STUDIES OF THE RECEPTOR FOR PHAGE A25 IN GROUP A STREPTOCOCCI: THE ROLE OF PEPTIDOGLYCAN IN REVERSIBLE ADSORPTION*

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The first step in the life cycle of a bacteriophage is its attachment to an outer surface of the bacterial cell. In gram-positive bacteria the cell wall peptidoglycan, or this molecule complexed with other macromolecules, often serves as the phage receptor (1). Previous reports have suggested that the group-specific carbohydrate may play a role as receptor site for the virulent group A streptococcal phage, A25. The inhibition of adsorption by group A antisera and the inability of group A variant cells to adsorb phage A25, provided indirect evidence supporting this view (2). Direct evidence, the isolation and characterization of the receptor site, was not obtained because heat-killed or mechanically disrupted cells failed to inactivate phage particles irreversibly, even though the potential to adsorb this phage reversibly was retained (2, 3).

Because it is now clear that phage A25 can infect and transduce antibiotic resistance between different groups of streptococci, such as groups C and G, it seemed essential to identify the phage A25 receptor in group A organisms (4–6). In contrast to earlier reports, the findings reported here demonstrate the irreversible attachment of phage A25 to heat-killed group A, G, and A-variant cells. The complexity of the adsorption process is exemplified by the fact that mechanical disruption of heat-killed cells completely destroyed the irreversible nature of this process. Receptors for the reversible attachment of phage A25 survive mechanical disruption, and experiments including electron microscopy are described which demonstrate the specificity of reversible adsorption and identify the responsible receptor as peptidoglycan. The evidence suggests that adsorption of phage A25 to group A streptococci occurs in a two-step reaction, the first step is the reversible attachment of the phage tail to peptidoglycan exposed on the cell surface, followed by the irreversible inactivation of the phage particle. The latter is still not understood.

Materials and Methods

Bacterial Strains and Bacteriophages. The virulent phage designated A25 was originally described and kindly supplied by Dr. W. R. Maxted (7, 8). This virulent phage was used for all adsorption experiments and has been further described by Malke (5) and Read and Reed (9).

The group A streptococcus used for a majority of the adsorption experiments was strain K56 (T-

* This work was supported by a research grant from the U S. Public Health Service (AI 08724) Dr Wannamaker is a Career Investigator of the American Heart Association

type 12, M-12) obtained from Dr. E. Kjems (10). Other strains tested were: strain 71-568, a group G streptococcus originally isolated at Red Lake, Minn.; strain 70-996, a group B streptococcus isolated from the wound of a patient at University of Minnesota Hospitals; strains B346/94/3, D315/87/1, 6108, D336/56/1, T11/137/1, group A-variant streptococci provided by Dr. R. Lancefield (11, 12); the pair of strains B349 (group A streptococcus) and B346/94/1 (group A-variant streptococcus) furnished by Dr. V. Fischetti (2); and strain 486, group A when grown at 30°C and group A-variant when grown at 37°C, supplied by Dr. E. Ayoub (13). The propagating strain for phage A25 was 9440, a group A streptomycin-resistant streptococcus (6).

The strains of streptococci used in this study, including variants, were grouped by the method of Lancefield (14), classified by the T agglutination method of Griffith (15, 16) and the M precipitin method of Lancefield (14, 16) or the immunodiffusion method described by Rotta et al. (17).

Media. The two basic broth media used in adsorption experiments were No. 1 broth and No. 3 broth, both described previously (6). No. 3 broth contained hyaluronidase (Sigma Chemical Co., St Louis, Mo), 40 μg/ml, and was used for the propagation of phage A25, as reported earlier (6). No. 1 broth was used as growth and adsorption medium for all test strains with the exception that the NaCl concentration was increased to 0.4 M for the growth of group G strains to be used in adsorption experiments and for the adsorption of these strains.

A second adsorption medium consisted of modified Weigle's buffer (MWB)[1] containing 0.01 M $MgSO_4$, 0.006 M Tris-HCl at pH 7.4, 0.4% NaCl, 0 02% $CaCl_2$, and 0.005% gelatin. The buffer was prepared and autoclaved without $CaCl_2$ which was autoclaved separately in a concentrated solution and added to the sterile buffer. Gelatin was omitted from the buffer when adsorption mixtures were to be examined with the electron microscope.

N6 agar, used to assay plaque-forming units (PFU), consisted of proteose peptone No. 3 (Difco Laboratories, Detroit, Mich.) 4%, yeast extract (Difco Laboratories) 2%, agar (Difco Laboratories) 1%, NaCl 0.6%, Na_2HPO_4 0.07%, $CaCl_2$ 0.02%, glucose 0.05%, and hyaluronidase (Sigma Chemical Co.) at a final concentration of 68 μg/ml. Separately autoclaved solutions of $CaCl_2$, glucose, and concentrated hyaluronidase solution which had been sterilized by filtration were added to sterile basal medium. Soft agar for pour plates containing 0.7% agar in N6 broth was also used

Preparation and Assay of Phage Lysates. Lysates containing phage A25 were prepared on strain 9440 grown in No. 3 broth as previously described (6) For plaque assays, strain 9440 was inoculated in No 1 broth and incubated 18 h at 30°C. 5-ml cultures were treated 15 min at 37°C with 3.4 mg of hyaluronidase. Samples, 0.1 ml, of appropriately diluted phage were added to 0.1 ml of indicator culture, and the mixture was incubated 15 min for adsorption to take place. After the addition of 2.5 ml soft agar to the mixture, the entire contents were poured onto the surface of a warmed N6 agar plate. Plates were incubated 18 h at 34°C before enumerating PFU.

Adsorption Assays. Irreversible adsorption assays measured the inactivation of phage by streptococcal cells without sedimentation of the bacterial-phage mixtures Overnight cultures grown at 34°C were killed by incubation at 56°C for 30 min. Diluted lysate, 1×10^5 PFU in 0.1 ml of broth or buffer, was added to 0.9 ml of the heat-killed cells or 0.9 ml of broth for controls. The adsorption mixtures were incubated at 37°C and 0.05-ml samples removed at intervals. To stop further adsorption, the samples were diluted 1:100 in chilled broth. Samples of this dilution were assayed for PFU by agar pour plates using strain 9440 as described. Reversible adsorption (RA) was assayed in the same manner with the exception that samples were centrifuged at 10,000 rpm for 10 min before assaying PFU in the supernate.

Cell Wall Preparation Overnight cultures in 100 ml of No. 1 broth were sedimented and suspended in 10 ml of MWB. Cells were disrupted by agitation with an equal vol of 0 015 inch diameter glass beads in a Mickle disintegrator or Vibrogen cell mill for 30 min. The broken cell debris was removed from the beads and suspended in 100 ml of 0 06 M phosphate buffer, pH 7.3, containing 10^{-3} M $MgCl_2$. The cell fragments were then digested with DNase and RNase (1 μg/ml) at 37°C for 30 min. After centrifugation the sediment was washed, first with phosphate buffer pH 7.3, containing 0.85% NaCl and then with sterile distilled water. After the washes, the sediment was suspended in 30 ml of 0.06 M phosphate buffer, pH 8.0, containing 0.2% trypsin (Difco Laboratories, 1.250). This mixture was shaken for 5 h at 37°C and then incubated 18 h at 4°C

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[1] *Abbreviations used in this paper:* IRA, irreversible adsorption, MWB, modified Weigle's buffer; PFU, plaque-forming units, RA, reversible adsorption; WB, Weigle's buffer

Trypsin-digested walls were washed twice with sterile water and once with MWB before they were finally suspended in 5 ml of this buffer for storage at 4°C.

Peptidoglycan Preparation. Peptidoglycan was prepared from cell walls (18) extracted with formamide as described by earlier workers (19, 20). Both the formamide residues and ethanol-precipitated peptidoglycans were tested for adsorptive properties. No difference was observed between the residue and the ethanolic precipitate with respect to residual rhamnose or phage adsorption activity. However, based on optical density measurements at 280 nm, the insoluble residue appeared to be heavily contaminated with protein. For this reason, the residue was separated from the formamide before the addition of acidified ethanol to the supernate and except where otherwise noted, adsorption experiments made use of the ethanol-precipitated peptidoglycan. The resulting precipitate was washed and finally suspended in distilled water. Lyophilization completely destroyed the receptor activity of peptidoglycan; therefore, all preparations were stored suspended in distilled water at 4°C. The residue and the ethanolic precipitate were resuspended in MWB for adsorption studies. Some preparations were repeatedly extracted with hot formamide as follows: the ethanolic precipitate from the first extraction was resuspended in 100 ml of formamide, extracted for 45 min at 165°C, and again the peptidoglycan was precipitated by the addition of acid ethanol. This process was repeated as many as four times.

Protoplasts were prepared by incubating washed, heat-killed streptococci with reduced phage lysin in a 7% NaCl solution for 30 min as described by Gooder and Maxted (21)

Preparation of Phage-Associated Lysin. Phage-associated lysin was prepared from group C streptococci infected with phage C1 by the method of Fischetti et al. (22).

Electron Microscopy Adsorption mixtures contained 0 1 ml of peptidoglycan at the indicated concentration suspended in MWB and phage A25, 5×10^9 to 5×10^{10} PFU, in 0 9 ml Weigle's buffer (WB). After a 30-min adsorption period at 37°C the mixture was sedimented by centrifugation at 10,000 rpm for 10 min, and the pellet was gently resuspended in 0.2 ml WB. This suspension was immediately fixed on grids for electron microscopic observation.

A drop of suspended mixture was placed on copper grids covered with a formvar film coated with carbon that had been exposed to a glow discharge for 2–3 min to facilitate spreading. After 3 min the excess fluid was removed with filter paper. A drop of 2% phosphotungstic acid adjusted to pH 7.0 with 1 N KOH was immediately added to the grid and wicked off after 15 s. Electron micrographs were taken with a Siemens Elmiskop I electron microscope at an accelerating voltage of 80 kV

Chemical Assays. Rhamnose was assayed by the method of Dische and Shettles (23). Total phosphorus was determined according to the Lowry method (24). Amino acid analysis was performed on a Beckman Model 120B amino acid analyzer. Weighed samples of peptidoglycan were hydrolyzed in 4 N HCl at 110°C for 18 h. Authentic samples of muramic acid and *N*-acetylglucosamine were treated in the same manner as peptidoglycan. The yields from authentic samples were used to correct for losses incurred during the hydrolysis of peptidoglycan.

Results

Irreversible Adsorption of Phage A25 to Heat-Killed Cells. The first step toward identification of the A25 phage receptor was the demonstration of adsorption to heat-killed streptococcal cells. Incubation for 30 min at 56°C proved adequate to sterilize an 18-h culture of strain K56 with the preservation of receptor activity. Phage particles were irreversibly inactivated by heat-killed cells at a rate somewhat slower than that of live cells, rate constants of $K = 1.5 \times 10^{-10}$/min and $K = 1.7 \times 10^{-10}$/min, respectively (Fig. 1). Reduction of the incubation temperature to 4°C during adsorption decreased the rate of adsorption. The temperature-sensitive nature of the irreversible process was consistent with the possibility that inactivation of phage A25 required an enzymatic reaction.

If irreversible adsorption (IRA), as measured in our system (see Materials and Methods), is a precursor to phage infection, then the specificity of IRA should be

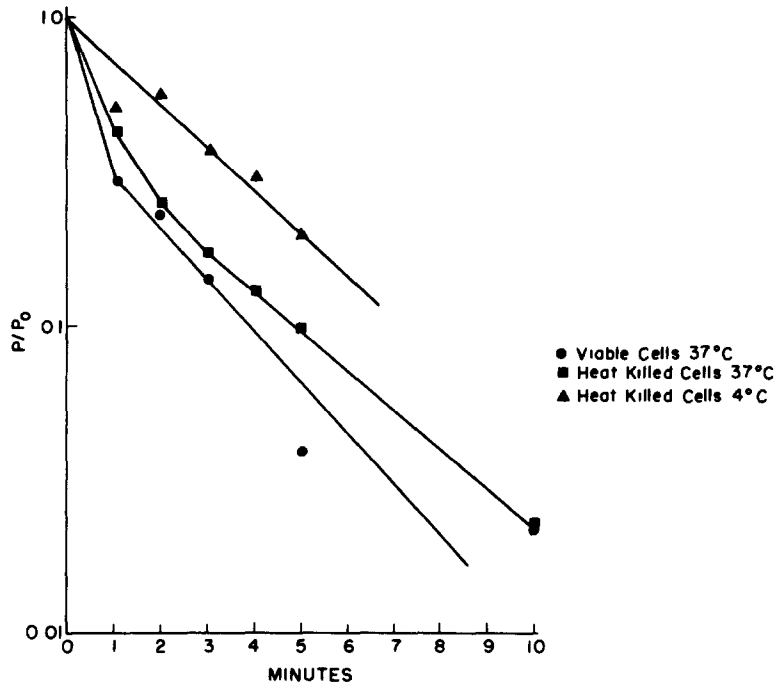

FIG. 1 Comparison of adsorption kinetics of heat-killed cells to viable cells at 37°C and 4°C. The experiment was carried out as described in the Materials and Methods section with the exception that diluted samples were vortexed 10 s with chloroform to prevent the formation of bacterial infectious centers

reflected by the host range of phage A25. As expected, both *Staphylococcus aureus* and *Sarcina lutea* cells failed to adsorb the phage (Table I). Likewise, phage A25 did not adsorb to group B streptococci, strain 70-996, nor did it form plaques on lawns of this culture. Heat-killed cells from strain K56, strain 71-568, and strain $T25_3$ readily inactivated phage particles. Although A25 particles were irreversibly inactivated by group G cells, the plaque-forming efficiency of this culture was reduced 100-fold when compared to K56 cells. Phage A25 could be easily adapted to these cells with an increased plating efficiency, suggesting that the originally reduced plating efficiency was due to a DNA restriction modification system (unpublished data). Overall the host range of phage A25 reflected the potential of the phage to adsorb irreversibly.

Digestion of heat-killed cells with hyaluronidase or the proteolytic enzymes trypsin, pepsin, and pronase did not alter their ability to adsorb phage, whereas treatment of these cells with phage lysin destroyed 99% of the receptor activity. The susceptibility of phage receptors to phage lysin suggested that intact cells and/or cell walls were required for adsorption.

Reversible Adsorption of Phage A25. Initial attempts to isolate the phage A25 receptor from K56 cells were thwarted by the instability of IRA to mechanical disruption of the cells. Disruption of heat-killed cells with a Braun homogenizer, Mickle disintegrator, or Vibrogen cell mill completely destroyed their ability to inactivate phage particles irreversibly. Variations in the suspending

TABLE I
Adsorption of Phage A25 to Heat-Killed Cells of Various Bacterial Strains and Efficiency of Plating on These Strains

Strain or species	Streptococcal group	Percent irreversible adsorption*	Efficiency of plating‡
K56	A	99 0	1.00
$T25_3$	A	96.4	ND
71-568	G	96.5	$7\ 7 \times 10^{-3}$
70-996	B	4 2	$2\ 0 \times 10^{-7}$
S. aureus	–	0	ND
Sarcina lutea	–	0	ND

ND, not done.

* The percent irreversible adsorption is equal to the number of PFU inactivated divided by the total number of PFU added to the adsorption mixture, approximately 5×10^6 PFU; this fraction multiplied by 100. IRA and RA are defined in the Materials and Methods section. The incubation is 30 min.

‡ The efficiency of plating is equal to the number of PFU on the test culture lawn divided by the total PFU added to the lawn, which is determined on the phage sensitive lawn, strain K56

media and periods of disruption were unsuccessful in preserving receptor activity. Although disruption completely destroyed IRA activity, cell fragments retained the potential to adsorb phage A25 reversibly. RA was assayed under the same conditions as IRA, except the cells or cell fragments with attached phage were removed by low speed centrifugation before assaying PFU in the supernate. As indicated in Table II, cell fragments or isolated cell walls prepared from disrupted cells of strain K56, 6108, and 71-568 reversibly adsorbed A25 particles. To examine the possibility that cell fragments were nonspecifically trapping phage particles, the specificities of RA and IRA were compared. Neither *Staphylococcus aureus, Sarcina lutea,* nor group B streptococci exhibited RA or IRA activity (Tables I and II). The group B cell walls, although usually completely devoid of RA activity, were somewhat variable and occasionally reversibly bound as much as 23% of the added phage particles (Table II).

The reversible nature of adsorption to cell fragments was verified by resuspending cell fragments and attached phage particles in buffer after their sedimentation. This resulted in the elution of approximately 50% of the attached phage with each resuspension (Table III). In contrast, very few PFU could be eluted from the whole heat-killed cells.

Isolation of the Phage A25 Receptor. From the preceding experiments a reversible reaction was postulated to be a requirement for irreversible adsorption of phage A25 to a somatic component of the streptococcal cell. For this reason subsequent experiments were directed toward the isolation and purification of the receptor responsible for this reaction. Peptidoglycan was isolated from group A cells (K56) and from group B cells (strain 70-996). Isolated cell walls were treated with hot formamide, and the peptidoglycan was precipitated from the formamide-water phase by the addition of acid ethanol. Three or more separate preparations of peptidoglycan from both strains have been tested for receptor activity, and representative results are shown in Table IV. The specificity of RA was preserved in the peptidoglycan that was relatively free from group

TABLE II
Irreversible and Reversible Adsorption of A25 to Mickle-Disrupted Cells and Trypsinized Cell Walls

Strain or species	Streptococcal group	Percent IRA to Mickle-disrupted cells*	Percent RA to Mickle-disrupted cells	Percent RA to trypsinized cell walls
K56	A	0	99.0	97.5
6108	A-variant	0	96.9	99.0
71-568	G	0	98.7	97 0
70-996	B	0	0	23.0
S. aureus	–	0	0	ND
Sarcina lutea	–	0	0	ND

ND, not done.
* See Table I and Materials and Methods section for the definition of terms. Cell fragments and cell walls were suspended in WB at the original cell concentration, approximately 5×10^8 colony-forming units (CFU)/ml

TABLE III
Elution of Reversibly Adsorbed A25 Phage Particles

Group A streptococcus strain K56	PFU/ml added	Total PFU adsorbed	Fraction of adsorbed phage eluted	
			Wash I	Wash II
Fragments from Mickle-disrupted heat-killed cells	2.28×10^5	$1\ 53 \times 10^5$	5.0×10^{-1}	5.1×10^{-1}
Heat-killed cells	2.28×10^5	$2\ 25 \times 10^5$	$8\ 0 \times 10^{-4}$	2.0×10^{-4}

Heat-killed cells and cell fragments in WB are incubated 30 min at 37°C with phage particles. Bacterial material with adherent phage is then sedimented by centrifugation, and the supernate is assayed for PFU The sediment is resuspended in MWB by gentle vortexing after which time the bacterial phage mixture is again sedimented and the supernate assayed for PFU. This process is repeated once more for the final elution test.

TABLE IV
Reversible Adsorption to Various Cell Preparations of Group A and Group B Streptococci

Preparation	Percent reversible adsorption	
	Group A (K56)	Group B (70-996)
Whole heat-killed cells*	99.2	0
Mickled cells	97.2	0
Trypsinized cell walls	96 8	0
Peptidoglycan	93.4	7.7
Protoplasts	0	ND

ND, not done
* Whole cells, mickled cells, and cell walls are suspended in MWB in amounts equivalent to the original concentration, 5×10^8 CFU/ml. The K56 peptidoglycan (2.2 mg/ml) and strain 70-996, group B peptidoglycan (3.8 mg/ml) contained 5 and 2.7% rhamnose, respectively Both were suspended in MWB. Protoplasts were suspended in MWB with 7% NaCl. Preparation of the cell fractions is described in the Materials and Methods section.

A carbohydrate. The preparations obtained from strains K56 and 70-996 which were used in this experiment (Table IV) contained 5.0 and 2.6% rhamnose, respectively. Mickled cell fragments, cell walls, and peptidoglycans from K56 cells all exhibited RA. The same cell fractions from strain 70-996 were devoid of this activity. Protoplasts, obtained from strain K56 by phage lysin digestion of whole cells and preserved in 7% sodium chloride, were unable to adsorb phage A25 reversibly (Table IV). Controls indicated that this sodium chloride concentration did not interfere with adsorption. The peptidoglycan extracted from K56 cells retained RA activity, but was not completely free of rhamnose and the teichoic acid content was unknown. For this reason, peptidoglycan was again prepared from K56 cells and extensively extracted with hot formamide and trichloroacetic acid. Peptidoglycan extracted four times with hot formamide retained receptor activity. The series of extractions removed 96 and 98% of the rhamnose and phosphorus, respectively, that was originally associated with the cell walls (Table V). This indicated that there was little or no remaining group carbohydrate or teichoic acid after the fourth extraction.

Trichloroacetic acid extraction of K56 peptidoglycan further established that teichoic acid did not constitute part of the RA receptor (Table VI). After three trichloroacetic acid extractions of the peptidoglycan, 99.8% of the cell wall phosphorus had been removed; yet, the peptidoglycan retained most of its original adsorptive capacity. Thus, it was clear that peptidoglycan, free of teichoic acid and group A carbohydrates, could reversibly adsorb phage A25.

To confirm that the receptor material was indeed peptidoglycan the amino acid content of a purified preparation was examined. Amino acid analysis of the most nearly pure preparation of peptidoglycan, 4× formamide extracted, revealed the expected amino acid composition; muramic acid, glucosamine, alanine, glutamic, lysine, in the following ratios: 1:1:5:1:1 (Table VII). When corrected for losses of muramic acid and glucosamine during the hydrolysis, the ratio was 1:1:2:1:1. Thus, the amino acid content of our peptidoglycan was similar to that previously reported (20, 26).

The Sensitivity of Phage Receptors to Muralytic Enzymes. Phage lysin, an amidase that attacks the alanine-muramic acid linkage of peptidoglycan (27), significantly reduced the receptor activity of K56 cells and cell fragments (Table VIII). The reduction in receptor activity was more striking with the mickled cell fragments than the intact group A cells. Because the enzyme was a crude unpurified preparation, it was necessary to test this activity on group G cells, strain 71-568, known to be resistant to the endopeptidase activity of phage lysin, yet able to adsorb phage A25 (unpublished data). Group G cells and their fragments reversibly adsorbed phage A25, but the receptor activity of the group G preparations (both whole cells and mickled-disrupted cells) proved much more resistant to phage lysin (Table VIII). Thus, the muralytic activity in the phage lysin preparation was responsible for the destruction of receptor activity of K56 cells and cell fragments.

Intact group A streptococci are known to be refractive to egg white lysozyme. However, formamide-extracted cells or cell walls are partially sensitized to this enzyme (20, 28). Although incubation of K56 cells or purified peptidoglycan suspensions for 18 h in the presence of 1 mg/ml lysozyme had little effect on the

TABLE V

Residual Adsorption, Rhamnose, and Phosphorus in Formamide-Extracted Peptidoglycan Preparations From Group A Streptococcus (Strain K56)

Cell fraction	Percent adsorption per mg*		Rhamnose		Phosphorus	
	Reversible	Irreversible	μmol/mg	Percent residual‡	μmol/mg	Percent residual
Cell walls	60 5	ND	0 74	100 0	3 85	100 0
Peptidoglycan, 1st extraction	51 3	5 8	0 38	51 0	1 25	32 4
Peptidoglycan, 2nd extraction	69 7	2 5	0 16	21 3	0 55	14 3
Peptidoglycan, 3rd extraction	63 8	6 5	0 08	10 6	0 15	3 9
Peptidoglycan, 4th extraction	46 3	12 8	0 03	4 2	0 09	2 3

* See Table I and Materials and Methods section for definition of terms In this table the percent adsorption has been normalized to dry weight (the percent adsorption divided by the milligram dry weight of the cell fraction in the adsorption mixture)

‡ The percent residual is equal to the original concentration of rhamnose or phosphorus in the cell walls divided by the remaining concentration of rhamnose or phosphorus after extraction this fraction multiplied by 100 Rhamnose made up 1% by dry weight of the peptidoglycan (four times extracted)

TABLE VI

Residual Adsorption and Phosphorus in Trichloroacetic (TCA) Extracted Peptidoglycan

TCA-extracted peptidoglycan	Percent reversible adsorption per mg*	Phosphorus‡	
		μmol per mg	Percent residual
No extraction	85.0	0.25	6.4
1st extraction	85.0	0.02	0.5
2nd extraction	58.0	0.01	0.2
3rd extraction	69 0	0.01	0.2

* See footnote in Table V.

‡ The peptidoglycan used in this experiment has been extracted three times with hot formamide; therefore, it retained only 6.4% of the phosphorus originally present in the cell wall. The percent residual phosphorus is equal to the micromoles of phosphorus retained after the TCA extraction divided by the micromoles of phosphorus present originally in the cell walls The extraction procedure is that described by Matsuno and Slade (25).

TABLE VII

Amino Acid Composition of Purified Peptidoglycan

Amino acid	Total nmol	Molar ratio	Corrected molar ratio*
Muramic	33	1	1
Glucosamine	48	1.4	1 6
Alanine	183	5.5	2.1
Glutamic	51	1.5	0.6
Lysine	47	1.4	0 5
Glycine	6	0 2	0.1

* Other amino acids accounted for 2% of the total dry weight. The molar ratios are corrected for losses of muramic acids and glucosamine during the hydrolysis.

TABLE VIII
Effect of Bacteriolytic Enzymes on Receptor Activity

Cells or cell fraction*	Group	Enzyme‡	Percent reversible adsorption		Percent inhibition
			Without enzyme	With enzyme	
K56 cells	A	Phage lysin	99.7	65 5	34 3
K56 mickled cells	A	Phage lysin	82.5	23 4	71 7
71-568 cells	G	Phage lysin	81.0	67 0	17.3
71-568 mickled cells	G	Phage lysin	65.0	58.0	10.8
K56 cells	A	Lysozyme	92.6	Variable	Variable
K56 peptidoglycan	A	Lysozyme	57.0	7.5	86.8

* See Table V

‡ Phage lysin, 0.2 ml as prepared by Fischetti et al. (22), is added to 5 ml of heat-killed cells or mickled cell fragments suspended in MWB. The mixture is incubated at 37°C for 1 h, washed free of enzyme, and suspended in MWB at the original concentration. For the lysozyme digestion, cells and peptidoglycan are suspended in phosphate buffer containing 1 μg/ml egg white lysozyme and incubated 18 h at 37°C Digested material is washed and resuspended in MWB. Adsorption is assayed as described in the Materials and Methods section.

optical density of these suspensions, reversible adsorption to peptidoglycan was severely impaired (Table VIII). Since infection requires the initial attachment of phage A25 to peptidoglycan, then whole cells may have patches of exposed peptidoglycan on their surfaces which may be vulnerable to the lytic action of lysozyme. Repeated experiments testing the lysozyme sensitivity of receptor activity on intact cells were variable. In three different experiments lysozyme destroyed 24, 43.2, and 100% of the RA receptor activity. Although our conclusion must be considered tentative, these results suggested that the RA receptor was composed of lysozyme-sensitive bonds and that these bonds were exposed on the surface of the streptococcal cells.

Electron Micrographic Observations of Phage Peptidoglycan Complexes. As final confirmation that cosedimentation of A25 particles with peptidoglycan was a specific interaction, as opposed to nonspecific trapping, peptidoglycan with adherent phage particles was sedimented and negatively stained for electron microscopic observation. Two K56 peptidoglycan preparations were examined. The pure preparation, 4% residual rhamnose (4× formamide extracted, Table V), is shown without added phage particles in Fig. 2 A. The peptidoglycan appeared as homogeneous, translucent broken spheres, very similar to the original pictures published by Krause and McCarty (20). When phage was added to the preparations that had been twice extracted (21% residual rhamnose), many phage particles were seen adhering tail first to the peptidoglycan spheres (Fig. 2 B). The most nearly pure peptidoglycan spheres (four times extracted, 4% residual rhamnose) were also seen with phage particles bound to their surface; however, the number of phage particles per sphere was reduced (Fig. 2 C). This reduction was also reflected by the biological assays of adsorption (Table V) and probably reflected damage due to the harsh treatment inflicted by four formamide extractions.

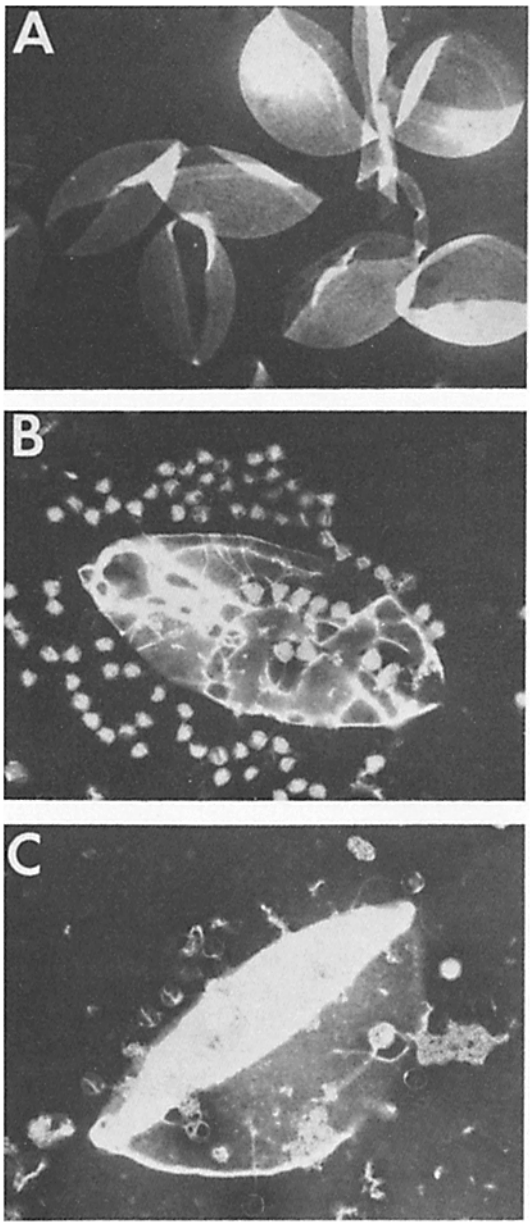

FIG 2 Electron micrographs of peptidoglycan phage A25 mixtures. (A) peptidoglycan extracted 4× with hot formamide (20,000 ×); (B) peptidoglycan extracted 2× with hot formamide mixed with phage A25 (40,000 ×); (C) peptidoglycan extracted 4× with hot formamide mixed with phage A25 (25,000 ×).

Fischetti and Zabriskie presented evidence implying that group A carbohydrate participated in the adsorption of phage A25 to viable cells (2). Although our data indicated that this somatic antigen was not essential for reversible adsorption to peptidoglycan, experiments were performed to examine its possi-

TABLE IX
Adsorption to Group A-Variant Strains

Strain	Group	Percent irreversible adsorption	Percent reversible adsorption
K56	A	98.7	98.2
B346	A	98.2	99.6
6108	AV	99.0	ND
B346/94/3	AV	99.5	99.5
D315/87/1	AV	99.4	98 7
D336/56/1	AV	8.0	15 4
T11/137/1	AV	11 7	36.5
B346/94/1	AV	19.4	15 5
486 (22°C)	A	99.2	97.8
486 (37°C)	AV	99.6	99.6

ND, not done.

ble function in the irreversible attachment of phage A25 to heat-killed cells. Six strains that synthesize A-variant carbohydrate were analyzed for IRA activity (Table IX). Before adsorption tests all cultures were grouped and all proved to be either A or A-variant cells as expected. Three A-variant strains, 6108, B346/94/3, and D315/87/1, had IRA activity equivalent to the group A strains K56 and B346. Three other A-variant strains, D336/56/1, T11/137/1, and B346/94/1, exhibited considerably less activity. With regard to strains B346 and B346/94/1, these results were identical to those previously reported (2). Much to our surprise, however, strain B346/94/3 and strain B346/94/1, which differed only in the number of passages each culture had received in laboratory media subsequent to mouse passage (Fischetti, personal communication), showed strikingly different capacities for adsorbing phage A25. The inability of strain B346/94/1 to adsorb phage A25 must depend on some factor other than the A-variant nature of the group carbohydrate, and this factor must have been lost during growth on culture media.

One additional experiment made use of an unusual strain reported by Ayoub and Dudding, strain 486; A-variant carbohydrate is synthesized by this strain when grown at 37°C, however, when grown at 30°C this strain forms a group A carbohydrate (13). This characteristic was verified in our laboratory, and cells grown at both temperatures were tested for the potential to inactivate A25 particles. As shown in Table IX, cells grown at both temperatures were equally capable of irreversible adsorption. This experiment and the lack of correlation between variation in the group A carbohydrate and sensitivity to phage A25 supports the view that this polysaccharide does not participate in the adsorption of phage A25 to streptococcal cells.

Discussion

The integrity of the bacterial cell wall is dependent on peptidoglycan, which comprises 50–90% of the dry weight of walls isolated from gram-positive cells (29). From these organisms the peptidoglycan is often found to be complexed with polymers of teichoic acid, carbohydrates, and protein which can impart

immunological specificity, and in some organisms serve as the receptor for infecting bacteriophage (1, 29).

A macromolecular complex composed of teichoic acid covalently linked to peptidoglycan has been found to inactivate various staphylococcal phages, reversibly or irreversibly (30, 31). Chemical or enzymatic hydrolysis of this structure in most cases abolished its phage-inactivating capacity (1). Although part of the receptor, purified teichoic acid alone is regularly without receptor activity (1, 31, 32). Thus, the configuration and the chemical composition of the peptidoglycan-teichoic acid complex are important determinants of the bacteriophage receptors in *S. aureus*.

Conclusive data regarding streptococcal bacteriophage receptors are limited. The group-specific carbohydrate, covalently linked to peptidoglycan and the basis for serological grouping of most streptococci, has been thought to form all or part of the receptor for certain group A and group C streptococcal phages (2, 3, 33). Inhibition of adsorption of phage C1 to group C streptococci by group C-specific antisera and the irreversible inactivation of this phage by enzymatically prepared group C carbohydrate has been considered that the receptor is composed of this carbohydrate (2, 3). This interpretation is debatable for two reasons: first, antibody bound to carbohydrate could sterically hinder adsorption by masking adjacent or underlying receptors; secondly, chemically prepared group C carbohydrate, free of peptidoglycan was devoid of receptor activity, suggesting that if group C carbohydrate is involved at all, a peptidoglycan-carbohydrate complex must be the true receptor (2, 3). Similar results were also obtained for a group A temperate phage, B940. Again enzymatically prepared carbohydrate exhibited activity, but when freed of peptidoglycan by formamide extraction it lost the potential to inactivate phage B940 (2).

Previous attempts to identify the receptor for virulent group A bacteriophages A25, A6, and A12 were foiled by the fact that heat-killed or mechanically disrupted cells were completely devoid of receptor activity (2, 3). Indirect evidence provided by this earlier study suggested that group A carbohydrate was required for adsorption of phage A25. The authors of this report showed that group A antibody inhibited adsorption of phage A25 to group A cells, and that with the conversion by mouse passage of a group A strain to an A-variant strain, phage receptors were either lost or became unreceptive to the phage (2). Zabriskie et al. suggested that the conversion of the A-variant phenotype was due to a temperate phage presumably acquired during animal passage (27). Aside from altering somatic antigens, the acquisition of a prophage can also change the adsorptive capacity of a cell (1). The above changes in group carbohydrate and phage sensitivity may well be phenotypically independent. One such pair of strains, a group A parent culture and its A-variant counterpart, were obtained from Dr. Fischetti and they behaved with respect to group carbohydrate synthesis and phage adsorption as previously reported (2). Other A-variant strains, however, were able to adsorb phage A25. One novel strain, known to synthesize group A carbohydrate at 30°C and A-variant carbohydrate at 37°C, adsorbed phage A25 when grown at either temperature. In addition to our findings, Krause also reported the susceptibility of a group A and A-variant pair of strains to infection by phage A25 (3).

As reported by others, our purified preparations of peptidoglycan, which

retained receptor activity, agglutinated when added to group A antisera (34). This cross-reaction between the *N*-acetyl-glucosamine moiety of group A carbohydrate and cell wall peptidoglycan could explain the inhibitory action of group A antisera on phage adsorption which was observed by Fischetti and Zabriskie. The amount of group A carbohydrate in peptidoglycan preparations indicated by the residual rhamnose concentration was too low to account for the intense agglutination reactions we observed. These results and the fact that phage A25 is now known to form plaques on other groups strongly suggest that the group A carbohydrate does not participate in the adsorption of phage A25 (Table I; references 4, 6).

In contrast to experiments reported by Fischetti and Zabriskie, in our experimental system heat-killed cells of group A strains K56, $T25_3$, and others displayed a marked adsorptive capacity almost equivalent to viable cells. These conflicting results can possibly be explained by the fact that growth media and suspending media for heat inactivation of cells differed in these two investigations (2). Our growth media lacked beef extract and was supplemented with horse serum. In addition, our assays made use of fewer PFU, which resulted in a more sensitive test for adsorption.

Mechanical disruption of heat-killed cells for the preparation of cell walls completely destroyed their capacity to inactivate phage A25 irreversibly, but, as originally described by Krause (3), cell walls appeared to retain the potential to adsorb this phage reversibly. A clear interpretation required the demonstration that this phenomenon was, indeed, the reversible specific attachment of phage particles to cell walls, as opposed to nonspecific entrapment of the phage by sedimented cell debris. The rapid release of attached phage from cell walls interfered with kinetic studies which might have distinguished entrapment from adsorption. Therefore, the host specificity of RA with respect to whole cells and various cell preparations and the direct observation of phage particles attached to peptidoglycan isolated from a group A strain (but not a group B strain) are the primary evidence that RA reflects the specific attachment of phage particles to cell receptors.

The muralytic enzymes, egg white lysozyme, and group C phage-associated lysin destroyed the receptor activity of purified peptidoglycan indicating that this structure must be intact. In order for phage A25 to attack K56 cells its tail structure must seek a path through the outer layers of peptides and polysaccharides to the peptidoglycan. Either the virus particle carries enzymes capable of exposing the underlying peptidoglycan or patches of this important exoskeleton are continually bare to the external environment as has been suggested by other studies (35, 36). Although our experiments were variable they suggest that the latter explanation is correct. Egg white lysozyme, although unable to lyse group A streptococci, can destroy receptor activity and as reported by others (37) can also cause the leakage of deoxyribonucleic acid from these cells.

In contrast to the staphylococcal system, teichoic acid is not a necessary component of the A25 receptor; highly purified peptidoglycan, devoid of phosphorus, retained its reactivity with phage particles.

Cell walls from both group G streptococci and group A streptococci adsorbed phage A25; however, group B walls lacked this property. One distinguishing

feature of group B cell walls compared to group A or group G walls is the amino acid content of the peptidoglycan. The interpeptide bridge of peptidoglycan from group A and G cell walls contains L-lysine-(L-alanine)$_{2 \text{ or } 3}$, whereas group B peptidoglycan contains L-serine-alanine (26). This suggests the interesting possibility that a minor chemical difference in the peptidoglycan could determine the host range of phage A25.

In many cases studied the adsorption of bacteriophage to a bacterial cell has been found to occur in a two-step reaction (1). The first step, reversible and temperature independent, precedes the second step which is irreversible and temperature dependent. Our results can best be explained by postulating a similar mechanism for the adsorption of phage A25 to group A streptococci. Initially, the phage attaches reversibly to peptidoglycan. Irreversible inactivation is subsequently triggered by a cellular component which is somehow destroyed by mechanical disruption of the cells.

Summary

Irreversible adsorption of a virulent phage, phage A25, to heat-killed streptococci, groups A, G, and A variant, has been achieved. Adsorption reflected the observed host range for phage A25 in that heat-killed group B cells were not able to inactivate the phage. Broken cells, cell walls, and peptidoglycan prepared from a group A strain K56 failed to adsorb the phage irreversibly, but retained the potential to carry out reversible adsorption. Experimental data including electron microscopy have demonstrated the specificity of reversible adsorption and have identified the peptidoglycan as a necessary cellular component of the receptor. The sensitivity of whole cells and purified peptidoglycan to muralytic enzymes suggests that the cell wall and peptidoglycan must be intact for optimal adsorption. In general the results are explained by postulating that adsorption of A25 phage particles to group A cells occurs by a two-step process; the first step involves recognition and reversible binding of the phage tail to the cell wall peptidoglycan, the second step is an irreversible reaction catalyzed by a yet unidentified cellular component which is destroyed when cells are ruptured.

We are grateful to Miss Donna Ritzi for performing the electron microscopy.

Received for publication 24 September 1976.

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