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An Early Effect of the S Component of Staphlylococcal Leukocidin on Methylation of Phospholipid in Various Leukocytes

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On incubation of rabbit polymorphonuclear leukocytes with the S component of staphylococcal leukocidin at 37°C, the ^{3}H-labeled methyl group of *S*-adenosyl[*methyl*-^{3}H]methionine was rapidly incorporated into phospholipid. Subsequently, the methylated phosphatidylcholine was degraded by activated phospholipase A_2. Complete blockage of the methylation of phospholipid by a mixture of erythro-9-[2-hydroxy-3-nonyl]adenine, adenosine, and L-homocysteine thiolactone markedly inhibited the activation of phospholipase A_2 by the S component. It also inhibited the binding of ^{125}I-labeled F component to the cells, but not that of the labeled S component. These results suggest that methylation of phospholipid in the cell membranes by the S component results in activation of phospholipase A_2, which induces the binding of the F component to the cells.

Staphylococcal leukocidin consists of two protein components (S and F) that act synergistically to induce cytotoxic changes in human and rabbit polymorphonuclear leukocytes. Previously (7, 10, 12, 14) we reported that the S and F components of leukocidin were preferentially bound and inactivated by G_{M1} ganglioside and phosphatidylcholine, respectively, in rabbit polymorphonuclear leukocyte membranes. The specific binding of the S component to G_{M1} ganglioside induces activation of cell membrane-associated phospholipase A_2, resulting in the release of prostaglandins and leukotrienes from the cells and then in enhancement of chemotaxis of polymorphonuclear leukocytes (11, 13). Furthermore, we found that there was no correlation between the susceptibility of cells to leukocidin and the number of binding sites for the S component in various cells (8, 9).

A receptor on the cell surface is defined as a molecule the specific binding of whose ligand transmits a signal that affects functions of the cell membrane, such as the activities of phospholipid methyltransferases, phospholipase A_2, and phospholipase C. Methylation of phospholipid is involved in one of the two pathways of phosphatidylcholine formation. In this pathway a methyl group is transferred enzymatically to phosphatidylethanolamine from the donor *S*-adenosylmethionine and then phosphatidylcholine is formed via mono- and dimethylated intermediates. Although the exact significance of methylation of phospholipid on the cell surface of leukocidin-sensitive cells is not yet clear, we report here that the S component of leukocidin induces rapid methylation of phospholipid that leads to an activation of phospholipase A_2 and that the susceptibilities of various cells to leukocidin may depend on the activation of both methyltransferases and phospholipase A_2 by the S component of staphylococcal leukocidin.

MATERIALS AND METHODS

Chemicals. Erythro-9-[2-hydroxy-3-nonyl]adenine was purchased from Burroughs Wellcome Co. (Research Triangle Park, N.C.). Purified cholera toxin was obtained from the Chemo-Sero-Therapeutic Institute (Kumamoto, Japan). *S*-Adenosyl[*methyl*-^{3}H]methionine (0.446 μCi/μl) was purchased from New England Nuclear Corp. (Boston, Mass.). Carrier-free Na^{125}I (4.23 Ci/μmol) was obtained from the Radiochemical Centre (Amersham, England). All other chemicals were of analytical grade.

Staphylococcal leukocidin. The S and F components of leukocidin were purified and crystallized as described previously (7).

Binding of ^{125}I-labeled leukocidin. The S and F components of leukocidin were iodinated with carrier-free ^{125}I by the chloramine-T method of Greenwood et al. (1) with a slight modification as described previously (5, 8). Preparations of the labeled S and F components had specific activities of 9×10^5 to 2×10^6 cpm/μg of protein. Binding of ^{125}I-labeled leukocidin to polymorphonuclear leukocytes was assayed as described previously (12).

Assay of phospholipid methylation. Polymorphonuclear leukocytes from humans and rabbits were purified by Ficoll-Hypaque density centrifugation (15). For examination of phospholipid methylation, 0.2 ml of the cell suspension (5×10^7 cells per ml of 0.01 M Tris hydrochloride-buffered saline, pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) was transferred to a test tube and the reaction was initiated by 4.5 μl of *S*-adenosyl[*methyl*-^{3}H]methionine (0.446 μCi/μl) at 37°C. The reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid containing 10 mM methionine. The mixture was centrifuged at 10,000 × *g* for 10 min, and the precipitate was washed with 0.5 ml of 10% trichloroacetic acid and then extracted with 3 ml of a mixture of chloroform and methanol (2:1, vol/vol). The extract was concentrated to 100 μl under N_2, spotted on Silica Gel 60 plates, and subjected to thin-layer chromatography in the upper phase of a mixture of chloroform, methanol, and water (70:30:5, vol/vol/vol). Radioactive peaks were scanned with a Berthold-Dünnschichit Scanner II, and regions of the plates corresponding to standard materials were scraped into scintillation vials containing 5 ml of Aquasol II (New England Nuclear Corp.) and counted in a liquid scintillation spectrometer. All experiments were carried out in triplicate.

Assay of phospholipase A_2 activity. Phospholipase A_2 was assayed by measuring the amount of radioactive lysophosphatidylcholine liberated from leukocytes (10^7 cells) labeled with L-α-[dipalmitoyl-1-^{14}C]phosphatidylcholine or liberated from cells (10^7) incubated with 4.5 μl of *S*-adenosyl[*methyl*-^{3}H]methionine (0.446 μCi/μl).

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RESULTS

Stimulation of phospholipid methylation by the S component. The time course of incorporation of the ^{3}H-labeled methyl group of *S*-adenosyl[*methyl*-^{3}H]methionine into phospholipid of polymorphonuclear leukocytes was examined. When polymorphonuclear leukocytes from rabbit peripheral blood were incubated with 5 ng of the S component, which was the minimum dose required for complete cytolysis of leukocytes (10^7 cells per 200 μl) in the presence of excess F component, rapid incorporation of the ^{3}H-labeled methyl group into their phospholipids was observed in the first 10 s. The amount of ^{3}H-labeled methyl group incorporated was about three times that in the control. The incorporation was maximal 15 s after addition of the S components (Fig. 1a). No increase in transmethylated nucleotides or proteins was observed under the same conditions. A concentration of the S component (0.5 ng) that caused destruction of <5% of the rabbit polymorphonuclear leukocytes (10^7 cells per 200 μl) in the presence of excess F component did not increase the amount of methylated phospholipids. These observations suggested that methylation of phospholipid depended on the concentration of the S component. Since G_{M1} ganglioside is also known to be a receptor of cholera toxin, the effect of cholera toxin on methylation of phospholipid was examined. No increase in methylated phospholipid was observed when rabbit polymorphonuclear leukocytes were incubated with 2 ng to 2 μg of cholera toxin (Fig. 1a). These results suggest that an increase in methylation of phospholipid was not induced by all molecules that bind to G_{M1} ganglioside, but was a specific effect of the S component of staphylococcal leukocidin.

TABLE 1. Inhibitory effects of EHNA, adenosine, and L-homocysteine thiolactone on methylation of phospholipid and phospholipase A_2 activation by the S component and destruction of leukocytes by leukocidin

Length of treatment (min)[a]	Phosphatidylcholine methylated by S component (%)[b]	Phospholipase A_2 activation by S component (%)[c]	Leukocyte destruction by leukocidin (%)[d]
0	100	100	100
5	91.7[e]	95.4	96.5
10	57.2	58.9	59.6
15	8.5	8.8	7.0
20	8.9	9.1	7.2

[a] Rabbit leukocytes (10^7 cells per 200 μl) were pretreated with EHNA (10 μM), adenosine (1 mM), and L-homocysteine thiolactone (0.1 mM).

[b] Phosphatidylcholine methylated by the S component (5 ng) in untreated leukocytes (10^7 cells per 200 μl) is defined as 100%. The total volume of the reaction was 205 μl.

[c] Activation of phospholipase A_2 by the S component (5 ng) in untreated leukocytes is defined as 100%. Rabbit leukocytes (10^7 cells per 200 μl) labeled with L-α-[dipalmitoyl-1-^{14}C]phosphatidylcholine were incubated with S component (5 ng) at 37°C for 60 s, and [palmitoyl-1-^{14}C]lysophosphatidylcholine liberated from the cells was measured as described previously (11). The total volume of the reaction mixture was 205 μl.

[d] Destruction of untreated leukocytes by leukocidin (S and F components) is defined as 100%. Rabbit leukocytes (10^7 cells per 200 μl) were incubated with excess F component (10^{-6} M) and S component (5 ng) at 37°C for 10 min. The total volume of the reaction mixture was 210 μl.

[e] Values are means of six determinations.

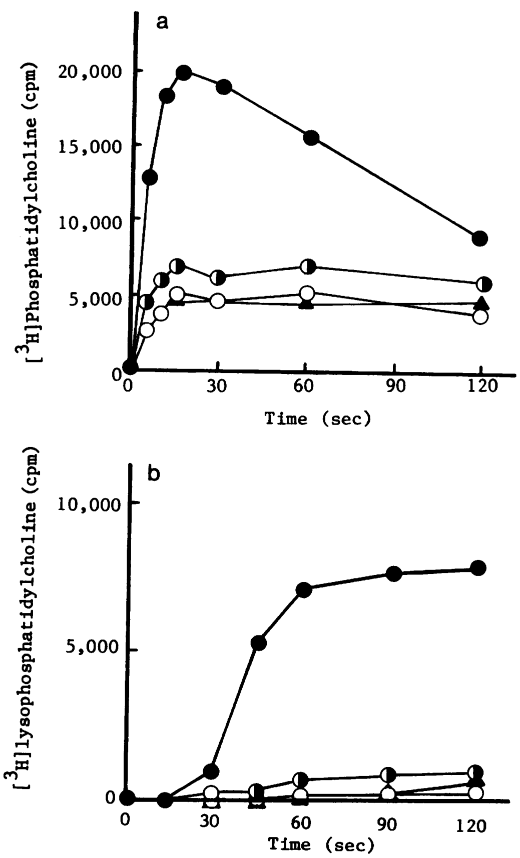

FIG. 1. Stimulatory effect of the S component on methylation of phospholipid and activation of leukocyte membrane-associated phospholipase A_2. Methylated phosphatidylcholine of rabbit polymorphonuclear leukocytes (a) and phospholipase A_2 activation (b) were assayed as described in the text. S component or cholera toxin was added at zero time with *S*-adenosyl[*methyl*-^{3}H]methionine. Symbols: ○, control; ●, 5 ng of S component; ◑, 0.5 ng of S component; ▲, 2 μg of cholera toxin.

Concomitant activation of phospholipase A_2 by the S component. When 5 ng of the S component was added to leukocidin-sensitive rabbit polymorphonuclear leukocytes (10^7 cells per 200 μl), a significant accumulation of [^{3}H]lysophosphatidylcholine was observed (Fig. 1b). The accumulation was observed 30 to 60 s after the addition of *S*-adenosyl[*methyl*-^{3}H]methionine and reached a plateau after 60 s. The time of this accumulation corresponded to that of the decrease in [^{3}H]phosphatidylcholine shown in Fig. 1. Addition of the S component (0.5 ng) or cholera toxin (2 ng to 2 μg) did not cause accumulation of [^{3}H]lysophosphatidylcholine (Fig. 1b). Since the S component activates phospholipase A_2 in the membrane of rabbit polymorphonuclear leukocytes, the peak of the release of [^{14}C]arachidonic acid and its metabolites from prelabeled phospholipids in the polymorphonuclear leukocyte membranes was observed in the first 75 to 90 s (data not shown). These results indicate that the decrease in [^{3}H]phosphatidylcholine and the increase in [^{3}H]lysophosphatidylcholine are induced by activation of phospholipase A_2.

Inhibition of phospholipid methylation reduced both activation of phospholipase A_2 and leukocytolysis. In a further examination of the relationship of phospholipid methylation, activation of phospholipase A_2, and leukocytolysis, we found that the methylation of phospholipid in rabbit polymorphonuclear leukocytes by the S component was inhibited by pretreating the leukocytes with various competitive inhibitors of *S*-adenosyl-L-methionine, such as erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA), adenosine, and L-

TABLE 2. Effects of treatment of various leukocytes with EHNA, adenosine, and L-homocysteine thiolactone on binding of ^{125}I-labeled S and F components of leukocidin[a]

Cells	Treatment[b]	Bound ^{125}I-S component (molecules per cell)	Bound ^{125}I-F component (molecules per cell)	
			Without S component	With S component[c]
Human leukocytes	−	4,900	<50	1,200
	+	4,700	<50	<50
Rabbit leukocytes	−	5,300	<50	1,300
	+	5,200	<50	<50
Rat leukemia	−	6,200	<50	1,400
DBLA-6 (DV) cells	+	5,900	<50	<50
Rat leukocytes	−	6,800	<50	1,300
	+	6,600	<50	<50
Mouse leukemia	−	4,100	<50	1,400
C1498 cells	+	4,000	<50	<50
Mouse leukocytes	−	8,200	<50	1,300
	+	7,800	<50	<50

[a] Various cells (10^7/200 μl) were incubated in duplicate with labeled S or F component (10^{-6} M) for 10 min, and bound components were assayed as described previously (12).

[b] Leukocytes were incubated in the presence of EHNA (10 μM), adenine (1 mM), and L-homocysteine thiolactone (0.1 mM) for 15 min at 37°C.

[c] The optimal amount of S component determined in Fig. 2 was present in each experiment.

homocysteine thiolactone. The degree of inhibition of phospholipid methylation depended on the period of preincubation with these compounds before exposure of the leukocytes to the S component (Table 1). A clear correlation was found among the inhibition of phospholipid methylation, activation of phospholipase A_2, and leukocytolysis. Inhibition of the methylation of phospholipid was maximal on incubation of the leukocytes with 10 μg of EHNA, 1.0 mM adenosine, and 0.1 mM L-homocysteine thiolactone for 15 min at 37°C. No inhibition of other transmethylations, such as the methylations of nucleotide and protein, was observed under these conditions.

Inhibition of phospholipid methylation reduced binding of the F component to leukocytes. Binding of the S and F components to leukocidin-sensitive cells, such as human polymorphonuclear leukocytes, rabbit polymorphonuclear leukocytes, rat leukemia DBLA-6(DV) cells, rat polymorphonuclear leukocytes, mouse leukemia C1498 cells, and mouse polymorphonuclear leukocytes, was examined and the results are shown in Table 2. The numbers of molecules of ^{125}I-labeled S component bound to these cells were calculated to be 4,900, 5,300, 6,200, 6,800, 4,100, and 8,200 per cell, respectively (Table 2). Pretreatment of these cells with competitive inhibitors of S-adenosyl-L-methionine for 15 min at 37°C did not appreciably affect the numbers of molecules of ^{125}I-labeled S component bound to these cells (Table 2). On the other hand, binding of the F component to leukocytes was reduced by inhibition of phospholipid methylation. Binding of the ^{125}I-labeled F component to rabbit polymorphonuclear leukocytes depended on the presence of the S component (5). No significant binding of the ^{125}I-labeled F component was observed in the absence of unlabeled S component, but when an appropriate amount of the S component was present, maximum binding of the ^{125}I-labeled F component (1,200 to 1,400 molecules per cell) to the leukocytes was observed (Table 2). The optimal amounts of the S component for maximum binding of the ^{125}I-labeled F component to human polymophonuclear leukocytes, rabbit polymorphonueclear leukocytes, rat leukemia DBLA-6(DV) cells, rat polymorphonuclear leukocytes, mouse leukemia C1498 cells, and mouse polymorphonuclear leukocytes were 0.3, 3.0, 400, 700, 1,000, and 3,000 ng, respectively (Fig. 2). In the presence of optimal amounts of the S component, 1,200 to 1,400 molecules of ^{125}I-labeled F component bound to these cells (Table 2). Treatment of all of these leukocytes with competitive inhibitors of S-adenosyl-L-methionine reduced the amount of bound ^{125}I-labeled F component to less than 50 molecules per cell (Table 2). Under these conditions, methylation of phospholipid and activation of phospholipase A_2 were also significantly inhibited (data not shown), as in the case of rabbit polymorphonuclear leukocytes (Table 1).

Relationship between phospholipid methylation by the S component and susceptibility of leukocytes to leukocidin. In further examination of the relationship between phospholipid methylation in the cells by the S component and cellular destruction by leukocidin, we measured the minimal doses of the S component required for complete destruction of cells, methylation of phospholipid, and activation of phospholipase A_2 in various cells. There was a significant correlation of the minimal doses of the S component required for these three actions in the cells (Table 3).

DISCUSSION

In previous papers (11–13), we reported that the binding of the S component to G_{M1} ganglioside on rabbit polymorphonuclear leukocyte membranes activated membrane-associated phospholipase A_2, resulting in an increase in the number of molecules of the F component bound to the cells. Inhibition of phospholipase A_2 by indomethacin had no effect on the number of molecules of the S component bound to G_{M1} ganglioside on rabbit polymorphonuclear leukocyte

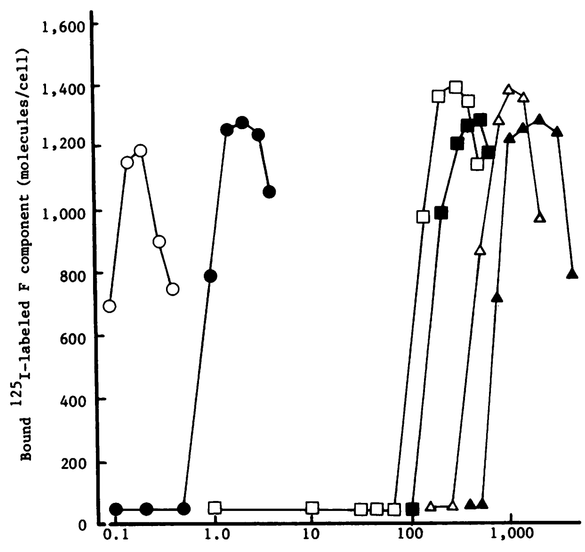

FIG. 2. Dependence of binding of ^{125}I-labeled F component to various cells on concentration of unlabeled S component. The indicated amounts of unlabeled S component (5 μl) were added to a mixture of the cells (10^7/200 μl) and ^{125}I-labeled F component (10^{-6} M). Bound components were assayed as described previously (12). Values are means of duplicate determinations. Symbols: ○, human polymorphonuclear leukocytes; ●, rabbit poymorphonuclear leukocytes; □, rat leukemia DBLA-6(DV) cells; ■, rat polymorphonuclear leukocytes; △, mouse leukemia C1498 cells; ▲, mouse polymorphonuclear leukocytes.

TABLE 3. Comparison of minimum concentrations of leukocidin and S component for cell destruction, methylation of phospholipid, and activation of phospholipase A_2 in various leukocytes

Cells	Minimum concn of S component (ng) required for:		
	Complete cell destruction[a]	Methylated phosphatidylcholine[b]	Activation of phospholipase A_2[c]
Human leukocytes	0.5 ± 0.1[d]	0.4 ± 0.1	0.5 ± 0.1
Rabbit leukocytes	5.0 ± 0.5	5.0 ± 0.5	5.0 ± 0.5
Rat leukemia DBLA-6 (DV) cells	750 ± 50	700 ± 50	750 ± 50
Rat leukocytes	1,250 ± 100	1,000 ± 100	1,200 ± 100
Mouse leukemia C1498 cells	2,500 ± 200	2,000 ± 200	2,200 ± 200
Mouse leukocytes	5,000 ± 500	4,200 ± 500	4,400 ± 500

[a] Various cells (10^7/200 μl) were incubated at 37°C for 10 min in the presence of excess F component (10^{-6} M) and various amounts of S component (5 μl). The total volume of the reaction mixture was 210 μl.

[b] Minimum amount of S component needed to cause incorporation of 20,000 cpm of the [^{3}H]methyl group from *S*-adenosyl[*methyl*-^{3}H]methionine into phosphatidylcholine. The cells (10^7/200 μl) were incubated at 37°C for 15 s in the presence of *S*-adenosyl[*methyl*-^{3}H]methionine (2 μCi/4.5 μl) and various amounts of S component (5 μl). The total volume of the reaction mixture was 209.5 μl.

[c] Minimum amount of S component needed to produce 7,500 cpm of [^{3}H]lysophosphatidylcholine. The cells (10^7/200 μl) were incubated at 37°C for 60 s in the presence of *S*-adenosyl[*methyl*-^{3}H]methionine (2 μCi/4.5 μl) and various amounts of S component (5 μl). The total volume of the reaction mixture was 209.5 μl.

[d] Values are means ± standard deviations for six determinations.

membranes, but markedly reduced the number of molecules of the F component bound to membrane phosphatidylcholines. These finding suggest that activation of phospholipase A_2 by the S component is an essential reaction for cytolysis of leukocytes by leukocidin. The S component rapidly stimulated phospholipid methyltransferases that catalyze the three-step methylation of phosphatidylethanolamine to phosphatidylcholine (Fig. 1) (2, 3). In leukocidin-sensitive cells, the phosphatidylcholine formed by the methylation was subsequently degraded by phospholipase A_2. This transmethylation is involved in linking receptor-mediated signals to cellular responses, since experiments showed that rapid methylation of phospholipid by the S component led to activation of phospholipase A_2 and that some of the processes could be blocked by methyltransferase inhibitors, such as EHNA, adenosine, and L-homocysteine thiolactone. These observations suggest that phospholipid methylation might be the primary step in activation of phospholipase A_2 by the S component of staphylococcal leukocidin, which leads to an increase in binding sites for the F component that induces leukocytolysis.

The receptor for cholera toxin is also G_{M1} ganglioside (4, 6) but cholera toxin did not stimulate methyltransferases or activate phospholipase A_2. This finding suggests that the increase in methylation of phospholipid is not a general effect of molecules that bind to G_{M1} ganglioside.

The maximal binding of the F component to leukocidin-sensitive cells was approximately 1,300 molecules per cell in the presence of an appropriate amount of the S component. With all cells examined, the binding of the F component was dependent on the amount of S component. These results suggest that the susceptibility of various cells to leukocidin depends on reactions induced by the S component, such as activations of methyltransferases and phospholipase A_2, but not on reactions induced by binding of the F component.

In cells that were highly sensitive to leukocidin, methyltransferases were activated by a low concentration of the S component, but in cells that were rather insensitive to leukocidin their activation required much larger amounts of the S component. These findings suggest that the degree of susceptibility of various cells to leukocidin depends on the primary effect of the S component in stimulating methylation of phospholipid in the cell membrane.

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