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Mode of Action of Staphylococcal Leukocidin: Relationship between Binding of ^{125}I-Labeled S and F Components of Leukocidin to Rabbit Polymorphonuclear Leukocytes and Leukocidin Activity

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The binding of ^{125}I-labeled S component to rabbit polymorphonuclear leukocytes was found to be concentration dependent and saturable at 37°C. Scatchard analysis of the binding curve gave a straight line, indicating that S component binds to a single population of sites. The dissociation constant, K_D, derived from the Scatchard plot was 5.57×10^{-9} M, and the number of binding sites per leukocyte was calculated to be approximately 5,300. Unlabeled S component (10^{-8} M) or subunit B of cholera toxin (10^{-7} M) readily competed with ^{125}I-labeled S component binding, and the labeled S component, preincubated with ganglioside G_{M1} at equimolar proportions for 5 min, lost the binding capacity to the leukocyte membranes. The binding number of ^{125}I-labeled F component to leukocidin-sensitive cells, such as rabbit polymorphonuclear leukocytes and the established human myelocytic leukemia cells, in the absence and in the presence of the unlabeled S component (2.1 nM), was calculated to be 50 and 1,300 molecules per cell, respectively. This increased binding of the labeled F component was time and temperature dependent. The binding number of labeled F component to other cell types comparatively insensitive to leukocidin, such as erythrocytes, adipocytes, intestinal cells, and HeLa cells, was calculated to be less than 50 molecules per cell in spite of the sufficient amount of unlabeled S component bound to their cells. These observations are consistent with the view that in rabbit leukocyte the S component, preferentially bound to the cell surface at 5,300 molecules per cell, contributes to enhance the F component binding up to about 1,300 molecules per cell and may thus play a role of synergistic action of both leukocidin components on the cell membranes in the leukocytolysis.

Known to be important in the pathogenicity of certain staphylococcal diseases, staphylococcal leukocidin consists of two protein components designated as F (fast) and S (slow) on the basis of their migration on carboxymethyl-cellulose columns (12, 17, 20, 21). These components are biologically inactive, but act synergistically to induce cytotoxic changes in polymorphonuclear leukocytes and macrophages of rabbits and humans (12, 20, 21). The toxic complex is highly specific, and no other cell type has been found to be susceptible.

In the previous paper (12), we reported that the molecular weights of crystallized F and S components were determined to be 32,000 and 31,000, respectively, and that the isoelectric points of the F and S components were pH 9.08 and 9.39, respectively. Since the primary action of leukocidin is exerted on the leukocyte membranes, in an attempt to elucidate the synergistic action of the F and S components, we studied their fixation and inactivation by some minor components of leukocyte membranes which play a key role in the mode of action of leukocidin. Thus, it has been suggested that phosphatidylcholine and ganglioside G_{M1} in the rabbit leukocyte membranes might be receptors for the F and S components of leukocidin, respectively (13). However, the mechanism by which each component of leukocidin interacts with and disrupts rabbit leukocyte membranes remains in question.

We report here the relationship between the bindings of ^{125}I-labeled S and F components to

either leukocidin-sensitive or -insensitive cells and the synergistic action of both components on cytolysis.

MATERIALS AND METHODS

Materials. Carrier-free [^{125}I]Na (4.23 Ci/μmol) was purchased from Radiochemical Centre, Amersham, England. Ganglioside G_{M1} was kindly supplied by Y. Nagai, Tokyo University. The purified cholera toxin subunit B was a gift from S. Ohtomo, Chemo-Sero-Therapeutic Institute, Kumamoto, Japan. All other chemicals were analytical reagent grade.

Purification and crystallization of the F and S components of leukocidin. The V8 strain of *Staphylococcus aureus* (ATCC 27733) was grown on yeast extract medium (19) with a reciprocating shaker at 37°C for 22 h. The culture filtrate was purified by precipitation with $ZnCl_2$, carboxymethyl-Sephadex C-50 chromatography, Sephadex G-100 gel filtration, and starch zone electrophoresis (12, 13). The F and S components of leukocidin were separated by a column of carboxymethyl-Sephadex C-50 with a linear gradient from 0.2 M to 1.2 M NaCl. The F and S components were crystallized by dialyzing against a saturated ammonium sulfate solution (pH 7.0) at 4°C for 16 h.

Iodination of the F and S components. The F and S components were iodinated with carrier-free ^{125}I by the chloramine-T method of Greenwood et al. (3) as modified by Kato and Watanuki (10). The standard reaction mixture contained 0.8 mCi of [^{125}I]Na, 40 μg of chloramine-T, and 0.5 mg of each component in 0.1 M sodium phosphate buffer (pH 7.4) in a total volume of 0.5 ml. The iodination reaction was allowed to proceed for 1 min at room temperature and was stopped by the addition of 0.1 ml of sodium metabisulfite (120 μg). Then 0.4 ml of 1% KI solution in buffer containing 0.05% bovine serum albumin was added, and each labeled component was separated from iodine and protein aggregates by passage through a Sephadex G-25 column. The column was equilibrated and eluted with 0.1 M phosphate-buffered saline solution (pH 7.6) containing 0.5% gelatin. A 1-ml fraction was collected, from which a 1-μl sample was taken for gamma-counting in a Packard auto-gamma scintillation spectrometer. Iodinated leukocidin retained 98% of its original leukocidin activity for rabbit leukocytes, and both preparations of the F and S components had specific activities between 9×10^5 and 2×10^6 cpm/μg of protein.

Leukocidin activity assay. Polymorphonuclear leukocytes (referred to in this paper as leukocytes) were prepared from rabbit peripheral blood with an isokinetic gradient of Ficoll (15). Leukocidin activity was assayed as described previously (12, 13). To standardize leukocidin activity, the microscopic slide adhesion method (2) was used. The development of the endpoints was determined as the smallest amount of leukocidin causing morphological changes in a standard leukocyte suspension (1×10^6 cells).

^{125}I-labeled leukocidin binding assay. All binding assays were performed with phosphate-buffered saline (0.01 M sodium phosphate buffer [pH 7.2]–0.15 M NaCl–2.7 mM KCl) containing 0.5% gelatin, referred to hereafter as assay buffer. Tubes for all incubations (Falcon Plastics, Oxnard, Calif.) were presoaked with assay buffer. The freshly isolated leukocytes (2×10^6) were suspended in 1 ml of assay buffer and incubated in duplicate with increasing concentrations (0.1 to 10 μg) of ^{125}I-labeled S component or F component. The mixture was incubated at 37°C, and at various times duplicate 0.2-ml samples of the cell suspension were removed and added to 10 ml of assay buffer which had been precooled to 4°C. The cell suspensions were centrifuged for 1 min at $1,000 \times g$, and the cell pellet was suspended in 2 ml of assay buffer after removal of unbound ^{125}I-labeled S component or F component by washing three times with assay buffer. The cells were filtered on a Pyrex microanalysis filter (Millipore Corp.) through a HAWP 25-mm filter pad (pore size, 0.45 μm). The cells were washed twice on the filter with 2-ml samples of assay buffer, the filter was added to a vial, and the radioactivity was assessed in a Packard auto-gamma scintillation spectrometer. Control samples were treated identically except that assay buffer, instead of leukocyte suspension, was added to the original 0.5 ml of leukocidin solution. The small number of counts retained by the filters during the control run were subtracted from the experimental results.

Preparation of cells. Rabbit intestinal epithelial cells were prepared from 30-cm segments taken immediately after anesthesia from the middle of the small intestine of 15-week-old rabbits (11). The gut segments were cut and washed free of visible mucus with Ringer's solution. The cell-rich fluid was poured through nylon mesh. The passed cells were sedimented at $200 \times g$ for 10 min and washed twice with Ringer's solution. More than 90% of the cells were viable, as judged by trypan blue exclusion and phase-contrast microscopy. They were suspended in phosphate-buffered saline (pH 7.0) containing 0.5% gelatin to give cell densities of 2×10^8 per ml.

Adipocytes were prepared by the method of Rodbell (16). Epididymal fat pads were removed from 15-week-old rabbits. Digestion was carried out in Krebs-Ringer phosphate buffer (pH 7.4) containing 4% (wt/vol) bovine serum albumin and 1 mg of bacterial collagenase (B grade, Calbiochem, La Jolla, Calif.) per ml. Following a 1-h digestion at 37°C in a shaking water bath, the cells were filtered through Japanese silk and washed three times with the albumin buffer in a laboratory centrifuge. The adipocyte suspension was made up to a 30% packed cell volume in round-bottom polypropylene tubes with assay buffer. The established HL-60 cells, derived from a patient with acute promyelocytic leukemia, and HeLa cells were grown in Eagle medium supplemented with 10% fetal calf serum in plastic petri dishes as described previously (9). All experiments were carried out 5 days after the initiation of subcultures when the density was approximately 2×10^6 cells per dish. Human and rabbit erythrocytes of peripheral blood were prepared as described previously (18).

RESULTS

Binding of ^{125}I-labeled S component to rabbit leukocytes. The binding of ^{125}I-labeled

S component to rabbit leukocytes occurred very rapidly. Binding equilibrium at between 15°C and 37°C was achieved within 5 min (data not shown). This rapid nature of the binding process is similar to the results obtained from the saturable binding process of cholera toxin to isolated rat fat cells and liver membranes. No discernible difference occurred in the rate or extent of binding of the S component to rabbit leukocytes at 15 compared with 37°C. This is of some importance since it is known that the exposure of leukocytes to the S component for just a few minutes, followed by washing of the cells to remove the free S component, is sufficient to elicit leukocytolysis by the synergistic actions of the S and F components. The binding of the S component was found to be concentration dependent and saturable (Fig. 1A). Scatchard analysis of the binding curve gave a straight line (Fig. 1B), indicating that the S component binds to a single population of the sites. The slope of the line gives the reciprocal of the equilibrium dissociation constant, K_D, and the intercept at the abscissa yields the number of binding sites. The data yield a value of 5.57×10^{-9} M for K_D and 5,300 molecules per leukocyte for the number of binding sites. The equilibrium dissociation constant can also be calculated from the data based on the competition of labeled S component and unlabeled S component for binding (Fig. 2). The addition of unlabeled S component to leukocyte cultures 10 min after the addition of ^{125}I-labeled S component resulted in a reduction of the specific binding by more than 90%, which yielded a dissociation constant, K_D, of 5.01×10^{-9} M derived from the Scatchard plot (data not shown). This result indicates that both the reversibility of the binding and the internalization of bound S component during the binding assay are minimal.

Competitive inhibition of ^{125}I-labeled S component binding by cholera toxin subunit B. Since cholera toxin subunit B binds specifically to cell surface receptor ganglioside G_{M1} (8), the leukocidin activity of the S component was competitively inhibited by cholera toxin subunit B in rabbit leukocytes. The data shown in Table 1 indicate that when cholera toxin subunit B was preincubated with rabbit leukocytes, the binding of ^{125}I-labeled S component was decreased by more than 95% by the competitive inhibition compared with the binding of the labeled S component to untreated cells. Although the cholera toxin subunit B bound to ganglioside G_{M1} had no influence on the binding of the labeled S component to the leukocytes, the binding capacity of ^{125}I-labeled S component pretreated with ganglioside G_{M1} was decreased by more than 95% compared with the labeled S component binding, and no leukocytolysis was induced by the labeled S component bound to ganglioside G_{M1} and the F component (Table 1).

Binding of ^{125}I-labeled F component to the rabbit leukocytes. The binding of the labeled F component was also characterized to

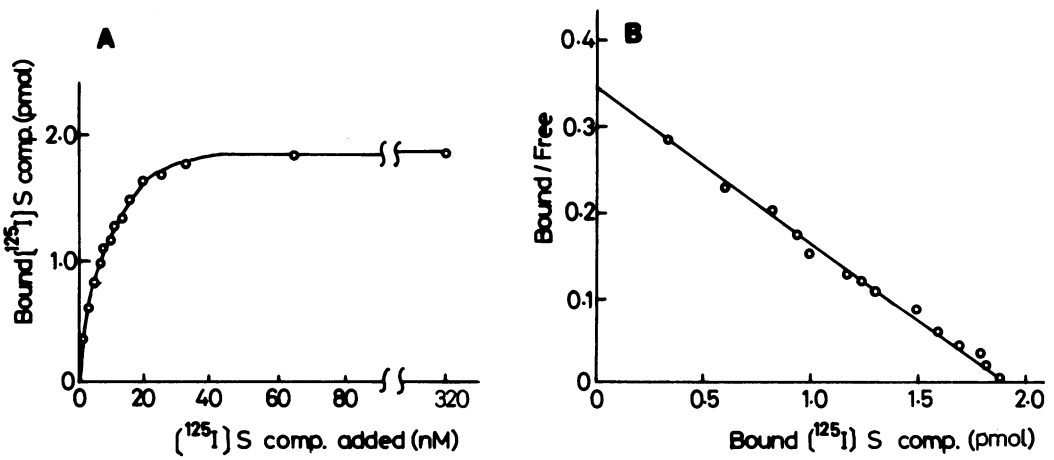

FIG. 1. *Binding of ^{125}I-labeled S component to rabbit leukocytes. (A) Duplicate microcentrifuge tubes containing 2.0×10^8 cells in 1.0 ml of assay buffer were treated with increasing amounts of ^{125}I-labeled S component. All tubes were incubated with gentle shaking for 10 min at 37°C. Counts associated with cells were determined by pelleting the cells, washing them three times, and transferring them to clear tubes for separate counting of pellets and supernatants in an auto-gamma scintillation spectrometer. Symbols show the mean of duplicate tubes. (B) Scatchard plot of binding data from (A). These data were used to calculate the apparent dissociation constant (K_D) and binding site densities.*

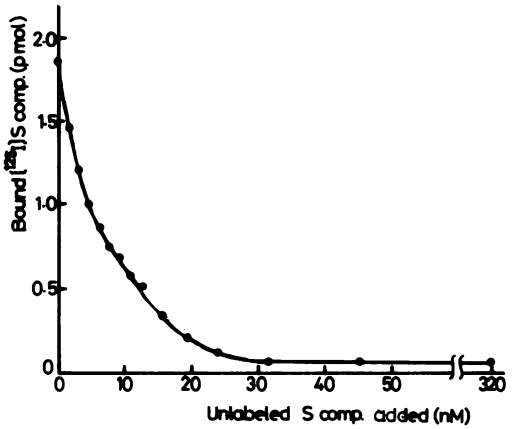

FIG. 2. *Effect of unlabeled S component on the binding of ^{125}I-labeled S component to rabbit leukocytes. Indicated quantities of unlabeled S component were added to duplicate polyethylene microcentrifuge tubes containing 1.0 ml of assay buffer and 2.0 × 10^8 cells and incubated at 37°C with gentle shaking for 10 min followed by the addition of 10 μg of ^{125}I-labeled S component to each tube. The cells were incubated for another 10 min at 37°C with gentle shaking and were then pelleted and washed three times and transferred to clear tubes. Pellets and supernatants were counted separately in an auto-gamma scintillation spectrometer.*

TABLE 1. *Inhibitory effect of ganglioside G_{M1} and cholera toxin subunit B on leukocidin activity and the binding of ^{125}I-labeled S component to leukocytes*[a]

Preincubation for 5 min with:	Addition at zero time of:	Leukocidin activity (%)	Binding of [^{125}I]S component (%)
[^{125}I]S	F	100	100
[^{125}I]S + G_{M1}	F	<5	<5
B	F + [^{125}I]S	<5	<5
B + G_{M1}	F + [^{125}I]S	94	96

[a] [^{125}I]S, ^{125}I-labeled S component (1.0 × 10^{-6} M); F, unlabeled F component (1.0 × 10^{-6} M); G_{M1}, ganglioside G_{M1} (1.5 × 10^{-6} M); B, cholera toxin subunit B (1.2 × 10^{-6} M). Rabbit leukocytes (2 × 10^8) were incubated in 1 ml of assay buffer.

aid in the interpretation of the synergistic effects of leukocidin on the lysis of the leukocytes. The binding properties of the F component to rabbit leukocytes were studied by the same procedure as was ^{125}I-labeled S component binding to the cells at 37°C. The number of binding molecules of the F component per rabbit leukocyte was calculated to be less than 50. The experiments in which it was tested under the same conditions as in Fig. 1A showed that the binding number of labeled F component was increased to approximately 1,300 molecules per cell and that leukocytolysis was induced by the synergistic actions of both leukocidin components (Fig. 3). Figure 4 shows that the binding reaction of ^{125}I-labeled F component was temperature and time dependent, indicating that metabolic events probably influence the extent of the binding.

Comparison of the binding of ^{125}I-labeled S and F components to various cells. For comparison of the binding of S and F components to various cells, either leukocidin-sensitive or -insensitive cells were used. The leukocidin-sensitive cells used were rabbit and human leukocytes and established human myelocytic leukemia cells, whereas rabbit and human erythrocytes, rabbit intestinal cells, adipocytes, and HeLa cells were used as the leukocidin-insensitive cells (Table 2). The binding number of ^{125}I-labeled F component to these cells was calculated to be less than 50 molecules per cell. The binding amount of ^{125}I-labeled S component to leukocidin-insensitive cells was greater than that to leukocidin-sensitive cells, but in rabbit and human erythrocytes there was an extraordinarily small number of binding sites of the S component, as was true also of binding sites of cholera toxin subunit B (1). The binding of ^{125}I-labeled F component to the leukocidin-sensitive cells was increased by the addition of unlabeled S component, but no increase occurred in the number of binding sites of the labeled F com-

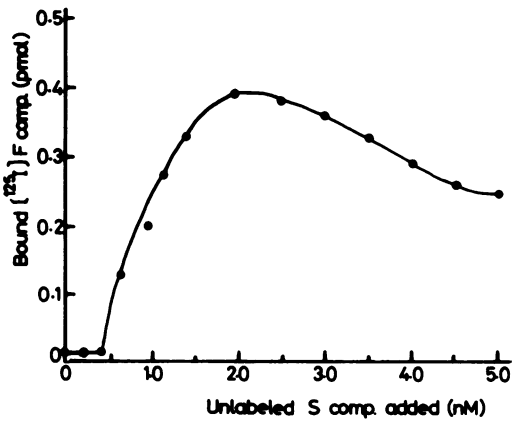

FIG. 3. *Dependence of increase of ^{125}I-labeled F component binding to rabbit leukocytes upon the unlabeled S component concentration. Indicated quantities of unlabeled S component were added to duplicate tubes containing 2.0 × 10^8 cells in 1.0 ml of assay buffer and 10 μg of ^{125}I-labeled F component. The tubes were incubated for 10 min at 37°C with gentle shaking. The cells were then pelleted and washed three times and counted as described in the legend to Fig. 1.*

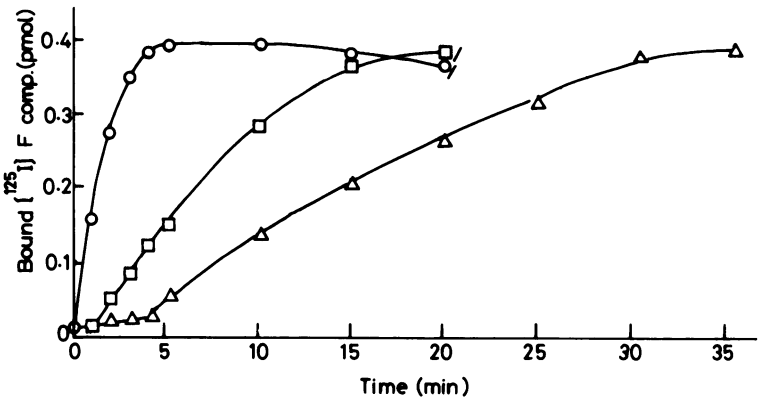

FIG. 4. *Temperature dependence of ^{125}I-labeled F component binding to rabbit leukocytes. Duplicate tubes containing 2.0 × 10^8 cells in 1.0 ml of assay buffer and 10 μg of ^{125}I-labeled F component with 65 ng of unlabeled S component were incubated for the indicated times at 37°C (○), 22°C (□), and 15°C (△).*

TABLE 2. *Comparison of the binding of ^{125}I-labeled S and F components to various cells*[a]

Cell	Bound [^{125}I]S component (cpm)	Bound [^{125}I]F component (cpm)	
		Without unlabeled S component	With unlabeled S component
Rabbit leukocyte	44,000	<50	1,040
Human leukocyte	50,600	<50	1,200
HL-60[b]	40,000	<50	1,100
Rabbit erythrocyte	<50	<50	<50
Human erythrocyte	<50	<50	<50
Rabbit intestinal cell	249,100	<50	<50
Adipocyte	160,200	<50	<50
HeLa cell	113,700	<50	<50

[a] The various cells (2 × 10^8) were incubated in duplicate with 10 μg of labeled F or S component in 1 ml of assay buffer as described in the text.

[b] HL-60, Human myelocytic leukemia cell.

ponent to the leukocidin-insensitive cells in the presence of the S component (Table 2).

DISCUSSION

It has been known that the primary action of staphylococcal leukocidin is exerted on the perturbation of leukocyte membranes. The previous studies (13) demonstrated the fixation and inactivation of the F and S components of leukocidin by the 2-position of the fatty acid moiety of 1,2-diacyl phosphatidylcholine and ganglioside G_{M1}, respectively. However, the order of binding priority of the F and S components of leukocidin to rabbit leukocyte membrane and the mode of action of both components on the cell membrane perturbation are still not clear.

We have shown in the present study that the binding of ^{125}I-labeled S component to rabbit leukocytes yields a straight line as derived by Scatchard plot and is inhibited competitively by subunit B of cholera toxin. These findings suggest that the binding of the S component appears to involve one type of site, presumably ganglioside G_{M1}, without cooperative interaction. Although the different numbers of binding sites of the S component molecules to the various cells were found to correspond to the marked difference in the content of ganglioside G_{M1} among the species (Table 2), the number of binding sites of the S component calculated in the present work for the various cells, such as leukocytes, intestinal cells, adipocytes, and erythrocytes, was of the same order of magnitude as that of labeled cholera toxin obtained for the various cells (1, 6, 7; Table 2).

The number of binding sites of labeled F component calculated for rabbit leukocytes (less than 50 sites per cell) was enhanced by the addition of unlabeled S component until the onset of the destruction of leukocyte membranes (about 1,300 sites per cell), and this binding reaction was time and temperature dependent (Fig. 3 and 4). However, no morphological change of leukocidin-insensitive cells occurred within the time scale of observation because the binding number of the F component to the cells was small (less than 50 molecules per cell) even in the presence of the S component (Table 2). The phosphatidylcholine is mainly located on the exterior side of the cell membranes with the polar head of the phosphorylcholine moiety on the outer surface (4). Since the F component is preferentially directed towards the fatty acid ester bond at the 2-position of phosphatidylcholine (13), the F component itself can hardly bind to the fatty acid moiety of phosphatidylcholine in the intact leukocyte membranes (less than 50 molecules per cell). We previously reported that

pretreatment of rabbit leukocytes with the S component results in rapid lysis of the leukocytes after the addition of the F component. Furthermore, the present work showed that the S component leads to an increase of the binding sites of the F component to the leukocidin-sensitive cell membranes to induce synergistically cytotoxic changes of the cells, whereas the binding of the S component to the leukocidin-insensitive cells is unable to enhance the binding sites of the F component to their cells (Table 2). We recently found that the S component had a potent ability for rabbit leukocyte chemotaxis, as well as that of a chemoattractant, *N*-formylmethionyl-leucyl-phenylalanine. Moreover, in a brief communication we reported that the S component activates rabbit-leukocyte-membrane-associated phospholipase A_2, which catalyzes the hydrolysis of the fatty acid ester bonds at the 2-position of the 1,2-diacyl phosphatidylcholine bilayer in cell membranes (5, 14). These findings suggest that when the S component preferentially binds to the ganglioside G_{M1} in the leukocyte membranes before the F component is bound, the F component may be capable of binding to the products of the split fatty acid ester bonds from phosphatidylcholine by the activation of phospholipase A_2 in the membranes. Further studies of the binding characteristics and synergistic action of the S and F components of leukocidin on rabbit leukocyte membranes are in progress. Detailed data of the stimulation of phospholipase A_2 activity by the S component will be reported elsewhere.

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