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Reaction of Human Immunoglobulin G against *K. pneumoniae* in the Presence of Complement

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The bacteriolytic action of human immunoglobulin G (IgG) and its modifications (pepsin-treated and sulfonated IgG) in the presence of complement was examined with *K. pneumoniae* and *S. aureus*. Lysis of *K. pneumoniae* was observed when concentrated IgG was added, while that of *S. aureus* was not. This lytic action against *K. pneumoniae* was unaffected by the above modifications of IgG. The results raise the possibility that IgG antibody an high concentration causes the lysis of *K. penumoniae* by activating the alternative pathway of the complement system and that such activation requires antibody specific to *K. pneumonia*.

Keywords——bacteriolysis; alternative pathway; *K. pneumoniae*; specific antibody; concentrated IgG

Higher animals are provided with the vital protective mechanism consisting of phagocytosis by macrophages, *etc.* and subsequent acquirement of immunity against invasive foreign material such as pathogenic microorganisms. Immunoglobulins (Ig's), which act as opsonins, are not able to destroy and remove antigens by themselves but can do so indirectly by activating the complement system or phagocytic system.[1)]

Many authors have reported that the serum of animals sensitized with gram-negative bacilli lyzes gram-negative bacilli in the presence of complement,[2–4)] but little work has been done on human immunoglobulin G (IgG) and modified IgG as regards bacteriolysis. The present study was designed to examine the complement-dependent bacteriolytic action of human IgG and its modifications (pepsin-treated and sulfonated IgG's) on *K. pneumoniae*.

Experimental

Material——Human IgG for *i.m.* injection was supplied by Hoechst AG (West Germany). SCD broth medium and heart infusion agar medium (HIA) were obtained from Eiken Kagaku Co., dried guinea pig complement from Kyokuto Seiyaku Co., ethylenediaminetetraacetic acid (EDTA) from Pure Chemicals Co., ethyleneglycolbis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) from Dojinyaku Kagaku Co., and pepsin, sodium bisulfite and $CuSO_4$ from Sigma Chemical Co.

Bacteria used were *K. pneumoniae* ATCC 10031 and *S. aureus* FDA 209 P as a control. Both strains were cultivated at 37 °C in SCD broth media overnight prior to the experiment.

Preparation of Modified IgG——IgG from which thimerosal had been removed by Sephadex G-25 gel filtration served as a starting material. Pepsin-treated IgG was prepared to a final concentration of 15 w/v% according to the method of Mandy and Nisonoff.[5)] Sulfonated IgG was prepared according to the method of Mueller.[6)]: 0.2 ml of sodium bisulfite (6%) and 0.2 ml of $CuSO_4$ (6%) were added to 5 ml of intact IgG solution containing 50 mg of IgG per ml, and the mixture was allowed to react at 4 °C for 30 min with occasional shaking; the reaction mixture was precipitated with 40% ammonium sulfate, filtered through Sephadex G-25 gel, and then concentrated to 15 w/v%.

Assay of Bacterial Agglutination Titer——IgG (5 w/v%) was serially diluted with phosphate buffer solution containing 0.9% NaCl (PBS, pH 7.2), and 0.5 ml of the obtained dilution was mixed with 0.5 ml of the bacterial suspension (absorbance at 650 nm = 1.5). After the mixture had been left at room temperature for 24 h, the bacterial

agglutination titer was determined and expressed as the reciprocal of the highest dilution.

Evaluation of Complement Fixation Activity——According to the method of Mayer,[7] the consumption of guinea pig complement by 3 IgG's which had been diluted to 5 w/v% was determined with gelatin veronal buffer (GVB) as the control.

Preparation of IgG Absorbed with *K. pneumoniae*——The precipitate of 5 ml of *K. pneumoniae* (5×10^{10}) was mixed with 5 ml of IgG (15 w/v%), incubated at 37 °C for 30 min and centrifuged at $1250 \times g$ for 20 min. The resultant supernatant was mixed with *K. pneumoniae* precipitates (5×10^{10}) and, in the same manner, incubated and centrifuged. This procedure was repeated five times. The finally obtained supernatant was adjusted to 15 w/v% solution, filtered through a sterile filter (0.45 μ), and designated as *K. pneumoniae*-absorbed IgG.

Immune Bacteriolysis——A 1 ml portion of 15 w/v% IgG solution and 1 ml of IgG serial dilutions from 15 w/v% were added to 1 ml of the bacterial suspension (1×10^7/ml) prepared by 2 washings with GVB-Ca^{2+}, Mg^{2+} at $1250 \times g$ for 20 min. After 30-min incubation at 37 °C, 0.5 ml of guinea pig serum (1 : 10 in GVB) was added and the whole was mixed and allowed to react at 37 °C for 90 min.

After the incubation, the bacteriolytic activity of IgG was evaluated by counting the number of surviving bacteria and by turbidimetry. The reaction mixture was diluted 5×10^5-fold with isotonic sodium chloride solution and 1 ml was mixed with 9 ml of HIA. After a 24-h incubation at 37 °C, the number of colonies on the agar medium was counted. The turbidity was measured at 650 nm.

The effect of IgG on bacteriolysis was also examined with 50 mM EDTA or EGTA in the same manner.

Statistical Analysis——All the obtained values were statistically analyzed by means of Student's *t*-test to compare the test sample and the control.

Results and Discussion

Bacterial Agglutination Titer and Complement Fixation Activity

Figure 1 shows the bacterial agglutination titers and complement fixation activities of intact, pepsin-treated and sulfonated IgG's.

When expressed as maximum dilution in the 2-fold serial dilutions, the agglutination titer was 2^7 against *K. pneumoniae* and 2^{12} against *S. aureus* for each of the three IgG's, and no significant difference was observed among the three. From the result that the bacterial agglutination titer for *K. pneumoniae*-absorbed IgG was 2^1, it appears that IgG antibody which participates in the agglutination is the specific antibody to *K. pneumoniae* and that the absorption treatment of IgG removed the specific antibody from it. Then, the complement fixation activity was examined by the use of guinea pig complement. The intact IgG showed high fixation ability, while modified IgG's showed activity as low as 10 CH_{50} or so.

These results indicated that pepsin treatment and sulfonation significantly suppressed the complement fixation activity of IgG without any effect on the bacterial agglutination ability. Since the anticomplement fixation test employed here[7,8] was considered to involve the reaction in the classical complement pathway, the low complement fixation activity suggested that the reaction does not involve the *Fc*-mediated pathway of the complement activation system. That is, it is unlikely that modified IgG might cause bacteriolysis by activating the classical complement pathway *via Fc*.

Influence of Fresh Guinea Pig Serum on Immune Bacteriolysis

The present study was designed to examine bacteriolysis due to human IgG in the presence of guinea pig complement. Since Schreiber *et al.*[9] have reported that guinea pig complement itself causes the lysis of bacilli, the direct effect of fresh guinea pig complement on *K. pneumoniae* and *S. aureus* was examined so as to determine the most suitable complement concentration for this study.

As Fig. 2 shows, fresh guinea pig serum decreased the vital bacterial count of *K. pneumoniae* dose-dependently up to a final dilution ratio of 1 : 16 but had no effect on *S. aureus*. This may be because complement did not affect the cell wall, which is thicker in *S. aureus* than in *K. pneumoniae*,[10] but the details remain unclear. In the experiments described hereafter, therefore, complement was diluted to a concentration having no direct effect on *K.*

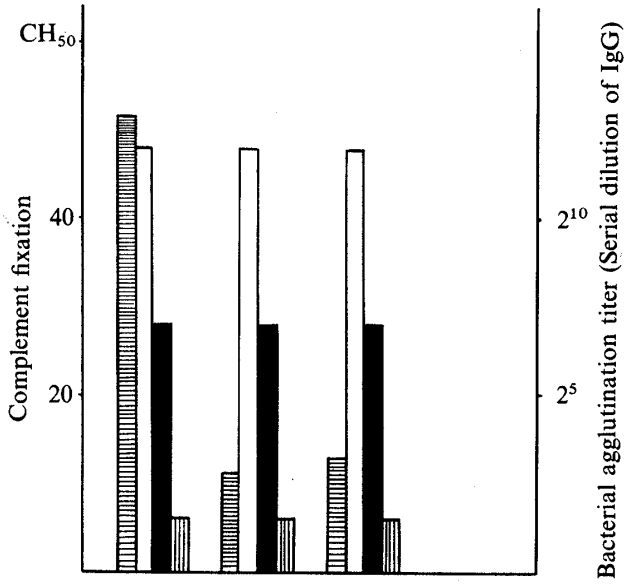

Fig. 1. Complement Fixation and Antibody Titer of the Three IgG's

▤, Complement fixation; ■, agglutination titer against *K. pneumoniae*; □, agglutination titer against *S. aureus*; ▥, agglutination titer of IgG absorbed with *K. pneumoniae*.

Each column represents the mean of two separate experiments. Each IgG concentration: 15 w/v%.

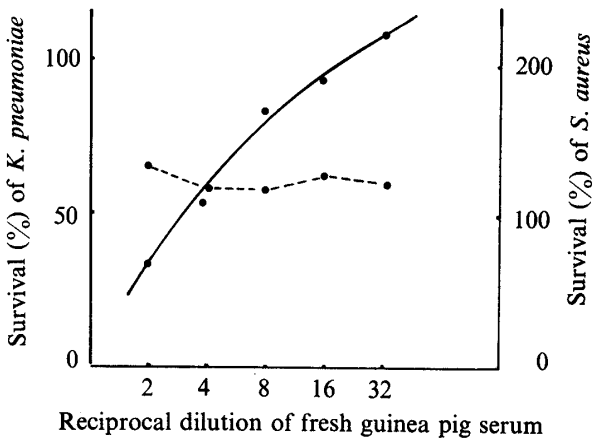

Fig. 2. Bactericidal Activity of Fresh Guinea Pig Serum without IgG

——, *K. pneumoniae*; ------, *S. aureus*.

Each value represents the mean of two separate experiments.

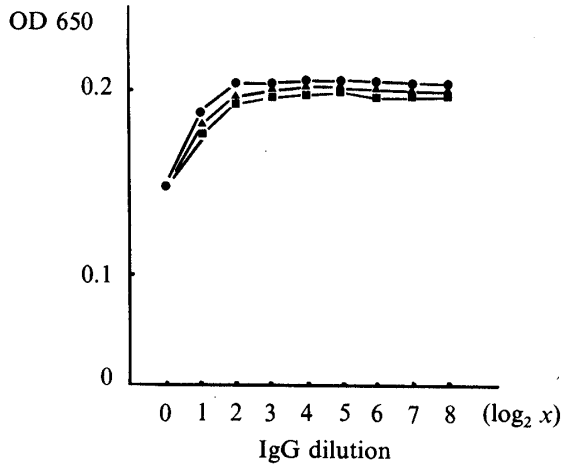

Fig. 3. Dose-Dependent Lysis of *K. pneumoniae* by IgG Combined with Complement

●, sulfonated IgG; ▲, intact IgG; ■, pepsin-treated IgG.

Each value represents the mean of three separate experiments. Initial IgG concentration: 15 w/v%.

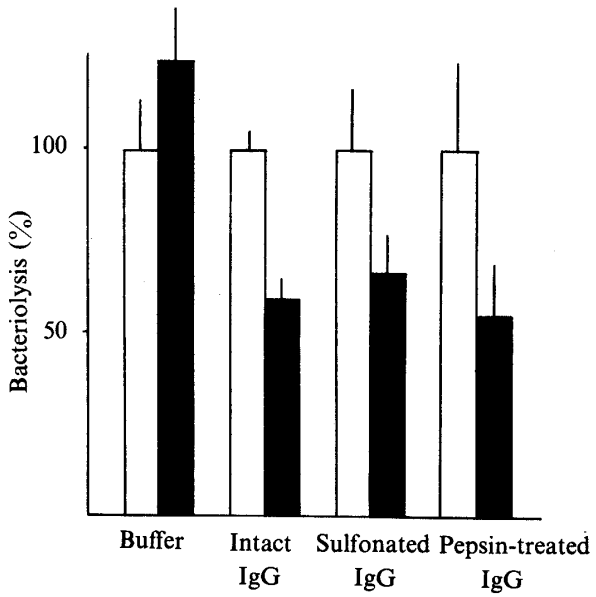

Fig. 4. Bacteriolytic Activity of Three IgG's against *K. pneumoniae*

■, with complement; □, without complement.

Each column represents the mean ± SD of three separate experiments.

pneumoniae, i.e., a final dilution of 1 : 25.

Immune Bacteriolysis

The effect of human IgG antibodies on *K. pneumoniae* was examined in the presence of guinea pig complement. As Fig. 3 shows, intact and modified IgG's decreased the turbidity of the reaction mixture at concentrations of 7.5 and 15 w/v% in the presence of complement, and no effect was noted at less than 3.75 w/v%. Thus, the effect of 15 w/v% IgG was examined in terms of the vital bacterial count. As shown in Fig. 4, the three IgG's at 15 w/v% significantly

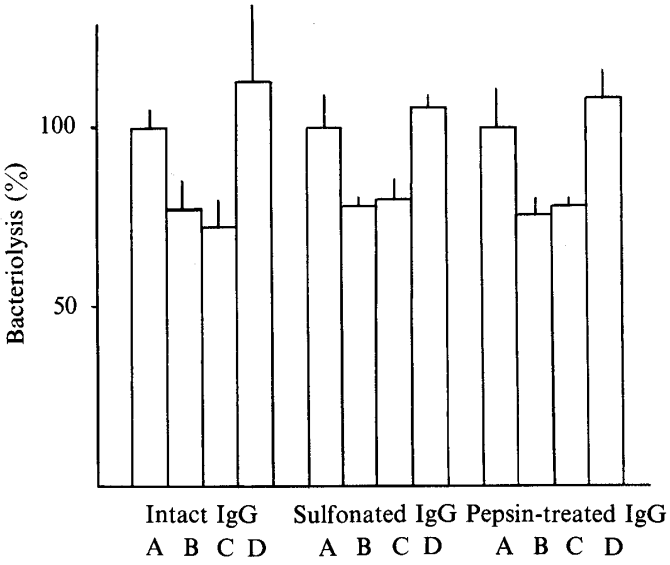

Fig. 5. Bacteriolytic Activity of Three IgG's against *K. pneumoniae* in the Alternative Pathway

A, IgG; B, IgG+complement; C, IgG+complement+EGTA; D, IgG+complement+EDTA.
Each column represents the mean±SD of three separate experiments.

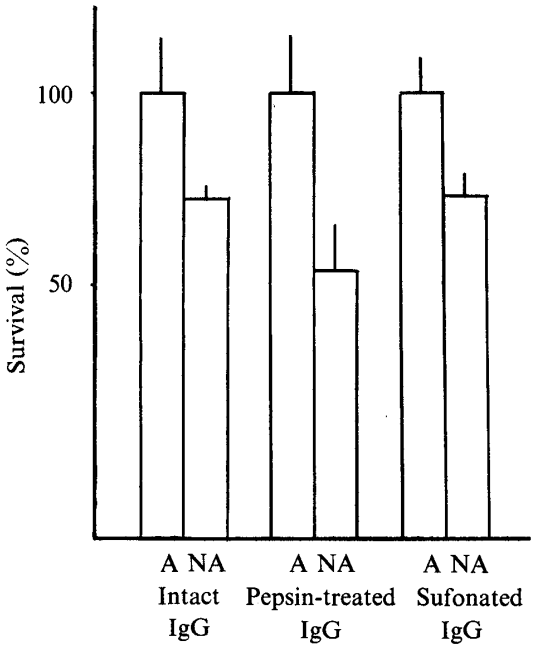

Fig. 6. Bactericidal Activity of *K. pneumoniae*-Absorbed IgG against *K. pneumoniae*

A, absorbed IgG; NA, nonabsorbed IgG.
Each column represents the mean±SD of three separate experiments.

reduced the number of vital *K. pneumoniae* in the presence of complement ($p<0.05$). Since the decrease in turbidity coincided with the vital cell count reduction, the turbidity decrease could be regarded as representing complement-mediated bacteriolysis by IgG.

The experiment on the complement fixation activity of intact and modified IgG's indicated that IgG and complement lyzed *K. pneumoniae* without the participation of the *Fc* of IgG. Activation of the classical pathway requires Ca ions and Mg ions and that of the alternative pathway, Mg ions.[11-13] The bacteriolytic reaction of IgG against *K. pneumoniae* was examined by the addition of EDTA (chelating Ca and Mg ions) or EGTA (chelating Ca ions) and the results obtained are shown in Fig. 5. EDTA completely inhibited the bacteriolytic activity of the three IgG's, suggesting that the bacteriolysis occurred in the presence of complement, while no inhibitory effect on bacteriolysis was noted on the addition of EGTA. These results imply that Mg ions are essential in the present bacteriolysis, *i.e.*, this phenomenon was caused by the participation of the alternative pathway. The $F(ab')_2$ of rabbit IgG has been reported to activate the alternative pathway of the complement system,[14,15] but few studies have been done on the activation of the alternative pathway by complexes of antigen/human IgG, or on sulfonated IgG from human or other species. As to the intact IgG, the alternative pathway has been reported to be activated by similar amounts of rabbit IgG and $F(ab')_2$ to the same degree[14] and by human IgG1, IgG2 and IgG3 in patients with autohemolysinic anemia,[16] but the latter, being a clinical finding, was considered to be an exceptional case. Therefore, our results described here represent the first experimental evidence indicating that intact, pepsin-treated and sulfonated IgG's strongly activate the alternative pathway using human IgG–*K. pneumoniae* complexes. In addition, our data support the finding of Steel *et al.*[15] that rabbit IgG lyzes vibrio cholera by activating the alternative pathway with increase of IgG concentration, and that of Johannsen *et al.*[17] that concentrated high-titer rabbit anti-RBC antibody and its IgG/$F(ab')_2$ activate mainly the alternative pathway to cause hemolysis. It appears the bacteriolysis observed in the present experiment is characteristic to species-nonspecific IgG and its modifications.

From the result that sulfonated human IgG elicited the same reaction as rabbit IgG/$F(ab')_2$, a similar bacteriolytic phenomenon would presumably be caused by the sulfonated specific IgG antibody from animal serum.

High-titer specific antibody is easily obtained from immunized animal serum. Human IgG is considered to be made up of natural antibodies and, therefore, the titer of the specific antibody from pooled human serum is far lower. For this reason, we used human IgG concentrated to a nonphysiological level of 15 w/v%. Since 15 w/v% human IgG might contain more nonspecific antibodies than specific antibody, the participation of nonspecific antibodies in the bacteriolytic phenomenon was examined with *K. pneumoniae*-absorbed IgG (nonspecific antibody). As Fig. 6 shows, *K. pneumoniae*-absorbed IgG did not cause lysis of *K. pneumoniae* even in the presence of complement and no significant difference was noted between the presence and absence of complement. These results indicate that the alternative pathway of the complement system is activated by the immune complex of an antigen and the specific antibody.

There is a good possibility that the specific antibody in human IgG may be purified through affinity chromatography or obtained by the hybridoma technique. If high-titer specific antibody can be prepared from human serum by a suitable method, the bacteriolytic phenomenon observed in the present study can presumably be studied at a physiological serum IgG level of about 1 w/v%. Furthermore, application of purified specific antibody to passive immunotherapy should contribute much in the future to the treatment of infectious diseases, and our result provide some basic data.

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