Review

Intravenous Immunoglobulin for Treating Bacterial Infections: One More Mechanism of Action

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Abstract: The mechanisms underlying the effects of γ-globulin therapy for bacterial infections are thought to involve bacterial cell lysis via complement activation, phagocytosis via bacterial opsonization, toxin neutralization, and antibody-dependent cell-mediated cytotoxicity. Nevertheless, recent advances in the study of pathogenicity in gram-negative bacteria have raised the possibility of an association between γ-globulin and bacterial toxin secretion. Over time, new toxin secretion systems like the type III secretion system have been discovered in many pathogenic gram-negative bacteria. With this system, the bacterial toxins are directly injected into the cytoplasm of the target cell through a special secretory apparatus without any exposure to the extracellular environment and, therefore, with no opportunity for antibodies to neutralize the toxin. However, because antibodies against the V antigen, which is located on the needle-shaped tip of the bacterial secretion apparatus, can inhibit toxin translocation, this raises the hope that the toxin might be susceptible to antibody targeting. Because multi-drug resistant bacteria are now prevalent, inhibiting this secretion mechanism is attractive as an alternative or adjunctive therapy against lethal bacterial infections. Thus, it would not be unreasonable to define the blocking effect of anti-V-antigen antibodies as the fifth mechanism for immunoglobulin action against bacterial infections.

Keywords: immunoglobulin; IVIG; LcrV; PcrV; translocation; type III secretory toxin; type III secretion system; V-antigen

1. Introduction

Non-specific intravenous globulin (IVIG) is a human polyclonal IgG from the pooled plasma of over 10,000 healthy blood donors. Because IVIG comes from so many donors, it is thought to contain antibody components capable of binding a large variety of antigens. In fact, the number of components far exceeds the antibody repertoire of a healthy individual [1-5]. With such an advantage, IVIG is used to treat severe infections and autoimmune diseases. Immunoglobulin in the body has a bioprotective activity that can prevent or cure infections. The mechanism involved in the IVIG effect against infectious diseases is understood to have four main features: (1) complement-associated immunolysis, (2) opsonization (immunophagocytosis), (3) toxin/virus neutralization, and (4) antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig.1). These four mechanisms of action are briefly summarized here.
(1) Complement-associated immunolysis: After the antibody binds to certain gram-negative bacteria, spirochetes, or other types of bacteria, the complement components react, pierce the cell membrane and destroy the bacteria through cell lysis.

(2) Opsonin action (immunophagocytosis): Neutrophils and macrophages have a receptor for the Fc portion of IgG, and can effectively phagocytose bacteria to which IgG antibody is bound via this receptor. IgG bound to bacteria induces active oxygen (O₂⁻) (a bactericidal substance) in the phagosome, and this O₂⁻ not only acts directly on the bacterium as an oxidant but also promotes phagosome to lysosome fusion. The phagocytosed bacteria are then effectively sterilized and digested by cooperation with lysosomal enzymes.

(3) Toxin neutralizing action: An antibody responds by binding to a toxin produced by a bacterium and neutralizing its activity. In the case of a viral infection, the antibody can bind to a virus particle to prevent the virus from entering the target cell.

(4) ADCC: When IgG antibodies bind to the virus-related antigens expressed on the surface of a virus-infected cell, a natural killer cell with Fc receptors can then bind to and damage the virus-infected cell. In doing so, it destroys the infected cell, which is the site of virus propagation and prevents the transmission of pathogenic viruses.

Figure 1. Mechanisms used by immunoglobulins to destroy infectious agents and toxins

The mechanistic effects of immunoglobulins against infectious diseases are understood to involve the following four main aspects: (1) complement-associated immunolysis, (2) opsonization (immunophagocytosis), (3) toxin/virus neutralization, and (4) ADCC. (5) The unmentioned fifth mechanism, which involves the blockade of toxin secretion by immunoglobulins, is discussed in this review.

During a bacterial infection, IVIG binds to the surface antigens on bacterial cells to promote bacterial lysis via complement, neutrophil- and macrophage-related phagocytosis, and the neutralization of bacterial toxins. Therefore, because the virus-neutralizing action and ADCC are related to the role of IVIG against viral infections, if limited to bacterial infections, the mechanism for IVIG can be focused on (1), (2), and a part of (3). In addition to the above-mentioned mechanisms, as a supplementary action, IVIG is thought to bind to the Fc-receptors on lymphocytes and suppress cytokine production via stimulation with toxins or cytokines [6-8].
However, beyond the above-mentioned mechanisms related to IVIG in Figure 1, recent advances in our understanding of the virulence mechanism used by gram-negative bacteria and the host’s immune response specific to the bacterial toxin secretion system (the type III secretion system) raise the possibility of there being an as yet unknown mechanism for IVIG-related pathogen targeting. After the next section, we overview the newly identified toxin-secreting function used by gram-negative bacteria and host immunity against it, and reconsider the mechanism of action for IVIG against pathogenic gram-negative bacteria.

2. The bacterial type III secretion system and its toxins

In the early 1990s, Swedish bacteriologists reported that in Yersinia, the bacterial toxin YopE is directly sent from the bacterial cytosol to the cytoplasm of the target cells through a specialized secretory apparatus [9-11]. Later, along with the progress obtained for the genome analysis of many pathogenic gram-negative bacteria, this new concept of toxin secretion was verified and homologous secretion systems were found in various pathogenic gram-negative species [12-15]. This type of toxin secretion, which was then called the type III secretion system, differs from the classical type I and II systems.

![Figure 2. Mechanisms of immunoglobulin-related action against infectious agents and toxins.](image)

(1) Type I & II secretion

(2) Type III & IV secretion

Gene-level analyses have revealed that the type III secretion system evolved from flagella [16,17]. In Legionella and Helicobacter pylori, toxin proteins and nucleic acids were found to be driven into target cells by a special secretion device that appears to have evolved from pili; subsequently, the system was named the type IV secretion system [18]. In both type III and IV secretion systems, toxins are injected directly into the cytoplasm of a target cell using a secretion device. Therefore, even in the presence of a neutralizing antibody against the toxin, because the toxin reaches the target cell without being exposed to the extracellular environment there is no possibility that antibodies can neutralize the toxin itself (Fig. 2). The fact that antibody immunization does not enable the host to offset the damage caused by the type III secretory toxins is alarming. Interestingly, toxins are produced in an inactive state in bacteria and only exert enzymatic activity in conjunction with a co-factor after being injected into eukaryotic cells. Hence, the inactive toxin only exerts its action after entering the target cell. Thus,
The toxin injection device, or 'injectisome', comprises many protein molecules. Toxins are called 'effectors', and the mechanism used by the toxins to move from the bacterial cytosol to the outside of the bacterial cell membrane is defined as 'secretion'. The bacterium also makes holes in the cell membrane of the target cell to drive toxins into the cytoplasm, a process called 'translocation'. Translocation involves a two-protein structure called the 'translocon', which is described later.

3. The V-antigen of the type III secretion apparatus and its specific antibody

About 70 years ago, in the 1950s, British researchers reported that the secreted protein components from Yersinia pestis contain an antigen recognized by the serum from infected mice that exerts a vaccine effect in a mouse model of Y. pestis infection [19-24]. This antigen was named the V-antigen. Later on in the 1980s, a set of proteins secreted from Yersinia (called Yop, Yersinia outer membrane proteins) under low-calcium conditions (named the low calcium-response, lcr) were found to contain the V-antigen protein (or LcrV). The LcrV gene was then found to be encoded by the pCD1 plasmid, which is essential for the pathogenicity of Yersinia [25], and passive immunity against LcrV was reported [26,27]. In the early 1990s, as mentioned above, it was reported that Yersinia injects some Yop proteins directly into its target cells through a special secretion apparatus, and that this apparatus is associated with a set of genes (called the Yop virulon) encoded on pCD1 [9,10]. Among the five Yop virulon-associated operons the lcrGVHyopBD operon, which encodes the five proteins (including LcrV) plays a role in toxin translocation. Key experiments then showed that a knockout mutant of the LcrV gene, lcrV, lost its toxicity and antibodies against it inhibited the toxicity [28,29].

In 1996, the exoenzyme S-related toxins were reported to be P. aeruginosa type III secretion toxins [30]. In 1997, in addition to two exoenzymes (ExoS and ExoT), the cytotoxic type III secretory toxin known as ExoU was newly discovered as a major lung injury factor in P. aeruginosa [31]. Consequently, a region called the exoenzyme S regulon in the chromosomal genome of P. aeruginosa was discovered (Fig 3, 1) [32]. Surprisingly, beyond the bacterial species lineage, the exoenzyme S regulon shares high homology with the Yersinia Yop virulon. In the exoenzyme S regulon, five operons encode the regulatory proteins, the secretion apparatus, and the translocon components (Fig 3, 2). The pcrGVHpopBD operon, which is homologous to the Yersinia lcrGVHyopBD operon, encodes five proteins associated with toxin translocation (Fig 3, 2) [33]. Then, as already seen with the LcrV vaccinations against Yersinia, in 1999 the effects of PcrV vaccination and passive immunization with anti-PcrV antibodies in animal models of P. aeruginosa pneumonia were reported [34]. In both Yersinia and P. aeruginosa, specific antibodies against LcrV and PcrV, respectively, reduced the infection pathology [28,34].

Translocation of Yersinia type III secretion involves two proteins—YopB and YopD—both of which are encoded by the lcrGVHyopBD operon [10, 35,36]. The homologs of these P. aeruginosa proteins, which are encoded by pcrGVHpopBD, are PopB and PopD [33]. YopB and YopD from Yersinia, and PopB and PopD from P. aeruginosa, are involved in pore formation in the eukaryotic cell membrane. The structural mechanism involving LcrV, YopB and YopD in Yersinia and PcrV, PopB, and PopD in P. aeruginosa involve a translocon (Fig 3, 3). The structural position of the V-antigen proteins in the type III secretion system was unknown, but in 2005, electron microscopic analysis...
showed that LcrV and PcrV are both cap-like structures located at the tip of the needle structure in the secretion apparatus [37,38]. Currently, V-antigens are thought to occupy the interface between the secretion needle and translocation as an essential component of the translocon.

**Figure 3.** Genome, gene, and protein structures associated with the *Pseudomonas aeruginosa* PcrV V-antigen

1. The *P. aeruginosa* PAO1 chromosomal genome contains a region, the exoenzyme S regulon, which shares high homology with the *Yersinia* Yop virulon.
2. In the exoenzyme S regulon, five operons encode regulatory proteins, secretion apparatus, and translocon components. The *pcrGVHpopBD* operon encodes five proteins associated with toxin translocation.
3. PcrV is a cap-like structure located at the top of the secretion needle, and PopB and PopD are involved in pore formation in the eukaryotic cell membrane. The structural mechanism involving three of the PcrV–PopB–PopD proteins involves a translocon.

**4. Blocking effects of antibodies against the bacterial type III secretion system**

A *pcrV*-deletional non-polar mutant of *P. aeruginosa* has lost its type III secretion toxicity in the same way as an *lcrV*-deletional non-polar mutant of *Yersinia* [34], but complementation of *pcrV* with a plasmid in trans restored the toxicity [35]. These observations indicate that a V-antigen, such as LcrV or PcrV, is essential for type III secretion intoxication [28,34]. Active immunization with recombinant PcrV improved mortality in a model of *P. aeruginosa* pulmonary infection [34], and passive immunization with a rabbit-derived anti-PcrV-specific polyclonal IgG against PcrV reduced the acute lung injury associated with *P. aeruginosa* type III secretion [39]. Additionally, intravenous administration of a polyclonal antibody F(ab’): significantly improved secondary sepsis in a rabbit.
6. pneumonia model [39]. The anti-PcrV-specific polyclonal IgG was also effective in a burned skin model of *P. aeruginosa* infection [40], and in a chronic *P. aeruginosa* pneumonia model also [41]. Consequently, the mouse-derived monoclonal antibody (Mab) 166 was developed [42], the administration of which reduced lung injury and mortality in the *P. aeruginosa* pneumonia infection model [43]. This effect was also observed for the Mab166 Fab (antigen binding fragment) [43]. This Mab166 monoclonal antibody was humanized for clinical use as KB001 [44], and phase II clinical studies with it have now been conducted [45-47]. Active immunization with recombinant PcrV also improved acute lung injury and mortality in *P. aeruginosa* pneumonia models [48-50]. In recent years, more researchers have reported on the clinical application of anti-PcrV antibodies that have been developed using different approaches.

5. V-antigen homologs

In addition to *P. aeruginosa* PcrV, *Aeromonas* AcrV, *Vibrio* VcrV, and *Photorhabdus* LssV have now been identified as *Yersinia* V-antigen homologs (Fig 4) [51]. It was reported that rainbow trout vaccinated with recombinant AcrV are not protected against infection with *Aeromonas salmonicida* [52,53]. *Aeromonas hydrophila* and *Vibrio* are infective to humans, but no detailed studies of AcrV and VcrV from each of them, respectively, have been performed. Thus, with the exception of LcrV and PcrV, the characteristics of V-antigen homologs from other species have not been adequately studied yet.

![V-antigen homologs diagram](image)

**Figure 4.** Phylogenetic tree of V-antigen homologs and orthologs


As shown in Fig 4, V-antigen orthologs have also been identified that share a genetic and functional association with V-antigen homologs; these include EspA from pathogenic *Escherichia coli*,...
Salmonella SipD and SseB, Shigella IpaD, Burkholderia BipD, Bordetella pertussis Bsp22, and Chlamydomphila CT1584 (Fig 4) [51]. These orthologs mostly take the form of a coated sheath-like structure around the needle of the type III secretion apparatus, unlike the cap-like structure of the V-antigen homologs on the needle tip. EspA from pathogenic E. coli is reportedly essential for type III secretion in this bacterium [54]. Regarding IpaD from Shigella [55], although the effects of vaccination with an IpaB–IpaD fusion protein have been reported, there have been no reports on other orthologs so far.

6. The antibody blocking mechanisms against type III secretion

Because both the whole IgG molecule and Fab fragment against P. aeruginosa PcrV can suppress the intoxication caused by type III secretion, it is possible that the Fc-domain of the antibody is not necessary for the blocking effect [39, 43, 56]. The binding site of the Mab166 monoclonal antibody is located at position 144–257 of the 294 amino-acid long PcrV molecule (Fig 5) [42]. Deleting one amino acid from the amino- or carboxyl-terminal of the PcrV fragment at position 144–257 results in the loss of Mab166 binding in immunoblotting, suggesting that this fragment (144–257) in PcrV is the minimal blocking epitope for this Mab [42]. In the three-dimensional structures of LcrV and PcrV, there are two coiled-domains in the center and carboxyl-terminal regions, respectively [57, 58]. These two-coiled regions form a double chain as a coiled-coil center shaft in the V-antigen, and the coiled-coil region forms a dumbbell-like structure with globular domains at both the amino- and carboxyl-terminal (Fig 6, 1) [57, 59, 60]. In serotype O8 of Y. enterocolitica, the carboxyl-terminal globular domain of LcrV contains a hypervariable region at position 225–232, and the antibodies generated against LcrV from this serotype are unable to protect against other Yersinia spp. carrying the alternative LcrV type [27]. Mab166 binds to the carboxyl-terminal globular domain of PcrV [42]. Perhaps deleting the 144–257 region of the fragment unravels the coiled-coil state, thereby impairing the three-dimensional protein structure. Therefore, the above observations imply that normal Mab166 binding is associated with the conformational structure of PcrV.

The humanized KB001 Fab antibody displays significantly higher binding affinity to PcrV than the original murine Mab166 does [44]. KB001 showed significant effects against infections in a dose-response study, suggesting that its inhibitory power against type III intoxication is significantly associated with the binding affinity of this antibody to its cognate antigen [44]. A detailed structure for LcrV has also been reported, and a pentagonal ring structure comprising an LcrV pentamer is evident on the tip of the secretion needle [59, 60]. Based on the homology between LcrV and PcrV, a three-dimensional structure for PcrV was described for the association with the translocon (Fig 6, 2) [62]. It is not certain yet, but antibody binding may occur in the vicinity of the central hole formed by the mushroom-like cap structure comprising the PcrV monomers and physically inhibits the passage of the toxin. These two coil regions form a double chain as a coiled-coil which forms a dumbbell-like structure with globular domains at both the amino- and carboxyl-terminals [57, 59].
Figure 5. Primary and secondary structures of PcrV and LcrV

*Y. pestis* LcrV is 326 amino-acids long and *P. aeruginosa* PcrV is 294 amino acids long. The channel-forming regions of PcrV and LcrV are shown in purple font. The blocking epitope of the anti-LcrV monoclonal antibody is located at position 135–275 (green font) in the LcrV molecule [28], and the binding site of the blocking Mab166 anti-PcrV antibody is located at amino acid position 144–257 in PcrV [42]. A V7-toll-like receptor-stimulating region was reported for LcrV [61], and a hypervariable region was reported for *Yersinia enterocolitica* LcrV [27]. Both LcrV and PcrV contain two coiled domains in their center and carboxy-terminal regions (designated by the letters ‘a’ and ‘d’).

Figure 6. Structures of PcrV and the translocon from *P. aeruginosa*

(1) The coiled-coil region of PcrV forms a dumbbell-like structure with globular domains at the amino- and carboxy-terminals. A hypervariable region was reported for *Yersinia enterocolitica* LcrV [27]. (2) The pentagonal ring structure consists of a PcrV pentamer located on the tip of the secretion needle in the *P. aeruginosa* type III secretion apparatus.
7. The blocking antibody fraction in human serum

It has been confirmed that a commercially available IVIG preparation contains a fraction that binds to PcrV and that administration of it reduces lung injury and mortality in a mouse model of P. aeruginosa pneumonia in a dose-dependent manner [63,64]. This effect was attenuated by removing the PcrV binding fraction in the IVIG by affinity chromatography depletion [63]. Additionally, a recent epidemiological study showed that anti-PcrV titers in sera from adult volunteers were significantly higher in approximately 10% of the tested volunteers [65]. When the therapeutic effects of the γ-globulin fractions obtained from the adults with high PcrV titers were tested in an animal model of P. aeruginosa pneumonia [66], high anti-PcrV titer-derived IgG was found to significantly improve lung injury and mortality, unlike the γ-globulin obtained from adult sera with low anti-PcrV titers [66]. These findings indirectly suggest that patients with high anti-PcrV antibody titers have acquired immunity against P. aeruginosa type III secretion toxicity. Therefore, the clinical administration of γ-globulin with a high antibody titer is expected to provide a prophylactic or therapeutic effect against P. aeruginosa infection.

8. The fifth mechanism of action of γ-globulin therapy and future research

Up until now, as far as bacterial infections are concerned and as mentioned above, four major mechanisms of IVIG action have been suggested: lysis by complement activation, opsonization and phagocytosis, toxin neutralization, and ADCC. However, with the type III secretion system, where bacterial toxins are injected directly into the cytoplasm of target cells by a special secretion apparatus, the toxin itself evades neutralization by the host’s antibodies, thereby offsetting protective immunity. The above-mentioned findings, which were obtained mainly from Yersinia and P. aeruginosa, have revealed that antibody binding to V-antigens can counteract bacterial type III intoxication in the host. Crucially in this respect, human sera and human sera-derived IVIG preparations contain antibody fractions that bind to PcrV [65,66].

The ability of these antibodies to inhibit a bacterial infection is independent of toxin neutralization, complement, or opsonization. Instead, their action seems to physically inhibit toxin secretion and/or translocation by recognizing the three-dimensional structure of the bacterial secretion apparatus. We do not know how significant this immunity is, especially in gram-negative infections. In the current situation where multi-drug resistant bacteria are common, application of a V-antigen homolog as a vaccine and IVIG therapy targeting V-antigen homologs is a potentially attractive alternative or adjunctive therapy against lethal bacterial infections [56]. Therefore, it seems reasonable to define the blocking effect of anti-V-antigen antibodies as the fifth mechanism of immunoglobulin action against bacterial infections (Fig 1, 5).

9. Conclusions

Four mechanisms of action have been suggested to explain the positive effects of γ-globulin therapy for bacterial infections: (1) the lytic action of complement activation, (2) the phagocytic action via opsonization, (3) toxin neutralization, and (4) ADCC. New toxin secretion systems like the type III secretion system have been discovered in many pathogenic gram-negative bacteria. With this system, the toxin (an effector) is directly injected into the cytoplasm of the target cell through a special secretory apparatus without being exposed to the extracellular environment, thus providing no opportunity for antibodies to neutralize the toxin. However, because antibodies can target the V-
antigen on the needle-shaped tip of the bacterial secretion apparatus and inhibit toxin translocation, this suggests that the toxin secretion-inhibitory effects of immunoglobulins should be considered γ-globulin’s fifth mechanistic action against bacteria.

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