STUDIES ON STREPTOCOCCAL BACTERIOPHAGES

II. Adsorption Studies on Group A and Group C Streptococcal Bacteriophages*

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(Received for publication 10 November 1967)

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The previous studies of Krause (1) had clearly demonstrated that the host range of certain streptococcal bacteriophages was confined to specific streptococcal groups: Group C bacteriophages propagating only on Group C streptococci, Group A bacteriophages only on Group A streptococci. Using cell wall fractions and enzymatic digestion of isolated cell walls, he further demonstrated that the Group C streptococcal bacteriophage C1 could be irreversibly inactivated by the carbohydrate moiety of the Group C streptococcus. However, these studies were performed on one Group C bacteriophage (C1). In addition only those carbohydrate preparations which had been made by the solubilization of streptococcal cell walls with muralytic enzymes were used. The previously noted differences in burst sizes of several different Group C bacteriophages (2) suggested that there might also be differences in the viral receptor sites of the Group C phages. It was therefore of interest to reexamine the question of the receptor site in Group C bacteriophages using both a number of different bacteriophages as well as a number of different carbohydrate preparations.

With respect to the Group A bacteriophages the problem of the viral receptor site was more complex. Krause's attempts to inactivate Group A bacteriophages with either isolated Group A cell walls or the group-specific carbohydrate were unsuccessful (1). In addition, preliminary experiments in our laboratory had indicated that any disruption of the intact living Group A streptococcus resulted in the loss of the viral receptor site for Group A bacteriophages. It was thus necessary to approach the problem of this viral receptor by employing techniques designed to alter or block specific cell wall constituents in the intact Group A cell.

The studies to be reported below indicate that whereas the Group C bacteriophages are specifically inactivated by Group C cell walls, only the C1 bacterio-

* This investigation was supported in part by the United States Public Health Service Grant HE 03919.

‡ Portions of this paper were submitted to Long Island University in partial fulfillment for a Master's Degree.

phage was blocked by muralytic preparations of group-specific carbohydrate. Furthermore, none of these bacteriophages were inactivated by chemically extracted group-specific carbohydrate. In contrast to the specific binding of Group C bacteriophages, virulent Group A phages were not inactivated by either isolated Group A cell walls or the carbohydrate preparations thereof. Temperate Group A phages differ from their virulent counterparts in that they were able to bind irreversibly to isolated Group A cell walls. However, only one temperate bacteriophage was inactivated by group-specific carbohydrate. While it was not possible to localize the specific viral receptor substance in Group A streptococci, the use of antisera to specific cell wall fractions, proteolytic digestion studies, and the use of streptococcal mutants suggest that the specific adsorption site of Group A bacteriophage resides in the cell wall carbohydrate. This site is related, at least in part, to the *N*-acetylglucosamine terminal unit of the group-specific carbohydrate.

Materials and Methods

Bacterial Strains.—The streptococcal and pneumococcal strains used in this study were from The Rockefeller University collection. Strains *Bacillus megatherium* and *Staphylococcus aureus* N. Y. 6 were obtained from Dr. Russell Schaedler and Dr. Stephen Morse respectively. The Group C streptococcal strain C88 was kindly obtained from Dr. Eugene Fox.

Bacteriophages.—Virulent phages A25, A12, and C1 were obtained from Dr. Richard M. Krause. Bacteriophages, ϕY and C343, were obtained from Dr. E. Fox. Stock phage lysates were prepared with the following strains: strain $T25_3$ (A25 phage), strain T12 (A12 phage) and strain A590 (A6 phage), strain 26RP66 (C1 phage) and strain C88 (ϕY and 343 phages). All lysates were filtered through a Coors No. 2 candle filter and stored at 4°C until use.

Temperate phages (T12gl) and (B276) were isolated from Group A streptococcal strains T12gl and B276 respectively. Temperate phage (B940) was obtained from Group A–variant strain B940. Lysogenic strains with these temperate phages were prepared in strain $T25_3$. Stock lysates of phages $T25_3$ (T12gl), $T25_3$ (B940), and $T25_3$ (B276) were obtained by the following method. Isolation of temperate phage from lysogenic streptococcal strains were obtained as previously described (3). The plaques obtained by this method were picked by repeated stabbings of the plaque centers and transfer of the adhering virus particles to 5 ml of dialysate broth. To 1 ml of these low titer phage stocks 0.1 ml of a 1:3 dilution of an 18 hr growth of strain $T25_3$ was added and the mixture then plated by the soft agar layer technique. Following 5 hr of incubation at 37°C in a candle jar, the plates were flooded with 2 ml of dialysate broth containing 0.005 M 2-mercaptoethanol. The soft agar layer was then removed by gentle scraping and the resulting suspension centrifuged at 8000 rpm for 15 min. Stock phage lysates containing 1×10^7 or 1×10^8 PFU/ml were obtained in this manner. 1 ml aliquots of these lysates were quick frozen in an alcohol and dry ice bath and stored at −70°C until use.

Media.—Methods for the preparation of broth media used for Group A and Group C phage-host experiments were as previously described (2).

Preparation of Agar Plates.—The preparation of Agar plates for Group A and Group C bacteriophages was as previously described (2).

Plating and Counting Bacteriophage.—Plating and counting of bacteriophages was as previously described (2).

Optical Densities.—All OD readings were carried out in 10×75 mm test tubes at 650 mμ in a Coleman Junior Spectrophotometer.

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Incubation.—All phage plates of the Group A system were incubated in a candle jar at 37°C for 18 hr. Plates for the Group C system were incubated at 37°C for 18 hr without the use of a candle jar.

Indicator Strains.—

Group A system: An 18 hr culture of strains $T25_3$, T12, or A590 in dialysate broth were centrifuged and resuspended in fresh dialysate medium to an OD of 0.04–0.06. 0.1 ml of these suspensions was added to the appropriate phage and added to the soft agar tubes at the time of plating. Strain $T25_3$ was used exclusively for the temperate phage experiments.

Group C system: An 18 hr culture of strain 26RP66 or C88 in Todd-Hewitt broth was centrifuged and resuspended to one third its original growth volume with fresh Todd-Hewitt broth. 0.1 ml was used in the soft agar at the time of plating.

Antisera.—Rabbit antiserum to the group-specific carbohydrate and polyglycerolphosphate was kindly supplied by Dr. Rebecca Lancefield and prepared as described (4). Purified Group A streptococcal antibody with *N*-acetylglucosamine specificity, kindly supplied by Dr. Maclyn McCarty, was prepared by adapting the procedure of Karush and Marks (5). β-aminophenyl-β-*N*-acetylglucosamide (6) was coupled with bovine fibrinogen, and this azoantigen was used to precipitate antibody from Group A streptococcal rabbit antiserum. The washed precipitate was redissolved in 2% *N*-acetylglucosamine and fractionated to recover the antibody globulin. The final preparation was 80–90% precipitable by Group A streptococcal carbohydrate.

Preparation of Phage-Associated Lysin.—Group C streptococcal phage-associated lysin was prepared by the method of Zabriskie and Freimer (7). The *Streptomyces albus* (SMA) enzyme was kindly supplied by Dr. Maclyn McCarty.

Preparation of Cell Walls.—The cell walls were prepared by the method of Salton (8) in which the streptococci were disrupted in a Braun disintegrator (9). The cell walls were separated from the remaining cellular material by centrifugation at 4500 rpm for 1 hr, washed four times in distilled water, lyophilized, and stored in a desiccator at 4°C until use.

Preparations of Cell Wall Carbohydrate.—Carbohydrate fractions were prepared from cell walls by either enzymatic digestion with *S. albus* or phage-associated enzymes, or chemically extracted by formamide or hot acid. Aliquots of the same lot of cell walls were extracted separately by all four methods so that a direct comparison could be made as to their ability to inactivate Group C phages. For the Group A phage studies extractions were made from cell walls isolated from the propagating strain of the phage under study.

The extraction procedures were as follows: digestion of isolated cell walls with the *S. albus* enzyme and isolation of the carbohydrate was by the method previously described (10). Lysis of the cell walls with phage-associated enzyme was carried out as follows: 50 mg of cell walls were resuspended in 5 ml of M/15 phosphate buffer at pH 6.6 with 0.05 M 2-mercaptoethanol. To this was added 2.5 ml of phage-associated lysin and a few drops of chloroform. The mixture was incubated at 37°C for 12 hr with mixing. The group-specific carbohydrate was then isolated by the same methods described for the *S. albus* enzyme extract (10).

Extraction of cell walls with hot formamide was by the method of Fuller (11). Acid extraction of cell walls was as previously described (4). All carbohydrate preparations were dialyzed against distilled water and dried from the frozen state. Preparations were checked for serological reactivity by the precipitin tube method with Group A or Group C antisera (4).

EXPERIMENTAL

Phage Adsorption Studies with Streptococcal Cellular Fractions.—In view of the observation that the propagation of Group A and Group C bacteriophages was a group-specific phenomenon(1), the inability of Group A bacteriophages

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to be inactivated[1] by isolated Group A cell walls or the carbohydrate moiety was puzzling. Either the Group A phage receptor site resided in another component of the streptococcus, as suggested by Krause (1), or the mechanisms for viral inactivation of Group A bacteriophages were more complex, involving perhaps several distinct factors. Accordingly, adsorption[1] experiments were performed with the major cellular fractions of Group A and Group C streptococci in order to determine both the specificity and the nature of the viral receptor sites for the Group A and Group C bacteriophages.

In a typical experiment streptococci, in a logarithmic phase of growth, are diluted in broth to an OD of 0.03. 0.1 ml of a phage lysate at a concentration of 5×10^7 PFU/ml is added to 0.9 ml of the whole cell suspension and the mixture is incubated at 37°C. At timed intervals, 0.1 ml aliquots are removed and diluted into 9.9 ml of the appropriate broth. This initial dilution step was found to be necessary in order to prevent any further phage-host interactions and to reverse any nonspecific viral adsorption. 1 ml aliquots of this 1:100 dilution are removed and centrifuged at 3000 rpm for 4 min to sediment the cells and the supernatant tested for residual phage by the soft agar layer technique.

For the experiments employing cell walls a concentration of 2 mg/ml of either Group A or Group C cell walls, prepared as described in Materials and Methods, are substituted for the whole cells. In adsorption experiments with disrupted cells, log phase streptococci are grown to an OD of 0.1, disrupted in a Braun disintegrator for 10 min, and the whole solution used as the substrate for phage adsorption.

The results of these experiments are summarized in Fig. 1 A. Line *c* confirms the observation of Krause (1) and demonstrates that the C1 bacteriophage is specifically inactivated by Group C cell walls. Furthermore, C1 phage does not adsorb to whole living Group A streptococci (line *a*). Similar results were obtained with Group C bacteriophages, ϕY and C343. In contrast, Fig. 1 B indicates that neither disrupted whole cell suspensions (line *a*), nor isolated cell walls of the Group A streptococcus (line *b*) are able to inactivate the A25 bacteriophage. Although not shown, A6 and A12 bacteriophages also failed to adsorb to these Group A streptococcal cellular fractions. The fact that these phages were capable of adsorbing to intact Group C cells (line *c*) suggested that the mechanism for Group A–virulent phage adsorption was different than that observed with the Group C bacteriophages. Experiments were therefore designed to determine whether A25 phage could adsorb to cells of other streptococcal groups as well as unrelated Gram-positive organisms.

Adsorption Studies of A25 Phage to Streptococcal Groups and Other Gram-Positive Organisms.—The adsorption techniques employed in these studies were essentially as described in the previous section. Representative strains from a number of streptococcal groups as well as other Gram-positive organisms were grown in Todd-Hewitt broth for 18 hr at 37°C. Log phase cultures for each

[1] The terms inactivation and adsorption are used interchangeably to denote the irreversible attachment of phage particles to streptococci or fractions thereof.

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strain were grown to an OD of 0.1. The strains were than individually tested for their ability to adsorb irreversibly the A25 bacteriophage.

In contrast to the specific adsorption of Group C bacteriophages to Group C streptococci, Table I indicates that A25 bacteriophage adsorbs not only to

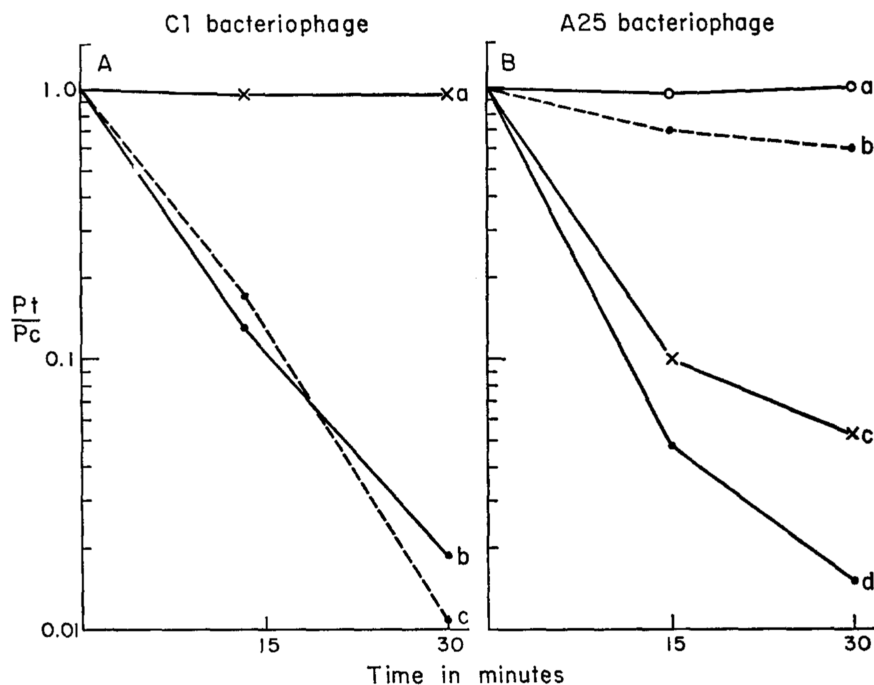

FIG. 1 A. C1 phage adsorption studies to whole cells and cell fragments.

(*a*) Lack of adsorption of C1 phage to Group A streptococcal cells. (*b*) Adsorption of C1 phage to Group C streptococcal cells. (*c*) Adsorption of C1 phage to isolated Group C cell walls.

FIG. 1 B. A25 phage adsorption studies to whole cells and cell fragments.

(*a*) Lack of adsorption of A25 phage to mechanically disrupted Group A streptococci. (*b*) Lack of adsorption of A25 phage to isolated Group A cell walls. (*c*) Adsorption of A25 phage to living Group C streptococcal cells. (*d*) Adsorption of A25 phage to living Group A streptococcal cells.

Abbreviations in this and following figures: Pt, plaque count at time of sampling; and Pc, plaque count at zero-time.

Group A streptococci but to streptococci of Groups C and G as well. Partial adsorption was also observed with strains of streptococcal Groups O and K as well as an encapsulated pneumococcal strain, but the percentage of adsorption never approached that observed with Groups C and G. While these results suggest that factors such as the configuration of surface components of these

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strains may be important in the adsorption of Group A bacteriophages, the failure of A25 phage to adsorb to unrelated Gram-positive organisms indicates that adsorption of this particular bacteriophage is not related to nonspecific adsorptive phenomena.

Comparison of the Adsorption of A25 and C1 Bacteriophages on Heat-killed Streptococcal Cells.—The evidence that A25 phage would not adsorb to cell wall fractions of the Group A streptococci (Fig. 1 B) suggested that an intact cell, perhaps coupled with a metabolic factor released by the cell, was necessary for irreversible adsorption of the Group A bacteriophages. In order to test this possibility, experiments were designed in which heat-killed streptococci were tested for their ability to adsorb these particular phages.

TABLE I

Adsorption of A25 Phage to Streptococcal Groups and Unrelated Organisms

Bacteria	Strain No.	Absorption of A25 phage as % of control*
Hemolytic streptococci		
Group A	$T25_3$	100
" C	26RP66	80
" G	D166B	87
" K	D34E	38
" K	D34B	35
" O	B357	21
Pneumococci		
Encapsulated	D39	25
Nonencapsulated	R36A	0‡
Bacillus megatherium	—	0
Staphylococcus aureus	N.Y. 6	0

* As observed after 30 min of phage-cell interaction.

‡ Less than 10% absorption.

Group A and C streptococci, in logarithmic phases of growth, were diluted in broth to an OD of 0.03. The cells were then heat-killed at 56°C for 30 min and used for phage adsorption experiments as described in the previous section.

In Fig. 2 A, the results of the adsorption of Group C bacteriophages to heat-killed Group C cells are plotted on semilogarithmic coordinates. The rate of adsorption of C1 phage to heat-killed Group C streptococci (line *a*) compares favorably with the results obtained with living cells (line *b*). The subsequent rise (noted in line *b*) is due to the normal phage cycle in the living cell. Fig. 2 B summarizes the results obtained with the A25 phage. Whereas the adsorption curve to living cells (line *b*) is similar to that obtained in the Group C system, heat-killed Group A streptococci are no longer capable of adsorbing the A25 bacteriophages (line *a*). Since the evidence indicated that A25 phage is inac-

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tivated only by whole living cells, identification of the Group A receptor site required experiments designed to alter or block known cell wall components on the intact living streptococcal cell.

Adsorption of Group A Phage to Enzymatically Digested Group A Cells.—The previous studies of Krause (1) and Friend and Slade (12) had indicated that the

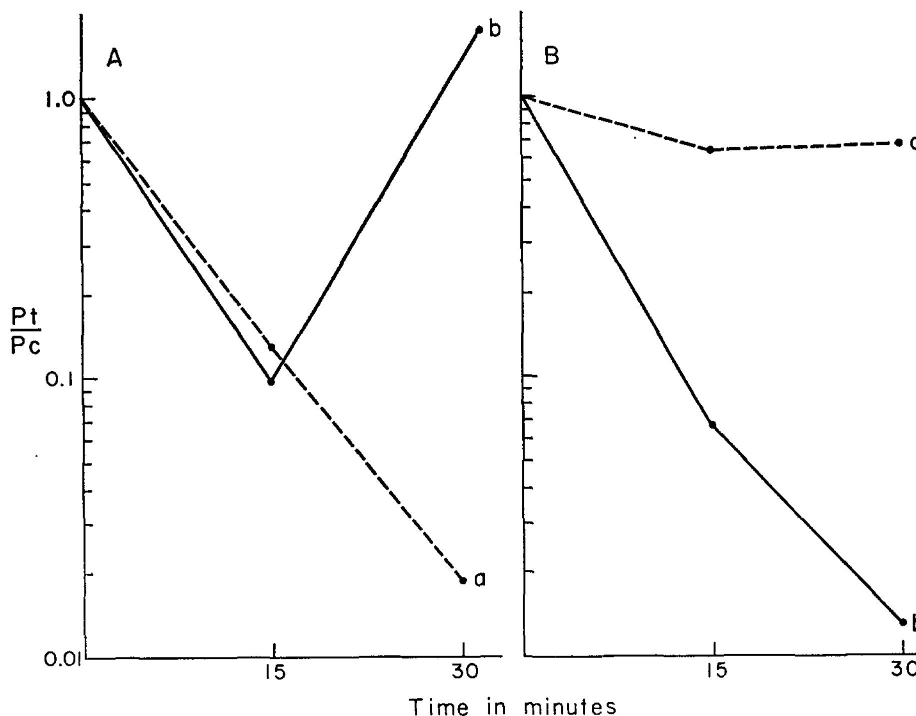

FIG. 2 A. The adsorption of C1 phage to heat-killed (*a*) and living Group C streptococci (*b*). Subsequent rise in line *b* is due to the normal phage cycle in the living cell.

FIG. 2 B. The lack of adsorption of A25 phage to heat-killed Group A streptococci (*a*) in contrast to adsorption to living Group A cells (*b*).

M protein, a major constituent of Group A streptococcal cell walls, did not play a role in the adsorption of Group A streptococcal phages. However, the possibility still existed that other proteins such as the T and R antigens, might play a role in viral inactivation (13). In order to rule out this possibility, protein digestion experiments on living streptococci were performed using a number of different enzyme preparations. In general the experimental procedure was the same for each enzyme preparation, only the pH and the buffer were varied in order to insure optimal activity for each enzyme.

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A typical experiment utilizing trypsin digestion of streptococcal cells was as follows: a 4 hr culture of $T25_3$ was centrifuged and resuspended in sterile M/15 phosphate buffer pH 7.5 to an OD of 0.4. Trypsin (Worthington Biochemical Corporation, Freehold, N. J.) was prepared in the same buffer at a concentration of 100 μg/ml; mixed with equal parts of the cell suspension, and incubated at 37°C with rotation. At the end of a 3 hr incubation period the suspension was centrifuged and the cells resuspended to the same volume with dialysate medium. 1 ml of this suspension was mixed with 1.0 ml of A25 phage containing 5×10^6

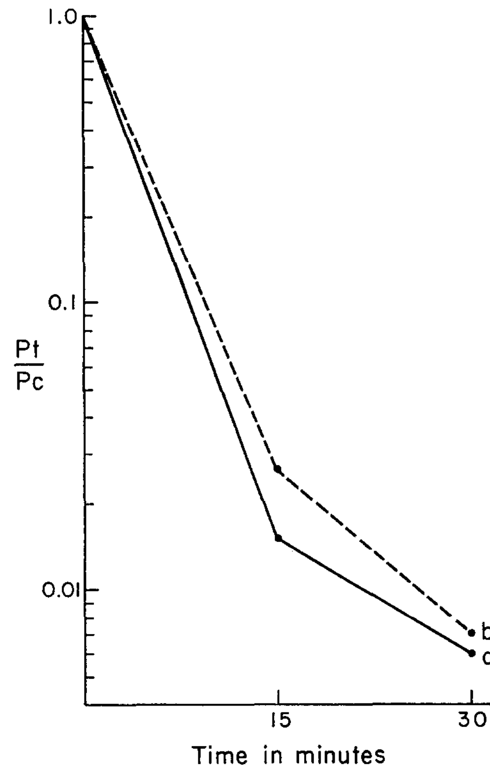

FIG. 3. Lack of adsorption of A25 phage to trypsin-digested Group A cells (*b*) as compared with the adsorption to undigested Group A cells (*a*).

PFU. At timed intervals 0.1 ml samples were removed and diluted into 9.9 ml of broth. 1 ml aliquots of this dilution were centrifuged and the supernatants plated by the soft agar layer technique. Identical experiments were performed with either papain or chymotrypsin solutions. In these experiments either 40 μg/ml of chymotrypsin in M/15 phosphate buffer at pH 7.3 or 40 μg/ml of papain in 0.01 M cysteine at pH 7.3 were substituted for the trypsin.

It can be seen in Fig. 3 that even after Group A cell walls have been digested with trypsin, no alteration of A25 phage adsorption was observed. Similar results were obtained with cells that had been digested with either papain or chymotrypsin.

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Blocking of A25 Phage Receptor Site by Absorbed Antisera.—Since protein digestion studies indicated that viral inactivation was not altered by proteolytic digestion, attempts to block the receptor site by the use of antisera to known cell wall consituents were utilized next.

Rabbit antiserum 1875, containing antibodies to both Group A carbohydrate and polyglycerolphosphate, were specifically absorbed in the following manner: 1 ml aliquots of the antiserum were mixed with Group A carbohydrate or polyglycerolphosphate at a concentra-

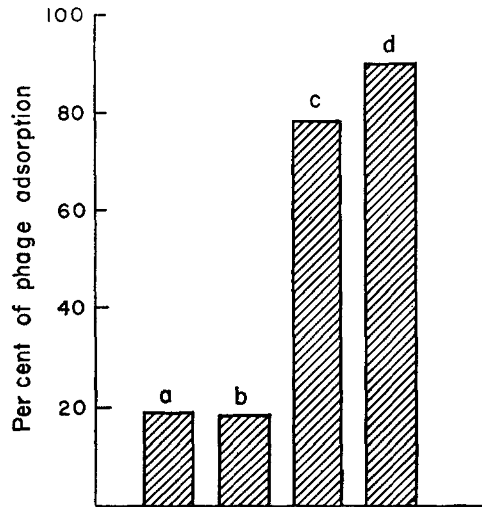

FIG. 4. The inhibition of A25 phage adsorption to Group A cells by unabsorbed Group A antiserum (bar *a*). No change is noted following absorption of the antiserum with polyglycerol-phosphate (bar *b*). Bar *c* designates the percentage of phage adsorption following absorption of the antiserum with Group A carbohydrate. Bar *d* designates the percentage of phage adsorption with preimmune rabbit serum. All results are expressed as the percentage of phage adsorption after 30 min of phage-host interaction.

tion of 50 or 100 μg per ml of antiserum. The mixture was incubated for 2 hr at 37°C and the resulting precipitates were removed by centrifugation. The supernatants were then tested for residual antibodies by the capillary precipitin method (4). If precipitation was again observed, the absorption procedure was repeated until all the antibody had been removed. The specifically absorbed antiserum as well as the unabsorbed control were then used in the following blocking experiment.

Group A streptococcal strain, $T25_3$, was grown in dialysate medium to an OD of 0.1. 1 ml aliquots were centrifuged and the cells resuspended in a 1:25 dilution of the absorbed or unabsorbed antiserum. Control cells were resuspended in a 1:25 dilution of normal rabbit serum. 0.9 of these mixtures were incubated at 37°C for 15 min at which time 0.1 ml of A25 phage at 5×10^7 PFU/ml was added to the suspensions. At timed intervals thereafter, 0.1 ml aliquots were removed; diluted 1:100 in order to stop further adsorption, and centrifuged to sediment the cells. The supernatants were then tested for residual phage by the soft agar layer technique.

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As illustrated in Fig. 4 unabsorbed antiserum directed against both the Group A carbohydrate and polyglycerolphosphate moieties of Group A streptococci inhibits more than 80% of phage adsorption (bar *a*). No change is noted when this antiserum is absorbed with polyglycerolphosphate (bar *b*). However, phage adsorption is almost completely regained when the antiserum is first absorbed with the Group A carbohydrate (bar *c*), and compares favorably with the normal rabbit serum control (bar *d*).

When a similar experiment was performed using purified antibody to Group A carbohydrate isolated by means of an *N*-acetylglucosamide azoantigen as

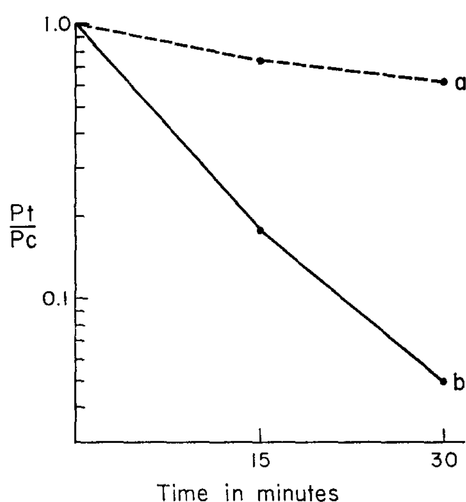

FIG. 5. The inhibition of A25 phage adsorption to Group A streptococcal cells previously treated with purified *N*-acetylglucosamine antiserum (*a*), as compared with the adsorption to cells treated with normal rabbit serum (*b*).

described under Materials and Methods, phage adsorption was again almost completely blocked (Fig. 5).

Adsorption of A25 Phage to Group A ard Group A–Variant Type 25 Streptococci.—The results of the antisera-blocking experiments indicated that the Group A carbohydrate, particularly the *N*-acetylglucosamine moiety, was intimately involved in the A25 phage adsorption site. In order to strengthen this evidence, mutant Group A streptococci lacking the *N*-acetylglucosamine moiety were utilized as the A25 phage host. These strains, termed A-variant, were available and the studies of McCarty (10), and Krause and McCarty (14) had demonstrated that they differed from the Group A strain in the amount of *N*-acetylglucosamine present in the cell wall carbohydrate. Whereas the Group A strain contained 25% glucosamine in the cell wall extracts, the A-variant strain contained only 1–3%. Accordingly, group A–variant strain B346/94/1 and the

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parent group A strain B346 (both type 25) were selected and tested for their ability to adsorb the A25 phage.

As illustrated in Fig. 6, when A25 phage are mixed with the Group A streptococcal strain, normal infection and maturation occurs (line *a*). However, when

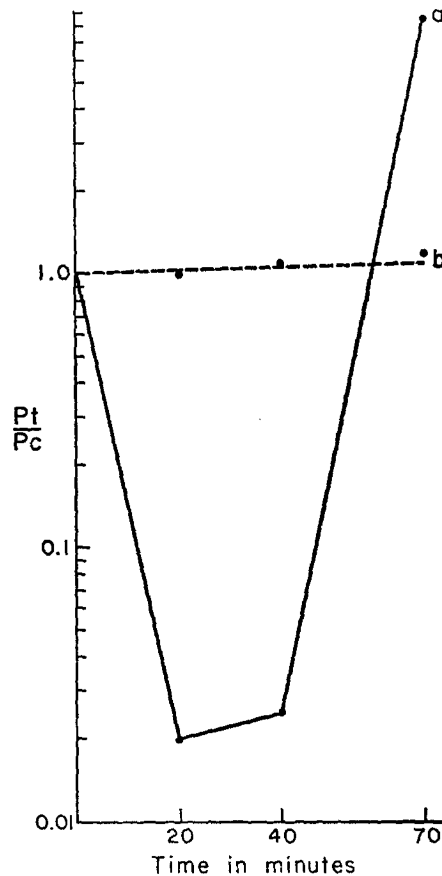

FIG. 6. The lack of A25 phage adsorption to Group A–variant, type 25 streptococcal strain B346/94/1 (*b*) as compared with the one-step growth cycle in the parent Group A type 25 strain B346 (*a*).

the group A–variant is utilized as the A25 phage host, no adsorption is observed (line *b*). These results again strongly suggest that *N*-acetylglucosamine is an essential factor for the irreversible adsorption of the A25 phage.

Differences in the Receptor Sites of Group C Bacteriophages.—As mentioned in the introduction, the early work in Group C bacteriophages was performed with only one bacteriophage and only muralytic preparations of Group C carbohy-

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drate were used. In an effort to elucidate further the nature of this receptor site, a number of different chemical and muralytic extractions of the group-specific carbohydrate were prepared. In addition, three Group C bacteriophages, isolated from different sources, were studied to determine whether differences existed in the receptor sites of streptococcal bacteriophages within a streptococcal group.

In general, the procedure for these adsorption studies was essentially as described above. Cell walls isolated from Group C streptococcal strain H46A were used at a concentration of 2 mg/ml in broth for the virulent Group C phage adsorption studies. In addition carbohydrate preparations from the same strain were prepared as described in Materials and Methods. Solutions containing 4 mg/ml of these carbohydrate preparations in broth were also utilized as the phage receptor substance.

TABLE II

Adsorption Patterns of Group C Streptococcal Bacteriophages to Isolated Group C Cell Walls and Various Carbohydrate Preparations

Substrates	Bacteriophage		
	C1	ϕY	C343
Group C cell walls	+*	+	+
SMA enzyme-extracted Group C carbohydrate	+	0‡	0
Phage-associated enzyme-extracted Group C carbohydrate	+	0	0
Formamide-extracted Group C carbohydrate	0	0	0
Acid-extracted Group C carbohydrate	0	0	0

* More than 90% inactivation.
‡ Less than 10% inactivation.

As can be seen from Table II all three virulent Group C phages adsorb readily to isolated Group C cell walls. However, only the C1 phage is inactivated by the phage enzyme and SMA prepared carbohydrates. In contrast no inactivation of any of the bacteriophages was observed when chemically extracted group-specific carbohydrate was used as the phage substrate.

Differences in the Receptor Sites of Group A Bacteriophages.—Since these results indicated that definite differences existed between the adsorption sites of the Group C streptococcal phages, it was conceivable that similar variations existed in the receptor sites of Group A bacteriophages. Furthermore most of the studies of the Group A phage-host system had been carried out with only the A25 bacteriophage (1, 12), and it was conceivable that the lack of cell wall inactivation of this bacteriophage was a phenomenon unique to this particular phage. Accordingly experiments utilizing other Group A virulent phages were

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undertaken in an attempt to determine whether or not all Group A phages exhibited similar adsorption patterns.

Three virulent Group A phages, A6, A12, and A25, propagating on their respective streptococcal strains were used for the adsorption experiments. For the cell wall studies 2 mg/ml of cell walls from each propagating strain served as the inactivating material. Carbohydrate preparations obtained by digestion with either SMA or phage enzymes were utilized at a concentration of 4 mg/ml.

In contrast to the results obtained with the Group C virulent phages, Table III indicates that only whole living Group A cells were able to inactivate the Group A virulent phages. No phage inactivation was observed with either isolated cell walls or any of the carbohydrate preparations.

TABLE III

Adsorption Patterns of Group A Streptococcal Bacteriophages to Group A Streptococci, Cell Walls, and Carbohydrate Preparations

Substrate	Virulent phages			Temperate phages		
	A6	A12	A25	$T25_3$ (T12gl)	$T25_3$ (B940)	$T25_3$ (B276)
Whole living Group A cells	+*	+	+	+	+	+
Group A cell walls	0‡	0	0	+	+	+
Phage enzyme–extracted carbohydrate	0	0	0	0	+	0
SMA-extracted carbohydrate	0	0	0	ND§	ND	ND
Acid-extracted carbohydrate	0	0	0	0	0	0

* More than 90% adsorption.
‡ Less than 5% adsorption.
§ ND, not done.

Adsorption Studies on Group A Temperate Phages.—Unlike the Group A virulent phage stocks which are stable from 3–4 wk in broth at 4°C, Group A temperate phages become inactivated in a matter of hours. It was of interest therefore to examine the adsorption patterns of these phages in an attempt to discover whether they were similar to those of the Group A virulent phages.

Similar adsorption studies as outlined for the Group A virulent phages were performed on temperate phages $T25_3$ (B940), $T25_3$ (B276), and $T25_3$ (T12gl). All were originally isolated from different streptococcal sources but were lysogenized with the same type 25 strain. Cell walls prepared from the propagating $T25_3$ strain was utilized at a concentration of 2 mg/ml. Carbohydrate, prepared as described, was isolated from the same cell wall preparation and used at a 4 mg/ml concentration.

The results of these experiments are tabulated in Table III. It can be seen that unlike the Group A virulent phages which adsorb only to living cells,

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Group A temperate phages are able to adsorb onto isolated cell walls. These results are quite similar to the patterns obtained with Group C bacteriophages. However, with respect to the adsorption patterns with the carbohydrate preparations, only the B940 phage, isolated from an A-variant strain, adsorbed to the phage enzyme carbohydrate. All of the temperate phages failed to adsorb to chemically prepared carbohydrate.

DISCUSSION

One of the most interesting findings of the studies reported above was the observation that Group C bacteriophages differed as to their inactivating sites on the streptococcal cell wall. While all three phages adsorbed to the isolated cell walls of a single streptococcal strain, only the C1 phage was inactivated by enzymatically prepared group-specific carbohydrate. These results would tend to suggest that the release of carbohydrate by the enzymatic digestion of cell walls results in the loss of additional factors necessary for viral inactivation for at least two bacteriophages. In addition, the failure of all three bacteriophages to adsorb to carbohydrate released by chemical extraction of streptococcal cell walls would suggest that more than the carbohydrate moiety per se is needed for phage inactivation. In this connection, it is known that enzymatic preparations of the carbohydrate contain elements of the mucopeptide that are absent from either acid or formamide-extracted carbohydrates (14). It would thus appear that in addition to the carbohydrate moiety the mucopeptide fraction of the cell wall is also crucial to Group C phage inactivation.

With respect to the Group A phages, the factors necessary for irreversible viral adsorption appear to be far more complex. All of the virulent phages studied failed to adsorb to either isolated cell walls or the group-specific carbohydrate. In contrast, all temperate Group A phages tested did adsorb to the isolated streptococcal cell wall. However, the need for additional factors for the complete viral inactivation of temperate Group A phages was again emphasized by the fact that 2 of 3 phages tested were not inactivated by group-specific carbohydrate preparations. In addition, the specificity of the receptor site of these bacteriophages must be questioned since temperate Group A bacteriophages were also inactivated by Group C cell walls.

While many hypotheses may be invoked to explain the differences in the adsorption patterns of these phages, the possibility that these differences may be related to the replicative cycles of these bacteriophages is attractive. For example, among the several virulent bacteriophages examined, only the C1 phage adsorbed to the soluble group-specific carbohydrate of the isolated streptococcal cell wall. This suggests that at the time of its release from the bacterial cell, this bacteriophage is exposed to a high concentration of materials capable

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of causing immediate readsorption. In order for the bacteriophage to survive under these conditions a high burst size would probably be required. In this connection, the burst size of the C1 bacteriophage was 10 times that observed for the other streptococcal bacteriophages (2). Although this concept might apply to the adsorption pattern of the C1 bacteriophage, it does not explain the adsorption patterns of the two other Group C bacteriophages which had low burst sizes (13 PFU/ml) and yet adsorbed to the isolated streptococcal cell wall. However, it must be remembered that Group C phages produce large amounts of phage-associated lysin which conceivably would solubilize any cell wall fragments released during the normal lysis of the cell. Thus the low phage yield of ϕY and C343 would be compensated for by the fact that these bacteriophages could not adsorb to the solubilized cell wall fragments. With respect to virulent Group A phages, all have low burst yields and do not, like the Group C bacteriophages, produce excessive amounts of phage-associated lysin. However, these adsorb only to intact Group A streptococci, thereby conserving the maximal number of infective units for further replication.

Although it was not possible to localize the specific receptor site for the majority of the Group A bacteriophages, the available data suggest that this site does reside in the streptococcal cell wall and is related to the *N*-acetylglucosamine terminal units of the carbohydrate. This evidence may be summarized as follows. First, an antiserum to both Group A carbohydrate and polyglycerolphosphate blocked the viral attachment of Group A phages to the host cell. This blocking action was not affected by prior absorption of the antisera with streptococcal polyglycerolphosphate but was affected by absorption with group-specific carbohydrate. Secondly, incubation of Group A streptococci with antisera to the purified *N*-acetylglucosamine moiety resulted in the inhibition of viral adsorption and indicated that the *N*-acetylglucosamine moiety was involved in viral attachment. Finally, the fact that the A25 phage failed to adsorb to a Group A–variant strain which lacked the terminal *N*-acetylglucosamine units was a strong point in favor of the participation of the cell wall carbohydrate, specifically the *N*-acetylglucosamine moiety.

Although the group-specific carbohydrate definitely plays a role in phage adsorption, the experiments with heat-killed streptococcal cells indicate that an additional factor must be present for irreversible inactivation of Group A virulent phages. The fact that this loss of adsorption occurs under mild elevations in temperature would tend to suggest this factor is dependent on active processes of the whole cell. While this is an unusual finding, evidence for this type of mechanism can be found in experiments with TI phage of the *Escherichia coli* system (15, 16), in which adsorption also took place only in the living intact cell. Until means can be devised to isolate this cofactor from a

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living cell, the question of the exact nature of the Group A phage receptor site must remain unanswered.

SUMMARY

Evidence has been presented that Group C bacteriophages differ as to their inactivating site on the streptococcal cell wall. While all three phages adsorb to isolated cell walls, only the C1 phage was inactivated by enzymatically prepared group-specific carbohydrate. None of the Group C phages were inactivated by chemically extracted group-specific carbohydrate. In contrast, all virulent Group A streptococcal bacteriophages adsorbed only to living Group A streptococci. However, Group A temperate phages were able to adsorb to isolated cell walls but not to group-specific carbohydrate.

While it has not been possible to identify the specific inactivating substance for the Group A virulent phages, certain pieces of evidence indirectly implicate the group-specific carbohydrate, specifically the *N*-acetylglucosamine moiety. The fact that Group A virulent phages failed to adsorb to heat-killed Group A streptococcal cells suggests that additional factors produced by the living cell are needed for complete viral inactivation.

The authors would like to express their deep appreciation to Dr. Emil C. Gotschlich and Dr. Maclyn McCarty for their interesting and stimulating discussions during the course of this work.

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