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The Growth Arresting Effect of Human Immunoglobulin for Intravenous Use Is Mediated by Antibodies Recognizing Membrane Glycolipids

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Intravenous human IgG (IVIg) given to patients with autoimmune disorders can result in significant clinical improvement in some patients. The mechanism(s) by which IVIg induces these improvements is(are) not known. We have previously shown that IVIg inhibited the proliferation of peripheral blood lymphocytes in allogeneic mixed lymphocyte reactions and of autonomously growing human and mouse cell lines. In an effort to identify the antigen(s) to which the human IgG binds, the human B cell line JY, whose proliferation was inhibited by IVIg, was incubated with IVIg, washed extensively with PBS, and lysed. Human IgG from these lysates was purified by protein A–Sepharose ($IVIg^{JY}$). $IVIg^{JY}$ binds to and inhibits the proliferation of JY cells and of peripheral blood lymphocytes stimulated in a MLR at a 1000- to 10.000-fold lower concentration compared to IVIg. $IVIg^{JY}$ was analyzed on a 5–15% gradient SDS/PAGE and only immunoglobulin heavy- and light-chain (run under reducing conditions) proteins were detected. Immunoprecipitation experiments from JY cell lysates with $IVIg^{JY}$ indicated that this IgG did not bind to a protein epitope. Thin-layer immunoblot experiments showed that the $IVIg^{JY}$ binds to glycolipids expressed by JY cells and lymphocytes. Furthermore, evidence is presented indicating that antiglycolipid antibodies are involved in IVIg-induced growth inhibition.

KEY WORDS: Immunoglobulin for intravenous use (IVIg); plasma membrane glycolipids; mixed lymphocyte reaction; growth inhibition and thin-layer chromatography.

INTRODUCTION

High-dose polyvalent immunoglobulin G for intravenous use (IVIg), purified from pooled plasma from healthy blood donors, has been reported to be an effective treatment in a variety of autoimmune disorders (1–7). Although, the precise mechanism of action of IVIg is still far from clear, several possibilities have been suggested: inhibition of autoantibody production (8), neutralization of pathogenic antibodies (9–13), and inhibition of antibody-dependent cellular cytotoxicity by Fc-receptor blockade of macrophages (14). Furthermore, it has been shown that IVIg administration enhances $CD8^+$ suppressor T-cell function (15–17), inhibits pokeweed mitogen-induced antibody production *in vitro* (18–21), decreases NK cell function (22), modulates complement activation (23, 24), and interferes with the cytokine network (25–27). We have shown that IVIg inhibits the proliferation of hematopoetic cell lines and of peripheral blood lymphocytes (PBL) in an allogeneic mixed lymphocyte reaction (MLR) (28). This antiproliferative effect of IVIg was also found for primary human fibroblasts and myoblasts, in which the growth arrest was accompanied with upregulation of the expression of the growth arrest-specific (GAS)-3 gene (I. N. van Schaik *et al.*, submitted). How IVIg achieves growth inhibition is not clear. In immunofluorescence experiments we have shown that IVIg did bind to the surface of cells whose proliferation was inhibited (28). Growth inhibition was also induced in cell lines that do not express receptors for IgG (FcγR's) on their surface, arguing against the involvement of FcγR's. $F(ab)_2$ fragments were shown to be superior to Fc fragments in mediating growth inhibition (29). Although intact IgG was more effective than $F(ab)_2$ fragments, the fact that $F(ab)_2$ fragments and not Fc fragments were the effective part of the polyclonal IgG suggests that an antigen(s) expressed by the cell line(s) may be involved in delivering a growth inhibitory signal. The main purpose of this study was to purify the fraction of IgG molecules from IVIg responsible for the growth inhibitory effect. In this paper we present results that

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show that IVIg contains IgG molecules that bind to glycolipids expressed by JY cells and PBL and that effectively inhibit the proliferation of these cells.

MATERIALS AND METHODS

Cells, Reagents, and Medium

Human peripheral blood lymphocytes (PBL) were obtained from buffy coats of healthy blood donors by Ficoll–Hypaque density centrifugation. The cells were used immediately or after cryopreservation in liquid nitrogen in a suspension containing 10% dimethylsulfoxide (Fluka, Brussels, Belgium). Cells were cultured in RPMI 1640 (GIBCO, Breda, The Netherlands) supplemented with 15% (v/v) heat-inactivated (30 min, 56°C) pooled human serum. The mouse hybridoma W6/32, which secretes an anti-HLA class I-specific monoclonal antibody (mAb), was obtained from the ATCC (Rockville, MD). The human EBV-transformed cell line JY was kindly provided by Dr. J. Borst, Netherlands Cancer Institute, Amsterdam. Bulk cultures of JY were performed in roller bottles in RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum (GIBCO). The hybridoma cells were grown in serum-free medium (GIBCO) and the mAb was obtained by concentrating the culture supernatant. SDS/PAGE analysis of a W6/32 mAb preparation showed that it contained >95% IgG. A mAb specific for human IgG was purchased from Beckton–Dickinson (San Jose, CA). Freeze-dried IVIg was obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam). Myeloma IgG was purified from the serum of a multiple myeloma patient by protein G–Sepharose according to the method described below. The gangliosides GM1, GM2, AGM1, AGM2, CTH, CDH, GQ1b, GT1b, GD1a, GD1b, Forssman, globoside, and galactocerebroside were purchased from Biocarb Chemicals (Lund, Sweden). Polyclonal anti-HLA-A_2-reactive human serum was kindly provided by Dr. A. Mulder, Academical Hospital Leiden, The Netherlands. All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Purification and SDS-PAGE Analysis of Human IgG from IVIg that Binds to JY Cells

JY cells (5×10^9) were incubated for 60 min at 4°C with IVIg (0.5×10^9 cells/ml, 30 mg IVIg/ml). Subsequently, the cells were extensively washed with phosphate-buffered saline (PBS) to remove nonbound IgG and lysed in lysis buffer (1×10^8 cells/ml; 1% NP-40, 50 m*M* Tris, 150 m*M* NaCl, 1 m*M* PMSF, 1 μg/ml pepstatin, 0.13 TIU/ml aprotinin, pH 8.0). Cell lysates were cleared of NP-40-nonsoluble material by centrifugation (30 min, 15,000 rpm; Beckmann Ultracentrifuge). The lysate was precleared twice at room temperature (rt) with CL-4B/Sepharose (Pharmacia, Uppsala, Sweden; 5-ml bead volume, 60 min, tumbling). IgG present in the lysate was purified by incubating the lysate o/n with 5 to 10-ml-bead volume protein A/Sepharose (Pharmacia) at 4°C, tumbling. Bound IgG on the protein A/Sepharose was eluted with 0.1 *M* glycine buffer pH 2.5. The eluate was neutralized with 2 *M* Tris buffer, pH 8.0, and concentrated in PBS to a final concentration of 2–3 mg/ml. This purified IgG ($IVIg^{JY}$) was analyzed on 5–15% gradient SDS-PAGE and stained with the silver stain reagents (Pharmacia) according to the manufacturer's directions. The $IVIg^{JY}$ was aliquoted and stored at 4°C for immediate use or for an extended time period at −20°C. Myeloma IgG was purified from the serum of a multiple myeloma patient by applying the serum to a protein G/Sepharose column (Pharmacia) at rt. Bound IgG was eluted from this column with 0.1 *M* glycine buffer, pH 2.7. The eluate was neutralized with 1 *M* Tris buffer (pH 8.0) and the myeloma IgG was freeze-dried and stored at −20°C until use.

Immunofluorescence Staining of Plasma Membrane Antigens

Cells (0.5×10^6) were incubated with appropriate concentrations of antibodies (IVIg, 30 mg/ml; IVIgJY, 30–0.3 μg/ml; polyclonal human anti-HLA-A_2 antiserum, 1/4 dilution) in a total volume of 50 μl at 4°C for 30 min. After each step the cells were washed twice with ice-cold PBS supplemented with 0.5% (w/v) bovine serum albumin (BSA; Sigma). Bound first-step human IgG was detected with FITC-labeled goat anti-human Ig (Nordic, Tilburg, The Netherlands). Stained cells were analyzed by FACScan (Beckton–Dickinson).

Proliferation Inhibition Experiments

An allogeneic mixed lymphocyte culture (MLR) was set up with peripheral blood lymphocytes (PBL) from two unrelated donors. The responder and stimulator PBL were mixed 1:1 and cultured (1×10^5 cells/well) in 96-well flat-bottom microtiter plates (Greiner). JY cells were seeded at 5×10^3 cells/well. Unfractionated IVIg was added at 30–0.001 mg/ml, and $IVIg^{JY}$ at 20–0.001 μg/ml. In addition, experiments that included $IVIg^{JY}$, which was absorbed to glycolipids isolated from JY

cells, were performed. The isolation of JY glycolipids and the adsorption procedure are described below. The supernatant of IVIg after incubation with JY cells was also tested at 30–0.01 mg/ml. Antibodies were added to the culture at the start of the experiment and remained present in the culture medium for the duration of the test. Control cultures were performed in culture medium without the addition of IVIg ($IVIg^{JY}$) or with the addition of control myeloma IgG (at 10 mg/ml). The final volume in each well was 150 μl. Culture plates were incubated in humidified air containing 5% (v/v) CO_2 at 37°C for 3 days. For the last day of culture 1 μCi ^{3}H-thymidine was added to each well of the culture plate, in a volume of 50 μl, and 20 hr later the cells were harvested on a filter and liquid scintillation counting was performed in a β-plate counter (LKB, Wallac 1205).

Immunoprecipitation from ^{125}I Surface-Labeled or ^{35}S-Methionine/Cysteine Metabolically Labeled JY Cells

JY cells (10×10^6) were surface labeled with 1 mCi $Na^{125}I$ (Amersham) using lactoperoxidase enzyme (Sigma) according to standard procedures or metabolically labeled with ^{35}S-methionine by incubation of 0.5×10^6 cells/ml with 5 μCi ^{35}S-methionine/cysteine (Amersham) in methionine/cysteine-free medium (GIBCO) for 5 hr at 37°C. After labeling, the cells were washed with PBS and lysed on ice for 60 min in 1% NP-40, 50 m*M* Tris, 150 m*M* NaCl, 1 m*M* PMSF, 1 μg/ml pepstatin, 0.13 TIU/ml aprotinin, pH 8.0, lysis buffer. Nonsoluble material was removed by centrifugation for 15 min at 15,000 rpm and 4°C in an Eppendorf centrifuge. Lysates were precleared 4× with 50-μl packed protein A/Sepharose beads. Immunoprecipitation (IP) was performed with 25 μl packed protein A/Sepharose with normal mouse serum (NMS; 10 μl) as a negative control and anti-HLA class I mAb W6/32 (10 μg) as a positive control. Test immunoprecipitation was done with 10 μg $IVIg^{JY}$. In some experiments a monoclonal antibody specific for human IgG was added (10 μg) in addition to the 10 μg $IVIg^{JY}$. After incubating the protein A/Sepharose antibody mixtures for 1 hr at rt, the beads were extensively washed with lysis buffer. The protein A/Sepharose beads were finally resuspended in sample buffer (SB) and boiled for 5 min. Subsequently, the beads were removed by centrifugation and the supernatants were run under reducing and nonreducing conditions on a 5–15% gradient SDS-PAGE. The gel was dried and exposed to X-ray film (Kodak) for 1–8 days at −70°C.

Isolation of Glycolipids from JY Cells and PBL and Adsorption of $IVIg^{JY}$ to Glycolipids from JY Cells

The gangliosides expressed at the surface of JY cells and PBL were isolated according to a procedure published previously (30). Briefly, cells were resuspended in 200 μl milli-Q purified water (aqua dest), and 750 μl methanol and 360 μl chloroform were subsequently added. This mixture was incubated for 30 min on a rollerbank, then centrifuged for 30 min at 2500 rpm, and the supernatant was collected (sup 1). The pellet was resuspended in 200 μl aqua dest and 800 μl chloroform/methanol mixture (1:2). This mixture was incubated for 30 min, subsequently centrifuged, as specified above, and supernatant was collected (sup 2). Sup 1 and sup 2 were pooled and aqua dest was added so that the final composition of the solution was chloroform:methanol:aqua dest = 1:2:1.4. The final solution was carefully mixed and allowed to separate (usually separation took 6 h). After separation the upper phase was collected (UF 1), and to the lower phase 200 μl methanol and 120 μl 0.01 *M* KCl were added. This was allowed to separate, and again the upper phase was collected (UF 2). UF 1 and UF 2 were mixed and freeze-dried. Freeze-dried glycolipids were redissolved in chloroform:methanol (1:2). For adsorption experiments 1 ml glycolipids from 100×10^6 cells dissolved in chloroform/methanol was added to a well of a 24-well tissue culture plate and dried. Subsequently, the well was extensively rinsed with PBS, and $IVIg^{JY}$ (3 μg/ml) was incubated in this glycolipid-coated well for 1 hr at rt. The negative control was $IVIg^{JY}$ incubated for 1 hr in a well not coated with the glycolipids. $IVIg^{JY}$ adsorbed in this way was tested for growth inhibition according to the method described above.

Thin-Layer Chromatographic Analysis of the Binding of $IVIg^{JY}$ to Glycolipids

Ten micrograms of the purified glycolipids was applied to plastic-backed, polygram Silica G, 0.25-mm thin-layer (TLC) plates of 8 × 8 cm (Machery-Nagel, Düren, Germany). A standard mixture of neutral glycolipids and a crude glycolipid mixture for thin-layer chromatography were purchased from BioCarb Chemicals (Lund, Sweden). The TLC plates were run at rt with 60:53:8 (v/v/v) chloroform–methanol–0.2% $CaCl_2$. After separation the glycolipids were visualized on the TLC plates with 0.1% naphthoresorcinol (Sigma, Deisenhof, Germany) in 20% H_2SO_4, diluted 1:1 in ethanol. For immunostaining the plates were air-dried and treated with 0.05% polyisobutylmethacrylate (Sigma) in hexane

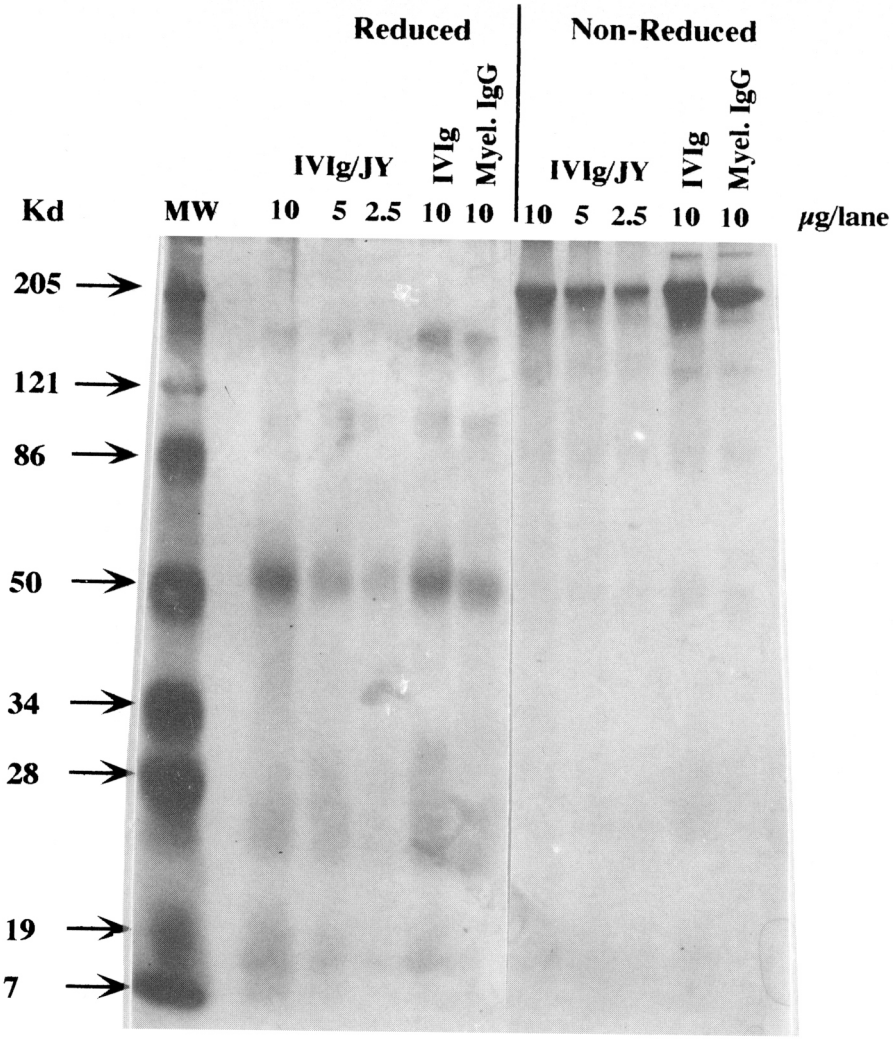

Fig. 1. SDS/PAGE analysis of $IVIg^{JY}$: 10, 5, or 2.5 μg IVIgJY was run on 5–15% SDS/PAGE under reducing conditions (left) and under nonreducing conditions (right). Unfractionated IVIg and protein A/Sepharose-purified myeloma IgG were run at 10 μg/lane. Proteins in the gel were visualised using a silver staining procedure (Farmacia).

for 1 min. After drying, the plates were folded in 50-ml tubes (Greiner) and placed on a roller apparatus for 1 hr at rt with 50 m*M* Tris, 0.14 *M* NaCl (pH 7.4), and 3% (w/v) nonfat dry milk (Oxoid Ltd, Bastingstoke, Hampshire, England). The plates were subsequently washed with PBS and antibody solutions of various concentrations (as specified in the figures) were added in PBS supplemented with 0.5% (w/v) BSA and 0.1% (w/v) NaN_3 (PBS/BSA). After an overnight incubation the plates were washed and bound human IgG was detected by incubation with biotinylated goat anti-human IgG followed by streptavidin/HRP. After each antibody/conjugate incubation the plates were washed 5× with 10 ml PBS. Subsequently, the plates were developed for 30 min in the dark with a freshly prepared solution of 0.56% (w/v) chloro-4-naphthol (Sigma). After development, the reagent solution was removed and the plates were washed in aqua dest.

RESULTS

In five independent experiments the IgG fraction in IVIg that binds to JY cells ($IVIg^{JY}$) was purified and the average yield was 1.78 ± 0.2 mg IgG/5 × 10^9 cells. The cells were incubated with a total amount of 300 mg IVIg, and of this 0.6% did bind to 5 × 10^9 cells (ca. 1.5 × 10^6 molecules of IgG bound/cell). This $IVIg^{JY}$ was analyzed, under reducing and nonreducing conditions, on 5–15%

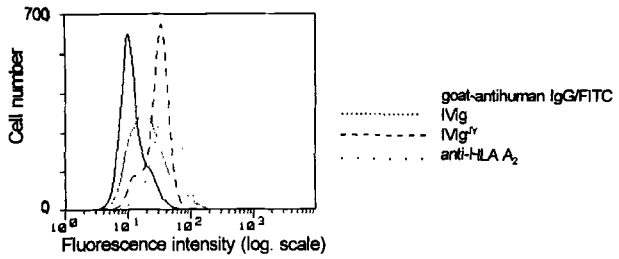

Fig. 2. FACScan analysis of $IVIg^{JY}$ binding to JY cells: 0.5×10^6 JY cells were incubated with 50 μl IVIgJY solution (- - - -; 10 μg/ml), IVIg (·····; 30 mg/ml), or anti-HLA-A2 serum (· · · · · · · ; 1/4 diluted) for 30 min at 4°C. Subsequently, the cells were washed and incubated with goat anti-human IgG-FITC (1/100 diluted). Nonbound FITC conjugate was removed by washing. A negative control was performed by omitting the first antibody incubation and subsequent staining with goat anti-human IgG-FITC (----------).

gradient SDS/PAGE. Proteins in the gel were visualized with a sensitive silver staining procedure (Fig. 1). The $IVIg^{JY}$ contained >95% IgG protein and no major other proteins could be identified. With unfractionated IVIg or monoclonal myeloma IgG, the only protein that was detected was also IgG. IgG subclass analysis of $IVIg^{JY}$ showed that all IgG subclasses were present. Moreover, the relative subclass composition was the same as that of the IgG fraction of normal human serum (66% IgG_1, 23% IgG_2, 7% IgG_3, 4% IgG_4).

The binding of $IVIg^{JY}$ to JY cells was next demonstrated with an immunofluorescence experiment using a FITC-labeled goat anti-human IgG to detect bound human IgG. The results of a representative experiment are shown in Fig. 2. The result of using $IVIg^{JY}$ at the optimum concentration of 10 μg/ml is shown in Fig. 2. Compared to unfractionated IVIg, 3000-fold less $IVIg^{JY}$ was needed to obtain maximal binding. Note that even at the optimal concentration of IVIg (30 mg/ml), the immunofluorescence signal was significantly lower and the peak broader than that obtained with $IVIg^{JY}$. Human serum that contains polyclonal anti-HLA-A_2 antibodies was included as a positive control and IgG from this serum clearly bound to the HLA-A_2^+ JY cells.

The capacity of $IVIg^{JY}$ to inhibit the proliferation of JY cells and of PBL in an allogeneic MLR was tested in a ^{3}H-thymidine incorporation experiment (Fig. 3). The results of this experiment clearly show that $IVIg^{JY}$ inhibited the proliferation of both JY and PBL in a dose-dependent way. Interestingly, $IVIg^{JY}$ completely inhibited the growth of the target cells at a concentration 3–4 logs lower than unfractionated IVIg. In this experiment the IVIg solution, after incubation with the JY cells, was also included and it is clear that is proliferation inhibition was not significantly different from that of unfractionated IVIg. This suggests that with 5×10^9 JY cells, only a fraction of the IgG molecules responsible for growth inhibition was removed from the IVIg solution containing a total of 300 mg IgG.

In an effort to identify the antigen(s) expressed by JY cells to which $IVIg^{JY}$ binds, immunoprecipitation experiments were performed with lysates from ^{125}I surface-labeled (three experiments) or ^{35}S-methionine/cysteine metabollically-labeled (three experiments) JY cells. Since it was completely unclear what the size(s) would be of the putative protein antigen(s), the immunoprecipitation samples were run, under reducing or nonreducing conditions, on a 5–15% SDS-PAGE gradient gel. Despite the fact that $IVIg^{JY}$ was purified based on its binding to protein A/Sepharose, it is possible that the binding affinity for protein A may be too low to be able to immunoprecipitate it complexed to its antigen. Therefore, in the immunoprecipitation experiments $IVIg^{JY}$ was used not only alone but also in combination with a mouse mAb against human IgG (Becton–Dickinson). This mAb was successfully used in immunoprecipitation experiments, indicating that it binds with sufficient affinity to protein A. In these experiments, however, it was never possible to immunoprecipitate any protein using $IVIg^{JY}$ alone or with anti-human IgG mAb added (data not shown). As a positive control in these experiments the anti-HLA class I mAb W6/32 was used, and with this mAb it was always possible to immunoprecipitate HLA heavy- and light-chain proteins.

The glycolipids expressed by JY cells and PBL were isolated and fractionated by thin-layer chromatography (TLC). The binding of IgG from IVIg or $IVIg^{JY}$ was tested on the TLC-fractionated glycolipids (Fig. 4). Direct staining with naphthoresorcinol revealed two distinct major bands in the JY and PBL preparations. One band ran just below AGM3 (Galb1–4Glcb1–ceramide) but above CTH (Gala1–4Galb1–4Glcb1–ceramide), while the other band ran in between CTH and globoside (GalNacb1–3Gala1–4Galb1–4Glcb1–1Cer). The latter band clearly bound $IVIg^{JY}$, while also with unfractionated IVIg, staining, although faint, could be seen. Furthermore, the JY and PBL preparations also contained a small amount of glycolipid, which appeared in the TLC plate at the position of galactocerebroside (Galb1–1ceramide), which was just visible after direct staining of the TLC plate. $IVIg^{JY}$ and, to a much lesser extent, unfractionated IVIg bound to this glycolipid. Neither $IVIg^{JY}$ nor IVIg bound to the glycolipids present in the standard mixture and the myeloma IgG did not bind to any of the glycolipids extracted from the cells. In order to test directly whether the antiglycolipid IgG was responsible for the $IVIg^{JY}$-induced growth inhibition, it

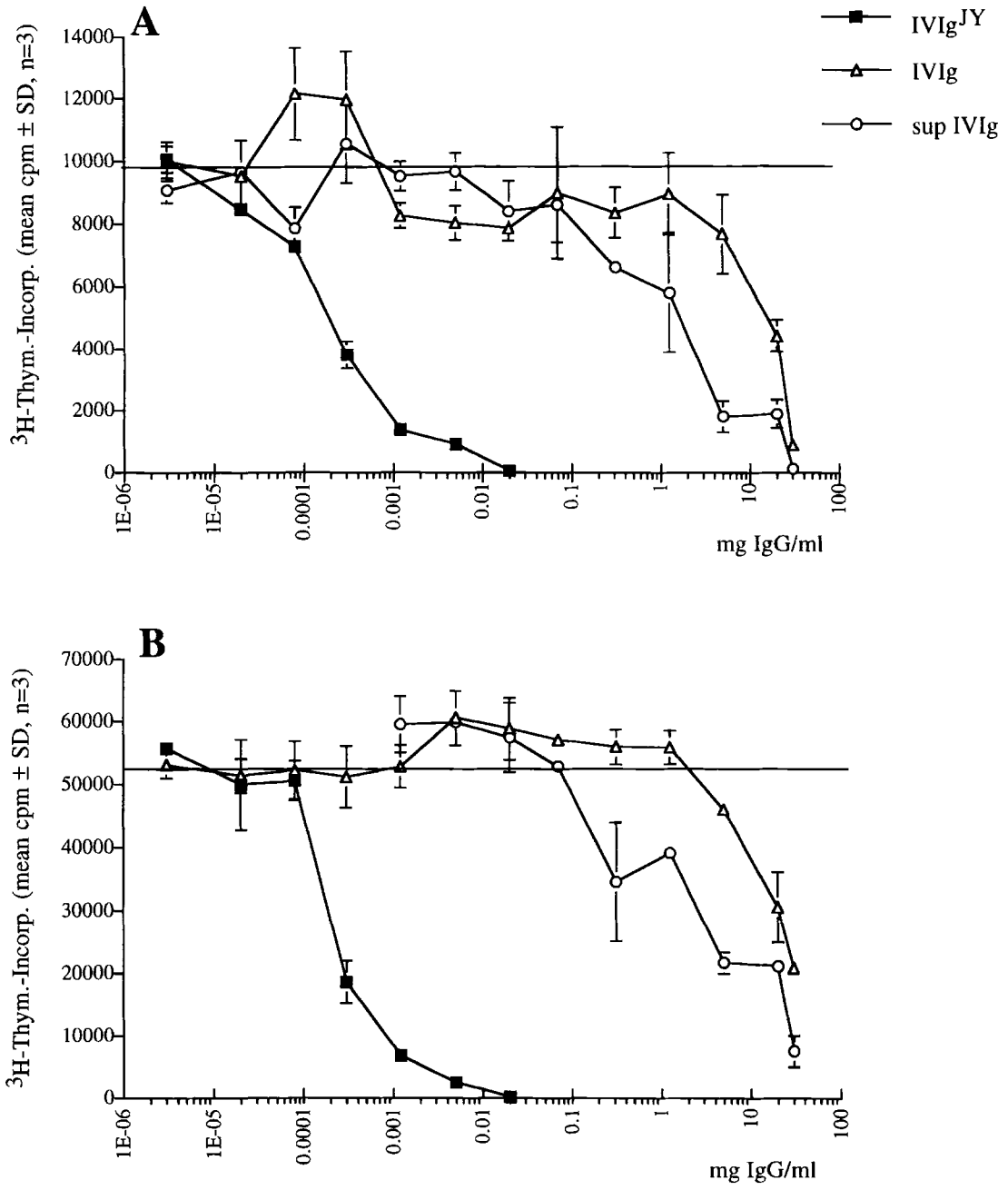

Fig. 3. $IVIg^{JY}$ inhibition of the proliferation of JY cells and lymphocytes in an allogeneic MLR. Density gradient-purified PBL from two unrelated donors were mixed 1:1 in culture medium and seeded in the wells of a 96-well tissue culture plate at 1×10^5 cells/well (A). Proliferating JY cells were harvested from an *in vitro* culture, washed once with medium, and seeded in the wells of a 96-well tissue culture plate at 1×10^3 cells/well (B). $IVIg^{JY}$, IVIg (unfractionated), and IVIg sup (after incubation with JY cells) were added to the appropriate wells at the indicated concentrations. Negative controls were performed with only culture medium or with the IgG purified from the serum of a multiple myeloma patient (10 mg/ml). After 2 days of culture at 37°C, in humidified air containing 5% (v/v) CO_2, 1 μCi of ^{3}H-thymidine was added to each well and culturing was continued for another 24 hr. Subsequently, the cells were harvested and processed for ^{3}H-thymidine incorporation. Indicated are the mean ± SD cpm of triplicate cultures. ^{3}H-Thymidine incorporation of control cultures (without IgG or with myeloma IgG) is indicated by the horizontal line in both graphs.

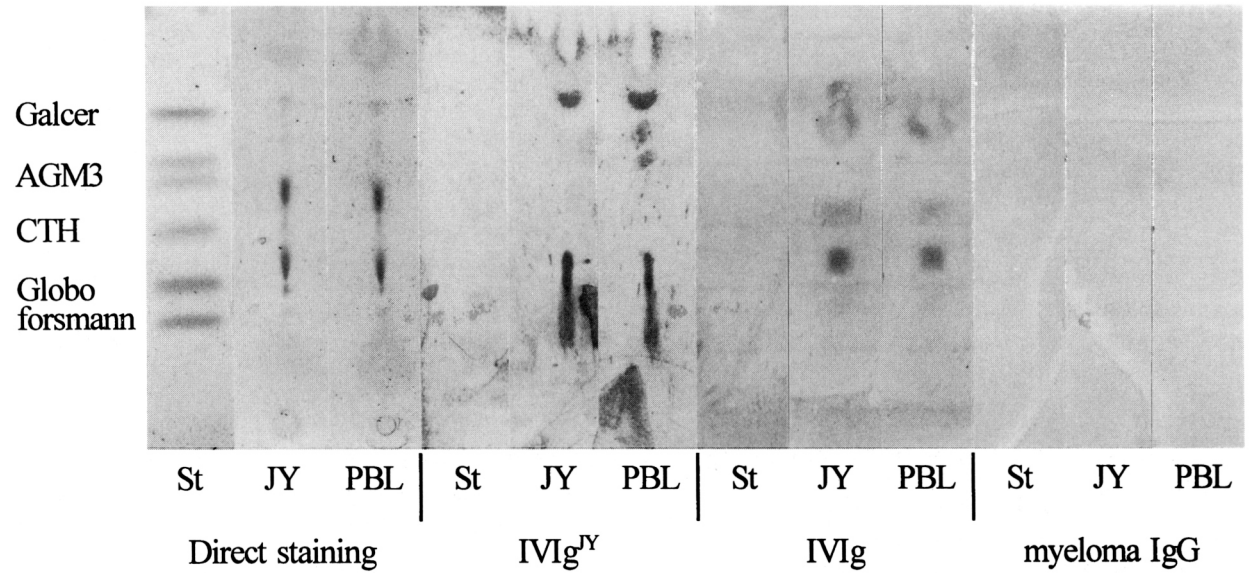

Fig. 4. Binding of $IVIg^{JY}$ and IVIg to glycolipids on thin-layer chromatography (TLC) plates. The first lane (St) in each panel contains a standard mixture of neutral glycosphingolipids: GalCer, galactocerebroside; AGM3, galactose (Gal) 1–4-glucose (Glc) 1–1-ceramide (Cer); CTH, Gal 1–4Gal 1–4Glc 1–1Cer (trihexosylceramide); globo, galactosamine (GalNac) 1–3Gal 1–4Gal 1–4Glc 1–1Cer (globoside); Forssman, GalNac 1–3GalNac 1–3Gal 1–4Gal 1–4Glc 1–1Cer. The second and third lanes in each panel contain glycolipids which were isolated from JY cells and PBL as described under Materials and Methods. Ten micrograms of each was applied to plastic-backed, silica gel, thin-layer plates. The plates were developed at room temperature with chloroform–methanol–0.2% $CaCl_2$, 60:53:8 (v/v/v). The glycolipids in the first panel (direct staining) were visualized with 20% H_2SO_4 and 0.1% naphthoresorcinol diluted in ethanol 96%, 1:1. The other panels were incubated overnight with $IVIg^{JY}$ (20 μg/ml), IVIg (0.6 mg/ml), or myeloma IgG (20 μg/ml) and immunostained with biotinylated goat anti-human IgG followed by streptavidin/HRP.

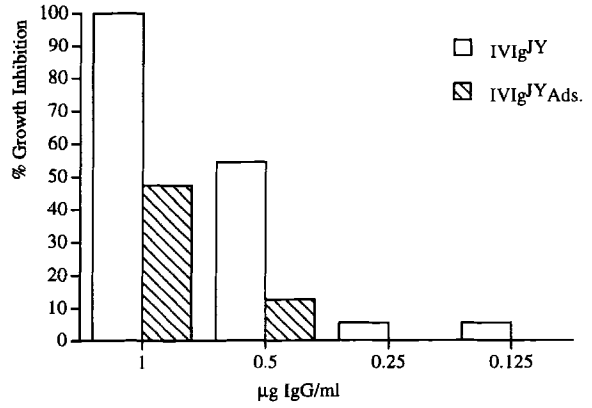

Fig. 5. Growth-inhibitory activity of $IVIg^{JY}$ is diminished by depleting antiglycolipid IgG. JY cells were harvested from a growing culture, washed once with medium, and seeded in the wells of a 96-well tissue culture plate at 1×10^3 cells/well. $IVIg^{JY}$ incubated in a well not coated with glycolipids ($IVIg^{JY}$) or $IVIg^{JY}$ adsorbed to glycolipids from JY cells ($IVIg^{JY}$ ads) was added to the appropriate wells at the indicated concentrations. After 2 days of culture at 37°C, in humidified air containing 5% (v/v) CO_2, 1 μCi of ^{3}H-thymidine was added to each well and culturing was continued for another 20 h. Subsequently, the cells were harvested and processed for ^{3}H-thymidine incorporation. Indicated is the percentage growth inhibition relative to the growth inhibition obtained with control $IVIg^{JY}$ of duplicate cultures. Maximal ^{3}H-thymidine incorporation in this experiment was 86,753 ± 12,867 cpm (n = 16).

was adsorbed to a well that was coated with the glycolipids from JY cells. The result of the proliferation experiment is given in Fig. 5. The adsorption of $IVIg^{JY}$ to the glycolipids diminished its ability to inhibit the proliferation of JY cells by approximately 50%. After one adsorption step the growth inhibition was not completely abolished, suggesting that not all the antiglycolipid antibodies were removed.

DISCUSSION

The mechanism by which IVIg inhibits the growth of cell lines and of lymphocytes in an allogeneic MLR is still obscure. Previous data from our laboratory indicated that the IVIg-induced growth inhibition was mediated by the binding of IgG to cells whose proliferation was inhibited (28). Therefore, experiments were performed to purify and characterize the IgG from IVIg that is responsible for growth inhibition. In this paper we present results that point to glycolipids as the target antigens on cells whose proliferation is inhibited by IVIg.

Our results show that $IVIg^{JY}$ (a) was >95% pure IgG, (b) bound to JY cells, (c) did not immunoprecipitate a protein antigen from JY cells, (d) inhibited the growth of

cell line cells and of lymphocytes in a MLR, and (e) contained antiglycolipid antibodies which were involved in the growth inhibition. The purification of $IVIg^{JY}$ proved to be very reproducible and SDS/PAGE analysis of this material on a 5–15% gradient gel showed that it comprised >95% IgG (Fig. 1). Importantly, this analysis did not show a protein antigen which might still be bound to the IgG since the isolation procedure would not necessarily break the IgG/antigen interactions. Immunofluorescence experiments (Fig. 2) showed that $IVIg^{JY}$ stained JY cells much more efficiently than did IVIg. Optimal staining with $IVIg^{JY}$ was achieved at 10 μg/ml (3000-fold lower concentration than needed with IVIg), was more intense, and resulted in a sharper peak than staining with an optimal concentration of 30 mg/ml IVIg. The reason for the more efficient staining with $IVIg^{JY}$ is not clear. However, this result again suggests, as did the SDS/PAGE analysis, that no antigen remained bound to the $IVIg^{JY}$ since this would prevent it from binding its antigen on JY cells. The results of the proliferation experiment show (Fig. 3) that $IVIg^{JY}$ inhibited the growth of JY cells and of PBL in an MLR at 3–4 log lower concentrations compared to unfractionated IVIg. In this experiment the IVIg solution after incubation with JY cells was also included and the growth inhibitory activity was still present at the same level as in the preabsorbed IVIg solution. This clearly indicates that the adsorption experiments were performed with an excess of IVIg. These results confirm previous findings from our and other laboratories with regard to IVIg-induced growth arrest (28, 29, 31–33). The growth inhibitory activity of $IVIg^{JY}$ was also observed on a $CD4^+$ T-cell clone which was isolated from the joint of a rheumatoid arthritis patient and which proliferated in response to stimulation with the exudate from an arthritic joint (M. Maurice and C. Verwey, personal communication).

Immunoprecipitation experiments with $IVIg^{JY}$ showed that it was not possible to immunoprecipitate a protein antigen from either ^{125}I surface-labeled or ^{35}S-methionine metabolically labeled JY cells (data not shown). With the control monoclonal anti-HLA class I antibody W6/32, it was always possible to immunoprecipitate heavy- and light-chain HLA class I molecules from labeled JY cell lysates. The immunoprecipitation experiments were also performed with the addition of a monoclonal antibody to human IgG that binds to protein A with a high affinity. However, this also did not result in a positive immunoprecipitation. The negative results, obtained in six independent immunoprecipitation experiments, suggest that $IVIg^{JY}$ does not bind to a protein antigen expressed by JY cells.

There is increasing evidence that glycolipids expressed by lymphocytes and nonhematologic cell lines may be involved in transmembrane signal transduction and lymphocyte activation (5, 34, 35). The expression of asialo-GM1 of lymphocytes, thymocytes, and natural killer cells has been shown to be correlated with activation and reflect a change in the level of exposure and glycolipid sialylation rather than a change in membrane lipid composition (34–39). With thin-layer chromatography, $IVIg^{JY}$ and also, although to a lesser extend, IVIg were shown to bind to two unidentified neutral glycolipids which ran between CTH and globoside and one which ran at the same position as did galactocerebroside (Fig. 4). In a proliferation experiment in which $IVIg^{JY}$ was used after adsorption to glycolipids isolated from JY cells, it could be demonstrated that removal of antiglycolipid antibodies from the $IVIg^{JY}$ solution also diminished the growth-inhibitory effect (Fig. 5). However, using this method it was not possible to remove all growth-inhibitory activity. An explanation for this result could be that the binding affinity of the IgG for isolated glycolipids is lower compared to the affinity for the native membrane-bound glycolipids. This would make it difficult to remove all antiglycolipid IgG. An alternative explanation would be that not only antiglycolipid antibodies are responsible for growth inhibition but also that antibodies with so far unidentified specificities, albeit nonprotein, are involved.

Our results point to a possible mechanism by which IVIg may inhibit the proliferation of cell line cells and of primary lymphocytes in a MLR. The growth-inhibitory effect of IVIg may also be operative in achieving immunosuppression, which is a well-documented effect of IVIg (5, 6, 29). However, we do not postulate that IVIg binding to glycolipids on the surface of lymphocytes and thereby exerting a growth inhibitory effect is the only mechanism by which IVIg achieves its beneficial effect in the treatment of autoimmune disorders. It is even conceivable that in different autoimmune diseases in which IVIg has been shown to be beneficial, different mechanisms or different combinations of mechanisms may be operative. Nevertheless, our data are the first to show an antigen target expressed by lymphocytes for IVIg. The possible signal transduction mediated by $IVIg^{JY}$ through surface glycolipids will have to be addressed in future experiments.

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