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Interaction of Purified Lipoteichoic Acid with the Classical Complement Pathway

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Glycerophosphate-containing lipoteichoic acids (LTAs) interact with the first component of the classical complement pathway (C1). This resulted in the activation of the classical complement pathway in serum, shown by the consumption of C1, C2, and C4. The dose-dependent interaction of LTAs with purified $C\bar{1}$ and C1q was dependent on the negative charges of the phosphate groups of LTA. It was reduced by charge compensation through D-alanine ester substituents and by sterical hindrance through di- and trihexosyl residues linked to position 2 of the glycerol moieties. The charge density of LTA may also play a role: poly(digalactosylglycerophosphate) LTAs, in which the phosphate groups are in a greater distance from each other, were less effective, and the loss of micellar organization by deacylation of LTA drastically reduced the complement activation capacity.

Most gram-positive bacteria contain lipoteichoic acids (LTAs) in their cytoplasmic membrane (11, 26, 41). These are high-molecular-weight amphiphiles consisting of a hydrophobic glycolipid moiety and a hydrophilic chain which is either a 1,3-linked poly(glycerophosphate) or, in a limited number of bacteria, a poly(dihexosylglycerophosphate) (24; K. H. Schleifer, J. Kraus, C. Dvorak, R. Killper-Bälz, M. D. Collins, and W. Fischer, Syst. Appl. Microbiol., in press). Native LTAs may be unsubstituted, but usually the glycerol moieties are substituted with D-alanine ester or glycosyl residues (or both) to various extents (11). LTAs seem to be concentrated in the outer layer of the cytoplasmic membrane and, by extending through the wall complex, may reach the surface of the cell (17, 32, 38, 41).

Depending on the degree of substitution with positively charged alanyl residues, the hydrophilic chain displays a more or less anionic character, as shown by chromatography on DEAE-Sephacel (15). Although various polyanionic compounds (27) and among them wall teichoic acids of gram-positive bacteria (39, 40, 42, 43) have been shown to activate the complement system, glycerophosphate-containing LTAs have not yet been tested for this potential adequately. There are only two short notes on complement activation by streptococcal and *Staphylococcus aureus* LTA (18, 37). From results obtained with cell-free preparations from *Streptococcus mutans* BHT, Silvestri et al. (33) suggested that LTA might bind to C1 and consume complement in whole serum. Recently the activation of the alternative pathway by pneumococcal LTA has been reported (22), but this polymer, containing dipolar ionic choline-phosphate residues (4, 5), is structurally quite different from the common glycerophosphate-containing LTAs.

In this study we therefore tested the main types of native glycerophosphate-containing LTAs for their capacity to activate the classical complement pathway in serum and especially their interaction with purified $C\bar{1}$ and C1q. In addition, modified LTAs were used to elucidate the structural requirements.

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MATERIALS AND METHODS

Bacteria and LTAs. Bacteria were grown as previously described (14, 24) and harvested at late logarithmic growth. LTA I was prepared from *S. aureus* DSM 20233, LTA II was prepared from *Streptococcus faecalis* ATCC 9790 (obtained from G. D. Shockman, Temple University, Health Science Center, Philadelphia, Pa.), LTA IIIa was prepared from *Streptococcus lactis* Kiel 42172, and LTA IIIb was prepared from *Streptococcus garvieae* NCDO 2155. LTAs were extracted with hot phenol-water from mechanically disrupted bacteria and purified by hydrophobic interaction chromatography on octyl Sepharose as previously described (12). The elution profile from the column indicated that the preparations were free of lyso compounds and deacylation products (12). The structure of the purified LTAs was characterized as in previous work (16, 24). The glycolipid moieties were identical to those described earlier (10, 16). Concentrations of LTAs, measured as phosphate and calculated on the basis of repeating units per chain (16), were 24, 20, 12, and 13 for LTAs I, II, IIIa, and IIIb, respectively.

The compounds Ia, Ib, and Ic were derivatives of the D-alanyl LTA I. The alanine-free derivative (Ia) was prepared by mild alkaline treatment (pH 8, 37°C, 24 h) and purified by dialysis. The *N*-acetyl derivative (Ib) was prepared as previously described (25). Compound Ic was prepared from Ia by enzymatic deacylation as described elsewhere (12).

Hemolytic complement assays and components. The methods for preparing sheep erythrocytes (E) sensitized with rabbit immunoglobulin G (IgG) antibody (A) and loaded with complement components to form the cellular intermediates (EA, $EAC\bar{1}$, EAC4, $EAC\bar{1}$,4) and imidazol-buffered saline with or without EDTA have been described by Rapp and Borsos (30).

Samples of pooled normal human serum were stored at −70°C. Partially purified $C\bar{1}$ was prepared by zonal ultracentrifugation of the euglobulin fraction of serum as described by Colten et al. (7). C1q was purified by the method of Stemmer and Loos (37) from pooled normal human serum

TABLE 1. Complement titers in normal human serum after incubation with LTAs[a]

LTA prepn	Effective molecules of complement (2.12×10^9)/ml of serum[b]		
	C1	C4	C2
I	8,081 ± 2,283 (41)	149 ± 17 (1.3)	3 ± 1 (4)
Ia	9,047 ± 2,177 (46)	103 ± 19 (0.9)	4 ± 2 (5)
Ib	1,521 ± 167 (8)	11 ± 3 (0.1)	4 ± 4 (5)
Ic	11,395 ± 2,697 (58)	337 ± 33 (3)	7 ± 2 (9)
II	18,066 ± 6,495 (92)	8,273 ± 1,759 (73)	61 ± 7 (78)
IIIa	16,772 ± 3,895 (86)	1,161 ± 152 (10)	19 ± 4 (24)
IIIb	17,948 ± 4,116 (92)	4,748 ± 1,617 (42)	38 ± 6 (49)
Whole serum	19,564 ± 5,189 (100)	11,311 ± 2,603 (100)	78 ± 9 (100)

[a] Serum was incubated with 1 mmol of phosphate containing a native LTA preparation or LTA modified in structure or charge. Results are mean values of four experiments.

[b] Values in parentheses indicate percentage of serum concentration.

and was immunochemically and protein chemically (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) proven to be free of IgG and IgM. Complement titrations were performed as described by Rapp and Borsos (30); C1q reconstitution for hemolytic functional titration was as described by Golan et al. (19).

RESULTS

To test the interaction of LTA with the classical complement pathway, three types of native LTA were used: (i) the LTA of *S. aureus* H, which is substituted with D-alanine ester (alanine/glycerol ratio, 0.63) (15, 16); (ii) the LTA of *S. faecalis* ATCC 9790, which is to a large extent substituted with mono-, di-, and triglucosyl residues (glycosylated glycerol/total glycerol ratio, 0.84) (16, 25); and (iii) the LTAs of *S. lactis* Kiel 42172 (IIIa) and *S. garvieae* (IIIb), both of which contain a poly(digalactosyl galactosylglycerophosphate) chain (24; Schleifer et al., in press). Since the digalactosyl residues of LTA III are integrated between the glycerophosphate moieties, the phosphate groups are in a greater distance from each other than in the 1,3-linked poly(glycerophosphate) structures of LTAs I and II (1.63 versus 0.75 nm) (24).

Also included were three modifications of LTA I: the alanine-free derivative (Ia), the *N*-acetylalanyl derivative (Ib), and the alanine-free deacylation product (Ic) which had lost the capacity of forming micelles.

In the first set of experiments we tested the interaction of these LTAs with the classical complement pathway. Equal volumes (50 μl) of normal human serum and LTA solutions (16.7 ± 0.2 mM phosphorus) were incubated for 15 min at 30°C. Then all samples were tested for remaining functionally active C1, C4, and C2 molecules. The results, expressed as complement consumption per micromole of LTA phosphorus, are shown in Table 1. The data indicate that all LTAs and derivatives activated the classical pathway because the classical complement components C1, C2, and C4 were consumed. This suggests that LTAs interact with macromolecular C1 whereby C1 is activated, causing the generation of $C\bar{1}$ esterase, which then acts on its substrates C4 and C2. Alanyl LTA (I) and the three LTA derivatives (Ia, Ib, Ic) displayed a high capacity of binding to C1; as a result, C2 and C4 were almost completely consumed (Table 1). LTAs II, IIIa, and IIIb, which have in common a high carbohydrate content, showed a comparatively weak interaction with C1 and a diminished consumption of C2 and C4.

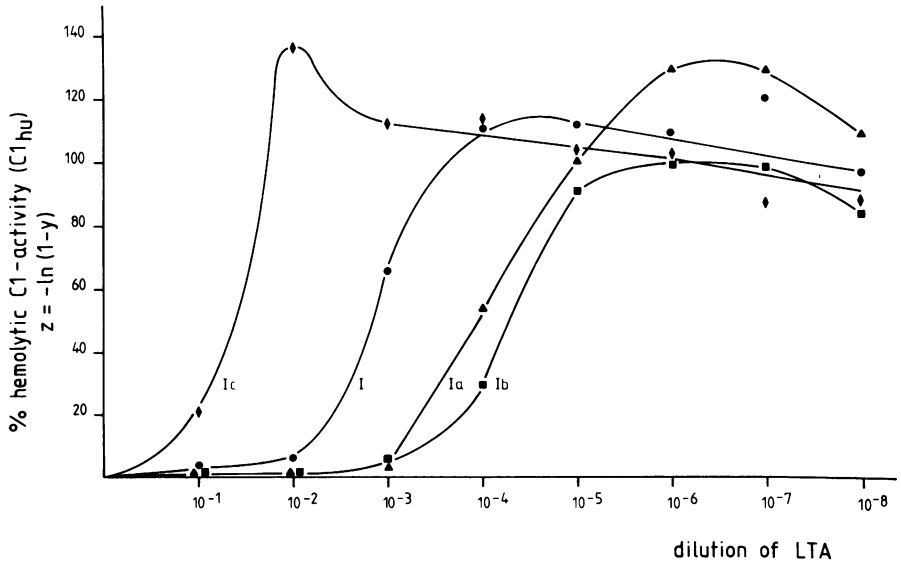

FIG. 1. Interaction of $C\bar{1}$ which had been purified from normal human serum with native D-alanyl LTA (I), the alanine-free derivative (Ia), the *N*-acetylalanyl derivative (Ib), and the alanine-free deacylation product (Ic). For the dose-response assays LTA solutions (16.7×10^{-3} M phosphorus) were diluted from 1:10 to $1:10^8$. The functional hemolytic activity of the C1 molecules was determined by $z = -\ln(1 - y)$ and calculated as hemolytic C1 activity (percentage of control).

To get more information on the interaction of LTAs with macromolecular C1 and its subcomponent C1q, dose-response experiments were carried out. In the first set a limited amount (i.e., 1 effective molecule per EAC4) of C1, purified from normal human serum, was incubated with serial dilutions of alanyl-LTA (I) and its derivatives (Ia, Ib, Ic). Native alanyl-LTA (I) displayed an intermediate inhibitory effect on the hemolytic C1 activity (Fig. 1). The effect increased remarkably when the positively charged alanine ester was removed (Ia) or converted into the uncharged *N*-acetyl derivative (Ib). These observations suggest that the negatively charged phosphate groups play an important role in the interaction of LTA with macromolecular $C\bar{1}$. However, deacylated alanine-free LTA (Ic) was much less effective, although it has the same number of negative charges per chain as compounds Ia and Ib. This result may be interpreted to indicate either that the hydrophobic fatty acid chains interact themselves with $C\bar{1}$ or that the micellar organization of LTA stimulated the binding because in the micelle the negative charges are concentrated as compared with the monomolecular solution of deacylated LTA.

From the curves in Fig. 1 the LTA concentrations effecting 50% inhibition were calculated. There are more than 3 orders of magnitude between the concentration required for *N*-acetylalanyl LTA (Ib) and deacylated LTA (Ic) (Table 2). In similar dose-response assays the carbohydrate-rich LTAs (II, IIIa, IIIb) were tested for their interaction with $C\bar{1}$ (Fig. 2). In agreement with the results obtained with whole serum (Table 1), all three preparations showed a weak interaction. The concentrations required for 50% inhibition were similar to that of alanyl LTA (I) (Table 2). Space-filling models (not shown) suggest that the negative charges of the phosphate groups of LTA II are shielded by the adjacent bulky di- and triglucosyl residues. In the case of LTAs IIIa and IIIb the greater distance of the phosphate groups from each other (see above) may play the predominant role.

The obvious significance of the negative charges for the effect of LTAs on macromolecular $C\bar{1}$ suggested an electrostatic binding to the positively charged subcomponent C1q. This was confirmed by dose-response experiments with C1q isolated from normal human serum and from guinea pig serum. The concentrations of LTAs necessary for 50% inhibition were calculated and compared (Table 2) with those obtained for inhibition of macromolecular $C\bar{1}$. With both C1q preparations the lowest concentration required was that of the alanine-free LTA derivative (Ia). The *N*-acetylalanyl derivative (Ib), although equally negatively charged, was less effective, suggesting that the *N*-acetylalanyl groups adjacent to the phosphodiester weaken the interaction with C1q moderately by sterical hindrance. The positively

TABLE 2. Concentrations of LTAs effecting 50% inhibition of the hemolytic activity of purified $C\bar{1}$ and C1q[a]

LTA	50% Inhibitory concn of LTA (mol/liter) for:		
	$C\bar{1}^{hu}$	$C1q^{hu}$	$C1q^{gp}$
I	9.2×10^{-6}	2.3×10^{-7}	6.2×10^{-5}
Ia	1.2×10^{-6}	2.1×10^{-8}	2.2×10^{-9}
Ib	2.3×10^{-8}	1.8×10^{-7}	2.8×10^{-7}
Ic	8.3×10^{-5}	7.6×10^{-5}	1×10^{-3}
II	8.9×10^{-6}	ND[b]	ND
IIIa	1.2×10^{-5}	ND	ND
IIIb	6.4×10^{-6}	ND	ND

[a] LTA concentrations were calculated from phosphate values on the basis of chain length as described in the text.
[b] ND, Not determined.

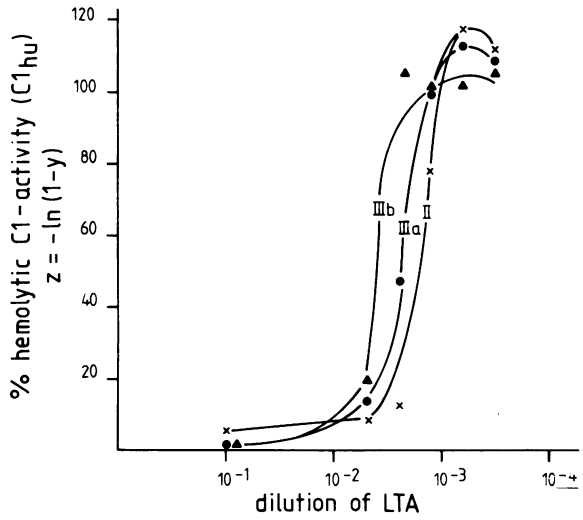

FIG. 2. Dose-response effect of carbohydrate-rich LTAs (II, IIIa, IIIb) on the hemolytic activity of $C\bar{1}^{hu}$. LTA solutions (16.7×10^{-3} M phosphorus) were diluted to 10^{-4}. The functional hemolytic activity of C1 was determined by $z = -\ln(1 - y)$ and calculated as hemolytic C1 activity (percentage of control). For LTA structures, see the text.

charged alanine ester of native LTA (I) increased the concentration effecting 50% inhibition of $C1q^{gp}$ by more than 3 orders of magnitude. Deacylation again showed the greatest effect (Ic): the concentrations required for 50% inhibition of $C1q^{hu}$ and $C1q^{gp}$ were 3.6×10^3 and 4.5×10^5 times higher, respectively, than that of the acylated parent compound (Ia). Even though there is no doubt that C1q interacts with certain forms of LTA, we have at present no explanation for the differences in dose response between human and guinea pig C1q.

DISCUSSION

These results indicate that LTAs possess the capacity to activate the classical complement pathway and to bind directly to purified $C\bar{1}$. Since LTAs interact directly with the C1 subcomponent C1q, the most basic protein of serum, an antibody-independent activation of the clasical complement pathway is most likely similar to bacterial lipopolysaccharide. This interpretation is supported by the fact that the net negative charge of the hydrophilic chain of LTA is of obvious importance for binding. Charge compensation by D-alanine ester substituents and shielding of the charge by bulky carbohydrate substituents reduce the binding effect. In this connection the greatly reduced efficiency of deacylated LTA may be seen as a dilution of negative charges resulting from the loss of micellar organization, although hydrophobic interaction between the fatty acids and C1 and C1q cannot be excluded. It was not until recently that critical micellar concentrations of LTAs were measured. The values reported by Courtney et al. (8) and Wicken et al. (40) are in the order of 10^{-6} and 10^{-7} M, respectively. One reason for this difference may lie in the fact that different dyes were used in these experiments. Further studies are therefore necessary to interpret the present results on the basis of critical micellar concentrations. Moreover, Courtney et al. (8) suggested that their LTA preparations contained only one fatty acid per molecule. The LTAs used in the present study have,

however, been established to contain two and three fatty acids (9, 13, 24), which would be expected to lower the critical micellar concentration by several orders of magnitude (20, 35).

The effect of LTAs is reminiscent of the earlier observed interaction of various polyanions with the complement system (27). Like other polyanions (27), LTAs bind to C1q (Table 2). However, only a few of the previously tested polyanions that interacted with C1q had the capacity of activating the C1 esterase. That LTAs belong to this active group has been demonstrated by the concomitant consumption of C1, C2, and C4 in serum (Table 1).

The biological significance of complement activation by LTAs is not yet clear. LTAs anchored in the cytoplasmic membrane may extend through the peptidoglycan layer and reach the surface of the cell (17, 32, 38, 41). Despite this, in intact bacteria complement activation by LTA seems to be of minor importance because peptidoglycan itself is a potent activator (39, 42, 43). Moreover, the ribitol phosphate-containing wall teichoic acid of *S. aureus* being a highly effective activator in vitro (39, 43) had no detectable effect in the whole cell, as shown with a teichoic acid-lacking mutant strain (39, 42). However, when the peptidoglycan is lost through the action of lysozyme or under the treatment with penicillin and other cell wall inhibitors, LTA is exposed in its full length on the surface of the protoplast or L-form variant. Moreover, L forms of group A streptococci have been found to be no longer able to incorporate alanine into LTA (6), so that the LTA of the L form is lacking alanine ester in contrast to the LTA of bacterial-phase cells (29, 34). It is of interest in this context that L variants of *S. aureus* and *S. faecalis* have been reported to activate complement via the alternative pathway, which resulted in death of these cells (31). Although the membrane component responsible for complement activation remains to be definitely identified, indirect evidence points to LTA.

The complement activation by LTA may also play a role in pathogenicity. From various bacteria LTA is spontaneously released (23, 28), and this process may be stimulated during treatment with penicillin and other cell wall inhibitors (1, 21). If the lipid anchor remains intact, excreted LTA forms micelles which, as shown in this study, may consume complement. Excreted LTA may also be inserted into the cytoplasmic membrane of host cells (2, 3) and kill them by complement activation; e.g., erythrocytes exposed to pneumococcal LTA are lysed by activation of the alternative complement pathway (22).

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