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The Effect of Leukocyte Hydrolases on Bacteria. XI. Lysis by Leukocyte Extracts and by Myeloperoxidase of a *Staphylococcus aureus* Mutant Which is Deficient in Teichoic Acid, and the Inhibition of Bacteriolysis by Lipoteichoic Acid[1] (40297)

M. N. SELA, I. OFEK, M. LAHAV, AND I. GINSBURG

Department of Medical Microbiology, School of Medicine, and the Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, founded by the Alpha Omega Fraternity, Jerusalem, Israel

In previous publications, we have shown that *Staph. aureus,* which had been harvested from the logarithmic phase of growth, was readily lysed by human leukocyte extracts (ENZ) and by myeloperoxidase (MPO). On the other hand, bacteria obtained from the stationary phase of growth were highly resistant to degradation by these agents (1–8). It was further demonstrated that the lysis of the bacteria by the leukocyte factors was probably caused by the activation of autolytic systems and not by the direct effect of lysosomal hydrolases on the bacterial walls (8). It was suggested that one of the reasons for the resistance to degradation of the stationary phase bacteria was not due to the lack of autolytic enzymes in the old cells, but to the much thicker cell walls of such cells (7, 8).

It is well established that Gram positive bacteria possess teichoic acid (TA) as an integral part of the cell wall and a membrane-associated lipoteichoic acid (LTA) (9). Since TA was claimed to deter the interaction of lysozyme with the peptidoglycan, thus conferring resistance to bacteriolysis (10), and since LTA has been implicated in the inhibition of autolytic enzymes in bacteria (11, 12), it was of interest to test the effect of TA and LTA on bacteriolysis induced by leukocyte factors. The data presented show that a mutant of *Staph. aureus,* which completely lacks ribitol TA but nevertheless possesses membrane-associated LTA, is much more susceptible to lysis by ENZ and by MPO than the parent strain. It will also be shown that while LTA strongly inhibited the lysis of *Staph. aureus* by ENZ and by MPO, TA was not inhibitory.

Materials and methods. Microorganisms. The following *Staph. aureus* strains were employed: The parent strain SH (Str^r) and the mutants 52A5 and 52A2. The mutant (52A5) lacks ribitol teichoic acid in the cell wall and no ribitol phosphate polymer was detected in any other cell fraction or in the spent medium. The lack of ribitol teichoic acid in the cell wall is caused presumably by some defect in the membrane or in some unknown factor required in the polymerization or attachment step of the teichoic acid to murein. The mutant 52A2 lacks *N*-acetylglucosamine on the cell wall ribitol teichoic acid. All these strains are also known to be deficient in protein A (for details see reference 13). These strains were kindly supplied by Dr. D. Mirelman from the Department of Biophysics, the Weitzmann Institute, Rehovoth, Israel (13). In addition we have employed *Staph. aureus* strain Cowan I which is known to be rich in protein A. The bacterial strains were cultivated either in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI) or in BHI which contained 0.5 μCi/ml of uniformally labeled [^{14}C]D-glucose, specific activity 150–250 mCi/mmol (New England Nuclear, Boston, MA) as described (2). All the bacterial cells were harvested either from the logarithmic phase of growth (after 3 hr of incubation, OD = 280 Klett units at 540 μm in a Klett Summerson colorimeter) or stationary phase of growth (after 18 hr of incubation, OD = 620 Klett units at 540 μm). The cells were washed several times in saline and were resuspended in distilled water.

Lipoteichoic acid (LTA). Lipoteichoic acid (LTA) was isolated from *Strep. mutans* (SE 1895/74), group A streptococci (strain C203S) and from *Staph. aureus* (SH, 52A5, 52A2 and cowan I) by phenol (Mallinckrodt Inc., St. Louis, MO) or by lysozyme (Sigma,

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Chemical Co., St. Louis, MO) (14). The bacterial extracts were dialyzed with six changes of distilled water and were then lyophilized, such preparation did not contain any traces of phenol. Teichoic acid (TA) was isolated from *Staph. aureus* SH by TCA (BDH, England) according to a method described by Matsuno and Slade (16).

Deacylation of LTA. was performed as described by Knox and Wicken (9).

Lipopolysaccharide (LPS). LPS from *E. coli* 0127:88 was purchased from Difco Laboratories (Detroit, MI). The LPS was dissolved in saline to the desired concentration.

Production of anti-LTA serum. Antibodies against LTA were prepared by immunizing rabbits either with *Strep. mutans* SE 1895/74, with *Staph. aureus* Cowan I or with group A type 3 streptococci, according to a procedure described in detail (14).

Determination of LTA activity. LTA was determined quantitatively by its ability to sensitize human RBC to agglutination in the presence of a standard anti-LTA serum as described (16).

Bacteriolysis. The lysis of staphylococci was performed as described in detail (2). Briefly, [^{14}C]labeled bacterial suspensions containing 1–5 × 10^3 cpm/100 Klett units per ml were incubated for 18 hr at 37° in 0.1 *M* acetate buffer pH 5.0 either with freeze and thaw extracts of human blood leukocytes containing 10–500 μg/ml of protein or with purified myeloperoxidase which was kindly supplied by Dr. I. Olsson from the Department of Internal Medicine University of Lund, Sweden, as well as with nuclear histone (Sigma Chemical Co., St. Louis, MO). MPO was used here as a cationic protein and not as a bactericidal agent which in collaboration with H_2O_2 and halide is a strong bactericidal agent.

The degree of lysis was determined by measuring the percentage release of soluble radioactivity from the standard labeled bacterial suspension (2).

The inhibition of bacteriolysis. Radio-labeled bacteria were incubated for 15 min at 37° with various amounts of LTA (phenol or lysozyme extract) with deacylated LTA (9) with TA or with LPS. Following incubation, the cells were lysed by leukocyte factors, and the degree of inhibition of lysis was determined as described (2). The results were expressed as the percentage of inhibition of the release of radioactivity from a standard suspension of [^{14}C]labeled staphylococci.

Results. The lysis of staphylococci by leukocyte factors. Table I shows that when stationary bacteria were employed, only strain 52A5 (which is deficient in TA) underwent massive lysis following treatment with human leukocyte extracts (ENZ). On the other hand, all the bacterial strains employed were equally susceptible to lysis when harvested from the logarithmic phase of growth (young cells). It is important to note that identical results were obtained when a purified preparation of myeloperoxidase (MPO) or histone were used instead of the leukocyte extracts (not shown). Table I also shows that strain 52A5 looses a somewhat higher percentage of radioactivity when incubated in buffer alone

TABLE I. THE LYSIS OF DIFFERENT STAPHYLOCOCCAL STRAINS BY LEUKOCYTE EXTRACTS.

Bacterial strain	Presence or absence of			% release of radioactivity from[a]			
				logarithmic phase bacteria		stationary phase bacteria	
	Protein A	TA	LTA[b]	Buffer	ENZ	Buffer	ENZ
SH	−	+	+	21	80	18	38
52A5	−	−	+	22	75	30	75
52A2	−	±[c]	+	28	78	15	35
Cowan I	+	+	+	25	92	10	30

[a] Radiolabeled bacteria (100 Klett units/ml 540 μm) suspended in 0.1 *M* acetate buffer pH 5.0 were incubated for 18 hr at 37° with 100 μg/ml of human leukocyte extracts (ENZ) and the soluble radioactivity was determined as described in Materials and Methods. Similar results were obtained with MPO or histone. The data are the mean of four experiments.

[b] LTA was extracted from the bacteria either with phenol or by lysozyme as described in Materials and Methods.

[c] This mutant lacks *N*-acetylglucosamine in its TA. .

(spontaneous lysis) as compared with the other strains. Since all the bacterial strains employed were found to possess LTA (Table I), it is postulated that TA, but not the membrane-associated LTA, may play an important role in the protection of old bacteria against lysis by leukocyte factors.

The inhibition by LTA of the lysis of staphylococci. LTA was recently shown to be a potent inhibitor of autolytic enzymes in *Strep. faecalis* (11) and *Diplococcus pneumoniae* (12). Since we have recently postulated (8) that the lysis of *Staph. aureus* by leukocyte extracts and by membrane-damaging agents like MPO and Phospholipase A_2, was due to the activation of autolytic enzymes, it was of interest to examine the possibility that LTA will also inhibit bacteriolysis induced by leukocyte factors and by MPO. Table II shows that when LTA (derived either from staphylococci or from streptococci) was added to staphylococci (SH and 52A5) in the presence of an inducer of lysis like ENZ or MPO, a strong inhibition of lysis occurred. It is also seen that H_2O_2 did not modify either the lytic effect of MPO or the inhibitory effect of LTA on bacteriolysis induced by MPO. The Table also shows that neither deacylated LTA nor TA nor LPS had any inhibitory property. It is also shown that none of the inhibitors employed lysed the bacteria. In other experiments (not shown) we found that the lysis of staphylococci by ENZ could not be inhibited by cytoplasmic fractions or cell walls derived from group A streptococci, when used at similar concentrations.

TABLE II. THE EFFECT OF LTA, DEACYLATED LTA, TA AND LPS ON THE LYSIS OF STAPHYLOCOCCI LEUKOCYTE FACTORS.

Reaction mixture[a]	% Release of radioactivity after 18 hr from:[b] Strain SH	Strain 52A5
Buffer alone	21	22
Lysozyme 100 μg	30	32
Leukocyte extracts 100 μg	80	75
MPO 100 μg	82	75
LTA[c] 250 μg	20	20
H_2O_2 0.3 μg	19	ND[d]
Leukocyte extracts + H_2O_2 0.3 μg	79	ND
Leukocyte extracts + LTA 150 μg	30	35
Leukocyte extracts + LTA 250 μg	25	28
Leukocyte extracts + LTA 500 μg	17	20
Leukocyte extracts + LTA 500 μg + H_2O_2 0.3 μg	14	ND
Leukocyte extracts + Deacylated LTA 150 μg	76	75
Leukocyte extracts + Deacylated LTA 250 μg	70	76
Leukocyte extracts + TA 250 μg	80	75
Leukocyte extracts + LPS 500 μg	78	72
MPO + H_2O_2 0.3 μg	72	ND
MPO + LTA 250 μg	23	25
MPO + LTA 500 μg	17	22
MPO + LTA 500 μg + H_2O_2 0.3 μg	15	ND

[a] The reaction mixtures were added to the labeled bacteria (logarithmic phase) in 0.1 *M* acetate buffer pH 5.0.

[b] Lysis was determined as the percentage of the solubilized radioactivity as described in Materials and methods. The data are the average of five experiments.

[c] All LTA preparations (see Materials and methods) behaved similarly.

[d] ND—Not done.

Discussion. The data on the higher susceptibility to lysis of the TA-deficient mutant by leukocyte factors and by MPO and the inhibition of bacteriolysis by LTA, further contribute to the understanding of the possible role which may be played by TA and LTA in the biology of the staphylococci.

Since TA was claimed to deter the interaction of lysozyme with the peptidoglycan (10) it may be postulated that the lack of this wall component from the mutant 52A5 rendered the cell more susceptible to bacteriolysis. Since however, the lysis of *Staph. aureus* by leukocyte enzymes was found not to be lysozyme-dependent (3, 5) and since the TA-less mutant was not more susceptible to lysis by lysozyme than the parent strain (Table II), one should seek other explanations for the higher susceptibility of the mutant to lysis by leukocyte factors.

It may be postulated that since old staphylococci (shown to be resistant to degradation) (Table I), possess much thicker cell walls (17) and since TA forms the bulk of the staphylococcus cell wall, it is possible that the lack of TA from the mutant renders the "thinner" cell wall of these microorganisms more susceptible to degradation by the autolytic enzymes, which are activated by the leukocyte factors (8). Thus TA may be essential for

the stabilization of the cell wall not only against lysozyme but also against the autolysins.

LTA, has been shown to be a potent inhibitor of autolytic enzymes in a variety of bacterial species (11, 12). The findings that exogenous LTA can inhibit the lysis, by ENZ and MPO (Table II) of staphylococci which possess endogenous LTA (Table I) is intriguing. To explain this phenomenon one may postulate that since leukocyte extracts, lysozyme and histone were shown to remove the bulk of LTA from bacterial cells (14), the lysis of the staphylococcus cells by leukocyte factors may involve, first the removal of endogenous LTA from the bacterial cells by the leukocyte factors, then the release from inhibition of the autolytic enzymes, and finally the facilitation of the activity of the autolytic systems.

As shown in Table II lysis of staphylococci (microorganisms known to produce H_2O_2) could be induced by MPO. These results are of interest, since neither KCN nor NaN_3, which are hemeprotein inhibitors, could inhibit bacteriolysis by MPO (unpublished observations). It thus points to the possibility that MPO (a cationic substance) like other membrane-damaging agents, (e.g. LCP, histone, phospholipase A_2, polymyxin B, colimycin), may interact with the protoplast membrane and through perturbation, activate the membrane-associated autolytic systems, through the removal of LTA (14). This may also explain teleologically why PMN contain large amounts of MPO.

The fact that H_2O_2 did not modify the inhibitory effect of LTA, on the lysis of staphylococci by MPO (Table II), further supports the assumption that MPO in this system acts as cationic protein.

The reasons for the use of acid buffers in the bacteriolytic system are based on our previous findings (5, 18) that optimal killing and lysis of staphylococci by leukocyte extracts and histone took place at pH 5.0, whereas only a slight effect was obtained at nutral pH.

The interrelationships among TA, LTA, autolytic systems and leukocyte factors in relation to the degradation of microbial cell wall constituents in inflammatory sites merit further examination.

Summary. A *Staph. aureus* mutant (52A5) which is deficient in wall teichoic acid (TA) was found to be highly susceptible to lysis by leukocyte extracts (ENZ) and by myeloperoxidase (MPO) when harvested from the stationary phase of growth, On the other hand, a staphylococcus mutant, which is deficient in *N*-acetyl glucosamine in its TA (52A2), the parent strain SH and a protein A rich strain Cowen I, could be lysed by the leukocyte factors only when harvested from the logarithmic phase of growth.

The lysis of all the bacterial strains by ENZ or by MPO was strongly inhibited by lipoteichoic acid (LTA) derived either from staphylococci or from streptococci. On the other hand, deacylated LTA, TA, LPS, cytoplasmic or cell wall components derived from streptococci had no inhibitory effect on bacteriolysis. It is concluded that TA may be important in the protection of old bacterial cells against degradation by leukocyte factors, and that LTA may be involved in the control of autolytic enzymes in staphylococci. The role of MPO in bacteriolysis is also discussed.

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