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## Abstract

Antibody activity, especially that involved in the reaction of antibody-dependent cell-mediated cytotoxicity (ADCC), of five commercially available human gammaglobulin preparations (standard, pepsin-treated, plasmin-treated, polyethylene glycol-fractionated and S-sulfonated gammaglobulin) was measured. All these gammaglobulin preparations had high titers of hemagglutination inhibition and neutralizing antibody against measles virus. In ADCC reaction, the pepsin-treated gammaglobulin preparation showed no antibody activity. The standard gammaglobulin preparation showed weak activity only when highly diluted. The remaining three preparations showed high activity. Though the S-sulfonated gammaglobulin preparation showed no activity in ADCC reaction, it showed high activity after reconversion by means of oxidation and reduction in vitro. The plasmin-treated gammaglobulin preparation showed greater activity than the polyethylene glycol-fractionated preparation of the optimal concentration. In ADCC tests using the plasmin-treated gammaglobulin preparation, K cell activity was strongly inhibited by Hg (thimerosal), while, in those using the standard gammaglobulin preparation, the activity was hardly influenced by Hg, suggesting that the low ADCC activity of the standard gammaglobulin preparation of high concentrations was due to the inhibitory effect of aggregated immunoglobulin G molecules.

**KEYWORDS:** antibody-dependent cell-mediated cytotoxicity, measles, immunology, gammaglobulin preparation

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# Cell-Mediated Cytotoxicity-Supporting Activity of Various Human Gammaglobulin Preparations

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Antibody activity, especially that involved in the reaction of antibody-dependent cell-mediated cytotoxicity (ADCC), of five commercially available human gammaglobulin preparations (standard, pepsin-treated, plasmin-treated, polyethylene glycol-fractionated and S-sulfonated gammaglobulins) was measured. All these gammaglobulin preparations had high titers of hemagglutination inhibition and neutralizing antibody against measles virus. In ADCC reaction, the pepsin-treated gammaglobulin preparation showed no antibody activity. The standard gammaglobulin preparation showed weak activity only when highly diluted. The remaining three preparations showed high activity. Though the S-sulfonated gammaglobulin preparation showed no activity in ADCC reaction, it showed high activity after reconversion by means of oxidation and reduction *in vitro*. The plasmin-treated gammaglobulin preparation showed greater activity than the polyethylene glycol-fractionated preparation of the optimal concentration. In ADCC tests using the plasmin-treated gammaglobulin preparation, K cell activity was strongly inhibited by Hg (thimerosal), while, in those using the standard gammaglobulin preparation, the activity was hardly influenced by Hg, suggesting that the low ADCC activity of the standard gammaglobulin preparation of high concentrations was due to the inhibitory effect of aggregated immunoglobulin G molecules.

*Key words:* antibody-dependent cell-mediated cytotoxicity, measles, immunology, gammaglobulin preparation

Human gammaglobulin preparations contain several kinds of measles antibodies, *e.g.*, neutralizing (NT) antibody, hemagglutination inhibition (HI) antibody and complement fixing (CF) antibody, and they have been used to prevent or lessen the severity of measles infections. Previously, we reported that the prophylactic effect of a gammaglobulin injection in the early stage of measles infection strongly depends on antibody-dependent cell-mediated cytotoxicity (ADCC) (1). At present, several types of human gammaglobulin preparations are available, including standard gammaglobulin preparations for intramuscular injection and gammaglobulin preparations for intravenous injection, such as pepsin-treated, plasmin-treated polyethylene glycol-fractionated and S-sulfonated gammaglobulin preparations. There are several advantages and disadvantages relating to these preparations which should be considered. For example, standard gammaglobulin preparations can be injected only in small dosages because they cannot be injected intravenously, pepsin-treated gammaglobulin can easily penetrate into tissue,

but its half life in the blood stream is very short because it is a small molecule, and S-sulfonated gammaglobulin takes about 24 h to be reconverted to native immunoglobulin G *in vivo* (2, 3).

In the present study, we compared the antibody activity of various commercial gammaglobulin preparations in ADCC reaction.

## Materials and Methods

*Human gammaglobulin preparations.* The preparations used were two lots of a standard gammaglobulin preparation (intramusuclar injection, 150mg/ml, Green Cross Co., Ltd.), one lot of a standard gammaglobulin preparation not supplemented with Hg (thimerosal) that was kindly supplied by Green Cross Co., Ltd., and the following intravenously injectable gammaglobulin preparations: one lot of a pepsin-treated gammaglobulin preparation (2.5g/vial; Hoechst Co., Ltd.), six lots of a plasmin-treated gammaglobulin preparation (2.5g/vial; Green Cross Co., Ltd.), six lots of a polyethylene glycol-fractionated gammaglobulin preparation (2.5g/vial; Green Cross Co., Ltd.), and two lots of an S-sulfonated gammaglobulin preparation (2.5g/vial; The Chemo-Sero-Therapeutic Inst.). All gammaglobulin preparations were dissolved to 50mg/ml gammaglobulin concentration.

*Reconversion of the S-sulfonated gammaglobulin preparation in vitro.* S-sulfonated gammaglobulin was reconverted to the native IgG form by treating with glutathione and oxidized glutathione, according to a modified method of Kato *et al.* (4), as follows: S-sulfonated gammaglobulin (24.8mg) was dissolved in 5.0ml of 0.1M Tris (trihydroxymethylethane) -HCl buffer (pH 8.8), and the solution was incubated with 24.0mg of glutathione at 37°C for 2 h. The solution was dialyzed three times in 0.1M Tris-HCl buffer (pH 8.5) for 3 h each time. After dialysis, 1.0mM of oxidized glutathione was added, the solution was incubated for 2 h at 37°C, and then dialyzed three more times in 0.9% saline. The concentration of gammaglobulin was adjusted to 5.0mg/ml. Reconverted gammaglobulin was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 1).

*Target cells.* A HeLa cell subline persistently infected with Toyoshima strain measles virus (HeLa/MV cell), established by T. Minagawa (5, 6), was kindly provided by Dr. S. Chiba, Department of Pediatrics, Sapporo Medical College, Sapporo. The nature of HeLa/MV cells has been described by T. Minagawa previously (5, 6). Measles virus antigens were detected in more than 90 persent of the cells (7). HeLa/MV cells were maintained in Eagle's minimum essential medium (MEM, Nissui) supplemented with glutamine, sodium bicarbonate and 10% heat inactivated fetal calf serum (MEM-10% FCS). Forty-eight hours before the ADCC test, HeLa/MV cells were subcultured. Just prior to the ADCC test, HeLa/MV cells were removed from the culture flask by treatment with 0.25% trypsin, and washed three times with

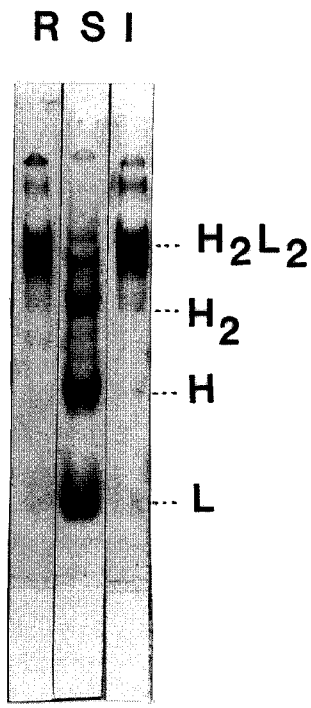


Fig. 1 SDS-polyacrylamide gel electrophoretograms (6% gel, 10mA/column) of three gammaglobulin preparations stained with Coomassie brilliant blue. R: *in vitro* reconverted S-sulfonated gammaglobulin, S: original S-sulfonated gammaglobulin and I: intact immunoglobulin G (polyethylene glycol-fractionated gammaglobulin). Reconverted S-sulfonated gammaglobulin showed a band ($H_2L_2$) in the same position as intact IgG. The original S-sulfonated gammaglobulin did not show the $H_2L_2$ band, but showed heavy chain ($H_2$, H) and light chain (L) bands.

MEM-10% FCS. One ml of a single cell suspension ($5\times10^6$/ml) of HeLa/MV cells was incubated with 200 $\mu$Ci of $^{51}$Cr-Na ($Na_2CrO_4$ 0.9% NaCl, 50mCi/ml, 59mCi/mg Cr, JRIA) at 37°C in a 5% $CO_2$ atmosphere for 45 min as previously described (7). $^{51}$Cr-labelled target cells were resuspended in MEM-10% FCS to a concentration of $1\times10^5$/ml.

*Effector cells.* Peripheral blood mononuclear cells (PBMs) were used as effector cells for the ADCC test. Twenty to 30 ml of venous blood was taken into preservative-free heparin by venipuncture from a healthy adult donor and centrifuged at 1,500 r.p.m. for 30 min using the Ficoll-Conray density gradient method. PBMs on the interface were washed three times with Dulbecco's PBS(−) and resuspended in RPMI-1640 (Flow Laboratories) supplemented with sodium bicarbonate, penicillin G, streptomycin, glutamine and 10% heat inactivated fetal calf serum (RPMI-10% FCS). The cell suspension was put into a flat-bottomed plastic tissue culture flask and allowed to settle for 90 min at 37°C in a 5% $CO_2$ atmosphere in order to remove the adherent cells from the effector cell population. Adherent cell depleted PBMs were adjusted to $4\times10^6$/ml in RPMI-10% FCS. These PBMs contain approximately 90% lymphocytes and 10% monocytes morphologically and were more than 99% viable as determined by the trypan blue dye exclusion test. There was minor contamination from red cells and platelets.

*ADCC test method.* The ADCC test was performed using the modified method previously reported (7). Briefly, 100 $\mu$l of target cell suspension, 50 $\mu$l of effector cell suspension (E/T ratio=20/1) and 50 $\mu$l of diluted gammaglobulin preparations were mixed in each well of a 96-well, round-bottomed microtiter plate. After 4 h of reaction at 37°C in a 5% $CO_2$ atmosphere, the plate was centrifuged at 1,200 r.p.m. for 5 min, and 100 $\mu$l of supernate were harvested from each well for gammacounting. An E/T ratio of 20/1 and a 4h reaction time were confirmed to be optimal (data not presented) by preliminary studies. All samples were tested in triplicate, and cytotoxicity was calculated as % lysis as follows:

$$\%\ \text{lysis} = \frac{\text{experimental release(cpm)} - \text{spontaneous release(cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release(cpm)}} \times 100$$

The antibody activity in ADCC reaction was expressed as % specific ADCC: % specific ADCC =% lysis by ADCC−% lysis by NK.

Maximum release was obtained from the supernate of target cells frozen and thawed three times with dry ice in alcohol and a warm bath, spontaneous release was obtained from target cells cultured 4 h at 37°C in a 5% $CO_2$ atmosphere, and natural killer (NK) activity was obtained from 100 $\mu$l of target cells, 50 $\mu$l of effector cells and 50 $\mu$l of RPMI-10% FCS instead of gammaglobulin.

*Titration of neutralizing (NT) antibody.* NT-antibody was estimated by inhibition of the cytopathic effect (CPE) in tube culture. Briefly, two-fold dilutions of gammaglobulin preparations from 1 : 8 to 1 : 1, 024 were mixed with 100 TCID50/0.1 ml of measles virus isolated from HeLa/MV cells. After incubation at 37°C for 1 h, the mixture was inoculated onto a monolayer of Vero cells in a tissue culture test tube. The final dilution that showed no or significantly weak CPE 2 weeks later was judged to be the NT-antibody titer.

*Titration of hemagglutination inhibition (HI) antibody.* HI-antibody was estimated as follows: Caorin and monkey red blood cell-treated samples were diluted in two-fold steps from 1 : 8 to 1 : 1,024 and mixed with 4 HAUs of HA-antigen measles virus. After 1 h of incubation at room temperature, each sample was mixed with green monkey red blood cells and incubated for 2 h at 37°C. The final dilution at which hemagglutination was significantly inhibited was judged to be the HI-antibody titer of the samples.

## Results

*NT- and HI-antibody titer of gammaglobulin preparations.* The starting concentration of gammaglobulin preparations was 50mg/ml except for the reconverted S-sulfonated gammaglobulin (5mg/ml) (Table 1). The standard gammaglobulin, pepsin-treated gammaglobulin and polyethylene glycol-fractionated gammaglobulin preparations showed higher NT- and HI-antibody titer than the plasmin-treated and S-sulfonated gammaglobulin preparations, but these differences were not found to be significant.

**Table 1** Measles virus antibody of gammaglobulin preparations

| Preparations[a] | Antibody titer | | Antibody activity in ADCC reaction(%)[b] |
|---|---|---|---|
| | NT-Ab | HI-Ab | |
| G.G. | 1 : 128 | 1 : 128 | 18.1 |
| G.G. | 1 : 256 | 1 : 128 | 20.5 |
| G.V. | 1 : 128 | 1 : 128 | 2.9 |
| V.G. | 1 : 64 | 1 : 64 | 35.8 |
| V.G. | 1 : 64 | 1 : 64 | 34.7 |
| V.G. | 1 : 64 | 1 : 128 | 36.4 |
| V.G.-I | 1 : 128 | 1 : 128 | 23.2 |
| V.G.-I | 1 : 128 | 1 : 128 | 20.3 |
| G.G.-S | 1 : 64 | 1 : 64 | 5.0 |
| R-G.G.-S | 1 : 8 | 1 : 8 | 20.5 |

*a*: Starting concentration of gammaglobulin preparations was 50mg/ml except for the reconverted S-sulfonated gammaglobulin (R-G.G.-S, 5mg/ml).

*b*: Antibody activity in ADCC reaction was measured at optimal concentration of gammaglobulin preparations and expressed by % specific ADCC, as described in Materials and Methods.

Abbreviations: NT-Ab, neutralizing antibody; HI-Ab, hemagglutination inhibition antibody; G.G., standard gammaglobulin; G.V., pepsin-treated gammaglobulin; V.G., plasmin-treated gammaglobulin; V.G.-I, polyethylene glycol-fractionated gammaglobulin; G.G.-S, S-sulfonated gammaglobulin.

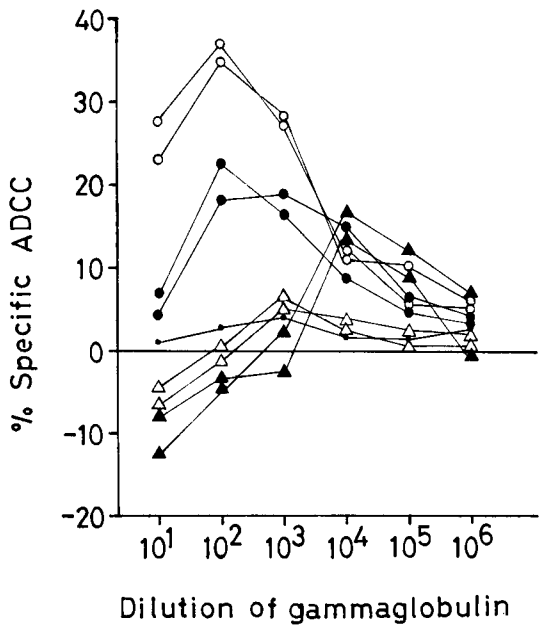


**Fig. 2** Antibody activity in ADCC reaction of various types of human gammaglobulin preparations. The plasmin-treated (○—○), polyethylene glycol-fractionated (●—●), pepsin-treated (•——•), S-sulfonated (△—△) and the standard (▲—▲) gammaglobulin preparations were tested at the concentration of 10~$10^6$-fold dilution.

*Antibody activity in ADCC reaction of gammaglobulin preparations.* Five gammaglobulin preparations were tested for antibody activity in ADCC reaction at various concentrations from 1 : 10 to 1 : $10^6$ using the ten-fold dilution method (Fig. 2). The plasmin-treated and polyethylene glycol-fractionated gammaglobulin preparations showed high antibody activity. The highest antibody activity in ADCC reaction of these two gammaglobulin preparations was at a 1 : 100 dilution, whereas at a 1 : 10 dilution the activity was lower, probably due to a prozone effect. Interestingly, the highest antibody activity was obtained with the plasmin-treated gammaglobulin preparation which contains a smaller amount of intact immunoglobulin G than the polyethylene glycol-fractionated gammaglobulin preparation (60% versus 100%). The S-sulfonated and pepsin-treated gammaglobulin preparations showed very little antibody activity in ADCC reaction at any concentration tested. ADCC activity was revealed to be extremely low when standard gammaglobulin preparations were used at high concentrations (1 : 10 and 1 : 100). Antibody activity in ADCC reaction was not parallel with the NT- and HI-antibody titer

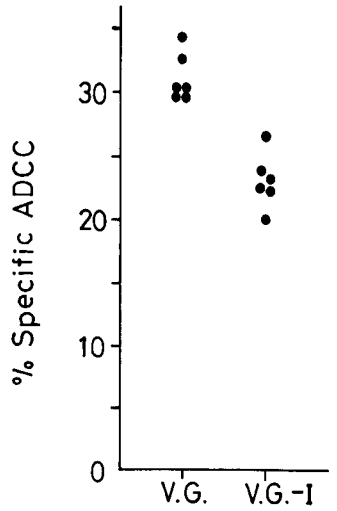


Fig. 3 Comparison of antibody activity in ADCC reaction between plasmin-treated (V. G.) and polyethylene glycol-fractionated (V. G.-I) gammaglobulin preparations. Six lots of each gammaglobulin preparation were tested at a final concentration of 1 : 100.

(Table 1).

*Comparison of antibody activity in ADCC reaction between the plasmin-treated and polyethylene glycol-fractionated gammaglobulin preparations.* Antibody activity in ADCC reaction was variable among the lots of the gammaglobulin preparation, but it was noteworthy that the plasmin-treated gammaglobulin preparation showed higher antibody activity than the polyethylene glycol-fractionated gammaglobulin preparation in all samples tested (Fig. 3). The reason for this result is not clear.

*Effects of mannitol, polyethylene glycol and plasmin on ADCC activity.* Polyethylene glycol and mannitol are contained in the polyethylene glycol-fractionated gammaglobulin preparation, but are not contained in the plasmin-treated gammaglobulin preparation which contains plasmin (Fig. 4). These three supplements were tested as to their inhibitory or enhancing effects on ADCC. Polyethylene glycol and mannitol were added to the plasmin-treated gammaglobulin preparation, and plasmin was added to the polyethylene glycol-fractionated gammaglobulin

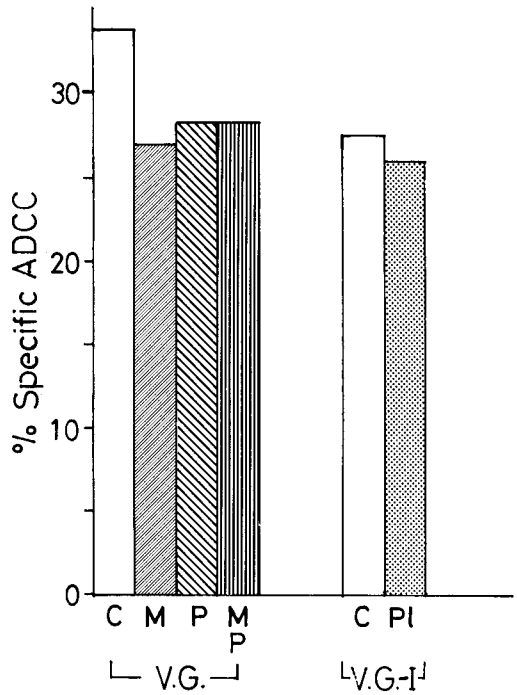


Fig. 4 Effects of mannitol, polyethylene glycol and plasmin on ADCC activity. Mannitol (M : 20mg/ml), polyethylene glycol (P : 5mg/ml) and both reagents (MP) were added to a plasmin-treated gammaglobulin preparation (V. G.), and plasmin (PI : 1CU/ml) was added to a polyethylene glycol-fractionated gammaglobulin preparation (V. G.-I). Antibody activity in ADCC reaction of each gammaglobulin preparation was tested at a final concentration of 1 : 100.

preparation. ADCC was compared to the original gammaglobulin preparation. Mannitol and polyethylene glycol inhibited ADCC, and plasmin did not enhance ADCC.

*Antibody activity in ADCC reaction of the reconverted S-sulfonated gammaglobulin preparation.* S-Sulfonated gammaglobulin, dissolved in distilled water, showed no antibody activity in ADCC reaction (Fig. 5). However, it is well known that S-sulfonated gammaglobulin is reconverted to native immunoglobulin G *in vivo* (3), so we tested antibody activity of *in vitro* reconverted S-sulfonated gammaglobulin. Reconverted S-sulfonated gammaglobulin showed as high antibody activity as the plasmin-treated gam-

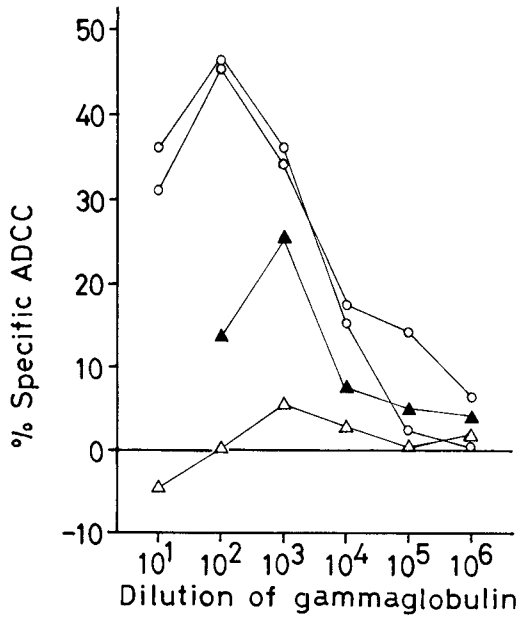


Fig. 5 Antibody activity in ADCC reaction of S-sulfonated gammaglobulin. An untreated S-sulfonated gammaglobulin preparation (△—△), an S-sulfonated gammaglobulin reconverted by oxidation and reduction (▲—▲), and two lots of plasmin-treated gammaglobulin preparation (○—○).

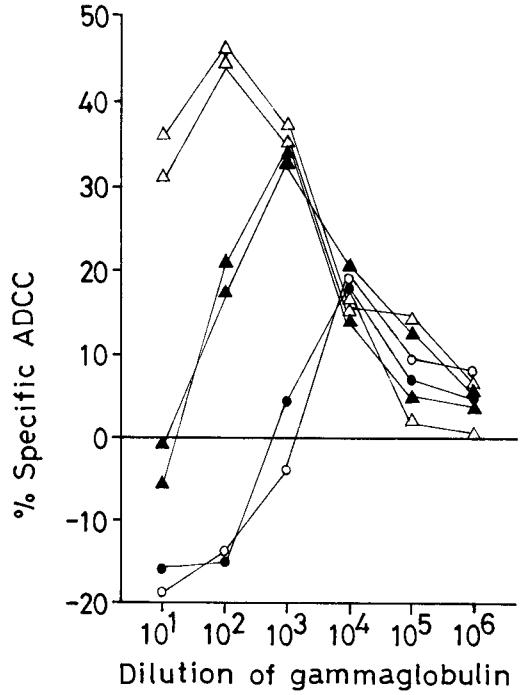


Fig. 6 Effects of Hg on ADCC activity. Hg(thimerosal) was added to two lots of the plasmin-treated gammaglobulin preparation to a concentration of 0.1mg/ml. The antibody activity in ADCC reaction of the plasmin-treated gammaglobulin preparation in the absence (△—△) and the presence (▲—▲) of thimerosal, and that of the standard gammaglobulin preparations in the absence (○—○) and the presence (●—●) of thimerosal.

maglobulin and polyethylene glycol-fractionated gammaglobulin preparations.

*Effects of thimerosal on ADCC activity.* Standard gammaglobulin preparations contain mercury in the form of thimerosal at the concentration of 0.1mg/ml (Fig. 6). Thimerosal has toxic effects on lymphocytes even at a low concentration when added to a lymphocyte tissue culture system. Thimerosal was added at a concentration of 0.1 mg/ml to both the plasmin-treated and standard gammaglobulin preparations, to which thimerosal had not been added. When thimerosal was added to plasmin-treated gammaglobulin, ADCC activity was markedly suppressed at high concentrations of 1 : 10 and 1 : 100. On the other hand, ADCC activity was shown to be much lower when standard gammaglobulin preparations were tested at the concentrations of 1 : 10 to 1 : 1,000 whether they contained thimerosal or not.

## Discussion

Human gammaglobulin preparations have long been used for the prophylaxis or treatment of infectious diseases. However, the impossibility of their intravenous administrations has hindered their applications in large doses. Recently, products providing high dosage gammaglobulin have been developed through enzymatic treatment to weaken the complement activation. However, there are some problems due to enzymatic treatment such as lowered biological activity and/or sl. ⁻tened half-life in the blood stream(2). Among these preparations obtained through enzymatic treatment, plasmin-treated gammaglobulin preparations contain about 60 per-

cent immunoglobulin G in the intact form with a half life as long as standard gammaglobulin preparations. New gammaglobulin preparations without enzymatic treatment are available which prevent activation of the complement system by inhibiting the aggregation of immunoglobulin G, and which can be injected intravenously. These preparations include S-sulfonated gammaglobulin (8) and polyethylene glycol-fractionated gammaglobulin (9). S-Sulfonated gammaglobulin is prepared by treating immunoglobulin G with sulfite and tetrathionate ions. Four interchain disulfide bonds are cleaved to give S-sulfonated groups. This form of immunoglobulin G profoundly weakens the complement-activating ability (3). S-Sulfonated gammaglobulin is reconverted to its original form of intact immunoglobulin G *in vivo* about 24 h after injection (2, 3). The polyethylene glycol-fractionated gammaglobulin preparation, on the other hand, is prepared by depleting the aggregated immunoglobulin G so as to collect only the immunoglobulin G monomer (9). Therefore, it has weak complement-activating activity, if any at all.

In the ADCC system, immunoglobulin G antibody is active only when it remains in its native form (10,11), *i.e.*, the pepsin-treated gammaglobulin preparation has no antibody activity in ADCC reaction as we have already reported (1). Very low ADCC and NK activities were shown when the standard gammaglobulin preparations were used at high concentrations. Mercury and aggregated immunoglobulin G contained in the standard gammaglobulin preparations are thought to be the inhibitory factors, with the latter being the major inhibitory factor. Not only dose pepsin-treated gammaglobulin have no antibody activity in ADCC reaction, but also its half-life in the blood stream is very short. On the other hand, it can easily penetrate into tissue by virtue of its small size. Moreover, pepsin-treated gammaglobulin preparation do not tend toward immunoglobulin accumulation in the blood stream, so that they do not cause inhibition of ADCC, NK activity (12,13) or other immunological reactions.

Three different types of gammaglobulin preparations are available at present which can act in the native form of immunoglobulin G *in vivo*. They are plasmin-treated, polyethylene glycol-fractionated and S-sulfonated gammaglobulin preparations. Among these three intravenous gammaglobulin preparations, plasmin-treated gammaglobulin contains 60 percent intact immunoglobulin G and 40 percent Fab and Fc fragments, while the other two consist of 100 percent intact immunoglobulin G. In terms of measles NT- and HI-antibody titer, the plasmin-treated gammaglobulin preparation had lower antibody titer, but its antibody activity in ADCC reaction was the highest. On the other hand, the polyethylene glycol-fractionated gammaglobulin preparation had higher NT- and HI-antibody titer, but lower antibody activity in ADCC reaction, than the plasmin-treated gammaglobulin preparation (Fig. 2). The reason for this peculiar phenomenon may be the inhibitory effect of both mannitol and polyethylene glycol contained in the polyethylene glycol-fractionated gammaglobulin preparation. Mannitol and polyethylene glycol, however, are secreted into the urine rapidly after injection, so that these inhibitory effects may be negligible *in vivo*.

S-Sulfonated gammaglobulin had no antibody activity in ADCC reaction *in vitro*, but it showed high activity after reconversion by oxidation and reduction *in vitro*. Therefore, it should have high activity *in vivo*.

Specific antibodies against various pathogens contained in gammaglobulin preparations are consumed quickly at the infection site after injection. On the other hand, nonspecific antibodies persist in the blood stream for a long time and might inhibit specific

immune reactions *in vivo* (13,14). Furthermore, repetitive injections may cause accumulation of non-specific antibodies in the blood stream for a long time. During that time, the patient may enter a relatively immune deficient state due to the inhibitory factor(s) in the injected gammaglobulin (15). Another suspected possible disadvantage of intact immunoglobulin G is the antigen-antibody complexes of the pathogen and its specific antibody, which if incompletly neutralized, will be phagocytosed by phagacytic cells in the blood and reticuloendothelial system. Thereafter, replication of the microorgainsms may occur in the phagocytic cells (16). Therefore, it must be emphasized that leukocyte viremia or bacteremia may occur and that the elimination of the pathogens may be delayed or persistent infection may occur. Pepsin-treated gammaglobulin preparations in which Fc and Fab portions are separated have a short half-life *in vivo* and hardly accumulate, so that the inhibitory effect(s) on the specific immune response may be weak if present at all. However, such preparations cannot act as an opsonin and have low antibody activity in ADCC reaction as described in this paper. For prophylactic use, a longer life span is more favorable for treating immune deficiency patients.

Gammaglobulin preparations should be administered to individual patients according to the type and the stage of the disease. Against measles virus infection, intact immunoglobulin G such as polyethylene glycol-fractionated and S-sulfonated gammaglobulin preparations appears to be the most suitable type to provide high antibody activity in ADCC reaction.

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