MAJOR ARTICLE

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, multilobar 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.

Received 7 May 2003; accepted 2 August 2003; electronically published 9 January 2004.

Presented in part: 102nd general meeting of the American Society for Microbiology, Salt Lake City, 21 May 2002.

Financial support: Programme Fondamental de Recherche en Microbiologie et Maladies Infectieuses et Parasitaires (Ministère de la Recherche et des Nouvelles Technologies).

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The Journal of Infectious Diseases 2004;189:346–53

0022-1899/2004/18902-0023$15.00

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-*

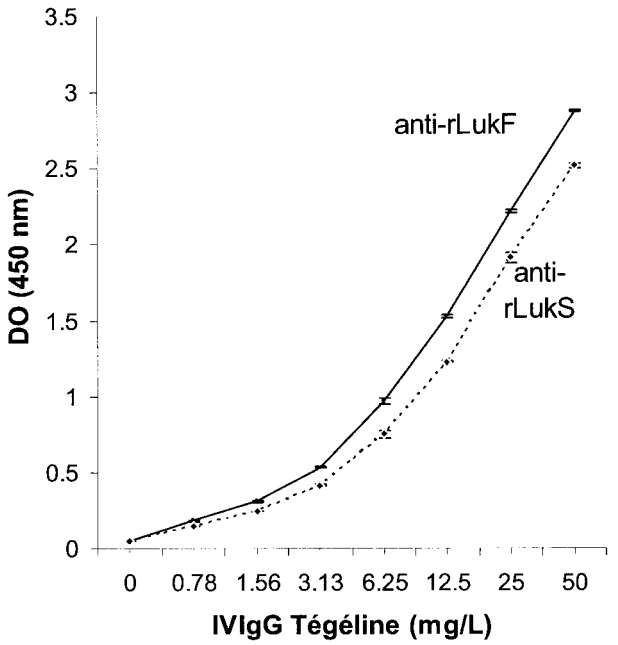

Figure 1. Binding of pooled IgG to LukS-PV and LukF-PV. The presence of IgG antibodies to recombinant (r) LukS–PV and rLukF-PV was tested by ELISA at the indicated dilutions (see Materials and Methods). Data are absorbance at 450 nm (mean ± SD of duplicate experiments).

PV$^+$, and *lukE lukD*$^+$), which was provided by Y. Kamio (Jutendo Sendai University, Japan), 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_2PO_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-thiogalactopyranoside (IPTG).

IVIg. Human intravenous polyclonal immunoglobulin (IVIg; Tégéline Laboratoire Français du Fractionnement et des Biotechnologies [LBF], autorisation de mise sur le marché [AMM]: 559895-3) was purchased from LBF. Tégéline is isolated 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) and native 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 AAT TCG AAT CTA AAG CTG ATA ACA ATA TTG AG-3′; and rlukF-1, 5′-CAG AAT TCG CTC AAC ATA TCA CTG 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 Doolitttle [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 rlukF-2, 5′-GCC TGC AGT TAG CTC ATA GGA TTT TTT TCC TTA G-3′) were chosen to overlap the stop codon of each gene. Restriction sites (*Eco*RI for rlukS-1 and rlukF-1; *Pst*I for rlukS-2 and rlukF-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 *Eco*RI and *Pst*I (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 sequencing. 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 manufacturer'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 mononuclear 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 removed by hypotonic lysis with 0.9% NH_4Cl for 15 min and followed by 3 washes in PBS (pH 7.2; BioMérieux SA). The final cell pellet was suspended and adjusted to 10^5 cells/mL in PBS. Fresh cells were prepared for each day's experiments.

Anti–LukS-PV and anti–LukF-PV immunoassays. 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–immunoglobulin 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 manufacturer's instructions (New England BioLabs). The wells of microtiter plates (Sigma) were coated with 125 μg of rLukS-

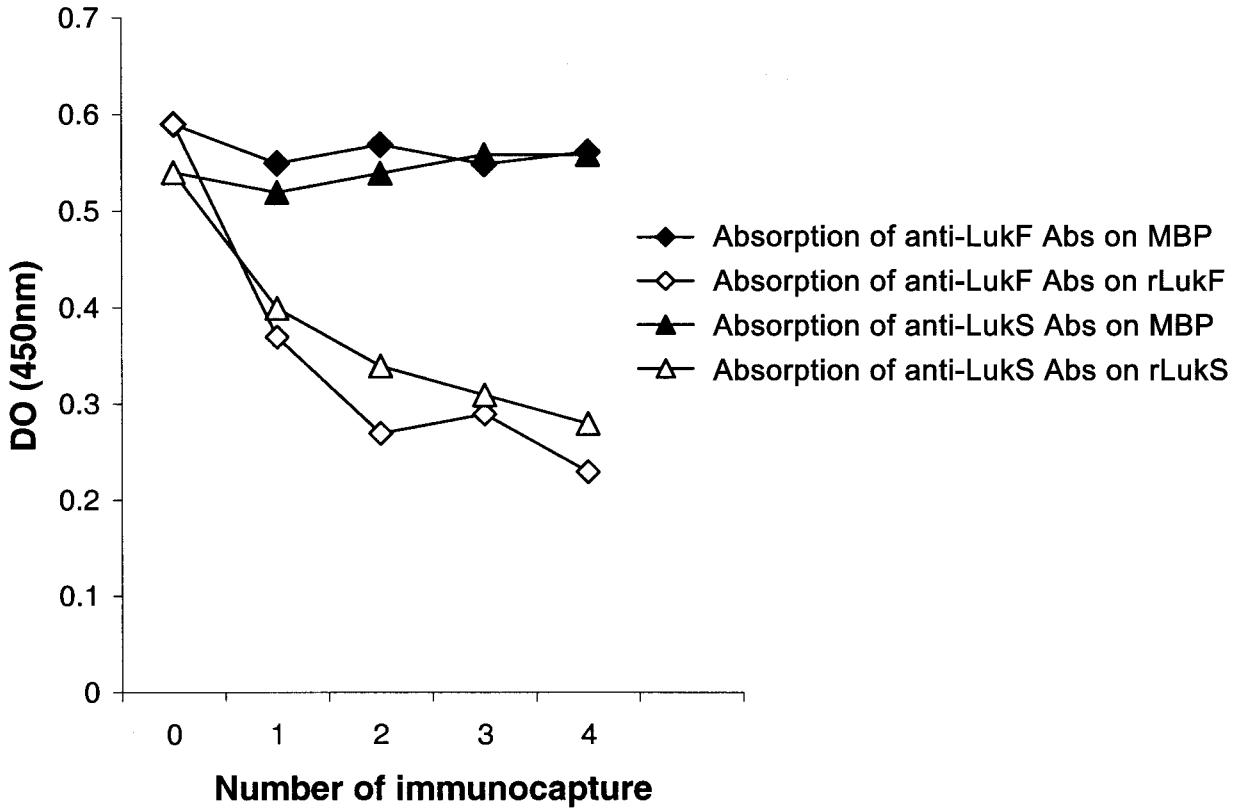

Figure 2. Effect of immunoabsorption of pooled IgG on maltose-binding protein (MBP), recombinant (r) LukS, and rLukF. The antibody reactivity of pooled IgG was examined by ELISA (as described in figure 1) after serial preincubations (0–4) for 1 h with MBP, rLukS, or rLukF (125 μg) coated on microplates.

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 H_2SO_4. 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 (3mg/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 (10^5 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) (λ_{EM} <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 (λ_{EM} >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% OsO_4–0.15-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

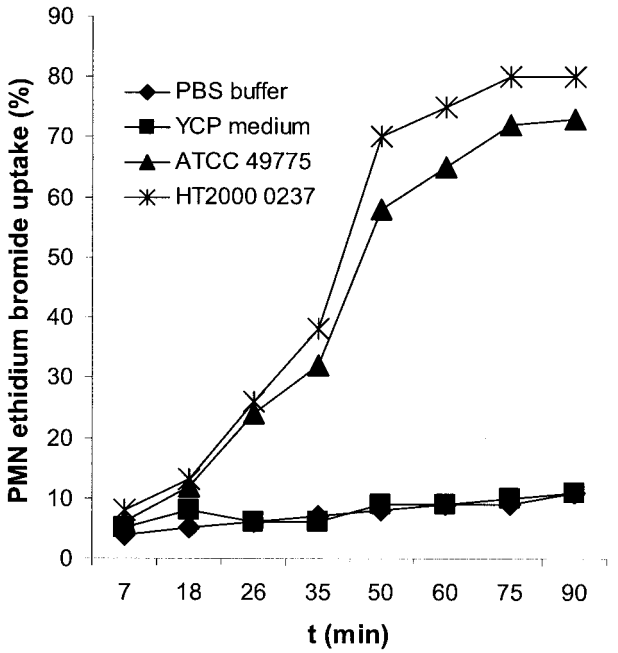

Figure 3. Kinetics of polymorphonuclear neutrophil (PMN) ethidium-bromide uptake induced by *Staphylococcus aureus* ATCC49775 supernatant, *S. aureus* HT2000 0237 supernatant, PBS, and YCP medium. The supernatants and media (5 μL) were added to leukocyte suspensions (10^5 cells/mL), together with ethidium bromide (4 μmol). At the time indicated, cell suspensions were analyzed by flow cytometry (3000 events) after electronic gating of the PMN population. Data are the percentage of PMNs stained by ethidium bromide.

was performed with Epon A (50%) plus B (50%) plus DMP30 (1.7%). Inclusion was obtained by polymerization of the Epon mixture (60°C; 72 h). Sections 60–80-nm thick were cut with an ultramicrotome (RMC MTX; Elexience) and were contrasted with uranyl acetate and lead citrate. Sections were observed with a Jeol 1200CX transmission electron microscope.

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 (Tégéline). 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 6*A*).

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

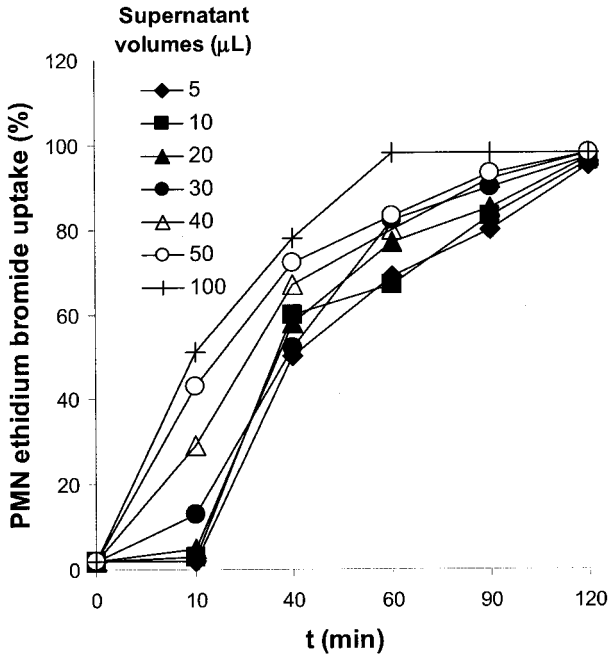

Figure 4. Concentration-dependent kinetics of polymorphonuclear neutrophil (PMN) ethidium-bromide uptake induced by *Staphylococcus aureus* HT2000 0237 supernatant. The indicated volumes of supernatant were added to leukocyte suspensions (10^5 cells/mL), together with ethidium bromide (4 μmol). Cell suspensions were analyzed as described in figure 3.

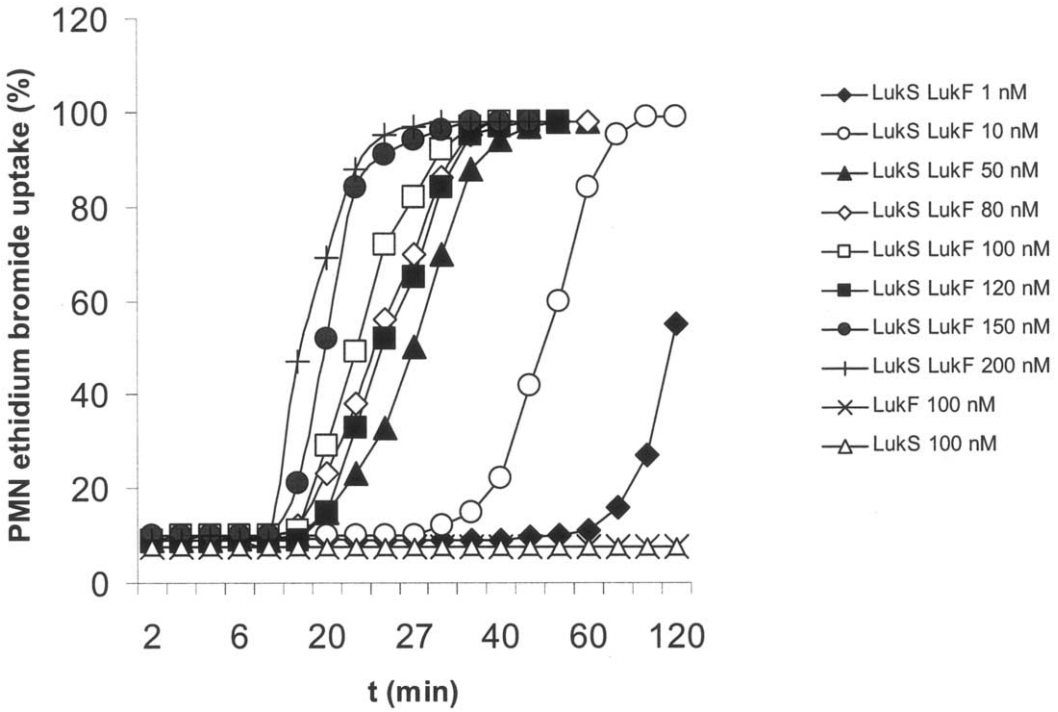

Figure 5. Time course of polymorphonuclear neutrophil (PMN) ethidium-bromide uptake induced by LukS-PV and LukF-PV. Solutions of maltose-binding protein (MBP)–LukS-PV and/or MBP-LukF-PV at the indicated concentrations were added to leukocyte suspensions (10^5 cells/mL), together with ethidium bromide (4 μmol), at the time indicated. Cell suspensions were analyzed as described in figure 3.

PMN necrosis (figure 6*B*). 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 6*C*). Lymphocytes were unaffected.

IVIg inhibits PVL leukotoxicity. The effect of IVIg on the cytopathic 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 transcription 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 6*A*–6*C*). Remarkably, necrosis was seldom observed, membrane alterations and cytoplasmic edema were limited in a given cell, and no nuclear/perinuclear defects were observed (figure 6*D*).

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

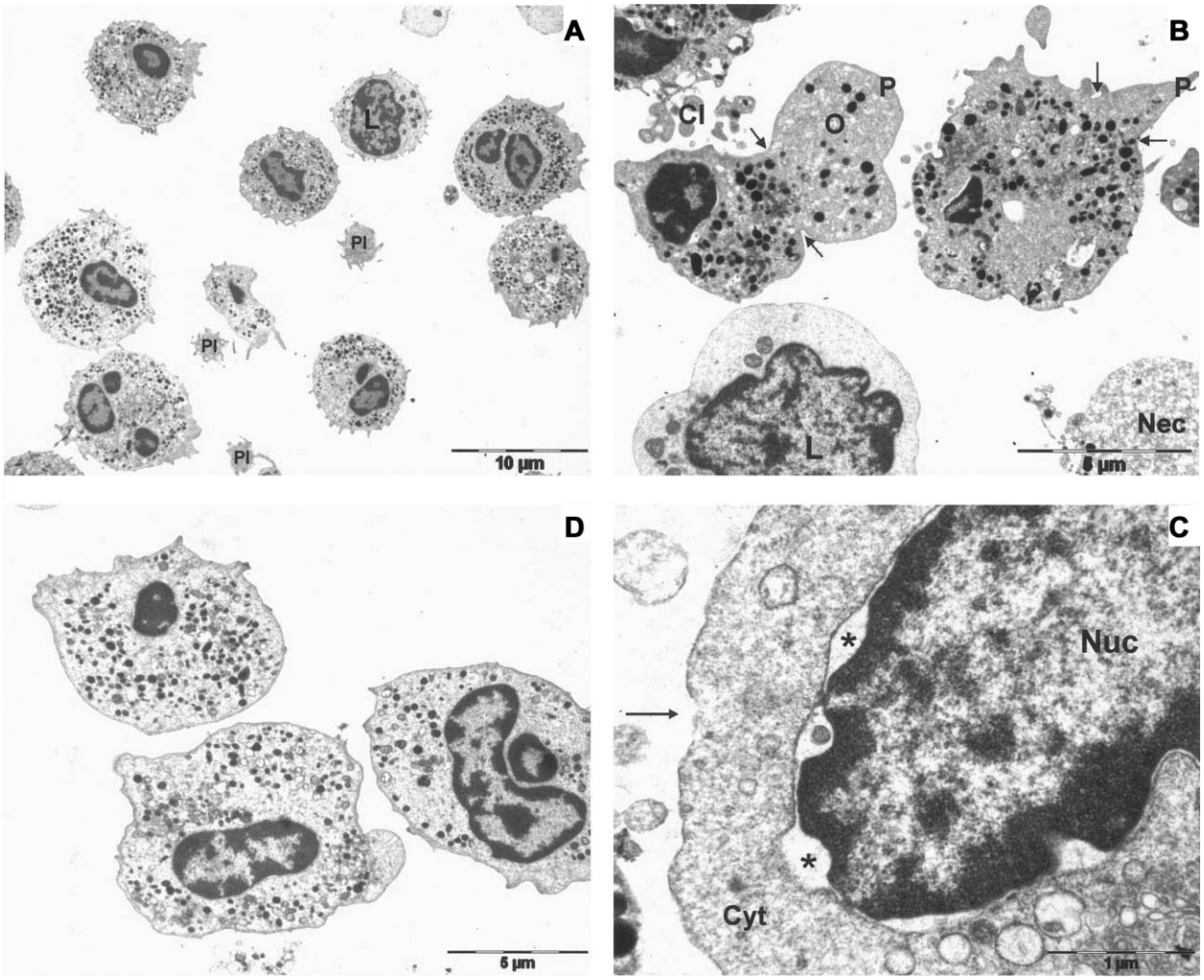

Figure 6. Transmission electron microscopy. *A,* Untreated and inactivated leukocytes, with a characteristic lobulated nucleus, numerous cytoplasmic granules, and few short pseudopods. One lymphocyte (L) and 3 platelets (Pl) are present among 8 neutrophilic polymorphonuclear cells. *B,* Leukocytes incubated with maltose-binding protein (MBP)–LukS plus MBP-LukF. Most polymorphonuclear neutrophils (PMNs) are necrotic (Nec) or clasmatocytotic (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.

hypervacuolization, loss of cytoplasmic granules, and thinning of the cytoplasmic membrane. IVIg inhibited pore formation and the cytopathic effect of both purified rPVL and crude *S. aureus* culture supernatants containing PVL and, potentially, other leukocidins, such as Hlg and LukE LukD. The protection conferred by IVIg was concentration dependent.

Interest in PVL has diminished in recent decades, probably owing to the low incidence of PVL-associated diseases in industrialized countries [2, 17]. However, PVL is currently an increasing focus of investigation with the description of newly identified syndromes, such as *S. aureus* necrotizing pneumonia and the worldwide spread of community-acquired skin and soft-tissue infections due to methicillin-resistant *S. aureus* carrying the PVL locus [3, 18, 19].

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

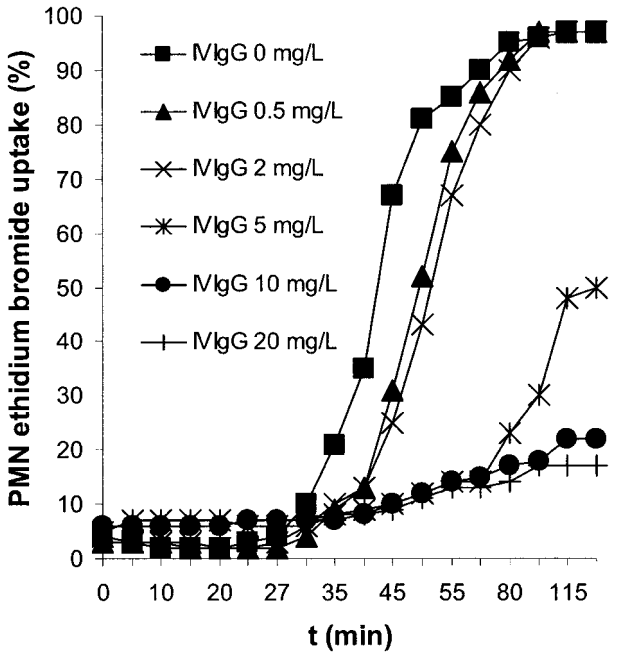

Figure 7. Intravenous immunoglobulin (IVIg) inhibition of polymorphonuclear neutrophil (PMN) ethidium-bromide uptake induced by LukS-PV/LukF-PV. Maltose-binding protein (MBP)–LukS-PV and MBP-LukF-PV (200 nmol) were incubated with IVIg at the indicated concentrations and then added to leukocyte suspensions (10^5 cells/mL), together with ethidium bromide (4 μmol). Cell suspensions were analyzed as described in figure 3.

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 Tégéline 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 nonspecific 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-

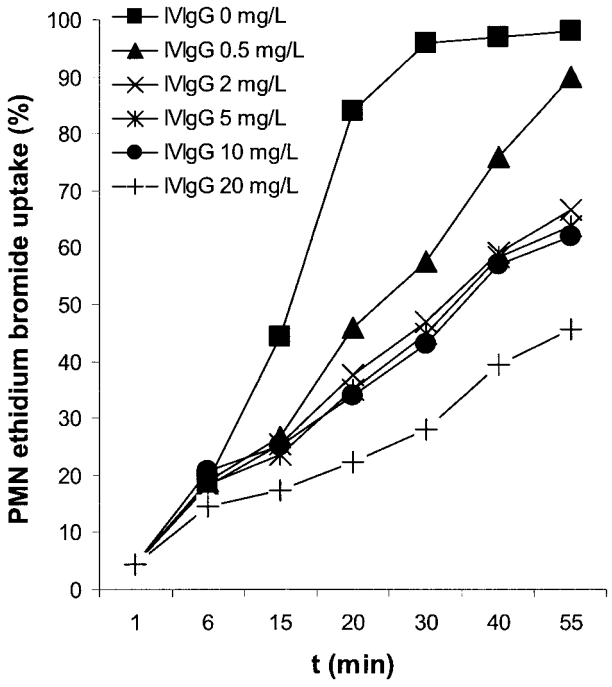

Figure 8. Intravenous immunoglobulin (IVIg) inhibition of *Staphylococcus aureus* supernatant-induced ethidium bromide uptake by polymorphonuclear neutrophils (PMNs). Culture supernatant (50 μL) of strain HT2000 0237 was incubated with the indicated concentrations of pooled IgG (IVIg) and then added to leukocyte suspensions (10^5 cells/mL) together with ethidium bromide (4 μmol). Cell suspensions were analyzed as described in figure 3.

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].

Acknowledgments

We are grateful to V. Chamouard, N. Violland, and C. Badiou for their technical assistance; D. Colin for technical advices on flow cytometric analysis; P.-A. Bryon for many helpful comments on transmission electron microscopy studies; and D. Young for editing the manuscript.

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