J Infect Chemother 24 (2018) 383–388

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Effective concentration of intravenous immunoglobulin for neutralizing Panton-Valentine leukocidin in human blood

Jun Tanaka [a, *], Fumihiro Hirayama [a, b], Sayuri Yanase [a], Shusei Uno [a], Takashi Nakae [a, b], Akihito Kamizono [a], Hideaki Hanaki [b]

[a] Central Research Laboratory, Japan Blood Products Organization, Kobe, Japan
[b] Research Center for Anti-infectious Drugs, Kitasato University, Tokyo, Japan

ARTICLE INFO

Article history:
Received 18 September 2017
Received in revised form
1 December 2017
Accepted 30 December 2017
Available online 1 March 2018

Keywords:
PVL
MRSA
Intravenous immunoglobulin
Neutralizing antibody titer
Toxin neutralization
Anti-toxin drug

ABSTRACT

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infects healthy individuals, although the precise cause remains unclear. CA-MRSA produces Panton-Valentine leukocidin (PVL), which often causes severe invasive infection; however, antitoxin drugs against PVL are limited. Intravenous immunoglobulin (IVIg) possesses antitoxin activity, but unfortunately, the optimal dose is unknown. Here, we measured the PVL neutralizing antibody titer in the plasma of Japanese individuals and sera of American donors. Next, we compared the cytotoxic effects of PVL on neutrophils in phosphate buffered saline (PBS) or whole blood to determine the effect of the neutralizing antibody. Finally, we evaluated the effective concentration of IVIg required to neutralize PVL in PBS and whole blood. We observed that the titer of PVL neutralizing antibody in healthy individuals polarized as high and low/none group. Additionally, the PVL neutralizing antibody titer considerably affected the concentration at which IVIg elicited its effect. This suggests that PVL-producing CA-MRSA might be involved in determining the severity of infection in healthy individuals without neutralizing antibody against PVL. The neutralizing effect of IVIg was observed in both PBS and whole blood. However, the optimal concentration of IVIg required for neutralizing PVL varied between PBS and whole blood. In addition, since the PVL-neutralizing activity of IVIg also largely depends on blood composition, such as neutralizing antibody concentration, the optimal dosage of IVIg as an antitoxin drug should be decided in a timely manner after considering the patient's medical background.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is known as a common nosocomial pathogen since 1961 [1,2] and is termed hospital-acquired MRSA (HA-MRSA). From 1997 to 1999, a new type of MRSA, called community-acquired MRSA (CA-MRSA), was reported in four cases of necrotizing pneumonia in the United States [3–5]. Although HA-MRSA infections generally occur more frequently among patients with weakened immune systems or in aged individuals, CA-MRSA infections occur in healthy individuals, especially children and adolescents, including athletes [1,2,6]. Despite ambiguity regarding the precise cause of CA-MRSA infection in healthy individuals, production of Panton-Valentine leukocidin (PVL) is one of the features of CA-MRSA infection [1,2,6,7].

PVL was first reported by Panton and Valentine in 1932 [8], and Finck-Barbabcon et al. [9] first purified it in 1991 from *Staphylococcus aureus* strain V8 (ATCC49775) isolated from a patient with chronic furunculosis. Cation-exchange chromatography elution profiles showed that PVL is a bi-component LukS-PV (32 kDa) and LukF-PV (34 kDa) protein [2,10–12]. PVL kills polymorphonuclear leukocytes, especially neutrophils, by forming pores on the cell surface, although PVL does not affect red blood cells, lymphocytes, and other tissues [13–15]. However, PVL produced by CA-MRSA often causes severe invasive infection, such as sepsis, necrotizing pneumonia, and osteomyelitis [1,2].

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; HA-MRSA, hospital-acquired MRSA; CA-MRSA, community-acquired MRSA; PVL, Panton-Valentine leukocidin; IVIg, intravenous immunoglobulin; GST, glutathione S-transferase; PCR, polymerase chain reaction; PBS, phosphate buffered saline.

* Corresponding author. Central Research Laboratory, Japan Blood Products Organization, 8F Kobe KIMEC Center Building, 1-5-2, Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo, 650-0047, Japan.
E-mail address: tanaka-jun@jbpo.or.jp (J. Tanaka).

https://doi.org/10.1016/j.jiac.2017.12.023

Generally, PVL-associated CA-MRSA infections are treated with antibiotics. Currently, abundant antibiotics are available for treating MRSA, and hence, the main focus of treatment is selection of optimal antibiotics. However, most CA-MRSA infections develop in non-nosocomial settings. Hence, PVL levels are already high in some cases by the time antibiotic treatment is initiated. In such cases, PVL persists in the body and continues to exert adverse health effects for certain time even if the administered antibiotics have completely eliminated the bacterial cells. Thus, removal of toxins along with bacteriolysis may be required for patients exhibiting severe symptoms; unfortunately the number of antitoxin drugs is limited compared to the abundance of antibiotics in the drug market.

Here, we focused on intravenous immunoglobulin (IVIg), a rare drug with multiple functions. IVIg is manufactured from the pooled plasma of more than ten thousand healthy people and contains various antimicrobial [16–20], and antitoxin [21–23] antibodies. Although IVIg has been used for treating infectious diseases, there is a large inter-country difference in the commonly used IVIg dosage. Thus, although the effect of IVIg on infectious diseases is recognized globally, the complete potential of IVIg has not been utilized properly so far.

The country-specific differences in IVIg dosage arises from the lack of knowledge about the optimal dosage required to effectively treat infectious diseases. One of the reasons is the dearth of methods for determining the optimum dose inside the body. Widely used methods for antitoxin evaluation are *in vitro* cytotoxic activity tests against target cells isolated from blood, or pharmacological tests on drug-administered infected animals [24]. However, since the *in vitro* assessments are performed in phosphate buffered saline (PBS), it is difficult to evaluate the effective concentration of the drug in the body. In contrast, *in vivo* evaluation is the ideal method for estimating the optimal drug dosage; however, the drug's sensitivity to the toxin often varies between experimental animals and humans. Therefore, assessment of the effective *in vivo* antitoxin concentration is challenging.

In this study, we established a method for evaluating antitoxin action that mimics the human body environment. We used PVL as a toxin and whole blood for mimicking the body environment. We estimated the effective concentration of IVIg required for neutralizing PVL cytotoxicity using the new evaluation method.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethics Committee on Human Research of Japan Blood Products Organization (Examination No. 2015倫理006), which conforms to the ethical guidelines of the Medical and Health Research Involving Human Subjects of the Ministry of Health, Labor, and Welfare of Japan.

2.2. Human blood and serum

Whole blood donations were obtained from healthy laboratory staff with informed consent. Plasma was obtained from whole blood anticoagulated with heparin by centrifugation. Normal human sera derived from eight American healthy donors were purchased from the Uniglobe Research Corporation (Reseda, CA, USA).

2.3. Production and purification of recombinant LukS-PV and LukF-PV proteins

We used the glutathione S-transferase (GST) fusion protein to produce and purify rLukS-PV and rLukF-PV, the two components of PVL. Genomic DNA was extracted from a MRSA clinical isolate collected in the Kitasato University Hospital. Primers used by Onishi et al. were used for constructing the vector [25]. The 5′ primer for *lukS-PV* (S2F, 5′-AAG GGA TCC GAT AAC AAT ATT GAG AAT ATT GGT G-3′) and *lukF-PV* (F2F, 5′-AAG GGA TCC CAA CAT ATC ACA CCT GTA AGT G-3′) were selected within the coding sequence of each gene (GenBank accession no. FJ895585), whereas the 3′ primer for *lukS-PV* (S2R, 5′-AAA GGC CGT CGA CTC AAT TAT GTC CTT TCA CTT TAA TT-3′) and *lukF-PV* (F2R, 5′-AAA GGC CGT CGA CTT AGC TCA TAG GAT TTT TTT CCT-3′) were selected to overlap with the stop codon of each gene. Comparison with the sequence registered in GenBank revealed two mutations in *lukS* (A527G and T663G) and one in *lukF* (G1729A). As A527G is a conservative mutation, we did not evaluate the precise effect of the mutation. The remaining two mutations were silent mutation. Restriction sites (*BamH* I for S2F and F2F; *Sal* I for S2R and F2R) were included in each primer. Polymerase chain reaction (PCR) fragments were digested with *BamH* I and *Sal* I (TaKaRa, Shiga, Japan) and were ligated (Ligation-Convenience kit; Nippon Gene, Tokyo, Japan) with the pGEX-6P1 expression vector digested with the same restriction enzymes. The plasmids were transformed in BL21 (DE3) pLysS competent cells (Novagen, Madison, WI, USA). The fusion proteins (GST-LukS-PV and GST-LukF-PV) were purified from lysates of transformed *E. coli* cells using the BugBuster GST Bind™ purification kit (Novagen) according to the manufacturer's instruction. LukS-PV and LukF-PV were stored below −60 °C. Both subunits were mixed at equimolar ratios immediately before addition to cells.

2.4. Neutralizing antibody titer of serum and plasma

Eight serum samples from the USA and eleven plasma samples from Japan were serially diluted two-folds. PVL of predetermined concentration (final concentration, 5 nM) was reacted with each diluted sample. After incubation, the agglutination activity of PVL in each sample was measured using the assay kit (Denka Seiken, Tokyo, Japan) and was expressed as residual PVL. The neutralizing antibody titers of the samples are expressed as the reciprocal of the highest two-fold serial dilution that demonstrated >90% neutralization. The neutralizing antibody titer of the sample was defined as 8, if the 1/2, 1/4, and 1/8 diluted samples neutralized more than 90% of the predetermined PVL.

2.5. Isolation of neutrophils

Neutrophils were isolated using the Polymorphprep (Alere Technologies AS, Oslo, Norway) in accordance with the manufacturer's instructions. The purity (>95%) of the isolated neutrophils was confirmed by microscopic observation and trypan blue exclusion test.

2.6. Measurement of PVL cytotoxic activity on isolated neutrophils and neutralizing activity of IVIg in PBS

PVL (final concentration, 0.1–100 nM) was added to isolated neutrophils in PBS with or without 1–10 mg/mL (final concentration) IVIg (Japan Blood Products Organization, Tokyo, Japan) or 10 mg/mL (final concentration) human albumin (Japan Blood Products Organization) and incubated for 15 min at 37 °C. After incubation, the neutrophils were allowed to stand on ice until viability was assessed by microscopic observation and trypan blue exclusion test.

2.7. Measurement of PVL cytotoxic activity on neutrophils and the neutralizing activity of IVIg in whole blood

Whole blood was added to 0.1–1000 nM of PVL (final concentration) with or without 1–10 mg/mL of IVIg (final concentration)