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Human immunoglobulin G reduces the pathogenicity of aquaporin-4 autoantibodies in neuromyelitis optica

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ABSTRACT

Neuromyelitis optica (NMO) pathogenesis involves binding of anti-aquaporin-4 (AQP4) autoantibodies (NMO-IgG) present in serum to AQP4 on astrocytes, which causes complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Human immunoglobulin G (hIgG) is effective for treatment of humorally mediated neurological autoimmune diseases and has been reported to improve disease outcome in a limited number of NMO patients. Here, we investigated hIgG actions on NMO-IgG pathogenicity using an *in vivo* rat model of NMO and *in vitro* assays. In rats administered NMO-IgG by intracerebral injection, the size of neuroinflammatory demyelinating lesions was reduced by ~50% when hIgG was administered by intraperitoneal injection to reach levels of 10–25 mg/mL in rat serum, comparable with human therapeutic levels. *In vitro*, hIgG at 10 mg/mL reduced by 90% NMO-IgG-mediated CDC following addition of NMO-IgG and human complement to AQP4-expressing cells. The hIgG effect was mainly on the classical complement pathway. hIgG at 10 mg/mL also reduced by up to 90% NMO-IgG-mediated ADCC as assayed with human natural killer cells as effector cells. However, hIgG at up to 40 mg/mL did not affect AQP4 cell surface expression or its supramolecular assembly in orthogonal arrays of particles, nor did it affect NMO-IgG binding to AQP4. We conclude that hIgG reduces NMO-IgG pathogenicity by inhibition of CDC and ADCC, providing a mechanistic basis to support further clinical evaluation of its therapeutic efficacy in NMO.

Introduction

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system (CNS) causing optic neuritis and transverse myelitis. A defining feature of NMO is the presence in serum of anti-aquaporin-4 (AQP4) autoantibodies (NMO-IgG) that bind to water channel AQP4 on astrocyte end-feet (Lennon et al., 2005). *In vitro* assays and animal models suggest that NMO pathogenesis involves NMO-IgG binding to AQP4 on astrocytes causing complement-dependent cytotoxicity (CDC), which is supported by findings of early loss of AQP4 and GFAP in human NMO lesions with perivascular immunoglobulin and complement deposition (Lucchinetti et al., 2002; Misu et al., 2007). The primary astrocyte cytotoxicity results in blood–brain barrier disruption, recruitment and degranulation of inflammatory cells (granulocytes and macrophages), and secondary oligodendrocyte injury and myelin loss (Papadopoulos and Verkman, 2012). Intracerebral injection in mice of NMO-IgG and human complement (Saadoun et al., 2010), or in rats of NMO-IgG alone (Asavapanumas et al., 2014), produces NMO-like pathology with astrocyte cytotoxicity, complement deposition, inflammation and demyelination. A significant role of antibody-dependent cellular cytotoxicity (ADCC) has also been demonstrated in NMO, as mice administered a mutated NMO-IgG lacking ADCC effector function showed reduced pathology as did mice treated with a Fcγ receptor (FcγR) blocking antibody (Ratelade et al., 2013).

The therapeutic efficacy of human immunoglobulin G (hIgG) administered intravenously was first reported in 1981 in the autoimmune disease idiopathic thrombocytopenic purpura (ITP) (Imbach et al., 1981). hIgG has since been used for the treatment of a broad range of immune-mediated demyelinating diseases of the nervous system including Guillain–Barré syndrome, chronic inflammatory demyelinating polyneuropathy, diabetic polyneuropathy, multifocal motor neuropathy, relapsing–remitting multiple sclerosis and myasthenia gravis (Gelfand, 2012). hIgG has been reported to have pleiotropic

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; AQP4, aquaporin-4; BSA, bovine serum albumin; CDC, complement-dependent cytotoxicity; CNS, central nervous system; dSTORM, direct stochastic reconstruction microscopy; EDSS, expanded disability status scale; FcγR, Fcγ receptor; GFAP, glial fibrillary acidic protein; hIgG, human immunoglobulin G; ITP, idiopathic thrombocytopenic purpura; MAC, membrane attack complex; MBP, myelin basic protein; NK-cell, natural killer cell; NMO, neuromyelitis optica; NMO-IgG, neuromyelitis optica immunoglobulin G antibody; OAP, orthogonal array of particles; PFA, paraformaldehyde; TIRF, total internal reflection fluorescence; WGA, wheat germ agglutinin.

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actions on the immune system, including accelerated clearance of autoantibodies, inhibition of complement deposition, interference with antigen recognition, and block of Fcγ receptors (Berger et al., 2013; Jacob and Rajabally, 2009). Other possible immunomodulatory actions of hIgG have been reported as well, including cytokine neutralization, inhibition of leukocyte migration, expansion of regulatory T cell populations, and dendritic cell activation (Jacob and Rajabally, 2009).

Limited reported data support the clinical benefit of hIgG in NMO (reviewed in Wingerchuk, 2013). hIgG has shown efficacy in the prevention of relapses in a small cohort of 8 NMO patients, with reduction in mean relapse rate from 1.8/year in the year before hIgG treatment to 0.006/year during a mean follow-up of 19.3 months (Magraner et al., 2013). The Expanded Disability Status Scale (EDSS) decreased from 3.3 to 2.6 in the hIgG-treated group. Other case studies also support a beneficial effect of hIgG in preventing relapse in NMO (Bakker and Metz, 2004; Okada et al., 2007). hIgG efficacy has also been suggested for treatment of acute NMO relapses, with clinical improvement seen in five out of 11 relapses in 10 patients reported in a retrospective study, with decreased EDSS from 7 to 6.5 at a median of 2 months after hIgG (Elsone et al., 2013).

Here, we tested the efficacy of hIgG in a rat model of NMO and investigated its potential cellular mechanism(s) of actions. *In vitro* studies of hIgG effects on each of the major steps in NMO pathogenesis suggested inhibition of CDC and ADCC as the principal mechanisms of hIgG clinical benefit in NMO.

Materials and methods

Rats

Lewis rats were purchased from Charles River Lab (Wilmington, MA). Experiments were done using weight-matched female rats (100–200 g), age 8 to 12-weeks. Rats were housed and bred in the animal laboratory resource center at the University of California San Francisco. Protocols were approved by the University of California San Francisco Committee on Animal Research.

Antibodies and sera

Recombinant monoclonal NMO antibody rAb-53, which recognizes extracellular epitope(s) on AQP4, was generated from a clonally expanded plasmablast population from the cerebrospinal fluid of an NMO patient, as described and characterized previously (Bennett et al., 2009; Crane et al., 2011). A chimeric NMO-IgG (NMO-IgG^{c}), provided by Dr. Jeff Bennett (Univ. Colorado Denver), was generated by cloning the sequence of the variable region of heavy and light chains of rAb-53 upstream of the constant region of mouse IgG2a. NMO serum was obtained from seropositive individuals who met the revised diagnostic criteria for clinical disease, with non-NMO (seronegative) human serum as control. In some studies IgG was purified from pooled NMO serum (NMO-IgG^{serum}) or control serum using a protein A-resin (GenScript, Piscataway, NY) and concentrated using Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA). Human immunoglobulin (Privigen®, CSL Behring, Kankakee, IL) was dialyzed against phosphate-buffered saline (PBS), which is the buffer used for the *in vitro* assays.

Rat model of NMO

Rats were anesthetized with intraperitoneal ketamine (75–100 mg/kg) and xylazine (5–10 mg/kg) and mounted in a stereotaxic frame, as described (Asavapanumas et al., 2014). Following a midline scalp incision, a burr hole of diameter 1 mm was made in the skull 3.5 mm to the right of the bregma. A 30-gauge needle attached to a 50-μL gastight glass syringe (Hamilton, Reno, NV) was inserted 5 mm deep to infuse 750 μg NMO-IgG^{serum} in a total volume of 10 μL (at 1 μL/min). Rats were administered 2 g/kg hIgG or bovine serum albumin (BSA) intraperitoneally 1 day before and 1 day after intracerebral injection of NMO-IgG (4 rats per group). Rats were anesthetized at 5 days following NMO-IgG administration and perfused through the left cardiac ventricle with 100 mL PBS and then 25 mL of PBS containing 4% paraformaldehyde (PFA). Brains were collected and processed for immunostaining. hIgG concentration in rat serum was measured with a human IgG ELISA kit according to manufacturers' protocol (Genway, San Diego, CA) and leukocytes were counted using a Hemavet 850 (Drew Scientific, Oxford, CT).

Immunostaining

Brains were post-fixed for 2 h in 4% PFA. Five micrometer-thick paraffin sections were immunostained at room temperature for 1 h with antibodies against AQP4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), GFAP (1:100, Millipore), myelin basic protein (MBP) (1:200, Santa Cruz Biotechnology), ionized calcium-binding adaptor molecule-1 (Iba1; 1:1000; Wako, Richmond, VA), albumin (1:200, Santa Cruz Biotechnology), neurofilament (1:200; Millipore), C5b-9 (1:50, Hycult Biotech, Uden, The Netherlands) or CD45 (1:10, BD Biosciences, San Jose, CA), followed by the appropriate fluorescent secondary antibody (1:200, Invitrogen, Carlsbad, CA) or biotinylated secondary antibody (1:500, Vector Laboratories, Burlingame, CA). Tissue sections were examined with a Leica (Wetzlar, Germany) DM 4000 B microscope. AQP4, GFAP and MBP immunonegative areas were defined by hand and quantified blindly by two different observers using ImageJ (http://rsbweb.nih.gov/ij/). Data are presented as area (mm^2) of immunonegative area.

Cell culture

Chinese hamster ovary (CHO) cells stably expressing human AQP4-M23 (named CHO-AQP4 cells) were generated as described (Crane et al., 2011) and cultured at 37 °C in 5% CO_2 95% air in F-12 Ham's Nutrient Mixture medium supplemented with 10% fetal bovine serum, 200 μg/mL geneticin (selection marker), 100 U/mL penicillin and 100 μg/mL streptomycin. Human natural killer cells (NK-cells) were purchased from Fox Chase Cancer Center (Philadelphia, PA) in which parental NK-92 cells (ATCC CRL-2407) were retrovirally-transduced to express the high-affinity 176V variant of the Fcγ receptor CD16 in pBMN-NoGFP (Yusa et al., 2002). Primary astrocyte cultures from rat neonatal brain were generated as described (Asavapanumas et al., 2014).

Quantification of cell surface AQP4

CHO-AQP4 cells or rat astrocytes were grown on coverglasses until confluence and incubated for 3 h with specified concentrations of hIgG or BSA at 37 °C. Cells were then washed extensively in cold PBS and blocked for 20 min in 1% BSA at 4 °C. Remaining AQP4 at the cell surface was labeled with 50 μg/mL NMO-IgG^{c} at 4 °C for 1 h. Subsequently, NMO-IgG^{c} was labeled by incubation for 1 h at 4 °C with goat anti-mouse IgG-conjugated Alexa Fluor 555 (1:200, Invitrogen), and the plasma membrane was stained using a fluorescent lectin, wheat germ agglutinin (WGA)-conjugated Alexa Fluor 488 (1:200; Invitrogen). Cells were then washed in cold PBS and fixed for 15 min in 4% PFA. Cell surface AQP4 was quantified as the (background-subtracted) ratio of red (surface AQP4) to green (plasma membrane) fluorescence using ImageJ, as described (Ratelade et al., 2011).

Super-resolution imaging

The size of plasma membrane AQP4 orthogonal array of particles (OAP) was measured by super-resolution (dSTORM) imaging, as described (Rossi et al., 2012; Smith et al., 2014). Briefly, CHO-AQP4 cells