 μ g/mL), TNFR1-Fc (5 μ g/mL), or complete culture medium, respectively, and subsequently used in neutrophil cultures. In other experiments, indicated concentrations of IVIg were incubated in complete culture medium in the presence and absence of neutrophils (5×10^6 /mL) for 1 hour. Neutrophils were subsequently removed by a centrifugation step at 2000 rpm for 5 minutes, and freshly isolated neutrophils (1×10^6 /mL) were cultured in supernatant with or without CH11. When treated in combination, CH11 was given 1 hour after addition of supernatant. Neutrophil death was assessed after 24 hours by uptake of 1 μ mol/L ethidium bromide and flow cytometric analysis (FACS Calibur).

Immunofluorescence analysis

Freshly isolated neutrophils were preincubated with the indicated concentration of IVIg for 30 minutes, washed once with PBS (4°C), and subsequently incubated for 15 minutes with fluorescein-coupled anti-CD95 (UB2) mAb (1:10) or fluorescein-coupled anti-CD16 mAb (1:50) in PBS at room temperature, followed by a single washing step with PBS (4°C). Staining of anti-CD95 and anti-CD16 mAb on neutrophils was measured by single flow cytometry (FACS Calibur). Isotype-matched control mAbs were used as negative controls. As positive controls for inhibition of binding, neutrophils were preincubated with nonconjugated CH11 (1 μ g/mL) and anti-CD16 mAb (5 μ g/mL) for 30 minutes at room temperature.

CD95 receptor RT-PCR

Total RNA of freshly isolated neutrophils (1×10^6) and neutrophils (1×10^6) cultured for 4 hours in the presence or absence of 30 mg/mL

IVIg was isolated according to the RNeasy protocol (Qiagen, Basel, Switzerland), which includes DNase digestion. RT-PCR and visualization of products were performed as previously described.¹⁴ Primers for CD95 receptor were sense primer: 5'-CACTTCGGAG-GATTGCTCAACA-3'; anti-sense primer: 5'-TATGTTGGCTCT-TCAGCGCTA-3'. Control amplifications were performed by using primers for glyceraldehyde-3-phosphate dehydrogenase.¹³

CD95 receptor protein expression

Freshly isolated neutrophils and neutrophils (1×10^6) cultured for 10 hours in the presence or absence of 30 mg/mL IVIg were washed with PBS and lysed with RIPA-buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS in PBS) supplemented with a protease inhibitor cocktail with frequent vortexing on ice for 40 minutes, including 2 freeze/thaw cycles with a methanol/dry ice bath. After a 10-minute centrifugation step to remove insoluble particles, equal amounts of the cell lysates were loaded on Tris-Glycine gels (Invitrogen Corp, Groningen, The Netherlands). Separated proteins were electrottransferred onto polyvinylidene difluoride membranes (Immobilion-P; Millipore, Bedford, Mass). The filters were incubated overnight with an anti-CD95 receptor mAb (clone 13, 1/500) at 4°C in Tris-buffered saline (TBS)/0.1% Tween-20/3% non-fat dry milk. For loading controls, stripped filters were incubated with an anti- β -actin mAb (1/10,000). Filters were washed in TBS/0.1% Tween-20 for 30 minutes and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1/3000; Amersham Pharmacia Biotech, Dübendorf, Switzerland) in TBS/0.1% Tween-20/5% non-fat dry milk for 1 hour. Filters were developed by an enhanced chemiluminescence technique (ECL-Kit; Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS

IVIg contains both agonistic and antagonistic anti-CD95 autoantibodies

Neutrophils are terminally differentiated cells that undergo constitutive apoptosis on in vitro culture in the absence of survival cytokines.¹⁵ Stimulation of these cells with agonistic anti-CD95 mAb accelerates the apoptotic process.^{7,15} With the CH11 mAb (1 μ g/mL), we observed approximately 20% specific cell death as a consequence of CD95 activation in 24-hour neutrophil cultures (Fig 1, A). Low IVIg concentrations (1 to 10 mg/mL) partially blocked CH11-mediated neutrophil death, whereby maximal inhibition was seen at 5 mg/mL. These data are in agreement with previously published work²⁻⁴ and implied the presence of antagonistic anti-CD95 autoantibodies in IVIg preparations. In the absence of CH11 mAb, however, IVIg dose-dependently induced neutrophil death, suggesting that they contain death-inducing factors besides death blockers. These death-inducing factors had additive effects when used in combination with the CH11 mAb (Fig 1, A) and were completely abolished in the presence of the pan-caspase inhibitor z-VAD-fmk (data not shown). Moreover, the antagonistic anti-CD95 autoantibodies were completely removed and the death-inducing factors partially removed by pretreatment of IVIg with neutrophils for 1 hour (Fig 1, B).

To determine whether the modulation of constitutive and CD95-mediated cell death by IVIg was apoptosis, phosphatidylserine redistribution, a hallmark of apoptotic

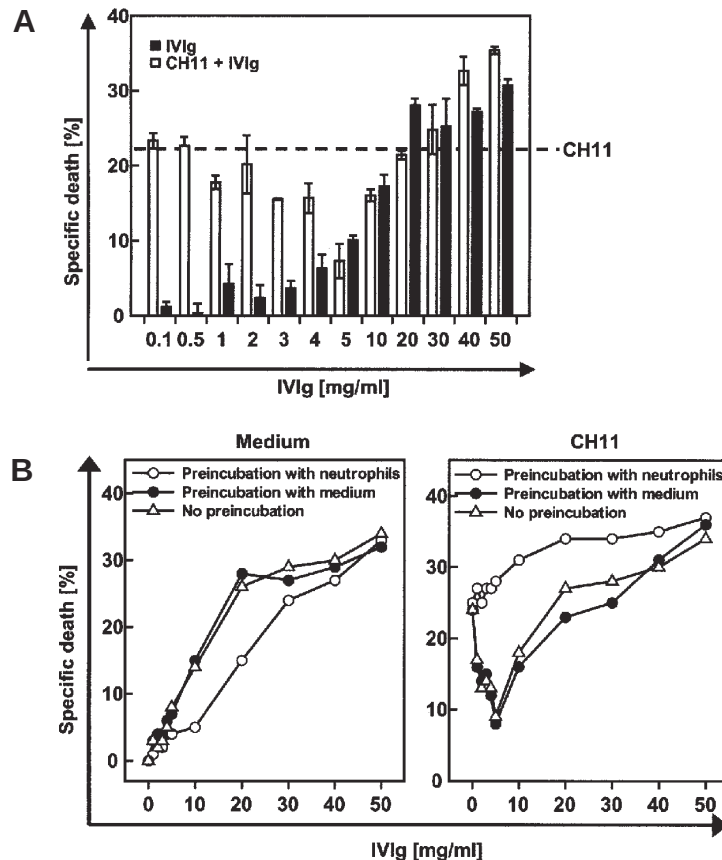


FIG 1. A, IVIg induced neutrophil death in a dose-dependent manner (black bars). However, low concentrations (1–10 mg/mL) of IVIg partially prevented anti-CD95 mAb (CH11)–mediated neutrophil death (white bars). The dashed line represents the mean level of neutrophil death specifically induced by CH11 treatment. Mean $\pm$ SEM are shown of 4 independent experiments (24-hour cultures). **B,** Pretreatment of IVIg with neutrophils (open circles) for 1 hour, but not medium (closed circles), partially removed the apoptosis-inducing autoantibodies (left panel) and completely allowed CH11-mediated neutrophil death (right panel). Compared to untreated IVIg (open triangles), preincubation with medium (closed circles) for 1 hour caused no significant differences in the apoptosis-modulating features of IVIg. Data are from 1 representative out of 4 independent experiments (24-hour cultures).

cells,^{13,16} was investigated in 6-hour neutrophil cultures (Fig 2). We observed inhibition of CH11-mediated apoptosis at low IVIg concentrations (1 to 10 mg/mL) and a dose-dependent induction of apoptosis in the absence of the CH11 mAb, confirming the data obtained with the ethidium bromide uptake test (Fig 1, A). The slightly higher IVIg concentrations required for the induction of apoptosis are likely the consequence of the much shorter culture period compared to the cell death assay. Washing the cells before CH11 treatment of the neutrophils had no effect on viability (Fig 1, A) or apoptosis (Fig 2). In additional experiments with the half-optimal concentration of CH11 (0.5 μ g/mL), we also obtained evidence for the presence of agonistic and antagonistic anti-CD95 autoantibodies in IVIg (data not shown).

Agonistic and antagonistic anti-CD95 autoantibodies recognize different epitopes

Together with the previously published work,^{2–4} the observation that low-dose IVIg blocks CH11-mediated neutrophil apoptosis in vitro suggested that the antagonis-

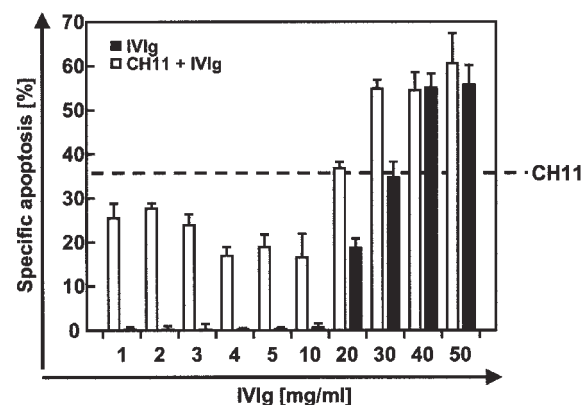


FIG 2. IVIg induced neutrophil apoptosis in a dose-dependent manner (black bars). However, low concentrations (1–10 mg/mL) of IVIg partially prevented anti-CD95 mAb (CH11)–mediated neutrophil apoptosis (white bars). The dashed line represents the mean level of neutrophil apoptosis specifically induced by CH11 treatment. Spontaneous apoptosis of neutrophils (baseline) was $25.0\% \pm 3.4\%$. Mean $\pm$ SEM are shown of 3 independent experiments (6-hour cultures).

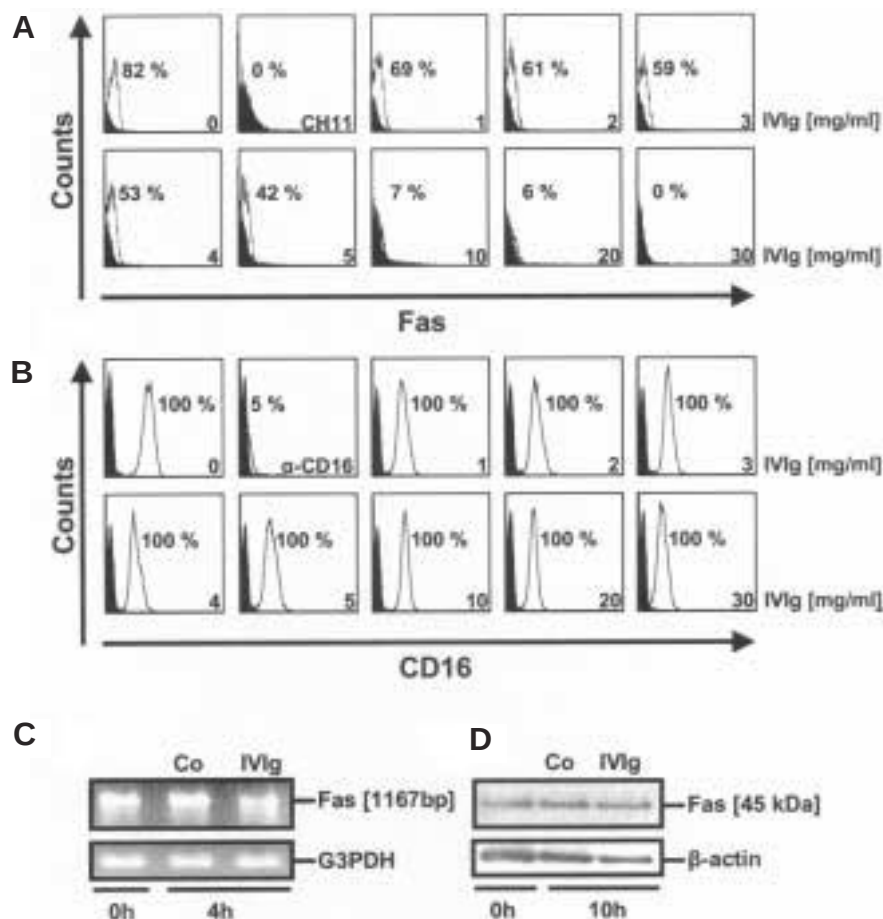


FIG 3. Inhibition of anti-CD95 mAb-mediated apoptosis is mediated by the presence of anti-CD95 receptor blocking autoantibodies present in IVIg and not the consequence of reduced CD95 receptor gene expression. **A**, IVIg dose-dependently inhibited FITC-UB2 staining of CD95 on neutrophils. **B**, IVIg did not prevent anti-CD16 staining of neutrophils, although the intensity of the staining appeared to be decreased at higher IVIg concentrations (greater than 30 mg/mL). Numbers in the right lower corner of the histograms represent the concentration of IVIg in mg/mL. **C**, CD95 receptor mRNA expression did not change in neutrophils cultured in the presence and absence (Co) of 30 mg/mL IVIg for 4 hours, as assessed by RT-PCR. **D**, CD95 receptor protein expression did not change in neutrophils cultured in the presence and absence (Co) of 30 mg/mL IVIg for 10 hours, as assessed by immunoblotting. Data represent at least 3 independent experiments in each panel.

tic anti-CD95 antibodies recognize the CH11 epitope. To obtain direct evidence for this assumption, we measured the binding of a FITC-conjugated anti-CD95 mAb (UB2) to CD95 on the surface of neutrophils in the presence and absence of different IVIg concentrations by flow cytometry. As a control, we added 1 μ g/mL CH11 mAb, which completely blocked binding of the UB2 mAb, suggesting that UB2 and CH11 target an identical epitope on the extracellular domain of CD95. IVIg also blocked UB2 binding in a dose-dependent manner (Fig 3, A). To exclude a potential unspecific IVIg effect, we tried to block binding of a specific mAb to CD16, which is another surface molecule expressed by neutrophils.¹⁷ Although IVIg was unable to prevent anti-CD16 mAb staining of neutrophils, slightly lower staining was observed at higher concentrations (greater than 30 mg/mL) (Fig 3, B). Thus, the data show that IVIg at the concentrations used does not gener-

ally prevent antibody binding to neutrophil surfaces, but the data also do not exclude the possibility that natural anti-CD16 autoantibodies are also present in IVIg.

Reduced CH11-mediated neutrophil apoptosis might also be the consequence of IVIg-mediated reduced Fas receptor expression. Culturing of neutrophils in the presence and absence of IVIg (range, 10 to 30 mg/mL) for up to 10 hours did not change Fas receptor mRNA (Fig 3, C) and protein (Fig 3, D) expression. Taken together, our data provide evidence that the partial block of CH11-mediated apoptosis is likely to be the consequence of antagonistic anti-CD95 autoantibodies present in IVIg that recognize the CH11 epitope.

The apoptosis-inducing effect of IVIg (up to 30 mg/mL) was inhibited by soluble CD95 (recombinant human Fas-Fc) (Fig 4, A) but not by soluble TNFR1 (recombinant human TNFR1-Fc) (data not shown).

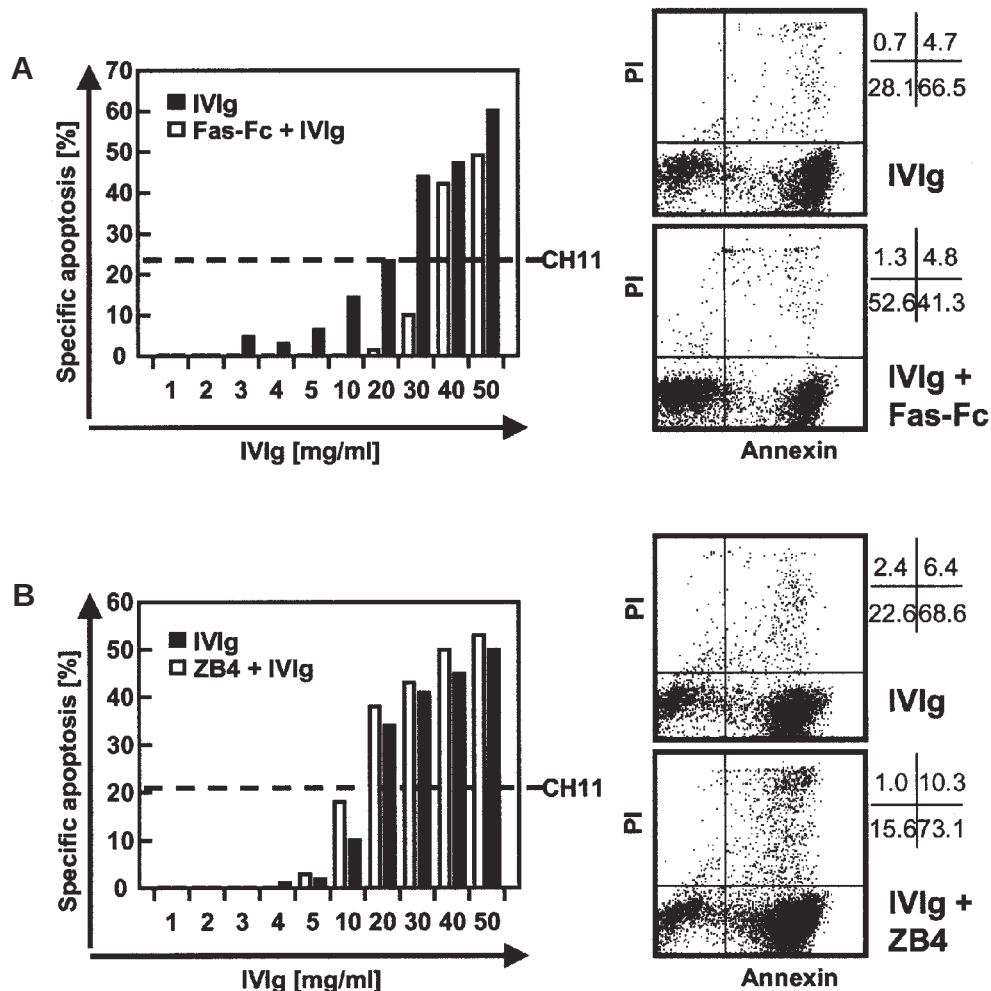


FIG 4. IVIg contains agonistic anti-CD95 receptor autoantibodies, which do not recognize the CH11 epitope. **A, Left:** Neutrophil apoptosis was dose-dependently induced by IVIg (black bars). Fas-Fc was able to block this effect up to IVIg concentrations of 30 mg/mL (white bars). The dashed line represents the level of neutrophil apoptosis specifically induced by CH11 treatment. Spontaneous apoptosis of neutrophils (baseline) was 22.3%. **Right:** Representative example of the apoptosis measurements (20 mg/mL IVIg). **B, Left:** In contrast to Fas-Fc, the CH11-blocking mAb ZB4 did not prevent IVIg-induced neutrophil apoptosis. ZB4 alone had no influence on the spontaneous apoptosis of neutrophils. The dashed line represents the level of neutrophil apoptosis specifically induced by CH11 treatment. Spontaneous apoptosis of neutrophils (baseline) was 29%. **Right:** Representative example of the apoptosis measurements (20 mg/mL IVIg). The quantitative analysis of these experiments is indicated at the upper right corner beneath each dot plot. Note that the numbers of annexin positive cells represent spontaneous plus IVIg-induced apoptosis. A total of 4 independent experiments are presented in this figure.

These data suggested that IVIg does not only contain anti-CD95 blocking but also agonistic anti-CD95 autoantibodies. In this system, however, Fas-Fc was no longer protective when 40 mg/mL or higher concentrations of IVIg were added, giving rise to the possibility that IVIg also contains other apoptosis-inducing autoantibodies, which do not recognize CD95. When we used an antibody (ZB4) that targets the same epitope as CH11 with higher affinity^{10,11} and completely blocks the pro-apoptotic effect of CH11 mAb^{10,11,18} instead of soluble CD95, the IVIg effects were not abrogated (Fig 4, B). Therefore, the agonistic anti-CD95 autoantibodies in IVIg appear to recognize an epitope(s), which differs from the epitope seen by the CH11 mAb.

DISCUSSION

There have been contrasting reports regarding anti-CD95 autoantibodies present in IVIg preparations. On the one hand, IVIg blocked CD95-mediated keratinocyte apoptosis possibly by the presence of antagonistic anti-CD95 autoantibodies.²⁻⁴ On the other hand, it was suggested that IVIg contains agonistic anti-CD95 autoantibodies able to induce apoptosis in lymphocytes and monocytes.^{5,6} By using neutrophils as target cells, we could clarify this apparent discrepancy in the literature and present evidence for the presence of both agonistic and antagonistic anti-CD95 autoantibodies within one IVIg preparation. Moreover, our study argues against the possibility that the

observed differences in the previous studies were due to the usage of different IVIg preparations.

The results of the current study have several implications for IVIg treatment, which is intended to target CD95. Clearly, besides toxic epidermal necrolysis,^{2,3} there are additional diseases (acquired immune deficiency syndrome, hepatitis, stroke, sepsis, graft-versus-host disease) in which unwanted CD95-mediated apoptosis plays a role in the pathogenesis.¹⁹ In diseases associated with immune hypersensitivity reactions, however, IVIg-mediated apoptosis via CD95 in inflammatory cells^{7,15,20} might be beneficial.

However, there are several issues that limit the enthusiasm for IVIg usage as primary therapeutic means in these diseases. First, the IVIg concentration required for the death-inducing effect in vitro is higher than the usual pharmacologic doses in vivo (1 to 10 mg/mL). Second, IVIg contains both agonistic and antagonistic anti-CD95 autoantibodies that could neutralize each other, resulting in marginal or no clinical efficacy. Clearly, the actual IVIg concentration and the CD95 sensitivity of the target cell likely determine the overall effect of such a mixed preparation of CD95 autoantibodies. Third, agonistic anti-CD95 autoantibodies might also cause unwanted additional effects when applied to patients. For instance, treatment with IVIg preparations has been associated with transient neutropenia²¹⁻²³ and with increased concentrations of liver enzymes in blood.²⁴

In spite of the difficulties in predicting the therapeutic value of IVIg preparations for an individual patient,²⁵ there is increasing evidence that they offer the possibility of modulating pathologic immune response by containing natural autoantibodies.¹ Because the balance between CD95 stimulating and blocking antibodies might vary among different IVIg preparations,²⁶ the neutrophil in vitro system reported in this study might be used as a bioassay to quantify CD95 autoantibodies. The anti-CD95 blocking antibodies have been shown to be clinically beneficial in diseases associated with unwanted CD95-mediated apoptosis. However, the concurrent presence of physiologic agonistic anti-CD95 autoantibodies in IVIg might be one of the limiting factors of the IVIg dose that can be used for a single patient.

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