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1 *Review*

2 Intravenous Immunoglobulin for Treating Bacterial 3 Infections: One More Mechanism of Action

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10

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

26 **Keywords:** immunoglobulin; IVIG; LcrV; PcrV; translocation; type III secretory toxin; type III
27 secretion system; V-antigen
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29 1. Introduction

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

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(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 (O_2^-) (a bactericidal substance) in the phagosome, and this O_2^- 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.

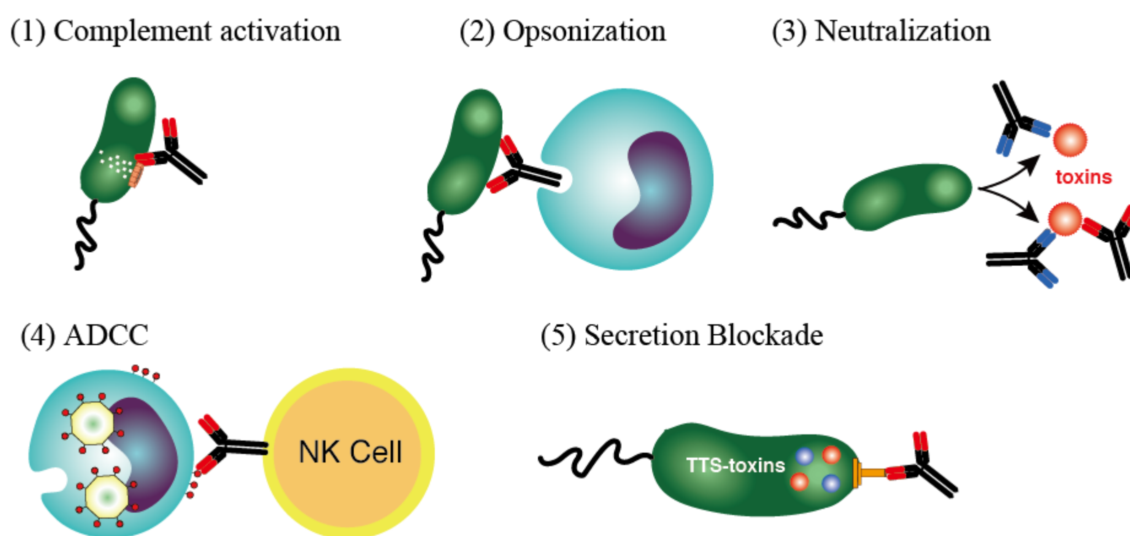

Figure 1. Mechanisms used by immunoglobulins to destroy infectious agents and toxins

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].

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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.

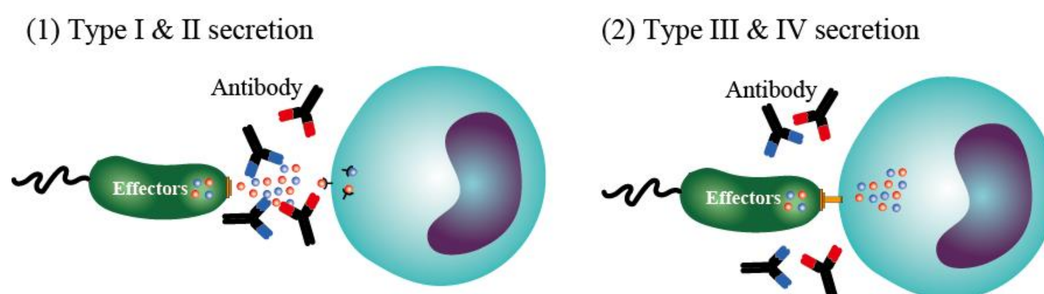

Figure 2. Mechanisms of immunoglobulin-related action against infectious agents and toxins. **(1)** In the classical type I and II secretion systems, bacteria secrete their toxins into the outside environment, and immunoglobulins are able to neutralize these toxins. **(2)** In the type III and IV secretion systems, the toxins are injected directly into the cytoplasm of the target cells using a special secretion device. Therefore, even when a neutralizing antibody against the toxin is present, because the toxin reaches the target cell without being exposed to the extracellular environment there is no opportunity for antibodies to neutralize the toxin itself.

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 exposure 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,

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although surprising, gram-negative bacteria are already equipped with a sophisticated stealth intoxication system capable of evading the host's acquired immunity in the form of antibodies.

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

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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')$_2$ significantly improved secondary sepsis in a rabbit

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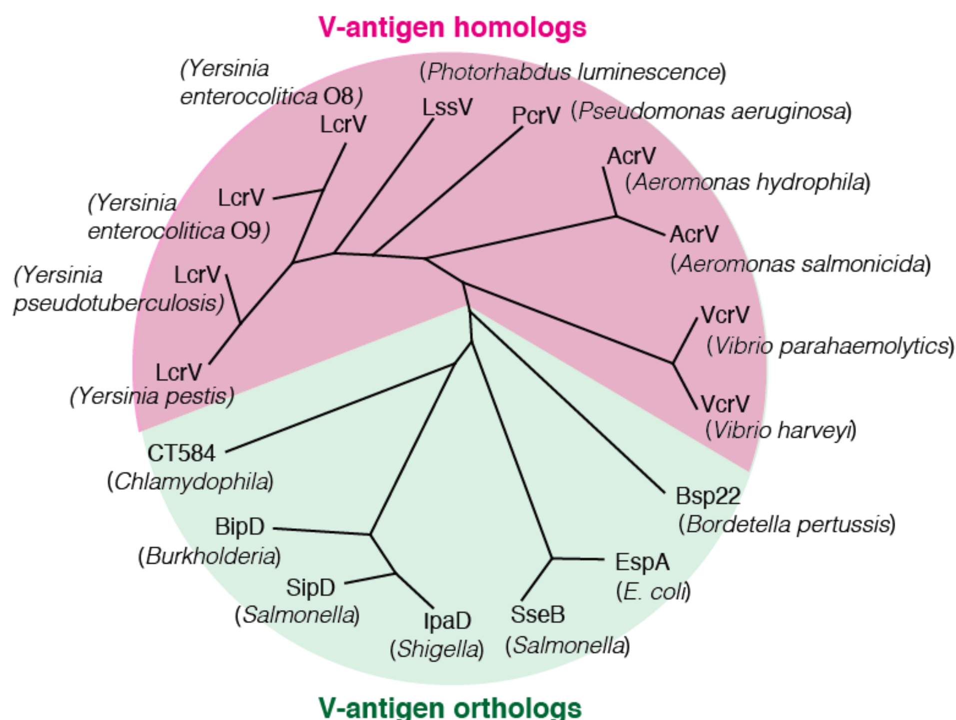
169 pneumonia model [39]. The anti-PcrV-specific polyclonal IgG was also effective in a burned skin
170 model of *P. aeruginosa* infection [40], and in a chronic *P. aeruginosa* pneumonia model also [41].
171 Consequently, the mouse-derived monoclonal antibody (Mab) 166 was developed [42], the
172 administration of which reduced lung injury and mortality in the *P. aeruginosa* pneumonia infection
173 model [43]. This effect was also observed for the Mab166 Fab (antigen binding fragment) [43]. This
174 Mab166 monoclonal antibody was humanized for clinical use as KB001 [44], and phase II clinical
175 studies with it have now been conducted [45-47]. Active immunization with recombinant PcrV also
176 improved acute lung injury and mortality in *P. aeruginosa* pneumonia models [48-50]. In recent years,
177 more researchers have reported on the clinical application of anti-PcrV antibodies that have been
178 developed using different approaches.

179 **5. V-antigen homologs**

180 In addition to *P. aeruginosa* PcrV, *Aeromonas* AcrV, *Vibrio* VcrV, and *Photorhabdus* LssV have now
181 been identified as *Yersinia* V-antigen homologs (**Fig 4**) [51]. It was reported that rainbow trout
182 vaccinated with recombinant AcrV are not protected against infection with *Aeromonas salmonicida*
183 [52,53]. *Aeromonas hydrophila* and *Vibrio* are infective to humans, but no detailed studies of AcrV and
184 VcrV from each of them, respectively, have been performed. Thus, with the exception of LcrV and
185 PcrV, the characteristics of V-antigen homologs from other species have not been adequately studied
186 yet.
187



188 **Figure 4. Phylogenetic tree of V-antigen homologs and orthologs**
189 *P. aeruginosa* PcrV, *Aeromonas* AcrV, *Vibrio* VcrV, and *Photorhabdus* LssV are *Yersinia* V-antigen
190 homologs, whereas *Escherichia coli* EspA, *Salmonella* SipD and SseB, *Shigella* IpaD, *Burkholderia* BipD,
191 *Bordetella pertussis* Bsp22, and *Chlamydophila* CT1584 are orthologs of the *Yersinia* LcrV V-antigen.
192 As shown in Fig 4, V-antigen orthologs have also been identified that share a genetic and
193 functional association with V-antigen homologs; these include EspA from pathogenic *Escherichia coli*,

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194 *Salmonella* SipD and SseB, *Shigella* IpaD, *Burkholderia* BipD, *Bordetella pertussis* Bsp22, and
195 *Chlamydophila* CT1584 (**Fig 4**) [51]. These orthologs mostly take the form of a coated sheath-like
196 structure around the needle of the type III secretion apparatus, unlike the cap-like structure of the V-
197 antigen homologs on the needle tip. EspA from pathogenic *E. coli* is reportedly essential for type III
198 secretion in this bacterium [54]. Regarding IpaD from *Shigella* [55], although the effects of vaccination
199 with an IpaB–IpaD fusion protein have been reported, there have been no reports on other orthologs
200 so far.

201 **6. The antibody blocking mechanisms against type III secretion**

202 Because both the whole IgG molecule and Fab fragment against *P. aeruginosa* PcrV can suppress
203 the intoxication caused by type III secretion, it is possible that the Fc-domain of the antibody is not
204 necessary for the blocking effect [39,43,56]. The binding site of the Mab166 monoclonal antibody is
205 located at position 144–257 of the 294 amino-acid long PcrV molecule (**Fig 5**) [42]. Deleting one amino
206 acid from the amino- or carboxyl-terminal of the PcrV fragment at position 144–257 results in the loss
207 of Mab166 binding in immunoblotting, suggesting that this fragment (144–257) in PcrV is the minimal
208 blocking epitope for this Mab [42]. In the three-dimensional structures of LcrV and PcrV, there are
209 two coiled-domains in the center and carboxyl-terminal regions, respectively [57,58]. These two-
210 coiled regions form a double chain as a coiled-coil center shaft in the V-antigen, and the coiled-coil
211 region forms a dumbbell-like structure with globular domains at both the amino- and carboxyl-
212 terminal (**Fig 6,** 1) [57,59,60]. In serotype O8 of *Y. enterocolitica*, the carboxyl-terminal globular domain
213 of LcrV contains a hypervariable region at position 225–232, and the antibodies generated against
214 LcrV from this serotype are unable to protect against other *Yersinia* spp. carrying the alternative LcrV
215 type [27]. Mab166 binds to the carboxyl-terminal globular domain of PcrV [42]. Perhaps deleting the
216 144–257 region of the fragment unravels the coiled-coil state, thereby impairing the three-
217 dimensional protein structure. Therefore, the above observations imply that normal Mab166 binding
218 is associated with the conformational structure of PcrV.
219 The humanized KB001 Fab antibody displays significantly higher binding affinity to PcrV than
220 the original murine Mab166 does [44]. KB001 showed significant effects against infections in a dose-
221 response study, suggesting that its inhibitory power against type III intoxication is significantly
222 associated with the binding affinity of this antibody to its cognate antigen [44]. A detailed structure
223 for LcrV has also been reported, and a pentagonal ring structure comprising an LcrV pentamer is
224 evident on the tip of the secretion needle [59,60]. Based on the homology between LcrV and PcrV, a
225 three-dimensional structure for PcrV was described for the association with the translocon (**Fig 6,** 2)
226 [62]. It is not certain yet, but antibody binding may occur in the vicinity of the central hole formed by
227 the mushroom-like cap structure comprising the PcrV monomers and physically inhibits the passage
228 of the toxin. These two coil regions form a double chain as a coiled-coil which forms a dumbbell-like
229 structure with globular domains at both the amino- and carboxyl-terminals [57,59].
230

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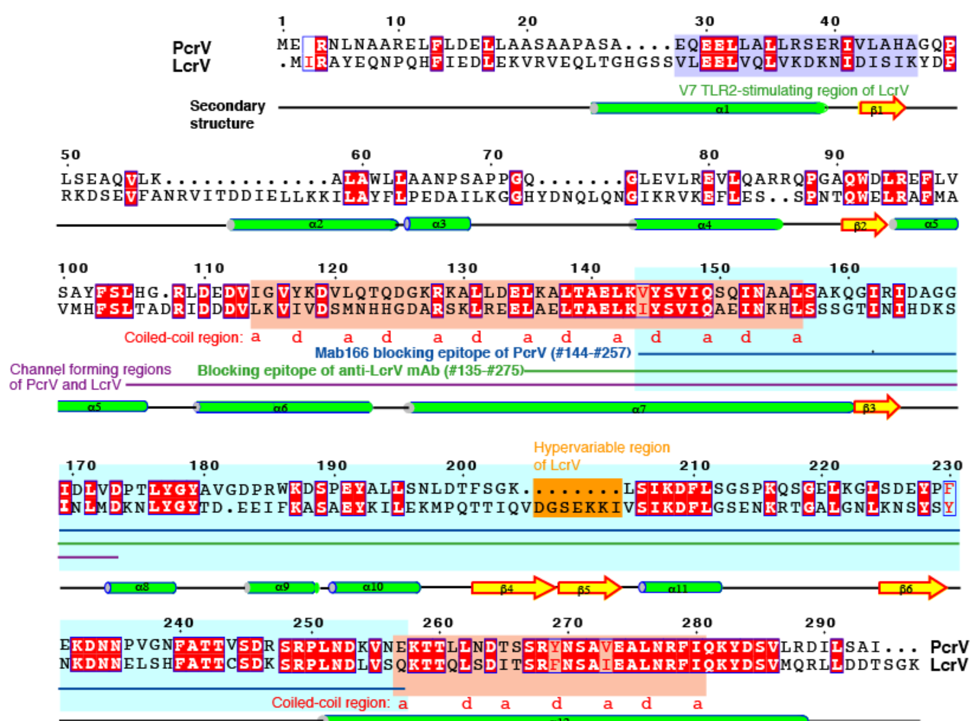
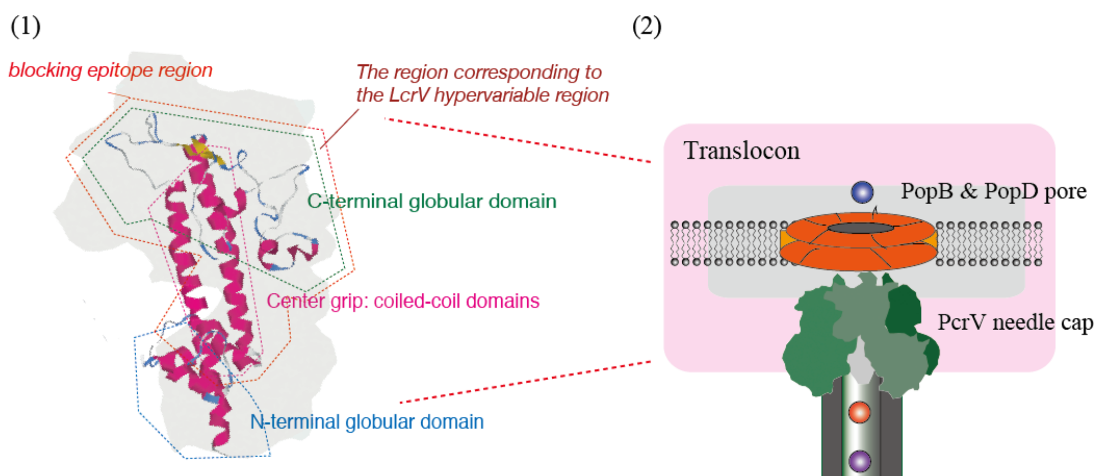


231 **Figure 5. Primary and secondary structures of PcrV and LcrV**

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



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

240 **(1)** The coiled-coil region of PcrV forms a dumbbell-like structure with globular domains at the
241 amino- and carboxyl-terminals. A hypervariable region was reported for *Yersinia enterocolitica* LcrV
242 [27]. **(2)** The pentagonal ring structure consists of a PcrV pentamer located on the tip of the secretion
243 needle in the *P. aeruginosa* type III secretion apparatus.

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244 **7. The blocking antibody fraction in human serum**

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

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

259 Up until now, as far as bacterial infections are concerned and as mentioned above, four major
260 mechanisms of IVIG action have been suggested: lysis by complement activation, opsonization and
261 phagocytosis, toxin neutralization, and ADCC However, with the type III secretion system, where
262 bacterial toxins are injected directly into the cytoplasm of target cells by a special secretion apparatus,
263 the toxin itself evades neutralization by the host's antibodies, thereby offsetting protective immunity.
264 The above-mentioned findings, which were obtained mainly from *Yersinia* and *P. aeruginosa*, have
265 revealed that antibody binding to V-antigens can counteract bacterial type III intoxication in the host.
266 Crucially in this respect, human sera and human sera-derived IVIG preparations contain antibody
267 fractions that bind to PcrV [65,66].
268 The ability of these antibodies to inhibit a bacterial infection is independent of toxin
269 neutralization, complement, or opsonization. Instead, their action seems to physically inhibit toxin
270 secretion and/or translocation by recognizing the three-dimensional structure of the bacterial
271 secretion apparatus. We do not know how significant this immunity is, especially in gram-negative
272 infections. In the current situation where multi-drug resistant bacteria are common, application of a
273 V-antigen homolog as a vaccine and IVIG therapy targeting V-antigen homologs is a potentially
274 attractive alternative or adjunctive therapy against lethal bacterial infections [56]. Therefore, it seems
275 reasonable to define the blocking effect of anti-V-antigen antibodies as the fifth mechanism of
276 immunoglobulin action against bacterial infections (**Fig 1,** 5).

277 **9. Conclusions**

278 Four mechanisms of action have been suggested to explain the positive effects of γ-globulin
279 therapy for bacterial infections: (1) the lytic action of complement activation, (2) the phagocytic action
280 via opsonization, (3) toxin neutralization, and (4) ADCC. New toxin secretion systems like the type
281 III secretion system have been discovered in many pathogenic gram-negative bacteria. With this
282 system, the toxin (an effector) is directly injected into the cytoplasm of the target cell through a special
283 secretory apparatus without being exposed to the extracellular environment, thus providing no
284 opportunity for antibodies to neutralize the toxin. However, because antibodies can target the V-

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285 antigen on the needle-shaped tip of the bacterial secretion apparatus and inhibit toxin translocation,
286 this suggests that the toxin secretion-inhibitory effects of immunoglobulins should be considered γ-
287 globulin's fifth mechanistic action against bacteria.

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289 manuscript preparation. KM checked and revised the manuscript. All authors have read and approved the final version of
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296 **Conflicts of Interest:** TS has a patent for immunization with PcrV from the Reagent of the University of
297 California (Berkeley, CA, USA).

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