Neutralization of Staphylococcus aureus Panton Valentine Leukocidin by Intravenous Immunoglobulin In Vitro

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Panton Valentine leukocidin (PVL) may be responsible for pulmonary necrosis in necrotizing Staphylococcus aureus pneumonia, a highly lethal infection. Commercial intravenous immunoglobulin (IVIg) preparations containing antibodies against PVL might have therapeutic value in this setting, as an adjunct to antimicrobial chemotherapy. To test this possibility, we determined anti-PVL antibody titers in commercial IVIg and the capacity of IVIg to prevent the cytopathic effects of PVL in vitro. Specific enzyme-linked immunosorbent assays based on purified recombinant PVL (rPVL) showed that IVIg contained specific anti-PVL antibodies. The cytotoxicity of rPVL and of crude culture supernatants of PVL-producing S. aureus strains were investigated by measuring ethidium-bromide incorporation by polymorphonuclear neutrophils (PMNs) in flow cytometric assays, as well as PMN ultrastructural changes by transmission electron microscopy. IVIg was found to neutralize pore formation and the cytopathic effect of both rPVL and S. aureus culture supernatants.

Panton Valentine leukocidin (PVL)–producing Staphylococcus aureus strains can cause primary skin and soft-tissue infections [1, 2], as well as in young immunocompetent patients, severe necrotizing pneumonia, which is characterized by high fever, hypotension, hemoptysis, multifocal alveolar infiltrates, and leukopenia [2, 3]. The mortality rate is ~75%, and autopsy reveals extensive necrotic and hemorrhagic lesions of the trachea, bronchi, alveolar septa, and parenchyma. The pathogenesis is uncertain, but several lines of evidence suggest that PVL plays an important role: (1) the strong epidemiological link with PVL-synthesizing S. aureus isolates [3]; (2) the high frequency of leukopenia, a known effect of PVL [3–5]; and (3) the necrotic lesions of the respiratory tract, which resemble the necrosis induced by purified PVL injection to rabbit skin [3, 6, 7]. Moreover, the frequent failure of antibacterial chemotherapy [3] points to a prominent role of bacterial toxins. Intravenous immunoglobulin (IVIg) has been proposed as an adjunct to antibiotics in severe staphylococcal and streptococcal toxemic syndromes, such as toxic shock syndrome [8, 9], the rationale being that the antitoxin antibodies contained in IVIg could neutralize the effects of the toxin and/or prevent binding to its target [8, 10]. To examine whether IVIg might be beneficial in necrotizing pneumonia, we tested commercial IVIg for PVL-specific antibodies and studied its capacity to prevent the cytopathic effect of PVL on polymorphonuclear cells in vitro.

MATERIALS AND METHODS

Bacterial strains and culture. S. aureus strain V8 (ATCC 49775; hlg”, hlgv”, lukS-PV lukF-PV”, and lukE lukD”) was used as the control PVL-producing strain [11]; this strain also produces γ-hemolysin (Hlg). S. aureus strain HT2000 0237 (hlg”, hlgv”, lukS-PV lukF-
IgG2 (34.1%), IgG3 (5.4%), and IgG4 (1.7%);

0.25% NaHPO$_4$, and 0.042% KH$_2$PO$_4$ [pH 7.0]) at 37°C.

yeast extract, 2% Bacto-Casamino Acids, 2% sodium pyruvate,

[12]. The strains were grown in YCP medium (3% [wt/vol]

glycerol, 12.5 mg/mL of Rif, 5 mg/mL of Amp, 100 mg/mL of 

ampicillin (100 mg/L), with or without isopropyl-

β-D-thiogalactopyranoside (IPTG).

PV, and lukE lukD), which was provided by Y. Kamio (Ju-
tendo Sendai University), was obtained by transducing 

ΦSLT into strain HT2000 0236 (a PVL-negative clinical isolate); 

this strain produces PVL, γ-hemolysin variant and LukE LukD

[12]. The strains were grown in YCP medium (3% [wt/vol]

yeast extract, 2% Bacto-Casamino Acids, 2% sodium pyruvate,

0.25% NaHPO$_4$, and 0.042% KH$_2$PO$_4$ [pH 7.0]) at 37°C

with vigorous shaking for 17 h [13]. After centrifugation, culture

supernatants were sterilized by filtration through 0.25 µm filters

and were stored at −20°C until used.

Escherichia coli TG1 (New England Biolabs) was used for

dNA cloning and genetic manipulations. It was grown in Luria

broth (LB) medium supplemented with glucose (2 g/L) and

ampicillin (100 mg/L), with or without isopropyl-β-D-thio-
galactopyranoside (IPTG).

IVIg. Human intravenous polyclonal immunoglobulin

(IVIg; Tégeline Laboratoire Français du Fractionnement et des

Biotechnologies [LBF]), was purchased from LBF. Tégeline is iso-

lated by fractionation of a pool of IgG collected from 20,000

donors. It contains >97.6% of IgG composed by IgG1 (58.8%),

IgG2 (34.1%), IgG3 (5.4%), and IgG4 (1.7%); <1.7% of IgA; and

traces of pepsine (http://www.biam2.org/www/Spe28008

.html#SpeThera).

Production and purification of recombinant (r) LukS-PV and LukF-PV proteins. We used pMAL-c2 (New

England Biolabs) to produce rLukS-PV and rLukF-PV (the 2

components forming the PVL) coupled to maltose-binding

protein (MBP). Primers were designed after identification of

suitable hybridization sites in lukS-PV and lukF-PV. The 5'

primers (rlukS-1, 5′-CAG TGC AAT CTA AAG CTG ATA

ACA ATA TTG AG-3′; and rlfkF-1, 5′-CAG TGC AAT CCT

CTC AAT TCA CTT T-3′) were chosen within the coding

sequence of each gene (Genbank accession no. X72700), omitting

the region predicted to encode the signal peptide published and

verified by hydrophobicity analysis, according to Kyte and

Doolittle [14], and SignalP V1.1 (World Wide Web Prediction

Server: http://www.cbs.dtu.dk/services/SignalP-2.0/). The 3′

primers (rlukS-2, 5′-GCC TGC AGT CAA TTA TGT CCT

TTC ACT TTA ATT TCA T-3′; and rlfkF-2, 5′-GCC TGC AGT

TAG CTA ATG TTT TTT TTC TCC TTA G-3′) were chosen to

overlap the stop codon of each gene. Restriction sites ( EcoRI for

rlukS-1 and rlfkF-1; PsiI for rlfkS-2 and rlfkF-2) were

included in each primer. DNA was extracted from ATCC 49775

and was used as a template for polymerase chain reaction (PCR)

amplification. PCR products and plasmid DNA were prepared

by use of the Qiagen plasmid kit. PCR fragments were digested

with EcoRI and PsiI (Promega) and were ligated (T4 DNA

ligase; Promega) with the pMAL-c2 expression vector digested

with the same restriction enzymes. The resulting plasmids were

transformed into E. coli TG1, and the integrity of the open-

reading frame of each construct was verified by DNA sequenc-

ing. The fusion proteins (MBP-LukS-PV and MBP-LukF-PV)

were purified from lysates of transfected E. coli cells by affinity

chromatography on amylose columns, according to the manu-

ufacturer’s instructions (New England BioLabs). The purity of

the fusion proteins was controlled by 7.5% SDS-PAGE. The

fusion proteins then were lyophilized and stored at 4°C.

Human leukocyte preparation. Peripheral blood mono-
nuclear cells (PBMCs) were collected from adult volunteers by

venipuncture, using EDTA (K3E 15% and 0.084 mL; Belliver

Industrial Estate) as the anticoagulant. Erythrocytes were re-

moved by hypotonic lysis with 0.9% NH$_4$Cl for 15 min and

followed by 3 washes in PBS (pH 7.2; BioMérieux SA). The

final cell pellet was suspended and adjusted to 10$^8$ cells/mL in

PBS. Fresh cells were prepared for each day’s experiments.

Anti-LukS-PV and anti-LukF-PV immunosassays. Anti-

LukS-PV and anti-LukF-PV antibodies were detected in IVIg

by use of specific ELISAs with solid-phase LukS-PV or LukF-

PV and revelation by a peroxidase-conjugated anti–immuno-

globulin antibody. The procedure was adapted from Current

Protocols in Molecular Biology [15]. In brief, recombinant LukS-

PV (rLukS-PV) and recombinant LukF-PV (rLukF-PV) were

purified from MBP-LukS-PV and MBP-LukF-PV by cleavage

with factor Xa and separation by Uno Q1-sepharose ion-

exchange chromatography (Biorad), according to the manu-

ufacturer’s instructions (New England BioLabs). The wells of

microtiter plates (Sigma) were coated with 125

$\text{g}$ of rLukS-

PV and were incubated with dilutions of IVIg. The plate was

washed and 200 $\mu$L of a solution of 1/1000 dilution of a

peroxidase-linked anti-human IgG conjugate (Promega) was

added. After washing, the plates were developed with a

substrate solution containing 0.01% (pH 7.5) H$_2$O$_2$ and

0.01% ABTS in potassium phosphate buffer. The absorbance

of the blue developed color at 450 nm (mean ± SD of duplicate

experiments) was measured. The absorbance values of the

samples were compared with a standard curve prepared with

human IgG. A non-parametric statistical analysis was used to

evaluate the difference between the means of the absorbance

values obtained with pooled IVIg and recombinant LukS-PV

and LukF-PV.

Figure 1. Binding of pooled IgG to LukS-PV and LukF-PV. The presence

of IgG antibodies to recombinant (r) LukS–PV and rLukF-PV was tested

by ELISA at the indicated dilutions (see Materials and Methods). Data

are absorbance at 450 nm (mean ± SD of duplicate experiments).
Figure 2. Effect of immunoabsorption of pooled IgG on maltose-binding protein (MBP), recombinant (r) LukS, and rLukF. The antibody reactivity of pooled IgG was examined by ELISA (as described in figure 1) after serial preincubations (0–4) for 1 h with MBP, rLukS, or rLukF (125 μg) coated on microplates.

PV or rLukF-PV in PBS overnight at room temperature, followed by incubation with a blocking solution of PBS-Tween (0.05%) and milk (10 g/L) for 30 min at 37°C. Unbound rLukS and rLukF was washed out twice with the blocking solution. Serial dilutions of IVIg were added to duplicate wells for 1 h at 37°C, and peroxidase-conjugated rabbit anti–human polyvalent IgG (Sigma) was added after 3 washes. The microplates were incubated for 1 h at 37°C and were washed before revelation by adding the substrate tetramethylbenzidine. The reaction was stopped with H2SO4. The plates were read at 450 nm in a Model 680 microplate reader (Biorad). Specificity of the antibody reactivity was examined by serial preincubations (0–4) for 1 h of IgIVG (3 mg/L) with MBP, rLukS, or rLukF (125 μg) coated on microplates before ELISA analysis.

Flow cytometric leukotoxicity assay. Polymorphonuclear neutrophil (PMN) membrane pore induction by MBP-LukS-PV and MBP-LukF-PV was measured by recording cellular ethidium-bromide incorporation in a flow cytometric method, as described elsewhere [16]. Appropriate dilutions of S. aureus supernatants or equimolar solutions of MBP-LukS-PV and MBP-LukF-PV were incubated for 15 min with serial dilutions of IVIg or PBS and then added to 1 mL of leukocyte suspension (10⁵ cells/mL) preincubated for 15 min with ethidium bromide (4 μmol). Kinetic measurements were made at room temperature by use of a FACscan flow cytometer (Becton Dickinson) equipped with 15-mW argon laser tuned to 488 nm. PMNs, monocytes, and lymphocytes were classically discriminated on the basis of their forward (FSC) and side (SSC) light scattering, and their fluorescence (FL1) (λem <530 nm) was recorded according to the experiment. Pore formation was determined by recording changes in fluorescence due to ethidium-bromide uptake. Ethidium-bromide fluorescence intensity was recorded in the FL3 channel (λem >650 nm). Data were analyzed with Cell Quest software (Becton Dickinson), and results were expressed as the percentage of ethidium bromide–positive PMNs.

Transmission electron microscopy. Cells (toxin-treated and untreated, with or without IVIg) were fixed, dehydrated, embedded, cut, and observed. In brief, cell suspensions were fixed by adding to the medium an equal volume of 4% glutaraldehyde (15 min) and by incubating for 30 min in 2% glutaraldehyde–0.1-mol Na cacodylate-HCl (pH 7.4). The cells then were washed 3 times in 0.2-mol Na cacodylate-HCl (pH 7.4) for 10 min and were postfixed with 1% OsO4–0.15-mol Na cacodylate-HCl (pH 7.4) for 30 min. The cells were dehydrated with an increasing ethanol gradient (30%, 50%, 70%, and 95%; 5 min) and pure ethanol (3 × 10 min). Impregnation...
**RESULTS**

**IVIg contains PVL-specific antibodies.** As shown in figure 1, anti–rLukS-PV and anti–rLukF-PV antibodies were detected in the commercial IVIg preparation (Tégléline). Signals were IVIg-concentration dependent from 0 to 50 mg/L and were slightly stronger with rLukF-PV than with rLukS-PV. The specificity of the anti–rLukS-PV and anti–rLukF-PV antibodies detected in our ELISA was confirmed by the signal reduction after IVIg preincubation with soluble rLukS-PV and rLukF-PV, respectively, but not with MBP (figure 2).

**Leukotoxic activity of PVL-producing S. aureus culture supernatants and of rLukS-PV and rLukF-PV.** Crude culture supernatants of *S. aureus* strains HT2000 0237 and ATCC 49775 were leukotoxic in amounts as small as 5 μL after 20 min (figure 3). No leukotoxicity was detected when PMNs were incubated with fresh YCP medium or with PBS (figure 3). The kinetics of pore formation increased with the supernatant volume used (figure 4). The recombinant MBP-LukS-PV and MBP-LukF-PV proteins failed to cause PMN pore formation when tested individually (figure 5). In contrast, when MBP-LukS and MBP-LukF-PV were used together at equimolar concentrations, their activity was concentration and time dependent (figure 5). The time required for the activity of the recombinant proteins to reach a plateau depended on their concentration (90 min with 10 nmol and 30 min with 200 nmol; figure 5). These latter conditions were chosen for electron microscopy studies.

**Ultrastructural changes in leukotoxin-treated cells.** Human PMNs not exposed to PVL had a diameter of 8–12 μm, a lobulated nucleus, and numerous intracytoplasmic granules; they accounted for ~60% of cells in peripheral leukocyte preparations. Their surfaces showed typical pseudopods (involving a limited area of the cell surface, with no alteration of intracellular organelles). Eosinophils, identified by their primary granules and secondary lysosomes, accounted for ~3% of cells. Lymphocytes appeared as round cells ~5 μm in diameter with a few small pseudopods, a single large nucleus, and low levels of cytoplasm. Very few monocytes were seen (figure 6A).

Preincubation of the cells for 30 min with 5 μL of culture supernatant of PVL-producing *S. aureus* or with 200 nmol of recombinant PVL caused alterations that are consistent with...
PMN necrosis (figure 6B). Analysis of multiple sections revealed that these alterations began with local membrane thinning, which was followed by the formation of large protrusions containing clarified cytoplasm and differing markedly from physiological pseudopods. Subsequently, the entire PMN showed signs of necrosis: the cells were rounded and enlarged, and the outer membrane was thinned. The cytoplasm appeared edematous and contained few granules. Most PMNs showed cytoplasmic hypervacuolization and translocation of azurophilic granules to the peripheral cytoplasm. Perinuclear spaces were enlarged, except next to nuclear pores (figure 6C). Lymphocytes were unaffected.

IVIg inhibits PVL leukotoxicity. The effect of IVIg on the cytotoxic activity of recombinant MBP-LukS-PV and -LukF-PV was examined. Preincubation of equimolar concentrations (200 nmol) of MBP-LukS-PV and MBP-LukF-PV with increasing amounts of IVIg (0.5–20 mg/L) inhibited leukotoxic cytotoxic activity in an IVIg concentration-dependent manner; moreover, the maximum plateau value was delayed for IVIg concentrations >2 mg/L (figure 7).

Culture supernatants (5 μL) of S. aureus strains ATCC49775 and HT2000 0237 then were preincubated with 10 mg/L of IVIg for 15 min before being tested on PMNs. The leukotoxic activity of ATCC 49775 was partially inhibited (data not shown), whereas that of HT2000 0237 was dramatically inhibited in the presence of IVIg (figure 8). To determine whether the inhibitory effect of IVIg was concentration dependent, the supernatant of strain HT2000 0237 was preincubated with increasing amounts of IVIg (0.5–20 mg/L). Inhibition started at 0.5 mg/L of IVIg and was concentration dependent; maximum of inhibition was achieved with 10 mg/L of IVIg (figure 8).

Finally, the inhibitory effect of IVIg was evaluated by transmission electron microscopy (TEM). Preincubation of the recombinant toxins (200 nmol) or crude culture supernatant of strain HT2000 0237 with IVIg (10 mg/L) for 15 min, before treatment of PMNs, showed a reduction in cellular alterations relative to controls (figure 6A–6C). Remarkably, necrosis was seldom observed, membrane alterations and cytoplasmic edema were limited in a given cell, and no nuclear/perinuclear defects were observed (figure 6D).

DISCUSSION

We investigated the presence and the effect of staphylococcal leukocidin-specific antibodies in a commercial IVIg preparation. We showed by use of ELISA that IVIg indeed contained such antibodies and demonstrated that IVIg inhibited the cytotoxicity of staphylococcal leukocidins on PMNs in vitro. Leukotoxicity was documented in 2 complementary ways: (1) a flow cytometric assay reflecting membrane pore formation and (2) transmission electron microscopy showing ultrastructural changes in PMNs, with cytoplasmic protrusion, cytoplasmic...
The high concentration of antibodies against PVL in a commercial IVIg preparation was not unexpected, as previous serological studies have demonstrated the presence of antibodies against the 2 proteins forming PVL LukS-PV and LukF-PV in most of human serum samples tested [20]. The presence in IVIg of antibodies to S. aureus toxins is usually attributed to natural exposure [10, 20]. However, whereas Hlg and LukE-LukD are produced by >99% and ~75%, respectively, of human S. aureus isolates [2, 18], PVL is generally produced by only 3%–5% of such isolates [2, 17]. All staphylococcal synergohymenotropic toxins are antigenically related [13]; hence, we suspect that most antibodies against PVL of IVIgG could be attributed to prior exposure to PVL, Hlg, or LukE-LukD of serum donors. Indeed, the prevalence of serum antibodies to

Figure 6. Transmission electron microscopy. A, Untreated and inactivated leukocytes, with a characteristic lobulated nucleus, numerous cytoplasmic granules, and few short pseudopods. One lymphocyte (L) and 3 platelets (Pl) are present among 8 neutrophilic polymorphonuclear cells. B, Leukocytes incubated with maltose-binding protein (MBP)–LukS plus MBP-LukF. Most polymorphonuclear neutrophils (PMNs) are necrotic (Nec) or clasmatoctytic (Cl), or show focal edema (O), large cytoplasmic protrusions (P), and loss of pseudopods; arrows indicate the sites of cytoplasmic constriction. C, At higher magnification, the cytoplasmic membrane is locally thinned (arrow), and the nuclear envelope is dilated (*). D, Leukocytes treated with MBP-LukS plus MBP-LukF preincubated with intravenous immunoglobulin. PMNs resembled untreated leukocytes (A). Cyt, cytoplasm; Nuc, nucleus.
PVL increases with age in healthy subjects, despite a lack of evidence of exposure to PVL-producing strains (authors’ data). Like S. Mudd et al. [20] in 1965, we found higher antibody titers against LukF than against LukS in IVIg. This difference might be caused by a higher antigenicity of LukF and/or by more-frequent exposure to toxins antigenically related to LukF than to LukS [20]. In our experience, we did not find variation between IVIg lots (data not shown), probably because of the large number of donors (20,000) used for IVIg isolation in Tége´line preparations. However, we could not exclude variation between commercial IVIg preparations.

Several mechanisms have been proposed to explain the inhibitory effects of IVIg on the immunological actions of bacterial toxins, including antigen-specific antibodies and non-specific blockade of PMN receptors [9]. We found that PMN preincubation with IVIg followed by IVIg elimination by washing did not alter PVL leukotoxicity (data not shown), which suggests that IVIg does not directly modulate PMN susceptibility to PVL. Thus, IVIg probably acted principally by interfering with PVL binding to PMNs. This may have been mediated by antibodies directed against epitopes of LukS (and probably of LukF), which are required for toxin binding to the putative LukS receptor (or against LukF epitopes required for binding to the LukS-receptor complex) on PMNs.

Rabbit immunization against PVL attenuates subsequent experimental PVL-induced skin necrosis [1, 6, 7]. Likewise, skin abscess formation after PVL injection was inhibited by concomitant injection of neutralizing anti-leukocidin antibodies [7]. Human serum titers of anti-PVL antibodies vary widely among individuals [20, 21] (authors’ data). Infected subjects fail to mount optimal humoral responses to PVL [20], but the severity and extent of S. aureus infection has been correlated with the serum anti-PVL titer [20, 21]. S. Mudd et al. [20] have tested PVL toxoid as an adjunctive treatment of S. aureus diseases. After PVL toxoid injection, they observed an increase in the anti-PVL titer and a beneficial effect in osteomyelitis and soft-tissue infection. However, PVL toxoid had adverse effects, such as inflammation at the injection site and frequent systemic reactions [1, 6, 20]. Anti-PVL antibody titers also could be increased by passive immunization with IVIg, particularly in patients with acute PVL-associated syndromes, such as the recently described necrotizing pneumonia. Interestingly, intravenous and intranasal administration of IVIg-IgG conferred protection against S. aureus challenge in leukopenic mice [22].

These findings warrant clinical trials of IVIg in staphylococcal necrotizing pneumonia. However, the concentration of PVL in lung and serum during necrotizing pneumonia (or PVL con-
centration during PVL-positive S. aureus infections) is not known. Until these parameters are determined, the general guideline proposed for IVIg in cases of bacterial toxemia could be proposed [8–10].

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