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Effect of intravenously Applicable Globulin on Phagocytosis of Bacteria by Human Polymorphonuclear Leukocytes

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Three gamma-globulin (IgG) preparations were prepared by enzymatic digestion with plasmin [IgG (plasmin)] or pepsin [IgG (pepsin)] and by polyethylene glycol (PEG) fractionation [IgG (PEG)] and their efficacy was compared in terms of the ability to promote phagocytosis *in vitro* of three strains of pyogenic bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, by human polymorphonuclear leukocytes. IgG (plasmin) had the composition of IgG: Fab: Fc=60: 26: 14; IgG (pepsin), IgG: $F(ab')_2$: Fc=8: 77: 15 and IgG (PEG), 90% or more IgG. In the phagocytosis test, IgG (PEG) was the most efficient and IgG (plasmin) more than IgG (pepsin) though agglutinin titers of these were much the same. The $F(ab')_2$ fragments were almost inefficient in spite of their agglutinin titer similar to those of the IgG preparations. The Fab and Fc fragments showed neither agglutinin titer nor the ability to promote phagocytosis. Since it has been confirmed by a previous study (ref. 16) on the phagocytosis mechanism that these differences in phagocytic effects result from a phagocytotic mechanism in which antigen bound to antibody at its Fab portion is phagocytized through receptors for Fc and complement present on the polymorphonuclear leukocyte surface, it may be concluded that an IgG preparation desirable for clinical use is that which contains intact IgG molecules as much as possible.

Keywords——immunoglobulin; phagocytosis; *Pseudomonas aeruginosa*; *Streptococcus aureus*; *Streptococcus pneumoniae*

Introduction

It is clear that phagocytosis in host defense against pyogenic bacteria such as *Pseudomonas aeruginosa* involves ingestion and killing of them by polymorphonuclear leukocytes (PMNs). Human PMNs possess surface membrane receptors for the Fc portion of the gamma-globulin (IgG) molecule and for component of complement (presumably C3b). IgG-coated bacteria are bound to these receptors by cell surface recognition by PMNs and readily ingested through these receptors and killed.

Recently, various IgG preparations for intravenous use have been developed to treat patients with an antibody deficiency syndrome as well as those with pathogens resistant to antibiotic therapy. The main component IgG of these preparations is enzymatically treated with pepsin or plasmin to reduce anticomplementary activity so as to enable intravenous application. There is a possibility therefore that these intravenous IgG preparations may be greatly reduced in effect to promote phagocytosis of bacteria by human PMNs as compared with untreated, intact IgG.

We prepared various IgG preparations consisting of enzymatically digested IgG and of intact (monomeric) IgG, and IgG fragments as well and compared their ability to promote phagocytosis of pyogenic bacteria by human PMNs.

Materials and Methods

Bacteria——*Pseudomonas aeruginosa* (Homma Serotype E), *Staphylococcus aureus* (ATCC 6538) and *Streptococcus pneumoniae* (Type I) were used.

1) Location: *Miyakojimaku, Osaka, 534 Japan.*

Preparation of IgG——IgG and albumin were prepared from human venous plasma by Cohn's cold ethanol fractionation method.[2] IgG was treated by enzymatic digestion with plasmin (human)[3] or pepsin[4] to obtain IgG (plasmin) and IgG (pepsin). IgG (plasmin) had the composition of IgG: Fab: Fc= 60: 26: 14 and IgG (pepsin), IgG: $F(ab')_2$: Fc=8: 77: 15. IgG (PEG) was prepared from human venous plasma by polyethylene glycol (PEG) fractionation method according to Coval.[5] In IgG (PEG), more than 90% of IgG were in the monomeric form and the rest IgG dimers.

Preparation of IgG Fragments by Enzymatic Digestion——Pepsin digestion was performed according to Mandy *et al.*[6] with minor modifications. IgG was dissolved in 0.1 M sodium acetate, pH 4.5, digested for 24 hr at 37° in the enzyme to protein ratio of 1: 100 and dialysed against several changes of 0.1 M phosphate buffer, pH 7.4. Papain digestion was performed according to Porter *et al.*[7] IgG was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.01 M cysteine, digested for 24 hr at 37° in the same enzyme to substrate ratio, and dialysed as described above. These digestion mixtures were immediately fractionated on a Sephadex G-200 column equilibrated with 0.2 M Tris-HCl, pH 8.0 containing 0.2 M NaCl to remove the undigested IgG. From the pepsin digest, $F(ab')_2$ fragments were isolated by chromatography on CM cellulose and then Fc fragments [Fc (pepsin)] on DEAE cellulose, whereas Fc fragments were isolated from the papain digest [Fc (papain)] on DEAE cellulose and then Fab fragments on CM cellulose according to Franklin *et al.*[8] These fragments were examined for purity by Ouchterlony tests and found free of contamination each other (Fig. 1-a and 1-b).

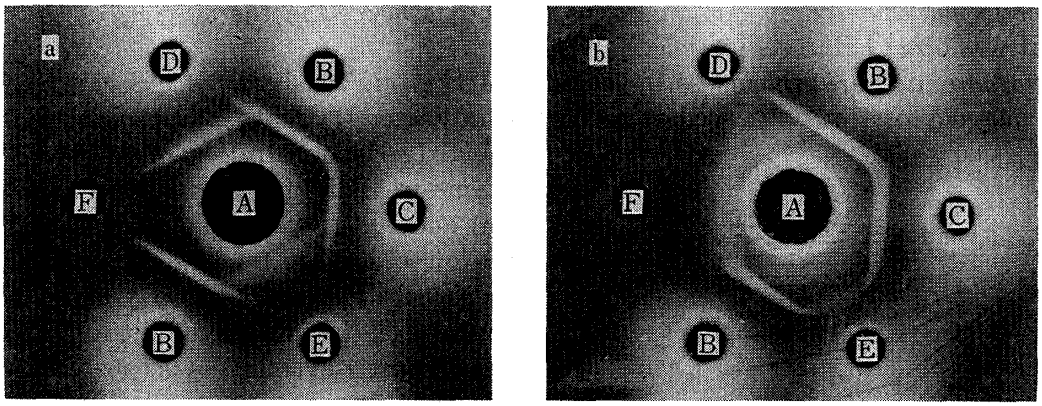

Fig. 1. Immunological Purity

[a] Gel-filtration plate which demonstrated precipitin lines formed between anti-IgG in center wells (A) and IgG(B), IgG[PEG] (C), F (ab,')$_2$(D) Fab[E], Saline [F] in peripherial wells, crossing-over each other. [b] Interaction of anti-IgG[A], IgG[B], IgG[PEG](C), $F(ab')_2$(D,) Fc[pepsin] [E], Fc[papain][F] were same as [a].

Agglutination Tests——Antibody activities against the three strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were determined by an agglutination test using the O-antigen prepared from formalin killed cells. For *Pseudomonas aeruginosa*, antibody activities against lipopolysaccharide (LPS) and original endotoxin protein (OEP) of the organism were also determined by using sheep erythrocytes sensitized with the LPS and OEP prepared by the methods of Boivin[9] and Homma,[10] respectively.

Complement——Human blood was collected by venipuncture and serum was separated. Test organisms were mixed with the serum to make a concentration of 10^9 cells/ml to remove natural antibody. The suspension of bacteria was shaken at 4° for 1 hr, centrifuged at 4° and the test organisms were added again to the supernatant. The procedure was repeated 3 times for complete removal of natural antibody to the test

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organism. Then, the serum was sterilized by filtration through a 0.45 mμ Millipore filter (Millipore Corp., U.S.A.) and used as complement.

Preparation of Human Polymorphonuclear Leukocytes (PMNs) Suspension——This was performed according to Young *et al.*[11] with minor modifications. Venous blood was drawn from healthy human adults (20 ml/adult) into a plastic syringe containing approximately 500 units of sterile heparin and mixed with 1/2 volume of isotonic 6% clinical dextran (M$\bar{w}$ 70000). The syringe was gently shaken and the erythrocytes were allowed to sediment at room temperature for 1 hr. The leukocyte-rich plasma was centrifuged at 500 ***g*** for 10 min. The leukocytes were washed twice with Hanks' balanced salt solution (HBSS) and resuspended in the same solution at a proper concentration. The suspension contained more than 85% PMNs.

Phagocytosis Tests——Reaction mixtures contained 0.1 ml each of a PMN suspension (1×10^6/ml), a bacterial suspension (approx. 3×10^5/ml) and one of 5—0.3% solutions of IgG preparations or fragments and 0.7 ml of the complement or HBSS. The mixtures were allowed to stand at 37° for 1.5 and 3.0 hr and centrifuged at 500 ***g*** for 5 min, and viable bacteria in the supernatant were counted by colony counting. Each experiment was repeated three times.

Results

Effect of IgG and Its Fragments on Phagocytosis by PMNs

The effect of IgG on phagocytosis of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae* by human PMNs was not observed in the absence of the complement but was observed in its presence.

Pseudomonas aeruginosa——In the intact IgG group consisting of IgG and IgG (PEG), marked phagocytosis was observed as a decrease in bacterial counts of approximately 2 $\log_{10}$ at the final IgG concentration of 0.5—0.03%. In the enzyme-treated IgG group consisting of IgG (plasmin) and IgG (pepsin), phagocytosis was noted at a little lower level of 2.0—1.7 $\log_{10}$ for IgG (pepsin) and at a greatly reduced level of 1.2—0.7 $\log_{10}$ for IgG (pepsin). Of the IgG fragments, F (ab')$_2$ showed a decrease in bacterial counts of 0.4—0.2 $\log_{10}$, which was a greatly reduced value as compared with that of IgG (pepsin). With Fc (pepsin), Fc (papain) and Fab fragments, there was no phagocytosis promotion as with albumin which was used as a control (Fig. 2).

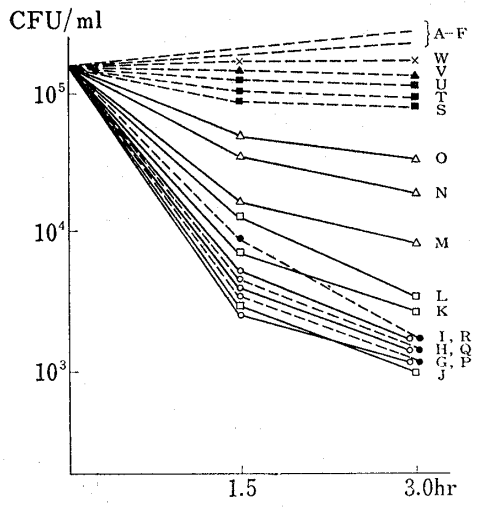

Fig. 2. Effect of IgG Preparations for Intravenous Use and IgG Fragments on Phagocytosis of *Pseudomonas aeruginosa* by PMNs

A) PMNs, B) FAS (Fresh absorbed serum), C) 0.5% IgG, D) PMNs+FAS E) PMNs+0.5%IgG, F) FAS+0.5%IgG, G,H,I) PMNs+FAS+0.5, 0.13, 0.03% IgG[PEG] respectively, J,K,L) PMNs+FAS+0.5, 0.13, 0.03% IgG[plasmin] respectively, M.N.O) PMNs+FAS+0.5, 0.13, 0.03% IgG[pepsin] respectively, P,Q,R) PMNs+FAS+0.5, 0.13, 0.03% IgG respectively, S.T.U) PMNs+FAS+;0.5, 0.13, 0.03% F(ab')$_2$ respectively, V) PMNs+FAS+0.5% Fab, W) PMNs+FAS+0.5%Fc.

Staphylococcus aureus——The results were similar to those of *Pseudomonas aeruginosa*. In the intact IgG group, there was a decrease of bacterial counts of approximately 1.1 $\log_{10}$, whereas in the enzyme-treated IgG group, IgG (plasmin) gave a value of approximately 1.1—0.4 $\log_{10}$ and IgG (pepsin) showed a greatly reduced value of 0.6—0.1 $\log_{10}$. Of the IgG fragments F (ab')$_2$ revealed a value of 0.2—0.1 $\log_{10}$, which was greatly lower than that of IgG (pepsin). With Fc (pepsin), Fc (papsan) and Fab fragments, there was no phagocytosis promotion (Fig. 3).

Streptococcus pneumoniae——The results were also similar to those of the above two strains. Phagocytosis was the most efficient in the intact IgG group, followed by IgG (plasmin) and they by IgG (pepsin). The IgG fragments failed to promote phagocytosis except F (ab')$_2$ which promoted phagocytosis slightly (Fig. 4).

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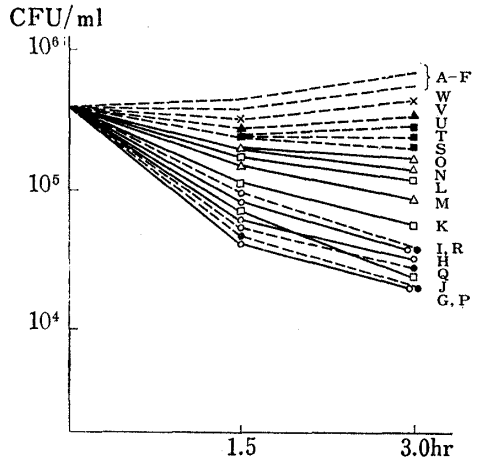

Fig. 3. Effect of IgG Preparations for Intravenous Use and IgG Fragments on Phagocytosis of *Staphylococus aureus* by PMNs

A) PMNs, B) FAS, C) 0.5%IgG, D) PMNs+FAS, E) PMNs+0.5% IgG, F) FAS+0.5%IgG, G,H,I) PMNs+FAS+0.5, 0.13, 0.03% IgG[PEG] respectively, J.K.L) PMNs+FAS+0.5, 0.13, 0.03% IgG [plasmin] respectively, M,N,O) PMNs+FAS+0.5, 0.13, 0.03% IgG[pepsin] respectively, P,Q,R) PMNs+FAS+0.5, 0.13, 0.03% IgG respectively, S,T,U) PMNs+FAS+0.5, 0.13, 0.03% $F(ab')_2$ respectively, V) PMNs+FAS+0.5% Fab, W) PMNs+FAS+0.5%Fc.

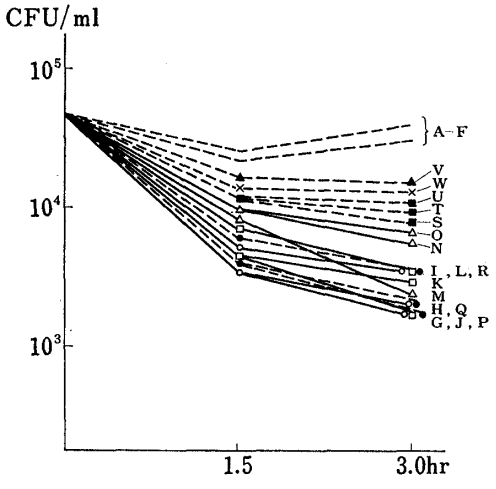

Fig. 4. Effect of IgG Preparations for Intravenous Use and IgG Fragments on Phagocytosis of *Streptococcus pneumoniae* by PMNs

A) PMNs, B) FAS, C) 0.5%IgG, D) PMNs+FAS, E) PMNs+0.5% IgG, F) FAS+0.5%IgG, G,H,I) PMNs+FAS+0.5, 0.13, 0.03% IgG[PEG] respectively, J,K,L) PMNs+FAS+0.5, 0.13, 0.03% IgG [plasmin] respectively, M,N,O) PMNs+FAS+0.5, 0.13, 0.03% IgG[pepsin] respectively, P,Q,R) PMNs+FAS +0.5, 0.13, 0.03% IgG respectively, S,T,U) PMNs+FAS+0.5, 0.13, 0.03% $F(ab')_2$ respectively, V) PMNs+FAS+Fab, W) PMNs+FAS+0.5%Fc.

TABLE I. Agglutinin titer of IgG and Its Fragments

IgG and fragments 5% protein conc. (w/v)	Agglutinin titer			Hemagglutinin titer *Ps. aeruginosa*	
	Ps. aeruginosa	*Staph. aureus*	*Str. pneumoniae*	LPS	OEP
IgG	2^{2-3} a)	2^5 a)	2^1 a)	2^4 b)	2^5 b)
IgG (PEG)	2^{2-3}	2^5	2^1	2^4	2^5
IgG (plasmin)	2^2	2^{4-5}	2^1	2^{3-4}	2^5
IgG (pepsin)	2^2	2^{4-5}	2^1	2^{3-4}	2^5
$F(ab')_2$	2^2	2^{4-5}	2^1	2^3	2^{4-5}
Fab	2^1	2^1	2^1	2^1	2^1
Fc (papain / pepsin)	2^1	2^1	2^1	2^1	2^1
Albumin	2^1	2^1	2^1	2^1	2^1

a) Agglutinin titers measured by using O-antigen prepared from formalin killed.
b) Passive hemagglutinin titers measured by using LPS and OEP coated sheep erythrocytes respectively.

Agglutinin titers of IgG and Its Fragments

In order to examine whether or not the antibody titer (mainly surface antibody titer for the microorganism) was involved in the differences in promotion of phagocytosis by PMNs between IgG and its fragments, agglutinin titers for cellular surface were determined (Table I). Although enzymatic treatment tends to reduce the antibody titer slightly, IgG (plasmin), IgG (pepsin), IgG (PEG) and $F(ab')_2$ fragments showed almost the same titer, varying in the range from 16—32 for *Staphylococcus aureus*, from 4—8 for *Pseudomonas aeruginosa* and being less than 2 for *Streptococcus pneumoniae*. The hemagglutinin titers of these preparations for LPS and OEP of *Pseudomonas aeruginosa* were 8—16 and 16—32, respectively. The

results indicated that IgG (PEG), IgG (plasmin), IgG (pepsin) and $F(ab')_2$ fragments which showed appreciable differences in phagocytosis tests differed only slightly in the antibody titer. Neither Fab nor Fc reacted.

Discussion

Host defense against pyogenic bacteria which grow outside the cell involves neutrophilic polymorphonuclear leukocytes, antibody and complement. In other words, the host's primary defense mechanism against pyogenic bacteria would depend on ingestion and subsequent killing of them by the interaction of PMNs with antibody and complement.[12] On the other hand, it has been accepted that *Mycobacterium* and *Listeria* which grow within the cell are not sufficiently treated by PMNs alone, but efficiently phagocytized and killed by macrophages.[13]

In the present study, we prepared three IgG preparations applicable to intravenous injection, IgG (plasmin), IgG (pepsin) and IgG (PEG) and compared the efficacy in terms of promotion of bacterial phagocytosis *in vitro* using the above described pyogenic bacteria-PMN system. As is evident from the above results with *Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pneumoniae*, phagocytosis was the most efficient with IgG (PEG), followed by IgG (plasmin) and then by IgG (pepsin).

Recently, it has been shown that the ingestion of microorganisms to PMNs involves their surface receptors for the Fc portion of the IgG molecule (Fc receptor) and for the activated third component of complement (complement receptor)[14,15]. This has been confirmed by our previous experiments on interaction of IgG molecules with phagocytic cells.[16] That is, since the ingestion of bacteria into PMNs is effected through their surface Fc receptor and complement receptor to which complexes of bacterium, antibody and/or complement are bound, it is necessary for antibody which coats bacteria to possess the Fc portion in its molecule.

In view of the above findings, it is considered that differences in their effect on phagocytosis of various IgG preparations prepared by different methods would depend on the rate of their interaction with the PMN surface receptors.

As IgG (PEG) prepared by polyethylene glycol fractionation method consists of more than 90% intact IgG molecules, all the molecules would have contributed to phagocytosis promotion. In the case of plasmin digested IgG [IgG (plasmin)], intact IgG molecules present in 60% would have worked to promote phagocytosis. In contrast, pepsin digested IgG [IgG (pepsin)] contains 90% or more $F(ab')_2$ fragments and little, if any monomeric IgG so that, even though the complex of bacterium and antibody could be formed, its lack of complement receptors and subsequent reduced binding to the Fc receptor and complement receptor on the PMNs would have resulted in less promotion of phagocytosis.

For the same reason, the Fab fragments obtained by papain digestion could be bound to bacteria but not to PMN membrane, resulting in the absence of the phenomenon of bacterial ingestion. On the other hand, slight promotion of phagocytosis with $F(ab')_2$ fragments would be due to weak activation of complement by the alternative pathway.[17]

In order to rule of the involvement of the antibody titer in the difference in phagocytosis among the IgG preparations and IgG fragments, agglutinin titers were determined for the three strains of *Pseudomonas seruginosa, Staphylococcus aureus* and *Streptococcus pneumoniae* and little difference was found. We consider therefore that the differences of

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the IgG preparations in phagocytic activity against bacteria could be ascribable to the structural element of IgG molecules contained in the preparations, and it is easily conceived that the difference of various IgG preparations in phagocytotic effect is reflected in differences in the therapeutic effect of the preparations in clinical practice.

On the basis of the above findings, it is concluded as a matter of course that the more intact IgG molecules an IgG preparation for clinical use has in its composition, the more effective it is as far as promotion of bacterial phagocytosis by PMNs is viewed as the interaction among the Fc portion of IgG molecules, the activated component of complement and PMN surface receptors for these.