In Vitro Investigation Regarding the Antibacterial Effects of Immunoglobulin Alone and in Combination with Antibiotics

Hideyo KATSUNUMA, M.D.

Department of Geriatric Medicine, Tokyo Medical College, Japan

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Introduction

Immunoglobulin G has a Y-shaped structure in which the H and L chains are bonded together by the S-S bond. This structure of immunoglobulin G was discovered for the first time by Porter and Edelman, later Valentein, Green and their co-workers demonstrated this Y-shaped structure of immunoglobulin G under an electron microscope, using DNP-octamethylene diamine as haptene. IgG was tried in many clinical cases of infectious diseases, but caused shock. This untoward side effect impeded further use of IgG. However, Schultz and Schwick⁶⁾ and their co-workers obtained F(ab')₂ fragment by digesting the Fc fragment of IgG with the aid of pepsin. This F(ab')₂ fragment which is free from untoward side effects has been in wide clinical use in the treatment of infectious diseases, achieving remarkable results⁹⁾¹⁰⁾. Today, preparations of immunoglobulin, such as sulfonated globulin and polyethylene glycol globulin, in which IgG is, both structurally and functionally, closer to native IgG, are drawing increasing attention and are in more common use.

IgG is presumed to act against infectious diseases by the following mechanisms. Firstly, IgG promotes the phagocytic activity of neutrophils and macrophages through the mediation of opsonin. Secondly, IgG destroys the cell membrane of microorganisms and acquires bacteriolytic activity through the classical or alternative pathway by activating the complement. IgG is believed to have no direct antibacterial action. On the other hand, however, there are reports (Fleming, Finland, Zwisler)^{1)~5)7)} that IgG exhibits an antibacterial action in vitro, when serum from patients, who have or have had an infection, is used alone or in combination with an antibiotic.

Because of these conflicting views, the present study has been conducted to determine whether IgG has a direct antibacterial action or not. In this study, pepsin treated immunoglobulin F(ab')₂ fragment, abbr. Pepsin-Ig, gamma-Venin (glycin free) which does not involve the mediation of neutrophils, macrophages or complements, were used alone or in combination with an antibiotic.

Materials and Methods

The species of microorganisms used in the present study consisted of gram-negative cocci (*K. pneumoniae*, *E. coli*, *P. aeruginosa*, *H. influenzae*, *N. gonorrhoeae*, *S. marcescens*) and gram-positive bacilli (*S. aureus*, *S. epidermidis*, *S. faecalis*). The culture media consisted of brain heart infusion (BHI) plus 2.5, 5.0 and 10% pepsin-Ig BHI, 5 and 10% albumin BHI, 12.5 µg/ml and 25 µg/ml AB-PC BHI, 5.0% pepsin-Ig + 12.5 µg/ml AB-PC BHI and 5% pepsin + 25 µg/ml AB-PC BHI.

The inoculum sizes ranged from 1.5×10^5 /ml to 2×10^6 /ml.

Results

I. Time Effect on the Viability of Incubated Gram Negative and Positive Bacteria

1. Changes in Live Cell Count in 5% Pepsin-Ig BHI Cultures

The time effect on the viability of gram-negative bacilli cultured in 5% pepsin-Ig BHI media is illustrated in Fig. 1. With the exception of *P. aeruginosa* and *S. marcescens*, the viability was seen to decrease in a step fashion after 1.5 hours of incubation. *N. gonorrhoeae* in particular had a remarkable loss of live cells, after 1.5 hours the live cell count had decreased to 2.0×10^3 cells/ml from the original inoculum size of 1.5×10^5 cells/ml. After 2.5 hours it was down to 3.5×10^2 cells/ml, and after 4 hours it had further decreased to 1.0×10^2 cells/ml.

Similarity with 10% pepsin-Ig BHI media, except for the 24-hours culture of *S. marcescens*, the viability of gram-negative bacilli decreased with time.

On the other hand, with BHI broth cultures, and cultures with 10% albumin BHI, shown in Figs. 2 and 3 respectively, all species of organisms showed a growth with time.

2. Time Effect on the Viability of Incubated Gram Positive Bacteria

The live cell count of *S. aureus* cultured in 5% pepsin-Ig BHI media decreased from 4.5×10^5 cells/ml to 2.5×10^5 cells/ml after 4 hours, but after 24 hours had increased to 5.5×10^8 cells/ml. With 10% pepsin-Ig BHI media, *S. epidermidis* alone showed a decrease in live cell count, from 7.5×10^5 cells/ml to 4.5×10^5 cells/ml after 4 hours and down to 2.0×10^4 cells/ml after 24 hours shown in Fig. 4. With BHI and 10% albumin BHI media, no change in live cell count was noted in any of the cultures.

3. Changes in Bacterial Viability in 5% Pepsin-Ig BHI Cultures after 24 Hours

Fig. 1 Proliferation-inhibitory effect on 5% pepsin-Ig BHI of gram-negative bacilli

*Pepsin Ig : Pepsin treated immcnoglobulin, F(ab')₂ fragment
*BHI : Brain heart infusion

- (1) : *Klebsiella pneumoniae*
- (2) : *Escherichia coli*
- (3) : *Pseudomonas aeruginosa*
- (4) : *Neisseria gonorrhoeae*
- (5) : *Hemophilus influenzae*
- (6) : *Serratia marcescens*

Fig. 2 Proliferation-of gram negative bacilli in the brain heart infusion (BHI) meduim

- (1) : *Klebsiella pneumoniae*
- (2) : *Escherichia coli*
- (3) : *Pseudomonas aeruginosa*
- (4) : *Neisseria gorrhoeae*
- (5) : *Hemophilus influenzae*
- (6) : *Serratia marcescens*

Fig. 3 Proliferation inhibitory effect on 10% albumin BHI of gram-negative bacilli

Fig. 4 Proliferation-inhibitory effect on 5% and 10% pepsin-Ig BHI of gram-positive cocci

The viability of cultured bacteria decreased considerably over the first 24 hours of incubation, but then began to rise. This fact suggests that pepsin-Ig retains its bacteriostatic action for 24 hours, but has no bactericidal action.

4. Changes in Bacterial Viability with Combinations of Antibiotics

Fig. 5 Shows the results of tests with BHI and pepsin-Ig used in combination with antibiotics. The combinations tested were BHI alone, 5% pepsin-Ig + BHI, 5% pepsin-Ig + 12.5 μ g/ml AB-PC + BHI, 5% pepsin-Ig + 25 μ g/ml AB-PC + BHI, 25 μ g/ml, and 12.5 μ g/ml AB-PC + BHI. The live cell counts after 1, 2, 4 and 6 hours of incubation were all found to be lower than with BHI alone. The live cell count with 5% pepsin-Ig + BHI alone was higher than when it was combined with 25 μ g/ml AB-PC, although the live cell count with 5% pepsin-Ig + BHI alone was lower than with 12.5 μ g/ml AB-PC BHI. The greatest reduction in live cell count was achieved with 5% pepsin-Ig + 25 μ g/ml AB-PC + BHI media. However, even with this combination the live cell count had increased to 7.5×10^6 cells/ml after 24 hours of incubation, as compared to the original inoculum size of 1×10^3 cells/ml.

II. Comparison of the Test Broth Turbidity between Test Tubes with Pepsin-Ig and Test Tubes without Pepsin-Ig

After 4 hours of incubation when the count of live bacterial cells began to decrease, the turbidity of test broth tubes with pepsin-Ig added was compared with that of test broth tubes containing no pepsin-Ig. The test broth tubes with pepsin-Ig were nearly transparent, while the tubes containing no pepsin-Ig were turbid, suggesting that without pepsin-Ig, bacteria proliferated.

III. Smear Staining Results

The morphology of *E. coli* with smear stain incubated with 5% pepsin-Ig revealed, after 4 hours of incubation, swollen round shape or thin elongated, shape forming filaments. In cultures without pepsin-Ig such changes were not noted.

Fig. 5 Proliferation inhibitory effect of 5% pepsin-Ig alone, combined with AB-PC and AB-PC alone on *K. pneumoniae* in vitro

- (1) Control BHI medium Culture
- (2) 5% Pepsin-Ig BHI
- (3) 5% Pepsin-Ig BHI + 12.5 μ g/ml ABPC
- (4) 5% Pepsin-Ig BHI + 25 μ g/ml ABPC
- (5) 12.5 μ g ABPC BHI
- (6) 25 μ g/ml ABPC BHI

Fig. 6 Coupling of pepsin-Ig to latex beads

Reagents

Pepsin Ig	Lot No. 341L0256
Latex Beads	$\phi=0.4-0.6 \mu\text{m}$ (Behringwerke)
Glycine saline	Glycine 7.51 g
	NaCl 5.85 g NaCl
	NaN ₃ 2 g
	Dissolved in 1800 ml D.D.W. and adjusted pH to 8.2 with 0.1N NaOH, then filled up to 2000 ml.

Coupling Method

{ 1 % Latex suspension in Glycine saline	5 ml
{ Glycine saline	15 ml
{ 1 % Pepsin Ig solution	5 ml
↓	
Rotated for 24 hr at 4°C	
↓	
Washed 3 times by centrifugation (10,000 rpm × 10 min., 4°C)	
↓	
Resuspended in 5 ml of Glycine saline (Pepsin-Ig - Latex)	

IV. Experiments to Detect the Pepsin-Ig Sensitized Latex-K. Pneumoniae Complex

Since it was shown that pepsin treated IgG had a bacteriostatic action, and produced cell swelling and filament formation when smears of *E. coli* cultures added with pepsin-Ig were stained, experiments were conducted to determine whether pepsin-Ig has a direct bactericidal action.

Experiments were designed as follows:

Experiment 1:

The pepsin-Ig coated latex was prepared by the procedure shown in Fig. 6. Briefly, 1% pepsin-Ig (glycine-free) was mixed with an equal volume of latex 0.4 to 0.6 μm in diameter and incubated at 37°C for 4 hours then, the agglutination titer of the pepsin-Ig coated latex measured against human IgG was 2⁸.

To 0.5 ml of pepsin-Ig coated latex, 2×10^8 cells/0.5 ml of *K. pneumoniae* were added, and the culture was incubated at 37°C for 15 min. This culture was negative stained for electron microscopy.

Experiment 2:

The procedure of Experiment 2 was the same as with Experiment 1, except that BSA was used instead of pepsin-Ig.

Experiment 3:

Uncoated latex was added to an equal volume of *K. pneumoniae* to obtain a control culture and incubated at 37°C for 15 min.

Experiment 4:

Latex was added to pepsin-Ig, in which the agglutination titer against *K. pneumoniae* was reduced to 2¹ by the procedure shown in Fig. 7, and the culture incubated. After incubation the culture was examined for a complex of the pepsin-Ig sensitized latex and *K. pneumoniae*.

Experiment 5: Detection of the complex of anti-IgG/Fab sensitized latex and pepsin-Ig treated *K. pneumoniae*

Anti-human IgG/Fab serum (Behring Werke) was obtained, using DEAE cellulose to obtain pure anti-

Fig. 7 Measurement of anti *K. pneumoniae* agglutination titer in IG100 and gamma-venin

Samples and Reagents

Bacteria	<i>Klebsiella pneumoniae</i> ATCC10031
IG100	Lot No. 1160
Gamma Venin (GV, Glycine free)	Lot No. 341L025, 341L026
PBS (pH 7.2)	

Method

1. Preparation of Bacterial Suspension
Overnight culture 150ml
↓
Washed 3 times by centrifugation (3,000 rpm×10min.)
↓
Resuspended in 30ml of PBS
↓
Diluted with PBS to adjust %T₆₅₀=4.5
2. Dilution of Samples
Serial ×2 dilutions were made from 5% solution by PBS.
3. Titration
Diluted sample 0.5ml
Bacterial suspension (%T₆₅₀=4.5) 0.5ml
↓
Mix
↓
Allowed to stand for 16h at R.T.
↓
Observed agglutination

Results

	Anti <i>K.p.</i> Agglut. Titer
IG100	2 ⁸
GV (341L025)	2 ⁸
GV (341L026)	2 ⁸

Fig. 8

IgG/Fab fragment and adjusted to a concentration of 1% with a saline solution of glycine. An equal volume of latex was added to this solution to obtain sensitized anti-human IgG/Fab latex. It was incubated with the addition of pepsin-Ig. Washed cells of *K. pneumoniae* were added to this culture and incubated again at 37°C for 15 min. After incubation the culture was studied under an microscope.

Experiment 6: Detection of the complex of anti-IgG/F(ab')₂ specific antibody sensitized latex and pepsin-Ig treated *K. pneumoniae*

F(ab')₂ obtained from gamma-venin by filtration to allow to be bound to Sepharose 4B and then added to anti-IgG/Fab and incubated to obtain specific anti-IgG/F(ab')₂. Latex sensitized with this specific anti-IgG/F(ab')₂ was incubated with the addition of *K. pneumoniae* previously exposed to pepsin-Ig. After 15 min of incubation at 37°C the culture was studied under an electron microscope.

Experiment 7:

Pepsin-Ig was directly added cultures of *K. pneumoniae* and incubated. After 15 min of incubation the cultures were studied under an electron microscope.

V. Electron Microscopic Findings with Neagative Stain

The morphology of coated latex sensitized with pepsin-Ig *K. pneumoniae* complex revealed in experiments 1, 5, 6, and 7 that cells of *K. pneumoniae* formed complexes with latex.

In the invaded cell, cell content is released into the space between the inner and outer cell membranes, and the cell membrane is swollen as shown in Fig. 8. Further, the cell membrane is fragmented in spots and cracked shown in Fig. 9.

Fig. 10 shows that in some affected cells the outer cell membrane is separated from the cytoplasm while in Fig. 11 exuding cell content can be seen. These morphologic changes of bacterial cells exposed to pepsin-Ig suggest that it has a bacteriolytic action.

The cultures directly added with pepsin-Ig also showed signs of bacteriolysis as seen in Fig. 12.

With *K. pneumoniae* exposed to anti-IgG/Fab, specific anti-IgG/F(ab') sensitized latex and pepsin-Ig, latex was also observed adhering to bacterial cells.

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Whereas in experiments 2, 3 and 4 where uncoated latex, BSA and absorbed latex were used, no latex was seen adhering to bacterial cells (Figs. 13, 14, and 15).

In electron micrographs (Figs. 16 and 17), obtained with ultra-thin sections by uranyl-acetate staining, the outer membrane and cell wall on one side of the bacterial cell had an indistinct structure, and cytoplasm exuded around the outer cell membrane. The other side of the cell retained an intact outer membrane and cell wall structure, but apparently cytoplasm moved to the injured side, so that the intact side was void of cytoplasm. The effect of loss of cytoplasm, is that the cell membrane shrinks giving the injured cell the appearance of a cast-off snake skin left only with the membrane.

Fig. 13

Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18 Possible mechanism of bacteriostasis or bacteriolysis by attack of cell membrane and cell wall due to $F(ab')_2$ fragment immunoglobulin (pepsin-Ig)

Discussion

Fleming obtained serum from a patient who had or had septic infection, and incubated it on an object glass with the addition of bacteria. Under these conditions he noted bacterial growth inhibition and, with the addition of an antibiotic of neutrophils, inhibition increased. Finland achieved bacterial growth inhibition both with γ -globulin containing a serum antibody derived from clinical material and with such γ -globulin plus an antibiotic and a sulfamide. In an *in vitro* experiment Zwisler exposed AB-PC resistant *Staphylococcus aureus* to pepsin-Ig and found that pepsin-Ig inhibited bacterial growth to a greater extent than Beriglobulin. The mechanism of inhibition by pepsin-Ig has not yet been clarified⁸⁾, however.

Human IgG is presumed to act against infectious diseases by promoting the phagocytic activity of neutrophils and macrophages through the mediation of opsonin, activating the complement, and destroying the cell membrane of microorganisms through the classical or alternative pathway, thus acquiring a bacteriolytic action. In the present study, to eliminate the effect of the complement, opsonin, neutrophil and macrophage, pepsin-treated human immunoglobulin (pepsin-Ig) was used. *In vitro* experiments pepsin-treated immunoglobulin F(ab')₂ fragment showed inhibition, and some electron microscopic findings which were morphologically considered compatible with bacteriolysis were obtained. This tendency was more marked with cultures exposed to pepsin-Ig plus antibiotics.

The findings obtained in this study suggest that pepsin-Ig adheres to the bacterial cell membrane (lipopolysaccharide) and acts on the cell wall in exhibiting a bacteriostatic action and even shows a bacteriolytic effect on some weak species of organisms. It still remains for further study to clarify the mechanism of action of pepsin-Ig, however, possible mechanism of action is shown in Fig. 18. The pepsin Ig, F(ab')₂ fragment attaches to the surface lipopolysaccharide of the outer membrane in sufficient numbers that this region of the bacteria is immobilized. Even at this stage, the bacteria can still move; such movement by other sides of the bacteria can lead to splitting of the immobilized membrane and cell wall, then through the inner membrane, resulting in cytoplasm leakage.

Conclusions

1) Pepsin-treated human immunoglobulin F(ab')₂ fragment, that is, pepsin-Ig, otherwise known as Gamma Venin (glycine-free) incubated containing brain heart infusion broth cultures exhibited a bacteriostatic action. When it was used in combination with antibiotics, it showed a bactericidal action.

2) When incubated cultures of pepsin-Ig sensitized latex and *K. pneumoniae* were examined under an electron microscope with negative stain, latex was found adhering to the cell membrane and injuring the cell wall. These findings were also seen in the cultures of sensitized anti-human IgG/Fab and specific anti-human IgG/F(ab')₂ latex and *K. pneumoniae*.

3) Electron micrographs obtained by ultra thin section and negative stain revealed pepsin-Ig injured bacterial cell membrane and cell wall, and cells releasing cytoplasm were noted. These findings were more remarkable with pepsin-Ig used in combination with antibiotics than with pepsin-Ig alone.

4) The morphological findings of cell membrane and cell wall studies of *K. pneumoniae* under electron microscopy strongly suggested that pepsin treated Immunoglobulin F(ab')₂ fragment attacked the cell membrane or cell wall of *K. pneumoniae* inhibiting proliferation, resulting in bacteriostasis and even showed a bacteriolytic effect on some weak species of organisms.

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試験管内における免疫グロブリン単独及び抗生物質併用 による抗菌効果に関する研究

東京医科大学老年病学教室

勝 沼 英 宇

要 旨

臨床的に細菌性感染症に効果を示す免疫グロブリンの作用は、(1) opsonin を介し、宿主の好中球、マクロファージの喰食能を促進、(2) classical 又は alternative pathway によって補体を活性化し溶菌作用を惹起、(3) 細菌の exotoxin を中和、することによるが、菌に対しては直接殺菌作用は有しないといわれている。本研究は pepsin 処理免疫グロブリン、F(ab')₂ fragment, glycin free のガンマベニン Behring werke 製（以下 pepsin Ig と略）を用い、免疫グロブリンの抗菌作用を検討した。培地は BHI、5 及び 10% pepsin Ig BHI、10% アルブミン BHI を使用、菌はグラム陰性桿菌及び陽性球菌を用い好中球と補体を加えない試験管内菌増殖抑制の有無を経時的に観察した。

BHI 及びアルブミン BHI 培地では菌は増殖し

たが、5.0 及び 10.0% pepsin Ig BHI 培地では ser-ratia を除き、グラム陰性桿菌は何れも 1.5, 2.5, 4, 24 時間と階段状に減少、特に *N. gonorrhoeae* は最も減少した。しかし、24 時間以後は再び増殖傾向を示した。形態学的には菌は spheroblast 化又は filament 化した。電顕的にはアルブミン及び pepsin Ig で感作していない latex には混合して培養した *K. pneumoniae* は附着していないが、pepsin Ig で感作した latex は菌の細胞膜に附着し、細胞内、外膜間に菌内容物が逸脱し、一部の菌では細胞外に菌内容物流出する所見が negative stain で証明された。透過電顕でも細胞膜消失、破壊断裂、菌細胞質の膜外流出が認められ、pepsin Ig による溶菌が示唆された。以上より 5.0 又は 10.0% 免疫グロブリンにより菌は直接的に増殖が抑制され、一部の弱い *K. pneumoniae* に対して溶菌作用を認めた。

別刷請求先：(〒160) 新宿区西新宿 6—7—1

東京医科大学老年病学教室 勝沼 英宇