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Establishment of a reference material for standardization of the anti-complementary activity test in intravenous immunoglobulin products used in Japan: A collaborative study

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ABSTRACT

Aggregates of human plasma-derived intravenous immunoglobulins (IVIGs) carries a risk of severe adverse events after nonspecific complement activation induced in humans administrated. Therefore, the anti-complementary activity (ACA) test is legally required in every batch of IVIGs in Japan. However, due to the intrinsic nature of this bioassay, there might be large differences in the results of ACA tests from laboratories, even when the same batch of IVIGs was measured. Our six laboratories evaluated whether there were such differences and argued for establishment of a reference material (RM) for standardization of the ACA test. Our results revealed inter-laboratory differences in ACA values, indicating a need to establish an RM. Therefore, after ACA values in candidate RMs were measured collaboratively, one RM was selected from two candidates and unit value-assigned. The RM in fact normalized the ACA test values for samples measured in parallel at almost all the laboratories, when the values were calculated relative to the assigned unit value of the RM. Thus, we established a first RM to standardize the ACA test in Japan, which enabled each laboratory to normalize ACA values constantly for IVIGs. This indicates that the establishment of an RM can contribute to quality control of IVIGs.

1. Introduction

A variety of intravenous immunoglobulin (IVIG) products are manufactured from human blood plasma. When IVIGs are administered to humans, immunoglobulin aggregates may nonspecifically activate serum complement, causing severe adverse events including anaphylaxis [1,2]. To reduce these risks and prevent these events, the anti-complementary activity (ACA) test is required before marketing [3–6]. This test is performed as one of the blood safety tests for IVIGs according to the Minimum Requirements for Biological Products (MRBP) [7] under Japanese Pharmaceuticals and Medical Devices Act. However, this test is a bioassay using sheep red blood cells (RBCs) and guinea pig complement and hemolysin (anti-sheep RBC antibody), therefore, it is difficult to obtain consistent ACA values in every test and laboratory [8].

To reduce immunoglobulin aggregates in all IVIGs used in Japan, plasma-derived IVIGs are treated in various ways during manufacturing: removal of the aggregates by processing with polyethylene glycol or ion-exchanging resin; inhibition of aggregate production by processing at pH 4; deletion or modification of the Fc portion by processing with pepsin or sulfonation. Every batch of these IVIGs is routinely tested for ACA values by every

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blood product manufacturer, and some IVIGs are also tested at the National Control Laboratory as a lot release test.

A standard method for the ACA test defined in the MRBP is based on Mayer's method [9]. In the European Pharmacopoeia, the ACA test results are expressed as a percentage reduction/consumption of complementary activity (CA) by each sample relative to that of control complement (defined as 100%) [10]. In Japan, ACA test values are expressed as CA values subtracted/consumed by each sample from that of control complement.

ACA is presumably affected by the particular batch of biomaterials (guinea pig complement, sheep RBCs and hemolysin), thus, it would be expected that intra-laboratory and inter-laboratory differences in ACA values would likely be significant. To normalize the values and minimize these differences, establishment of a reference material (RM) for standardization of the ACA test is required, but this has remained to be achieved.

In this collaborative study, we assessed intra-laboratory and inter-laboratory differences of the ACA test. We established an RM with an assigned ACA unit value, and demonstrated that the differences could be normalized by calculating ACA unit values of the test samples relative to those of the RM. Obtaining constant and reproducible ACA unit values using the RM in the quality control test will enable manufacturers and national authorities to standardize the ACA test, which will be useful in achieving consistent quality of IVIGs.

2. Materials and methods

2.1. Preparation of hemolysin-sensitized sheep RBCs

Twenty milliliters of sheep blood in Alsever's solution (Japan Lam Co. Ltd., Hiroshima, Japan) was suspended in saline and then centrifuged at 450×*g* at 4 °C for 10 min. After centrifugation, lymphocytes and lysed cells were removed from the blood by repeated washing at least three times to obtain RBCs alone. A rabbit anti-sheep RBC antibody, called hemolysin (Denka Seiken Co. Ltd., Tokyo, Japan), was also diluted to 1:100 with Geratin-barbital/veronal buffer (GVB) and then added stepwise to and mixed well with an equal volume of RBCs. After incubation of the mixture at 37 °C for 15 min, the cells were washed with GVB at least three times to remove lysed cells and to obtain the sensitized RBCs. RBCs were counted and adjusted to 5×10^8 cells/ml for the following procedure.

2.2. Measurement of ACA test values

The ACA test was carried out according to the MRBP in Japan. Approximately 20 CH_{50} (50% hemolytic unit of complement) guinea pig complements (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) were incubated with or without 10 mg IVIG sample in GVB (a total of 1 ml reaction mixture per sample) at 37 °C for 1 h. Reaction mixtures were diluted and incubated with 2.5×10^8 sensitized sheep RBCs in GVB (a total of 3.5 ml) at 37 °C for an additional 1 h. The incubated samples were centrifuged at 450×*g* at 4 °C for 10 min and the degrees of hemolysis (referred to as [y]) in supernatants obtained were measured at A_{541}. The remaining CA after incubation with or without globulin generally hemolyzed 10%–90% of RBCs. In the same manner, in a reaction mixture including distilled water or GVB, instead of guinea pig complement, degrees of hemolysis were measured as a background lysis or non-lysis control, respectively. Each [y]/(1−[y]) value (as the ordinate) was plotted at indicated dilution ratios of the complement (as the abscissa) on log–log graphs. In plotted graphs, the lines obtained with or without IVIG samples, which were fitted to the points around 50% hemolysis, ran parallel to each other. The complement dilution rate when 50% hemolysis was shown was calculated in each IVIG sample. To obtain the CA (CH_{50}) value, the dilution rate was converted by a scaling factor 2 because Mayer's modified 1/2 method was performed. The CA values (CH_{50}/ml) from data obtained with or without IVIG samples were referred to as [a] or [b], respectively.

Finally, an ACA value was determined from the difference between [b] and [a], and should not be more than 20 units (U)/ml, according to the Biological Products Standard in Japan.

2.3. Collaborative measurement of ACA values

To examine intra-laboratory and inter-laboratory differences in ACA values of IVIGs and assess whether establishment of an RM was needed to reduce the differences, six laboratories participated in this collaborative study and performed ACA tests.

Two lyophilized IVIGs, Kenketsu Veniron-I (Teijin Co. Ltd., Tokyo, Japan) and Kenketsu Glovenin-I (Takeda Co., Ltd., Osaka, Japan), were selected from among IVIGs commercially used in Japan The two products were randomly labeled A and B, and their corresponding product names were blinded when they were distributed to the participants. Simultaneously, the same batch of guinea pig complement (Batch No. EDAB9101, Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) was also distributed to them for this study.

2.4. Assignment of unit value to RM

Each laboratory measured ACA values of samples three times by using different sheep RBCs independently sensitized on 3 days, with their own routine methods, according to the World Health Organization guidelines for production of standard materials [11]. All raw data, including A_{541}, were collected by the National Institute of Infectious Diseases (the National Control Laboratory). The geometric means (GMs) and the geometric coefficient variations (GCVs) of ACA values obtained in candidate RMs at all the laboratories were calculated. Taking into account the given GMs and GCVs, we determined which candidate was suitable as the RM. The overall GM of ACA values obtained for the selected candidate was given to the RM as an assigned ACA unit value (U/ml).

2.5. Statistical analysis

ACA and CA values obtained in this study from all participants were assessed by analysis of variance with post multiple comparison procedure and $P < 0.05$ was considered significant. These analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Evaluation of intra-laboratory differences in complement batches and impact of differences on ACA values

ACA values are likely to be affected by differences in batches of the three biological materials used in the test: guinea pig complement, sheep RBCs, and hemolysin (anti-sheep RBCs antibody). Differences in the batches of complement are thought to have the greatest effect. Thus, selection of appropriate batches of complement may be important to achieve test repeatability.

To evaluate intra-laboratory differences in and the impact of differences in complement batches on ACA values, we compared the values for the same globulin measured using three complement batches (A, B and C) at the National Control Laboratory (Fig. 1). A total of 42 test measurements were made (18 in batch A, 6 in batch