Journal of Pharmaceutical and Biomedical Analysis 151 (2018) 317–323

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

In vitro evaluation of the biological activities of IgG in seven Chinese intravenous immunoglobulin preparations

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ARTICLE INFO

Article history:
Received 7 November 2017
Received in revised form 6 January 2018
Accepted 11 January 2018
Available online 16 January 2018

Keywords:
Intravenous immunoglobulin
Biological activity
Pathogenic antigen
Complement activation
Fcγ-receptors binding

ABSTRACT

The IgG activities of antigen recognition, Fc-mediated complement activation and cellular Fcγ-receptors (FcγRs) binding are critical for intravenous immunoglobulin (IVIg) immunotherapy in a variety of immune deficiency diseases. Further, these activities could be influenced by different plasma sources and the IVIg manufacturing processes of different manufacturers. This study evaluated and compared the biological activities of IgG in 7 IVIg preparations produced by different Chinese manufacturers. By using ELISA and two-dimensional immunoblotting, the binding capacity and antibody repertoire of IVIg against typical pathogenic antigens were investigated. Further, Fc-mediated complement activation and receptor binding activities were measured by the haemolysis method and flow-cytometric assay respectively. The results showed that all of the preparations tested have a broad spectrum of antibodies against the *E. coli* O157:H7 proteome, and each IVIg has its own distinct antibody repertoire. Compared to the European Pharmacopoeia IgG standard, the mean indices of the pathogenic antigen binding capacity, complement activation activity and FcγRs binding activity in Chinese preparations were 152%, 143% and 87%. The biological activities varied widely among the 7 IVIg preparations, and no significant differences were observed between the different batches of most IVIgs from the same manufacturer. This study will contribute to the improvement of the IVIg product quality evaluation system and an increased understanding of the variety of IgG biological activities in Chinese IVIg preparations.

1. Introduction

Intravenous immunoglobulin (IVIg, pH 4) is administered as an antibody replacement therapy for immunodeficiency syndromes and as an immunomodulatory therapy for inflammatory and autoimmune conditions. The major component of IVIg preparations is immunoglobulin G (IgG), which is produced by pooling plasma from thousands of healthy blood donors [1–4]. IgG mediates a large number of immune neutralization and immunomodulatory activities dependent on the structural integrity of the molecule, in particular the Fab and Fc portions. The Fab portion combines with antigenic determinants, representing the recognition function. Meanwhile, the Fc portion mediates complement activation and binding to cell receptors, representing effector functions [5,6]. Moreover, the genetic background and geographic distribution of donors could influence the diversity of antibodies in IVIg, and the manufacturing procedures involving purification, viral inactivation, ultrafiltration and filtration, and storage could alter the integrity of the IgG molecule and degrade its biological activity. This may result in a change in clinical efficacy and safety [7–9].

As the precise mechanism of action of IVIg is complex and not yet fully understood, *in vitro* assessing methods of IVIg biological activity are still not comprehensive. In the European Pharmacopoeia (EP) 9.0 [10] and the Chinese Pharmacopoeia (CP) v2015 [11], the Fc-mediated complement activation assay was required to evaluate the biological activity of IVIg. However, this assay has some disadvantages and its evaluation is not comprehensive. To further improve the IVIg product quality evaluation system, it is necessary to better understand the IVIg preparations produced by the Chinese industry and to provide evidence for some of the differences in the biological functions among Chinese IVIg preparations. In this work, by using four improved methods, we evaluated and compared the pathogenic antigen binding capacity, the IgG antibody repertoire against pathogenic antigens, the complement activation activity and the Fcγ-receptors (FcγRs) binding activity of IVIg preparations from 7 Chinese manufacturers.

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https://doi.org/10.1016/j.jpba.2018.01.021
0731-7085/

2. Materials and methods

2.1. IVIg preparations

Seven liquid IVIg preparations (5%, pH 4) were collected from 7 different manufacturers in China (preparation A to preparation G), and each preparation included 3 different batches. All of the preparations were obtained by ethanol fractionation based on the Cohn method [12,13]. The viral inactivation/removal steps of them are pasteurization (or nanofiltration) and terminal low pH (pH 4.0) incubation. The stabilizer for 6 IVIg preparations is maltose, and for preparation E is sorbitol. In all of the experiments performed, except for the analysis of IgG antibody repertoires, 3 batches of each IVIg preparation were tested.

2.2. Reference standard

The European Pharmacopoeia standard, "Human Immunoglobulin Biological Reference Preparation batch 2" (EP BRP), was used as the reference standard. It was obtained from the Council of Europe (Strasbourg, France).

2.3. Escherichia coli (E. coli) culture and protein extraction

E. coli O157:H7 were cultured in a sterile Luria-Bertani medium containing 5 g/L of yeast extract, 10 g/L of trypton, and 10 g/L of NaCl (pH 7.0) for 12 h at 37 °C. A bacterial pellet (1 ml) was collected and washed 3 times with 10 ml of phosphate-buffered saline (PBS; Hyclone, UT, USA). The pellet was then suspended in 50 mM of Tris-HCl (pH 8.5) containing 2 mM EDTA, 100 mM NaCl, 100 μg/ml lysozyme and 1 μM PMSF, and then physically ruptured by sonication for 15 min at 300 W using an ultrasonic processor (Vibra-Cell; Sonics & Materials, Newtown, CT, USA) on ice, followed by centrifugation at 15,000*g* for 10 min at 4 °C (Biofuge Stratos; Thermo Scientific, Osterode, Germany). Lastly, the supernatant was collected, aliquoted, and stored at −80 °C.

2.4. ELISA measurement of pathogenic antigen binding capacity of IVIg preparations

1 mg/ml of *E. coli* protein extraction in Tris-HCl was coated on a 96-well plate (Nunc MaxiSorp, Thermo scientific, Denmark). It was left overnight at 4 °C. EP BRP IgG standard and IVIg samples were diluted with PBS by a dilution factor of 1:300 based on preliminary experiments. The plate was washed 3 times with PBS containing 0.1% Tween-20 (PBS-T) after every incubation. After blocking with 0.5% human serum albumin (HSA, Taibang Biological Products, Guizhou, China) for 2 h at 37 °C, the diluted IgG standard and IVIg samples were loaded and left overnight at 4 °C. The plate was then incubated with HRP-conjugated goat anti-human IgG Fc polyclonal antibody (Abcam, UK). For color development, 3,3′,5,5′-tetramethylbenzidine (TMB, Solarbio, Beijing, China) was used and terminated with 2N H_2SO_4, and the absorbance was read at 450 nm on a plate reader (Synergy H1 Hybrid Reader, BioTeK, VT, USA). A total of 21 batches of IVIgs were tested 3 independent times in duplicate. The EP BRP IgG standard was used as a reference and positive control, and Tris buffer was used as a negative control.

2.5. Demonstration of the IgG antibody repertoire against E. coli in IVIg preparations

Two-dimensional gel electrophoresis (2-DE) and immunoblotting with protein extracts of *E. coli* O157:H7 were used to study the IgG antibody repertoire of the 7 IVIg preparations. The equipment and procedure that we used in this study were exactly as described previously [14].

2.6. Evaluation of the complement activation activity of IVIg preparations

A microassay for the measurement of the complement activation activity of IgG was taken based on the procedure proposed by CP v2015 by using the human diphtheria toxoid as an antigen [11]. A total of 21 lots of IVIgs were tested in triplicate 3 independent times.

Human group O blood was collected into an anticoagulant citrate dextrose (ACD) solution, red blood cells (RBCs) were separated by centrifugation (1000*g*, 10 min), washed 3 times with PBS and suspended at 1% (v/v) in PBS with 1.3 μg/ml tannic acid. After incubating at 37 °C for 15 min, cells were collected by centrifugation (1000*g*, 10 min), washed 3 times with PBS and resuspended at 1% (v/v) in PBS. The human diphtheria toxoid (SinoPharm, Beijing, China) was then added into the RBC solution to a final concentration of 50 Lf/ml and incubated at 37 °C for 30 min. Following centrifugation (1000*g*, 10 min), the cells were washed 3 times with a 0.75% (v/v) albumin barbital buffer (pH 7.2) and finally resuspended in appropriate volume of albumin barbital buffer such that the absorbance of the solution when diluted 1:10 was 1.0 ± 0.1 at 541 nm. IVIg preparations were diluted to 40 mg/ml in albumin barbital buffer and adjusted to pH 7.0 with NaOH. Each sample was prepared in triplicate. The EP BRP IgG standard was used as a reference and positive control, and albumin barbital buffer was used as a negative control. 900 μl of diluted samples were mixed with 100 μl of antigen-coated tanned RBC solution in microcentrifuge tubes, incubating at 37 °C for 15 min. The cells were then washed 3 times with albumin barbital buffer. After the final wash, the cells were resuspended with an albumin barbital buffer to the final volume of 800 μl. Haemolysis was monitored in a microtitre plate format [15]. A 160 μl aliquot of each sample of RBC suspension was transferred in duplicate to wells of a microtitre plate and pre-warmed in a plate reader at 37 °C. A 40 μl guinea pig complement (50 CH_{50}/ml, Sigma-Aldrich, MO, USA) pre-warmed for 30 s at 37 °C was added immediately to each well. The plate was returned to the plate reader and absorbance was monitored at 541 nm every minute for 30 min. The observed haemolysis curves were examined by electronic derivative analysis (Gen5, version 2.00; BioTeK, VT, USA) as described in the study of Vrdoljak et al. [15], involving the determination of the maximal rate of change of absorbance achieved at the inflection point. The index of complement activation activity was expressed as a percentage of the reference standard.

2.7. Detection of the FcγRs expressed by THP-1 cells

THP-1 cells (human monocytic cells, ATCC TIB-202) were cultured in a RPMI 1640 medium (Life Technologies, Paisley, UK) containing 10% fetal bovine serum (FBS, Thermo scientific, Rockford, IL, USA), 1% penicillin-streptomycin (Hyclone, UT, USA) and 50 μM β-mercaptoethanol (Life Technologies, Carlsbad, CA, USA). FITC-conjugated mouse anti-human CD16 antibody (clone 3G8, recognizes FcγRIII), FITC-conjugated mouse anti-human CD32 antibody (clone FLI8·26, recognizes FcγRII) and FITC-conjugated mouse anti-human CD64 antibody (clone 10·1, recognizes FcγRI), obtained from BD Biosciences (Heidelberg, Germany), were used for the analysis of FcγRs expression by THP-1 cells. Appropriate isotype control antibodies were included in all of the experiments.

2.8. Evaluation of the FcγRs binding activity of IVIg preparations

The FcγRs binding assay was taken based upon the previous description with some modifications [16,17]. Moreover, the rela-