INHIBITION OF BACTERIAL WALL LYSINS BY LIPOTEICHOIC ACIDS AND RELATED COMPOUNDS

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SUMMARY - Lipoteichoic acid (LTA) from several Gram-positive microorganisms, Forssman antigen (Fag) from Diplococcus pneumoniae R36A, and an acidic lipopolysaccharide (ALP) from Micrococcus luteus were examined for effects on four wall lysis systems. The LTAs inhibited the N-acetylmuramidases of Streptococcus faecalis and Lactobacillus acidophilus, and the amidase of Bacillus subtilis 168. Deacylated LTA failed to inhibit. LTAs failed to inhibit the amidase of pneumococcus. Fag inhibited the pneumococcal amidase, but had no effect on the other three systems.

Bacterial autolytic enzymes have been shown to be important both in cell division (1,2) and in the bacteriolytic (killing) action of penicillins and other wall inhibitory antibiotics (3). A search for the mechanism of cellular control of these enzymes lead to the detection of an autolysin inhibitory factor in S. faecalis (2). More recently, a powerful inhibitor of the pneumococcal autolysin (an N-acetylmuramyl-L-alanine amidase E.C.3.5.1.28) was identified as the choline-containing Forssman antigen (Fag: "lipoteichoic acid") of this bacterium (5). Also, Fag added to the medium of growing pneumococci, was found to protect cells against lysis by penicillin and vancomycin (5). These observations have considerable importance concerning the mechanism of action of bacteriolytic antibiotics. It also has been suggested (5) that lipoteichoic acids may play a physiological role in regulating autolysin activity.

We now report the results of experiments designed to test the possible general validity of the pneumococcal findings for other bacterial systems.

Fag is an amphipathic substance which contains structure bound lipids, is localized in the plasma membrane, and seems to have a primary structure analogous to that of the choline and ribitol containing wall teichoic acid of this

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Abbreviations: LTA = lipoteichoic acid; Fag = Forssman antigen; ALP - acidic lipopolysaccharide; SDS = sodium dodecyl sulphate

species (6,7). There are similarities in chemistry and cellular location between Fag and other amphipathic compounds found in other species of Gram-positive bacteria, notably lipoteichoic acids (LTA: 8,9) and the acidic lipopolysaccharide (ALP) of M. luteus (10,11). Conventional LTAs contain a linear chain of 25-30 glycerol phosphate residues covalently linked to a glycolipid, and are widely distributed as membrane associated components in Gram-positive bacteria (8,9). Instead of an LTA, M. luteus (lysodeikticus) has been shown to possess membrane associated ALP, which has been recently characterized as a succinylated mannan (10,11). We examined a variety of these amphipathic compounds, obtained from several sources, as potential inhibitors of 4 different, homologous and heterologous, bacterial autolytic systems. The results reported show that each of the autolytic systems can be inhibited by one or more but not all of the compounds tested, and are consistent with both the general occurrence of such inhibitory activities and a type of specificity for each system.

RESULTS AND DISCUSSION - Lysis of cell walls by the N-acetylmuramide glycan hydrolases of S. faecalis ATCC 9790 and L. acidophilus strain 63AM Gasser (12,13) was inhibited by low concentrations of LTAs (Table 1). Low concentrations of five different LTAs, isolated and purified from 4 different bacterial species (L. casei NIRD R094, L. fermentum NCTC 6991, S. lactis ATCC 9936, S. faecalis 39 and NCIB 8191) inhibited the S. faecalis system (Fig. 1). Irrespective of the degree of, or chemical nature of glycosyl substitution of the glycerol residues of the LTA (8), dose responses to all 3 streptococcal LTAs were indistinguishable when compared on the basis of ηmoles of LTA/mg wall (Fig. 1). Estimates of cellular content of LTA at 1 to 2% of bacterial dry weight (9), and of cell wall at 25 to 40% of bacterial dry weight (14) indicate that S. faecalis contains about 3 to 5 ηmoles of LTA/mg wall. Thus the LTA concentrations used to inhibit muramidase activity are well within amounts found in cells.

LTAs of two lactobacillus species also inhibited lysis of S. faecalis walls (Fig. 1). For these 2 LTAs only concentrations higher than 0.2 ηmoles/mg in-

TABLE 1

Effect of LTAs, Fag, and ALP on the Wall Lytic Systems of *S. faecalis* and *L. acidophilus*

	µg/ml	nmoles LTA per mg wall[2]	% of control lysis rate
A. On Lysis of Walls of S. faecalis ATCC 9790[1]			
LTA of *S. faecalis* 39 (Exp. 1)	0.7	0.1	86
	2.1	0.4	54
(Exp. 2)	4.5	0.7	31
	9.0	1.3	25
Deacylated[3] LTA of *S. faecalis* 39	24.3	5.2	100
Fag of pneumococcus[4]	25.3	-	139[5]
ALP of *M luteus*[6]	3.3	-	106
	6.7	-	101
	16.7	-	70
	34.2	-	101
B. On Lysis of Walls of L. acidophilus[7]			
LTA of *L. fermentum* NCTC 6991	4.6	0.9	99
	9.2	1.8	71
	23.0	4.6	9
LTA of *S. faecalis* 39	1.8	0.3	83
	3.6	0.5	48
	7.2	1.0	13
Deacylated[3] LTA of *S. faecalis* 39	24.3	3.6	82
Fag antigen of pneumococcus[4]	13.3	-	98

[1]The assay system as previously described (12) consisted of 1.3 to 2.0 mg dry weight of wall-enzyme complex from exponential phase cells in 3.0 ml of 0.01 M sodium phosphate, pH 6.8. The rate of wall lysis in control cultures was 0.40 to 0.52 hrs^{-1} determined from the slope of the first order reaction.

[2]Concentrations of LTA in nmoles were determined on the basis of their phosphorus content and the assumption of an average glycerol phosphate chain length of 27.5 (8). All LTAs were prepared by phenol extractions and gel filtration (18).

[3]Deacylation was carried out in 10 volumes of methanolic 0.2 M KOH at 37°C for 15 min. followed by passage through a Dowex 50 column as described (18). Dry weight was estimated from phosphorus content as acylated LTA equivalents.

[4]Fag was prepared as described previously (7).

[5]Stimulation of the rate of wall autolysis in the *S. faecalis* system was attributed to the probable presence of a small residue of trypsin used in the preparation of Fag (7). Low concentrations of proteinases are known to activate a latent form of the *S. faecalis* enzyme (12). A similar increase in wall lysis rate was not observed in the *L. acidophilus* system which is unaffected by trypsin (13).

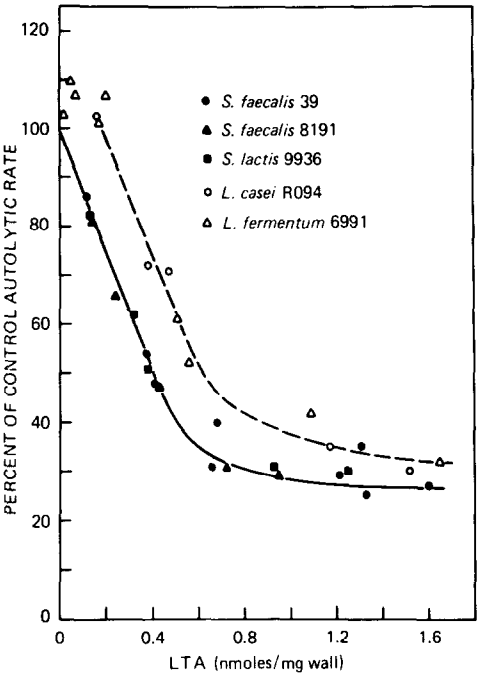

Figure 1. Inhibition of lysis of S. faecalis walls by purified LTAs of several bacterial species. Assay system and other details as described in Table 1.

hibited, and the slope of the concentration response paralleled that of the streptococcal LTAs. The L. fermentum LTA was also less effective than the streptococcal LTA on the L. acidophilus muramidase system (Table 1B). The reason for this discrepancy remains to be determined.

LTAs have a common backbone of polyglycerol phosphate, but differ in sugar substituents, and in the nature of their glycolipids (8). Chemical deacylation of several LTAs reduced or completely abolished inhibitor activity for both muramidases, even at high concentrations (over 24 µg/ml; Table 1). In contrast to the effect of LTAs, pneumococcal Fag failed to inhibit either of the muramidases,

[6]Preparations of ALP were kind gifts from Drs. Owen and Salton (assays at 3.3, 6.7 and 16.7 µg/ml, (10)) and from Drs. Pless and Lennarz (assay at 34.2 µg/ml, (11)).

[7]The assay system (13) consisted of 1.3 to 2.0 mg dry weight of wall-enzyme complex from exponential phase cells in 3.0 ml of 0.05 M sodium citrate, pH 5.0.

TABLE 2

Effect of LTA on the Action of Soluble S. faecalis N-acetylmuramidase on SDS-inactivated Walls[1]

	µg/ml	nmoles LTA per mg wall	% of control Lysis rate
LTA of S. faecalis 39 (Exp. 1)	4.3	0.7	41
	8.5	1.5	24
	21.3	3.7	11
LTA of S. faecalis 39 (Exp. 2)	6.8	1.1	32
" " " " " Autolysin pre-bound to wall (10 min., 0°C)	6.8	1.1	29

[1]Crude autolysin was obtained and activity determined in 0.005M sodium phosphate pH 6.7 as previously described (12).

even at a relatively high concentration (Table 1). ALP of M. luteus also failed to substantially inhibit the S. faecalis system (Table 1A).

LTA also inhibited the action of soluble S. faecalis muramidase on SDS-inactivated walls (Table 2). Prebinding of enzyme to walls did not affect the degree of inhibition observed. These results suggest that LTA does not affect binding of autolysin to its wall substrate.

LTAs of B. subtilis (crude) or L. fermentum (purified) did not affect the rate of lysis of the B. subtilis wall-enzyme complex (Fig. 2A). However, activity of crude B. subtilis amidase on SDS-walls (Fig. 2B) was inhibited by both crude LTA of B. subtilis and purified LTA of L. fermentum. Inhibition was particularly strong when the enzyme was preincubated with the LTAs (see legend to Figure). Preincubation of the crude enzyme alone produced no loss of activity. Neither Fag nor deacylated LTA of L. fermentum inhibited this system. A highly purified amidase of B. subtilis (15) was also inhibited by low concentrations of S faecalis 39 LTA (Table 3). In these experiments preincubation with LTA for 10 min. failed to increase inhibition, although the incubation resulted in recovery of only 68% of activity of an "enzyme alone" control. As with the muramidase of S. faecalis, LTA does not seem to affect binding of B. subtilis amidase to the wall substrate. At present, effects of LTA either directly on enzyme

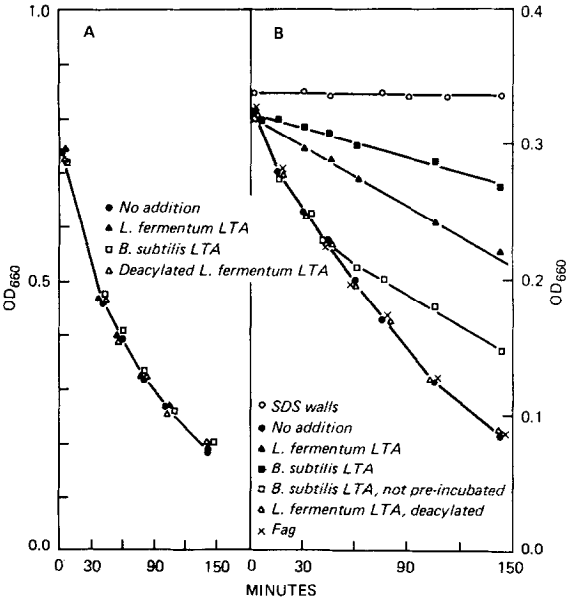

Figure 2. The effects of several LTA preparations and Fag on (A) lysis of a B. subtilis wall-enzyme complex and (B) on the action of crude B. subtilis amidase on SDS-inactivated B. subtilis walls.

(A). B subtilis wall-enzyme complex was isolated from cells grown in Spizizen's medium to 5 x 10^8 cells/ml, by mechanical disintegration and washing three times with cold 0.15M NaCl. Walls were suspended at an A_{660} of 0.75, in 1.0 ml (0.05M pH 8) Tris containing the indicated compounds at 100 µg/ml, and the turbidity was measured during incubation at 37°C.

(B). The supernate (15,000 x g, 30 min) of an autolysate of the wall-enzyme complex described above was used as a source of crude amidase. The various preparations (LTAs at 100 µg/ml, Fag at 190 µg/ml) were added to amidase (100 µl) in 1.0 ml of 0.05M Tris, pH 8. After 20 min. at 37°C, SDS-inactivated (80°C, 30 min.) walls (200 µl) were added and turbidity measured as in (A). In one tube (—□—), the SDS-walls were added at time zero. B. subtilis LTA was prepared by hot aqueous phenol extraction (18). The sources of all other preparations are given in Table 1.

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activity or on wall substrate seem equally possible.

The various LTA preparations failed to inhibit, and in some instances stimulated the amidase assay system of D. pneumoniae (Table 4). The concentrations used were at least 100 fold those used either in the muramidase or B. subtilis amidase assays. As reported previously, Fag inhibited the diplococcal amidase at 5 and 12.5 µg/ml (5). Addition of antipolyglycerolphosphate antibody (8) to the pneumococcal system did not affect Fag inhibition, suggesting

TABLE 3

Effect of LTA on the Release of [^{3}H]Glucosamine label from *B. subtilis* Walls by the Action of a Purified *B. subtilis* Amidase[1]

	µg/ml	nmoles LTA/µmole amino sugar	% of control lysis rate
LTA of *S. faecalis* 39	2.3	3.8	64
	4.5	7.6	37
	18.0	30.5	20
LTA of *S. faecalis* 39 preincubated with amidase, (10 min., 37°C)	2.3	3.8	72
Deacylated LTA of *S. faecalis* 39	19.5	33.0	100

[1]The assay system as previously described (15) consisted of 50 µl [^{3}H]glucosamine labeled walls (150 nmoles of amino sugar, 1.7 x 10^2 dpm) 2 µl of amidase, in 0.05 M Tris, pH 8.0, containing 0.05 M LiCl, 0.01 M $MgCl_2$ and 1mM EDTA in a total volume of 200 µl. The reaction was stopped after 60 min. at 37°C. Purified amidase and labeled wall substrate were kindly provided by Dr. L. Glaser. For LTA preparations, see Table 1.

TABLE 4

Effect of LTAs, Fag, and ALP on the Pneumococcal Amidase System[1]

	µg/ml	% of control
LTA of *S. faecalis* 39	500	121
LTA of *S. lactis* 9936	500	158
LTA of *L. casei* R094	500	135
LTA of *B. subtilis* 168	350	131
ALP of *M. luteus*	1000	100
Fag of Pneumococcus	5	37
	12.5	20
Fag of pneumococcus plus antipolyglycerol-phosphate antibody (6)	5	47
	12.5	29

[1]The assay system as previously described (5,19), consisted of [methyl ^{3}H]choline labeled walls (2.0 µg, 10^4 cpm) plus crude amidase (15 µg protein from about 1.5 x 10^6 cell equivalent units of cell lysate) in 0.05 M Tris maleate, pH 6.9 in a total volume of 220 µl. The reaction was stopped after 10 min. at 37°C. For materials used see Table 1.

that inhibition is not due to contaminating LTA.

The data presented indicate that, at low concentrations, polyglycerophosphate type LTAs can inhibit three autolytic enzyme systems. The unique

specificity of Fag for the pneumococcal amidase could be due to the absence of a polyglycerophosphate type of LTA. The presence and chemical nature of polyglycero phosphate containing polymers in pneumococci has not been carefully investigated.

Deacylation of LTA results in loss of several biological activities (8) in addition to loss of lytic inhibitory activity. Other amphipathic compounds which have properties in common with LTA, including association with membranes (i.e., ALP and Fag), were not effective inhibitors of the two muramidases. Further work will be required to unravel the specificities of the observed effects, especially since the degree of acylation of each compound or preparation is not precisely known. The membrane association of LTA as well as its occurrence in both acylated and deacylated forms (16,17), are appropriate for a role in in vivo regulation of autolytic activity.

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