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Identification of a Lysin Associated with a Bacteriophage (A25) Virulent for Group A Streptococci

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A phage-associated lysin was found in culture lysates resulting from the propagation of virulent bacteriophage A25 on the group A streptococcal strain designated K56. In contrast to the previously described group C streptococcal phage-associated lysins, A25 phage-associated lysin was more active on chloroform-treated cells, was not phage bound, and was active on some group G and H strains, as well as on group A and C strains. A25 phage-associated lysin had an optimum pH of 6.7 and was inactivated by 10^{-3} M *p*-hydroxymercuribenzoate. Group A cells exposed to penicillin were more susceptible to A25 phage-associated lysin, whereas chloramphenicol-treated cells became resistant to lysis. Release of lipoteichoic acid appeared to precede lysis, and cardiolipin treatment of cells reversed the effects of chloroform and penicillin treatments. These results suggest the possibility that A25 phage-associated lysin may have a mechanism similar to the mechanism of an autolysin or that cell lysis may be due to the activation of an autolysin.

Muralysins are enzymes that disrupt bacterial cells by attacking the peptidoglycan portion of the bacterial cell wall. These lytic enzymes have been associated with the ability to undergo genetic transformation (22-24), with cell growth and septation (10, 11, 29), with the lethality of some antibiotics (28, 31, 32), and with bacteriophage infection of and release from host bacterial cells (1, 7, 19, 26, 30). One of the best-studied phage-associated lysins (PAL) is induced during the infection of group C streptococci by the C1 bacteriophage (35). Although this group C bacteriophage does not infect group A streptococci, the group C PAL has a greater lytic activity for group A streptococci than for the group C streptococci, in which the lysin is produced (7).

Early reports from Maxted (18, 19) suggested the possible existence of a group A PAL, but until now such a lysin has not been recognized or described. A group A PAL is of interest because (i) the identification of a group A PAL is the first evidence that the release of bacteriophage progeny from infected group A streptococci could be due to the enzymatic lysis of the bacterial cells (18), (ii) the identification of a group A PAL supports the hypothesis that bacteriophages can induce the in vivo formation of streptococcal L-forms (19, 35) and (iii) some properties of the group A PAL suggest that susceptibility to this lysin is similar to the regulation of autolytic activity which has been described for some group D streptococci (28, 31, 32).

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MATERIALS AND METHODS

Bacterial strains and bacteriophages. All bacterial strains used in this study have designations from our culture collection, except *Streptococcus pyrogenes* ATCC 10402. The group A virulent phage A25 has been described by and was obtained from W. R. Maxted (18). Phage A25 was propagated and titrated on bacterial strain GT8761, which was originally obtained from E. Kjems as strain K56 (34).

The group C bacteriophage C1 was obtained from V. Fischetti (8) and was propagated on group C *Streptococcus* strain 73-004, which was supplied by V. Fischetti as strain 26 RP66.

Media. Bacteriophage A25 was propagated on strain K56 growing in a liquid medium developed for this study. This medium was designated L3 and contained (per liter) 40 g of proteose peptone 3 (Difco Laboratoreis), 2 g of yeast extract, and 9.5 g of the organic buffer HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Research Organics, Inc.). After the pH was adjusted to 7.3 with NaOH, L3 broth was sterilized by autoclaving and then aseptically supplemented with filter-sterilized solutions of the following: 0.25% glucose, 0.15% $CaCl_2$, and 68 μg of type 1-S bovine hyaluronidase (Sigma Chemical Co.) per ml. The titers of phage A25 stocks were determined by the soft agar overlay method on N6 plates, as described by Cleary et al. (2).

The group C bacteriophage was propagated on strain 73-004 growing in Todd-Hewitt broth under the conditions described by Fischetti et al. (8). C1 phage was titrated by the soft agar overlay method, using Todd-Hewitt broth as the base medium.

Preparation of lysins. The A25 PAL was pre-

pared in the same way that the phage was propagated; 1 drop of a frozen stock culture of host strain K56 was inoculated into 5 ml of L3 broth. This culture was incubated overnight at 30°C, diluted 1:100 with prewarmed L3 broth, and infected with phage A25 at a multiplicity of infection of 1.0. After 4 h of incubation at 37°C, the remaining cell debris was removed by centrifugation at 10,000 rpm for 10 min. Lysin activity was stabilized by adding 3 mM 2-mercaptoethanol (Eastman). In the experiments indicated, A25 phage particles were separated from lysin by ultracentrifugation with a Beckman model L ultracentrifuge at 100,000 × *g* for 2 h.

The C1 PAL was prepared in a similar manner by infecting strain 73-004 in Todd-Hewitt broth at a multiplicity of infection of about 0.5 and incubating at 37°C for 3 h.

Preparation of substrates for testing lysin activity. Various strains of streptococci were used as substrates for the PAL, and cultures of these strains were prepared by inoculating L3 broth with a 1% inoculum of a frozen stock culture and incubating overnight at 30°C. Substrate cells were removed from the growth medium by low-speed centrifugation and then suspended in 0.1 M phosphate buffer (pH 6.7) containing 0.005 M EDTA and 3 mM 2-mercaptoethanol. Each cell suspension was adjusted to a turbidity at 620 nm of 0.45.

Routinely, chloroform-killed strain K56 cells were used as the substrate for the PAL. Before these cells were removed from L3 broth cultures, chloroform was added to a concentration of 5% (vol/vol). After 3 h at 37°C, the cell suspensions were separated from the chloroform by decanting, and then the cells were sedimented and suspended as described above.

For preparations of cell wall fragments, strain K56 cells were disrupted with a Mickle disintegrator and then treated with nucleases and trypsin as described by Cleary et al. (2). Portions of the cell wall fragments were also heated to 63°C for 30 min or treated with chloroform as described above for whole cells. Turbidity reduction was measured at 450 nm as described below for the lysin assay.

Assay for muralysin activity. Lysin activity was determined by measuring the turbidity reduction of substrate cell suspensions at 620 nm with a Coleman Junior II spectrophotometer. A 1.5-ml volume of substrate cells was mixed with 0.5 ml of a lysin preparation in a tube (10 by 75 mm). Lytic activity was expressed as the decrease in optical density per minute at 37°C. Routinely, lytic rates for undiluted A25 PAL were determined for the initial 10 min of cell lysis, during which the reaction was essentially linear. C1 PAL was diluted 1:50 with L3 broth to reduce the rate of lysis to a level comparable to that of A25 PAL. In cases where lytic rates were very low, linear rates of turbidity reduction were determined from measurements taken for up to 1 h.

Effects of inhibitors and antibiotics on muralytic activity. Inhibition of PAL by *p*-hydroxymercuribenzoate (pHMB) (Sigma) was determined at concentrations of up to 10^{-3} M pHMB. Stock solutions of this inhibitor were prepared in 0.1 M glycyl glycine buffer (pH 7.5) and incubated with unreduced PAL for 10 min before substrate K56 cells were added; the method was as described above for the standard lysin assay, except that 2-mercaptoethanol was deleted from the assay buffer. Inhibition was measured by the decrease in turbidity reduction compared with a control containing untreated lysin.

Inhibition of substrate lysis by cardiolipin (diphosphatidyl glycerol) was determined at concentrations of up to 190 μg/ml. For this assay strain K56 cell suspensions in lysin assay buffer were adjusted to an optical density at 620 nm of 0.40. Cardiolipin was obtained from Sigma as an ethanolic solution (5.57 mg/ml) from bovine hearts and was incubated at room temperature with the substrate cells for 30 min before lysin was added. Inhibition was measured by the decrease in turbidity reduction compared with an ethanol control.

The effects of chloramphenicol and benzylpenicillin on substrate susceptibility to the PAL were determined by adding one of these antibiotics to late-log-phase cultures and incubating the cultures for an additional 1 h at 37°C before the cells were sedimented and suspended in lysin assay buffer as described above. Chloramphenicol (Sigma) was dissolved in 95% ethanol and used in final concentrations ranging from 40 to 400 μg/ml. Benzylpenicillin (1,675 U/mg; Sigma) was used in final concentrations ranging from 0.5 to 50 μg/ml. Each change in lytic activity was compared with the lytic activity of substrate cells not exposed to either antibiotic.

Assay for LTA. The lipoteichoic acid (LTA) released by PAL was measured by the semiquantitative passive hemagglutination assay described by Sela et al. (27). LTA spontaneously adsorbs to erythrocytes (RBC) (20, 21). RBC sensitized by adsorption of LTA agglutinate when sera having anti-LTA activity are added. Sera with hemagglutinating activity were prepared by immunizing rabbits with heat-killed group A streptococci (M-type 76, T-type 12). The LTA used to confirm the anti-LTA activities of sera was prepared by phenol extraction from group A strain 78-011 (M-type 3), as described by Moskowitz (20). Lipids interfering with hemagglutination were extracted from antisera with fluorocarbon 114B2 (Du Pont Co.).

The release of RBC-sensitizing material (presumably LTA) from strain K56 cells by A25 PAL was measured at intervals during cell lysis. Cell lysates were heated briefly to 80°C to inactivate muralysin and streptolysin O activities before sensitization of RBC was attempted. Amounts of cell lysate ranging from 3 to 100 μl were used to sensitize 0.5-ml portions of 1.0% suspensions of washed RBC in phosphate-buffered saline (pH 7.0). After 30 min of incubation at 37°C, sensitized RBC were washed twice with phosphate-buffered saline and then resuspended in phosphate-buffered saline. A 50-μl sample of the washed sensitized RBC was mixed with 0.2 ml of phosphate-buffered saline containing fluorocarbon-extracted antiserum in a 1:100 dilution. The release of LTA from strain K56 cells at various times during lysis by A25 PAL was measured as the amount of cell lysate required to sensitize RBC to hemagglutination.

RESULTS

Our initial attempts to demonstrate an A25 PAL were inconclusive because the rate of turbidity reduction of substrate cell suspensions

was extremely low. To rule out the possibility that the observed rate of lysis was due to phage replication, substrate cells were treated in several ways. No lysis was detected in cell suspensions which had been heat killed or treated with chloramphenicol to prevent protein synthesis. However, cells killed with chloroform lysed at a rate significantly greater than untreated cells, and this treatment ruled out the possibility that cell lysis was due to replication of the A25 phage.

The rate of lysis was increased further by adding reducing agents to the chloroform-treated cells. The highest lytic rate for cells suspended in 0.1 M phosphate buffer was at pH 6.7. We also found that the media in which substrate cells were grown affected their susceptibility to A25 PAL. Of the media tested, cells grown in Todd-Hewitt broth had the lowest rate of lysis, and cells grown in L3 broth medium had the highest rate.

Under these optimal conditions for assay of lysin, no lytic activity was detected in culture supernatants from uninfected cells, in mechanically disrupted preparations of uninfected cells, or in the soluble supernatant fractions of uninfected mechanically disrupted cells.

After the conditions for producing A25 PAL and for demonstrating lysis had been improved and standardized, the substrate treatments described previously for the C1 PAL were examined systematically for A25 PAL. Maxted reported that both untreated and chloroform-treated cells were equally suitable as substrates for the group C PAL (18). Table 1 shows that for group A *Streptococcus* strain K56, the rate of lysis was significantly greater for chloroform-treated cells. As with the group C PAL, cells heated at 56°C had a reduced rate of lysis with the A25 PAL, and when the substrate was heated to 85°C, there was no detectable lysis.

Chloroform treatment of cells before heat treatment at 56°C resulted in a higher rate of lysis than heat treatment alone or heat treatment before chloroform treatment. These results suggest that chloroform treatment involves a modification of the cells more complex than simple killing.

Cell wall fragments prepared by mechanical disruption have been used as the substrate for several types of muralytic enzymes (1, 5, 9, 12, 29, 30). Using the turbidity reduction method, we could not demonstrate lysis of cell wall fragments by A25 PAL, even after chloroform treatment of cell wall fragments.

It has been shown that the group C PAL is present both free in the supernatant fluid (soluble) and bound to bacteriophage particles (9). Table 2 shows that the A25 PAL was free and that there was no detectable muralytic activity

TABLE 1. *Effect of various treatments on the lysis of strain K56 by the A25 PAL*

Substrate	Rate of lysis[a]	Relative rate[b]
Whole cells		
Untreated	0.002	1.0
Chloroform treated[c]	0.0047	2.4
Chloroform treated (inactive lysin)[c, d]	0	0
Heated[e]	0.0007	0.35
Heated, chloroform treated	0.001	0.5
Chloroform treated, heated	0.002	1.0
Cell wall fragments[f]		
Untreated	0	0
Chloroform treated[c]	0	0
Heated[e]	0	0

[a] Rate of turbidity reduction (optical density at 620 nm) per minute at 37°C.

[b] Rate of turbidity reduction compared with untreated cells.

[c] Cells were incubated at 37°C for 2 h with 5% chloroform.

[d] A25 PAL was heat inactivated at 60°C for 10 min.

[e] Cells were heated at 56°C for 1 h in growth medium L3.

[f] Cell wall fragments were prepared as described in the text, and turbidity reduction was measured at 450 nm.

TABLE 2. *Separation of A25 PAL from phage A25 by ultracentrifugation and determination of stability*

Fraction	Storage conditions	Phage titer (PFU/ml)	Lytic activity[a]
Whole lysate[b]	Fresh	7.0×10^{8}	1.8×10^{-3}
	4°C, 1 week	7.1×10^{8}	0
	−20°C, 1 month	6.7×10^{8}	2.0×10^{-3}
Supernatant	Fresh	7.8×10^{6}	2.1×10^{-3}
	4°C, 1 week	8.1×10^{6}	0
	−20°C, 1 month	6.5×10^{6}	2.1×10^{-3}
Sediment[c]	Fresh	1.7×10^{9}	0
	4°C, 1 week	1.2×10^{9}	0
	−20°C, 1 month	2.0×10^{9}	0

[a] Turbidity reduction (optical density at 620 nm) per minute with chloroform-treated, strain K56 cells as substrate.

[b] Lysates were obtained by propagation of phage A25 and were subjected to ultracentrifugation at 109,000 × *g* for 2 h.

[c] Suspended in 1/10 the original volume of L3 broth.

associated with phage A25 particles sedimented by centrifugation. Our inability to demonstrate muralytic activity does not rule out the possibility that an inactive form of muralysin or a very small amount of lysin was bound to the phage particles.

Whole phage lysates and sedimented phage particles after storage at 4°C for 1 week had unchanged phage titers but were without detectable muralytic activity (Table 2). Soluble A25 PAL also became inactive after storage

under the same conditions but did retain its activity after storage at −20°C for 1 month.

The A25 PAL has properties which suggest that it is an enzyme. Table 1 shows that heating the lysin for 10 min at 60°C resulted in the loss of muralytic activity. As has been reported for the C1 PAL, incubation with trypsin also results in a loss of muralytic activity (18). Figure 1 shows that there was a proportional relationship between the rate of substrate cell lysis and the A25 PAL concentration. The non-linearity of the rate curve suggests that measurable lysis was the results of a complex reaction.

Figure 2 confirms the finding of Doughty and Hayashi (7) that C1 PAL was inhibited by 10^{-5} M pHMB in experiments in which group A cells were employed as the substrate. Figure 2 also shows that a higher concentration (10^{-3} M) of pHMB was required for complete inactivation of the A25 PAL. These inactivating concentrations of pHMB for the two PAL closely resemble the relative concentrations of pHMB which Kessler and Krause found allowed attachment but prevented infection by bacteriophages C1 and A25 in their respective hosts (16).

Table 3 shows the results of a survey of various strains of streptococci for susceptibility to lysis by the A25 and C1 PAL. For this survey, the C1 lysin was diluted 1:50 with L3 broth immediately before the assay so that the observed rates of cell lysis would be comparable. Also, muralytic activity was determined at the optimum pH (pH 6.7) of the A25 lysin, rather than the reported optimum pH (pH 6.1) of the C1 lysin (1, 7). At pH 6.1 the C1 lysin would have been about 50% more active. Maxted (18) reported that suspensions of streptococcal strains belonging to groups A, C, and E were readily lysed by the C1 PAL, whereas suspensions of group H streptococci cleared more slowly. Table 3 shows that the A25 PAL was active on all strains of group A streptococci tested, on an A-variant strain, on both strains of group H tested, on at least one of two group C strains, and, to a small degree, on one group G strain. Single strains of groups B, D, and N streptococci were not lysed by the A25 PAL.

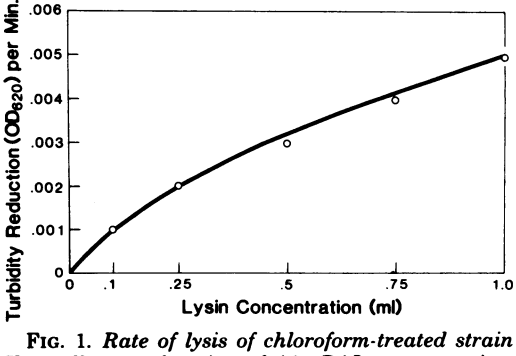

FIG. 1. *Rate of lysis of chloroform-treated strain K56 cells as a function of A25 PAL concentration. Lysis during the first 10 min of turbidity reduction was determined under the standard assay conditions described in the text. OD_{620}, Optical density at 620 nm.*

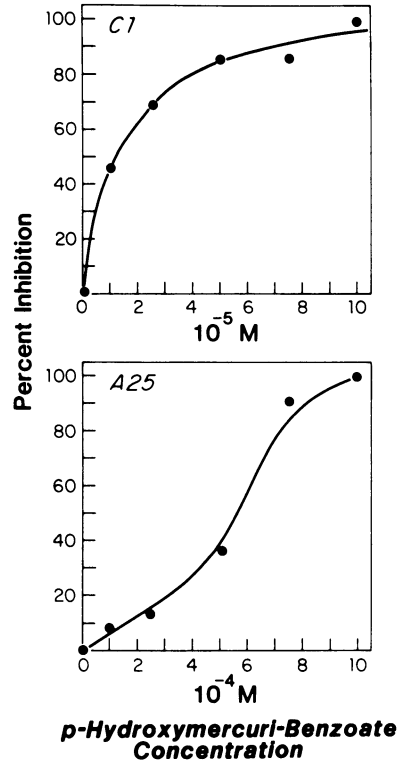

FIG. 2. *Inhibition of C1 PAL and A25 PAL as a function of pHMB concentration. Conditions were as described in the text.*

Table 3 also shows that for all group A strains examined and for all other strains, except one of the two group C strains, untreated cells were lysed by the C1 PAL at a significantly higher rate than chloroform-treated cells. On the other hand, the A25 PAL was more active on group A streptococcal cells that had been chloroform treated; for the other groups the lytic rates for chloroform-treated cocci were generally lower than the rates for untreated cells.

As reported in previous studies, the C1 PAL was more active on group A streptococci than on the strain of group C *Streptococcus* (strain 73-004) on which the C1 phage was propagated (7). In contrast, the A25 PAL was most active on strain K56, the group A *Streptococcus* used for the propagation of phage A25 (Table 3).

TABLE 3. *Survey of various strains of streptococci as substrates for group A and group C PAL*

Substrate cells			Relative rate of cell lysis[a]			
			A25 PAL		C1 PAL[b]	
Group	Type	Strain	Untreated[c]	Chloroform treated[c]	Untreated[c]	Chloroform treated[c]
A	12	GT8761 (K56)	1.0	2.4	2.5	2.0
A	12	79-194	0	0.9	2.0	1.0
A	6	GT9440smr	0.3	0.7	2.0	1.2
A	4	ATTC 10402	0.3	0.7	1.0	0.8
A-variant		GT6108	0.7	0.5	1.7	0.8
H		75-304	0.4	0.5	0	0
H	Challis	79-954	0.5	0.3	0.2	0
C		PF4603B	0.8	0.5	0.5	0
C		73-004	0.1	0	0.3	0.3
E		75-303	0	0	0.1	0
B		76-043B	0	0	0.1	0
G		74-447	0.2	0.1	0	0
D		75-335	0	0	0	0
N		75-323	0	0	0	0

[a] Relative rate of lysis compared with the activity of the A25 PAL against untreated strain K56 cells.
[b] Diluted 1:50 with L3 broth.
[c] Cell treatment.

Several types of muralytic enzymes are affected by antibiotic treatment of the bacterial cells serving as the substrate (7, 13, 14, 26, 28, 31-33). Table 4 shows that a relatively low concentration of penicillin (0.5 μg/ml) significantly increased strain K56 susceptibility to the A25 PAL, whereas no increase in susceptibility to the C1 PAL was detected at this concentration of penicillin. A 10-fold higher concentration of penicillin was required for a comparable increase in susceptibility to the C1 PAL. Strain K56 cells treated with 40 to 400 μg of chloramphenicol per ml became totally resistant to both the C1 PAL and the A25 PAL. The changes in susceptibility to the A25 PAL after treatment with either penicillin or chloramphenicol resembled the effects of these antibiotics on the autolytic systems of pneumococci and group D streptococci (28, 31, 32).

Independently, Shockman and collaborators (3-5, 15, 17, 28) and Tomasz and co-workers (4, 12-14, 31, 32) have reported that one effect of penicillin on a number of bacteria is that it causes the release of certain lipids and LTA. The release of these substances precedes cell lysis in those bacteria which have demonstrable autolytic systems (28, 32, 33). Group A streptococci do not lyse after penicillin treatment, and no autolytic systems have been detected in group A streptococci; however, penicillin treatment of group A streptococci is known to cause the release of certain phospholipids and LTA (13).

The effects of chloroform and penicillin treatments suggested the possibility that lipids could influence susceptibility of group A streptococci to the group A PAL. One of the lipids released by penicillin treatment of gram-positive bacteria has been identified as diphosphatidyl glycerol (cardiolipin) (3-5, 13). If cardiolipin is added to *Streptococcus faecalis* cells after penicillin treatment, the rate of cellular autolysis is greatly reduced. Figure 3 shows the effects of different concentrations of cardiolipin on the susceptibility of strain K56 cells to the A25 PAL. The data show that cardiolipin reduced susceptibility to the A25 PAL in untreated cells. Inhibition of lysis was more pronounced in cells that had been treated with either chloroform or penicillin. The greatest inhibition was found in cells treated with both agents.

LTA is thought to regulate autolytic systems either by interacting with autolysins or by interacting with the substrate of autolysins (28, 31, 32). Since penicillin treatment of cells increases susceptibility to the A25 lysin (Table 4) and it is

TABLE 4. *Effect of penicillin treatment on rate of lysis*

Penicillin concn (μg/ml)	Relative rate of lysis[a]	
	A25 PAL	C1 PAL
0	1.0	1.0
0.5	1.4	1.0
5.0	1.8	1.5
50.0	2.6	1.6

[a] Rate of lysis compared with the rate of lysis of untreated strain K56 cells.

known that penicillin treatment releases group A streptococcal LTA in the absence of lysis (13), we investigated the possibility that the release of LTA was a preliminary step in the lysis of cells by the A25 PAL. The release of LTA from strain K56 cells was measured by indirect passive hemagglutination. Table 5 shows that the release of LTA appeared to be a preliminary step in cell lysis, a finding which is compatible with observations that have been made in organisms with known autolytic systems.

DISCUSSION

Our data indicate the existence of a group A PAL which has some properties that set it apart from the group CPAL which has been described by others (1, 7-9, 18, 19, 35). A major difference between the A25 PAL and the C1 PAL is that group A streptococci appear to be at least 50 times more susceptible to the C1 PAL than to the A25 PAL. This may explain why the possible existence of a group A PAL was not investigated after Maxted (18) reported that some group A phage preparations cleared suspensions of group A cocci which were not infected by the phages in those preparations.

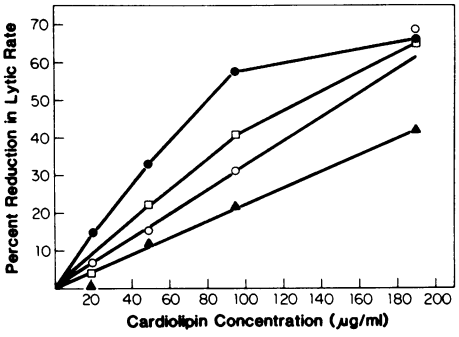

FIG. 3. *Reduction in the lytic rate of A25 PAL as a function of the cardiolipin concentration used to treat substrate cells. Before cardiolipin treatment, strain K56 cells were either not treated (▲) or treated with chloroform (○), penicillin (□), or penicillin plus chloroform (●). Details are described in the text.*

TABLE 5. *Relationship of turbidity reduction to release of LTA in group A cells treated with A25 PAL*

Treatment	Turbidity reduction (%)	Release of LTA with the following sensitizing amt of cell lysate:			
		100 μl	30 μl	10 μl	3 μl
A25 PAL					
1 min	0	+++[a]	+	−	−
15 min	21	+++	+++	+	−
30 min	42	+++	+++	++	+
45 min	55	+++	+++	++	+
60 min	63	+++	+++	++	+
75 min	67	+++	+++	+++	++
90 min	67	+++	+++	++	+
No lysin	0	−	−	−	−
Heated lysin	0	+	−	−	−

[a] Degree of passive hemagglutination compared with the degree of passive hemagglutination observed for purified LTA by the assay described in the text.

Both PAL are similarly affected by temperature, pH, reducing agents, and trypsinization. Comparable effects have been reported for other PAL and autolysins (1, 7, 8, 22-24, 29, 30). However, A25 PAL appears to have a more restricted activity than other lysins in that it has no apparent activity on cell wall fragments prepared by mechanical disruption. A possible explanation for this is that these fragments are similar to the product of A25 PAL activity on whole cells. This would be consistent with the observation that A25 phage progeny are released from infected cells by the splitting of cells along discrete lines, rather than through general lysis (25).

Susceptibility to inactivation by pHMB is shared by the C1 and A25 PAL; however, the level of pHMB which inactivated the A25 PAL (10^{-3} M) was much greater than the inactivating concentration for the C1 PAL (10^{-5} M) (7). The similarities between the respective pHMB concentrations that inactivate A25 PAL and C1 PAL and which prevent phage infection (16) suggest that these PAL could be involved in the initiation of phage infection. This is consistent with the finding that purified C1 particles have a muralytic activity (9); however, no muralytic activity was detected in association with infectious A25 particles after sedimentation by ultracentrifugation (Table 2). A possible explanation for this is that an A25 phage-bound PAL could be stabilized in an inactive form, since stored phage stocks remain infectious even after all detectable lytic activity is lost. Another possibility which has not been ruled out is that A25 phage particles are not directly lytic, but instead activate a latent autolytic system.

Maxted reported that untreated and chloroform-treated streptococcal strains belonging to groups A, C, E, and H were susceptible to a group C PAL (18). Table 3 shows that A25 PAL differs from C1 PAL in both group specificity and muralytic activity against chloroform-treated cells. Streptococci belonging to groups A, C, G, and H were susceptible to A25 PAL to various degrees. This range of susceptibility is consistent with the report that agar surface lawns of cocci belonging to serogroups A, C, G, H, and L were lysed by spot applications of phage A25 (6). One aspect of the specificity of A25 PAL is like the specificity of C1 PAL (7):

group A streptococci are more susceptible to both lysins than group C streptococci. However, within group A streptococci there appears to be considerable variation in the degree of susceptibility (Table 3).

Chloroform-treated group A streptococci were more susceptible to the A25 PAL than untreated cells, whereas the C1 PAL appeared to be more active against untreated streptococci. These results suggest the possibility that group A streptococci could have a chloroform-soluble component that blocks the A25 PAL. There is good evidence that several chloroform-soluble lipids are involved in the regulation of autolysis in pneumococci and some group D streptococci (3, 4, 13, 17, 28, 31, 32). Cardiolipin has been identified as one of the most potent inhibitors of autolysins. Figure 3 shows that strain K56 cells can be protected from A25 PAL by adding cardiolipin in the same way that *S. faecalis* has been protected from autolysins.

It has been established that penicillin kills some bacteria by inducing autolytic activity (28, 31, 32). The mechanism of induction seems to involve the release of certain lipids and LTA from the cell surface. Autolytic enzymes have not been identified in group A streptococci. Nevertheless, Horne et al. (13) have reported that although group A cocci do not lyse after penicillin treatment, there is an immediate and massive release of both old and newly synthesized lipids, which can also be extracted from cells with chloroform-methanol (2:1). Doughty and Hiyashi (7) reported that under certain conditions penicillin-treated cells were more susceptible to the C1 PAL. We found that whereas treatment with relatively high levels of penicillin increased susceptibility to the C1 PAL, only $\frac{1}{10}$ as much penicillin was required for a comparable increase in susceptibility to the A25 PAL (Table 4). The highest increase in penicillin-induced susceptibility was comparable to the increase obtained by chloroform treatment of the same streptococci. Adding cardiolipin to penicillin-treated cells significantly reduced susceptibility to the A25 PAL (Fig. 3).

Chloramphenicol has been shown to suppress the release of lipids and LTA from some bacteria, as well as reduce susceptibility to autolysins (24, 28, 31–33). In agreement with this, we found that strain K56 cells became resistant to both A25 PAL and C1 PAL after treatment with chloramphenicol. Reduced susceptibility to these lysins may involve an increase in cell wall material at forming cell wall septa as a consequence of chloroamphenicol treatment (10).

Our finding that chloroform-soluble lipids affected susceptibility to the A25 PAL suggested the possibility that LTA could also be involved in cell lysis. Using a passive hemagglutination assay for LTA, we found no evidence that LTA was released from streptococci by chloroform treatment; however, we did find that LTA seemed to be released from cells by the A25 PAL at the beginning of cellular lysis (Table 5). This result is consistent with the reported activity of autolytic systems and with the activity of a muralysin associated with the pneumococcal bacteriophage DP-1 (12, 26, 31, 32). Phage DP-1 cannot infect or be released from cells with a modified LTA, nor does phage DP-1 infect cells treated with chloramphenicol or amounts of LTA known to inhibit pneumococcal autolysins (26).

In recent studies in this laboratory, bacteriophage A25 has been propagated on a strain of group C *Streptococcus*. A lytic activity has been detected in this heterologous phage-host system, and attempts are currently in progress to determine whether this lytic activity is identical to the A25 PAL produced in group A streptococci. These studies may bear on the questions of whether A25 PAL is a phage product and whether A25 PAL is specified by the infected host bacteria. The latter possibility would be of special interest because autolytic systems have not been demonstrated in group A streptococci.

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