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Interaction of the Two Components of Leukocidin from *Staphylococcus aureus* with Human Polymorphonuclear Leukocyte Membranes: Sequential Binding and Subsequent Activation

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The sequential interaction between the two components S and F of leukocidin from *Staphylococcus aureus* and the membrane of human polymorphonuclear neutrophils has been investigated in the presence of 1 mM Ca^{2+}. With ^{125}I-labeled components, it has been shown that binding of the F component occurred only after binding of the S component. The kinetic constants of binding of both components were not statistically different (K_d, approximately 5 nM; B_m, approximately 35,000 molecules per cell), and both Hill coefficients were 1. The application of increasing concentrations of leukocidin provoked a dose-dependent secretion of the granule content, as determined by hexosaminidase and lysozyme activity measurements. Furthermore, the separate perfusion of S and F components on human polymorphonuclear neutrophils deposited on a filter induced secretion of the granules content only when the perfusion of the S component preceded that of the F component. We conclude, therefore, that (i) S-component binding is a prerequisite for F-component binding and for subsequent activation of polymorphonuclear neutrophils and (ii) there is a specific binding site for the S component in the plasma membrane.

Staphylococcus aureus produces numerous toxins which are more or less well characterized. In 1932, Panton and Valentine (13) described the biological activity of the so-called Panton-Valentine leukocidin (PVL) isolated from strain V8 from a case of chronic furunculosis and distinguished this toxin from hemolysins of staphylococcal origin. Woodin (19, 20) was the first to purify PVL and to characterize two synergistic proteins having molecular weights of 32,000 and 38,000, termed F and S, respectively, for fast and slow elution by column chromatography. Later, Noda et al. (11), using another purification technique, determined that PVL consisted of two proteins, also called F and S but having molecular weights of 32,000 and 31,000, respectively. In fact, molecular cloning and sequencing (4, 16, 18) of leukocidins from *S. aureus* have shown that these proteins along with gamma hemolysin correspond to very closely related toxins belonging to the same family of synergohymenotropic toxins (14).

The two components of PVL were purified to homogeneity in our laboratory from the V8 strain, and molecular weights of 38,000 and 32,000 were determined for F and S components, respectively (7). It appears that the apparent molecular weights of F and S components were reversed compared with those determined by Woodin (20) due to different purification techniques. Furthermore, immunosera obtained from rabbits allowed an epidemiological study of PVL-producing *S. aureus* strains which revealed a strong association between PVL and staphylococcal cutaneous infections such as furuncles and primitive abscesses (5).

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Subsequent work has shown that the synergistic action of the two components of PVL is related to the formation of pores in the membrane of human polymorphonuclear neutrophils (PMNs), pore size being dependent on divalent cation concentration (6). Woodin (21) has proposed that the synergistic action of the two components consists of the initial binding of the F component (32,000 Da) to the leukocyte membrane and the subsequent binding of the other component (38,000 Da).

The present study was designed to investigate PVL binding at physiological calcium concentrations and to confirm the sequential fixation of the two components of PVL, using labeled components of the toxin as well as the toxin-induced secretion of granule contents from PMNs as indicators of their activation.

MATERIALS AND METHODS

Toxin production. The two components of PVL were purified as described previously (7) by chromatography of the supernatant of *S. aureus* V8 (ATCC 49775) on a cation-exchange column.

Preparation of human neutrophils. Human PMNs were prepared from buffy coats of healthy donors, obtained from the Centre Régional de Transfusion Sanguine de Strasbourg (France) as described previously (6), and were resuspended in a buffer solution containing 140 mM NaCl, 5 mM KCl, 1.1 mM $CaCl_2$, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid], and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.3).

Radioiodination of PVL. The two components of PVL were iodinated by the chloramine-T method modified from Rosa et al. (17). The iodination mixture (400 μl) contained 40 μg of chloramine-T, 200 μCi of [^{125}I]NaI, and 0.1 mg of S or F component in sodium phosphate buffer (0.26 M; pH 7.5). After 30 s of incubation, the reaction was stopped by the addition of 100 μl of 0.01 mM sodium phosphate buffer (pH 7.5) contain-

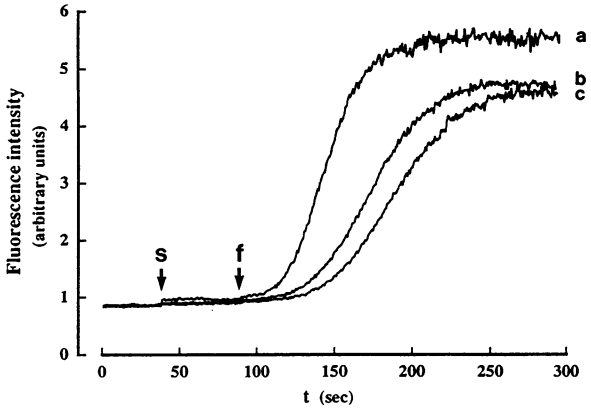

FIG. 1. Determination of toxic activity of ^{125}I-labeled PVL. Fura2-loaded human PMNs were incubated with unlabeled or labeled S and F components (1.2 nM each). Fluorescence variations were the ratio of measured emissions at 510 nm of 340/380-nm excitations. a, unlabeled S and F; b, labeled S, unlabeled F; c, labeled S and F.

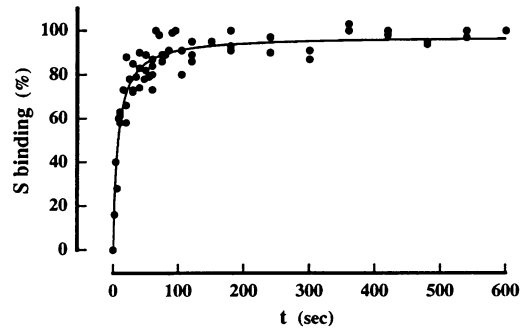

FIG. 2. Association kinetics of ^{125}I-labeled S component (1.2 nM) with 3×10^6 PMNs per ml. Data are from nine experiments.

ing 1 M NaCl, 60 mM KI, 210 mM $Na_2S_2O_5$, and 1% bovine serum albumin. The separation of free from bound ^{125}I was achieved by applying the mixture to a Sephadex G-25 column (Pharmacia, LKB, St. Quentin-Yvelynes, France) equilibrated with 0.5 M sodium phosphate buffer (pH 7.5) and 0.1% bovine serum albumin. The structural integrity of the labeled toxin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent autoradiography. Specific activity was determined by gamma counting of an aliquot and also, after electrophoresis, by comparing the staining intensity of the aliquot with that of a known quantity of an unlabeled sample of toxin, using an Ultroscan (LKB).

Toxic activity assay. Since it has been shown that the ability of leukocidin to form calcium channels through the PMN membrane is concentration dependent (6), this property has been used to verify the biological activity of the labeled toxin. PMNs were loaded with the fluorescent calcium probe Fura2 as described previously (6), and after application of the leukocidin components, the variations in fluorescence intensity were determined with a spectrofluorometer (Deltascan; PTI, Kontron, France). As shown in Fig. 1, the labeled S component retained more than 80% of its potency during the iodination process and the labeled F component was almost as potent as the native component.

Binding assays. All binding experiments were conducted at room temperature in order to avoid possible internalization of the toxin into the PMNs by endocytosis. Binding incubations were carried out in Eppendorf tubes (1.5 ml) containing 200 μl of the suspension solution for different times depending on the experiment. The relationship between binding and PMN concentration was linear up to 15×10^6 PMN per ml, and all subsequent incubations were performed in the presence of 3×10^6 PMN per ml. Incubation was stopped by the addition of 1 ml of cold (4°C) buffer solution used for PMN preparation followed by centrifugation at $600 \times g$ for 2 min. The pellet was then washed three times with the same solution and counted in a gamma counter (Beckman, Gagny, France). Each determination was performed in triplicate.

Secretion determination. Two types of experiments were performed. In the first experiment, 2×10^6 PMN per ml were incubated at 37°C for increasing periods of time with increasing concentrations of PVL in microtiter wells in a final volume of 200 μl. The plate was then centrifuged at $400 \times g$ for 5 min at 4°C, and supernatants were recovered for enzymatic assays. Total lysozyme and hexosaminidase activities were determined in 200 μl of PMN suspension solubilized by 0.2% Triton X-100.

In the second experiment, 10^7 PMN were deposited on a low-binding protein sterile filter (0.45-μm pore size; Millex-HV) which was placed in a perfusion apparatus consisting of a peristaltic pump (P_1; Pharmacia) and a fraction collector (7000 Ultrorac; LKB). Cells were perfused at 37°C with the suspension buffer containing 0.1 mg of bovine serum albumin per ml at a rate of 260 μl/min. PVL was introduced into the perfusion mixture for 5-min periods. Fractions were collected every minute for the enzymatic assays.

Primary granule release was monitored by measuring the amount of hexosaminidase activity in cell supernatants (7a). Cell supernatants (50 μl) were incubated overnight with 50 μl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.2 M sodium citrate (pH 4.5). After addition of 100 μl of 1 M Tris (pH 9), the plate was read at 405 nm in a Titertek Multiskan Mc (Flow Laboratories, Orsay, France). Data are expressed as either absorbance units or the percentage of total activity present in the supernatant of untreated cells solubilized by Triton X-100.

Lysozyme release from primary and secondary granules was assessed in 100-μl samples by monitoring the lysis of 0.15 mg of *Micrococcus lysodeikticus* (Sigma, St. Quentin-Fallardier, France) per ml in 1 ml of acetate buffer (pH 6.0) at 450 nm for 6 min. Lysozyme concentration in the samples was determined by comparison with solutions of known concentrations.

The lactate dehydrogenase activity released from lysed cells was determined spectrophotometrically by monitoring the reduction of pyruvate at 340 nm, which is related to the oxidation of NADH.

RESULTS

S-component binding. Figure 2 shows the association kinetics of the ^{125}I-labeled S component with PMNs. Approximately 80% of the binding was obtained after 1 min of incubation, and complete binding occurred within 5 min. The labeled S component (1.2 nM) was allowed to bind for 20 min and then diluted in a 1,000-fold excess of cold S component; its release from the receptor was investigated. Maximum dissociation did not exceed 50% (Fig. 3).

Saturation experiments were carried out in order to evaluate the affinity constants (K_d) and the maximal binding capacities (B_m) of components S and F to PMNs. In these experiments, nonspecific binding was evaluated in the presence of a 1,000-fold excess of cold toxin component. An example of such an experiment, with S component, is shown in Fig. 4. Since the

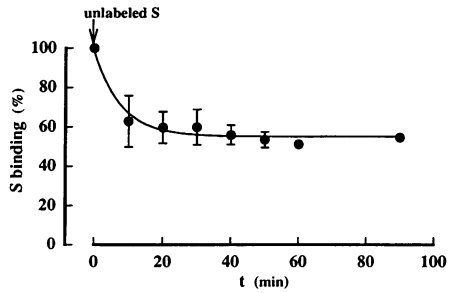

FIG. 3. Dissociation kinetics of ^{125}I-labeled S component (1.2 nM; 20-min incubation) with 3×10^6 PMNs per ml (unlabeled S, 1 μM) $n = 4$.

Hill coefficient determined for S or F binding was 1 (Table 1), indicating the presence of one class of binding sites, the curve fitting by non-linear regression analysis (Sigma-Plot, Jandel, Germany) of the data obtained from the saturation experiments was used to determine kinetic parameters. A K_d of 6.1 ± 0.9 nM and a B_m of 38,900 ± 11,000 molecules per PMN were obtained (Table 1).

F-component binding. In the absence of the S component, the observed binding of the F component was less than 20% of the total binding obtained in the presence of the S component (Fig. 5). Furthermore, when added alone, the F component did not provoke any pore formation (6) or induce any secretion in PMNs (this study). If the S component had previously been allowed to bind to the PMNs, consequent binding of the F component would occur, and K_d and B_m values which were not statistically different from the values obtained for the S component could be determined (Table 1).

Secretion. A single application of S or F component alone did not provoke any secretion of the granule content of the PMNs. However, in the presence of 2.3 nM S component, the application of increasing concentrations of F component resulted in concentration-dependent hexosaminidase and lysozyme secretions (Fig. 6A and B). At the end of this experiment, lactate dehydrogenase liberation did not exceed 5% of the total content.

When the two components of PVL were injected separately into the perfusion line of PMNs deposited on a filter, they had no effect on secretion. When they were injected successively and the perfusion sequence was S component-rinsing period-F component, hexosaminidase (Fig. 7A) and lysozyme (Fig. 7B) secretion could be detected. Conversely, when the perfusion sequence was F component-rinsing period-S component, no secretion occurred, but the subsequent simultaneous injection of the two components provoked hexosaminidase and lysozyme secretion, showing that PMNs retained their secretion potencies.

TABLE 1. Dissociation constants (K_d), maximal binding capacities (B_m), and Hill coefficients determined for components S and F (in the presence of S) on human PMNs[a]

Toxin component	K_d (nM)	B_m (kilomolecules per cell)	Hill coefficient
S	6.1 ± 0.9	38.9 ± 11.0	1.03 ± 0.04
F (+S)	2.0 ± 1.6	32.6 ± 11.3	0.96 ± 0.04

[a] Values are means ± standard deviations of three experiments in triplicate.

DISCUSSION

The ^{125}I labeling of the two components of PVL did not impair their toxic activity, showing that the binding experiments described in this study are representative of the binding behavior of the native toxin. All binding experiments were conducted at 20°C to avoid the internalization of the toxin by endocytosis, but the possibility that an internalization was responsible for the incomplete displacement of the radioactive toxin by the native toxin cannot be ruled out. This observation is probably relevant to the insertion of the S component into the membrane, which is irreversible.

Our experiments describe the concentration-dependent and saturable binding of both toxin components. We have shown that the initial binding of the S component (32,000 Da) is a prerequisite for the binding of the F component (38,000 Da). This observation is in agreement with that of Woodin and Wieneke (22), who proposed that binding of the 32,000-Da toxin component preceded that of the 38,000-Da component, although they termed them differently. Furthermore, an initial application of the S component was required for PMN activa-

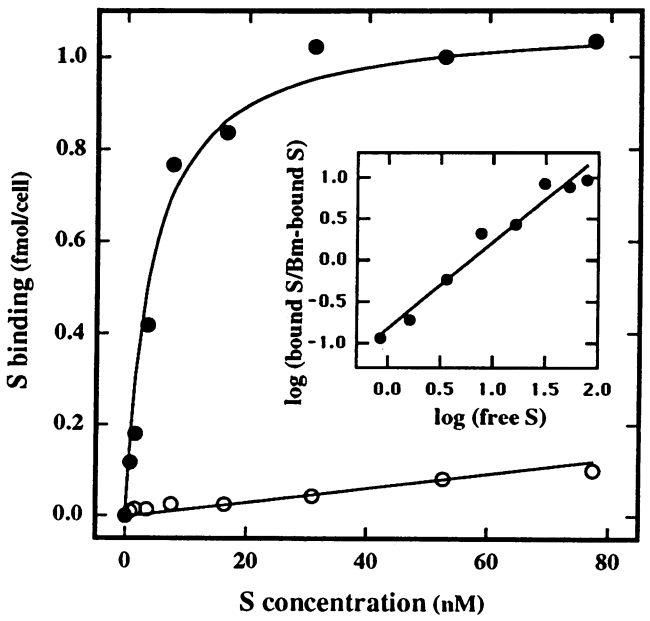

FIG. 4. Example of a saturation experiment with the S component and human PMNs (3×10^6 per ml). ●, specific binding (insert, Hill coordinates); ○, nonspecific binding.

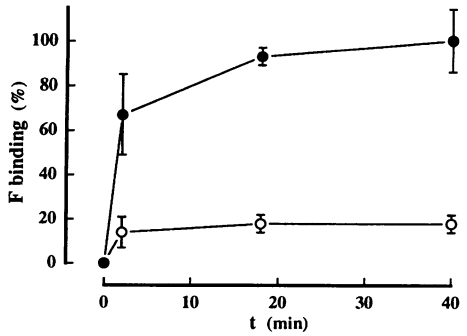

FIG. 5. Association kinetics of ^{125}I-labeled F component (1.2 nM) with 3×10^6 PMNs per ml: ○, in the absence of S component; ●, after 20 min of incubation with S component (3.5 nM). $n = 4$.

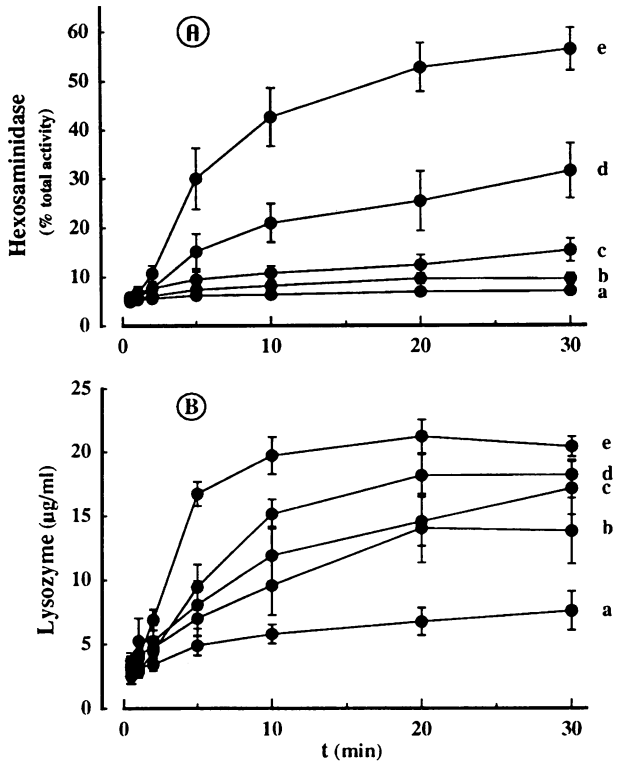

FIG. 6. Influence of S component (2.3 nM) and increasing concentrations of F component (a, 0 pM; b, 85 pM; c, 142 pM; d, 285 pM; e, 570 pM) on secretion of hexosaminidase (A) and lysozyme (B) by human PMNs (3×10^6 per ml) in the presence of Ca^{2+} (1 mM). n = 5.

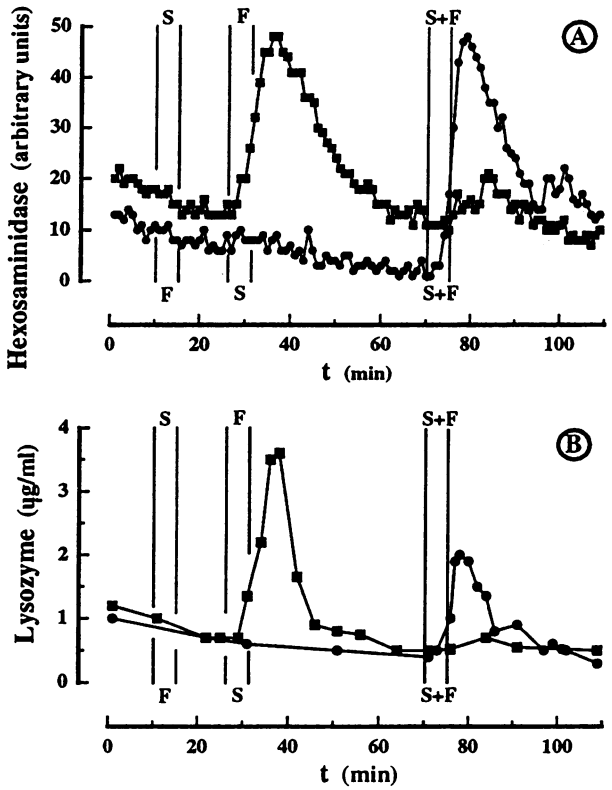

FIG. 7. Hexosaminidase (A) and lysozyme (B) secretion during sequential perfusion of 10^7 PMNs with PVL components. ■, sequence: S, F, S+F; ●, sequence: F, S, S+F. This experiment is the most representative of four different experiments.

tion, as demonstrated by the granule content secretion experiments. Under these conditions, the S component could facilitate F-component binding by unmasking a site included in the membrane, as suggested by Noda et al. (12); alternatively, the S component could be a part of the receptor, or the receptor itself, after a conformational modification, hence facilitating the incorporation of the F component into the membrane. Moreover, the results obtained by secretion experiments clearly show that preliminary aggregation of the two components of the toxin is not necessary for insertion of the toxin into the membrane.

Since it has been shown that in PMNs a rise in intracellular free Ca^{2+} is sufficient to cause secretion (3), it seems likely that the secretion observed in this study, which only occurs in the presence of Ca^{2+} (results not shown), is due to an increase in intracellular Ca^{2+} (6) provoked by the formation of the ion-sized pore after the binding of both toxin components. Furthermore, under these conditions, no leakage of intracellular proteins could be observed. Lysozyme liberation was more rapid than hexosaminidase liberation, which confirms the differential control of exocytosis of specific and primary granules (1, 2, 9, 10).

The present study shows that PVL, in addition to toxicity for PMNs, provokes the liberation of the granule content at physiological Ca^{2+} concentrations, which may participate in the necrosis of surrounding tissues in dermic PVL-positive injuries as well as in experimental PVL-induced lesions (5). Moreover, it has been shown that PVL is an inducer of the liberation of inflammation mediators (8).

In conclusion, the S and F components of leukocidin have high affinities for PMN membranes. The binding of the S component (32,000 Da) is an absolute requirement for the subsequent binding of the F component (38,000 Da) and subsequent PMN activation.

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