INFECTION AND IMMUNITY, Apr. 1977, p. 280-292

Vol. 16, No. 1
Printed in U.S.A.

# Possible Dual Function of M Protein: Resistance to Bacteriophage A25 and Resistance to Phagocytosis by Human Leukocytes

PAUL P. CLEARY* AND ZAIGA JOHNSON

*Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455*

Received for publication 19 October 1976

Spontaneous phage A25-resistant ($A25^R$) mutants of group A streptococci, strain K56, were isolated. The mutant cultures were unable to adsorb phage particles and hyperproduced M protein. Trypsin-digested $A25^R$ cells regained the ability to adsorb phage particles, but failed to become infectious centers. This failure indicated that the mutation created a double barrier to phage growth: (i) receptors were masked by M protein; (ii) irreversibly adsorbed phage were unable to multiply. Spontaneous variants of one $A25^R$ mutant, shown to be M negative ($M^-$) by electron microscopy, serological tests, and sensitivity to phagocytosis, rapidly adsorbed phage and were able to become infectious centers. Therefore, it was concluded that the mutant phenotype, $A25^R$, arose by a single mutation and genes coding for this trait and M protein synthesis were either genetically linked, controlled by a common gene or were biochemically interdependent. The $A25^R$ phenotype was unstable and, as expected for plasmid-coded properties, acridine orange induced segregation of this phenotype. The parental $M^+$, A25-sensitive ($A25^S$) cultures proved to be a mixed population. Infection at various multiplicities indicated that this culture was composed of phage $A25^S$ cells and cells more resistant to infection. Morphological comparison of thin sections of $A25^R$ and $A25^S$ cells by electron microscopy demonstrated striking differences. The $A25^R$ culture was composed entirely of cells uniformly covered with M protein, whereas the $A25^SM^+$ wild-type culture was a mixed population, the majority of cells devoid of M protein. Phagocytosis by human blood enriched the culture for the latter cell type, suggesting that differences in phage sensitivity in the wild-type culture were also determined by the presence or absence of M protein. Thus M protein can serve a dual function for the streptococcal cell by allowing it to avoid infection by bacteriophage and ingestion by human leukocytes.

The outer envelope of group A streptococcal cells is a complex of immunogenic polysaccharides and proteins linked to the structural matrix, peptidoglycan (8, 14, 17, 20). Although this complex has been depicted as distinct layers of M protein, T protein, R proteins, and group A carbohydrate (14), a mosaic network with these various components protruding to differing degrees and with patches of peptidoglycan exposed may be a more accurate picture. It would appear that the streptococcal cell has evolved these somatic structures in an attempt to preserve and protect its cell wall, the "Achilles' Heel" of the bacterial cell, from enzymatic attack. Recent findings have suggested another reason for the streptococcal cell to evolve these somatic coverings—peptidoglycan has been found to make up the receptor for the virulent bacteriophage A25 (P. Cleary, submitted for publication). One can easily imagine that deposition of a polypeptide such as M protein could protect the streptococcal cell from infection by viruses as well as ingestion by human phagocytes.

The M protein, which is located in a layer of fuzz on the surface of group A streptococci (27), has long been recognized for its antiphagocytic properties (15, 16), but only recently has the molecular structure and chemical composition been described. Studies reported by Fischetti et al. have established that the antiphagocytic component of this protein is created by the polymerization of type-specific peptides that are in some manner associated with the cell wall (6). Because the antigenic determinants are strain specific, the M protein is employed to classify group A streptococci, and presently approximately 70 types are recognized (23). In addition to resistance to phagocytosis, other properties associated with the M protein in-

clude fibrinogen precipitation (12), the long-chain phenomenon (26), and, in some types, the serum opacity reaction (31).

Genetic variation of virulence factors is common among bacterial pathogens. Resistance to phagocytosis, a variable trait of group A streptococci, although long recognized, has been difficult to study because useful biological characteristics associated with M protein have been unavailable. Recent genetic experiments from our laboratory have suggested that genes controlling the synthesis of M proteins reside on extrachromosomal deoxyribonucleic acid (DNA) (2). Evidence implicating the extrachromosomal nature of these genes relies on the phenotypic instability of the serum opacity factor which is found to vary concurrently with M protein. Growth of group A streptococci in media containing curing agents such as ethidium bromide and rifampin increased the rate at which genes controlling these phenotypes are segregated. Since attempts to isolate plasmid DNA from appropriate strains of group A streptococci have met with variable success up to this time (unpublished data), we have continued to approach the problem of M protein variation with genetic techniques.

A positive selection for genes controlling M protein synthesis would greatly facilitate the analysis of gene variability. One possible selective marker is mutations imparting resistance to the bacteriophage A25. Maxted has reported that phage A25-resistant variants overproduce hyaluronic acid and M protein, and are at the same time more resistant to phagocytosis and much more virulent for mice (22). The mechanisms of phage resistance and, of more interest to us, its relationship to M protein synthesis remain a mystery and, therefore, are the topics of this report.

Adsorption studies have shown that the mechanism of phage resistance is complex, involving two barriers in the phage growth cycle. The first blocks adsorption of phage particles by masking phage receptors; the second inhibits phage multiplication at some unknown step subsequent to adsorption. The genetic linkage of phage resistance to M protein synthesis is established and the variability of both phenotypes in wild-type cultures is described. Other experiments relate resistance to phagocytosis by human blood to phage resistance, inasmuch as resistance to one fortuitously results in resistance to the other.

## MATERIALS AND METHODS

**Bacteria.** The bacterial strain used throughout this study was strain K56, a group A streptococcus (T-12, M-12) originally described and obtained from E. Kjems (13) and shown to be $M^+$ by precipitin tests, phagocytosis assays, and electron microscopy (27). Strain CS46 was a glossy, $M^-$ variant isolated from strain K56. Other streptococcal strains used in this study were derivatives of strain K56 and their properties are described in Results. Bacterial cultures were routinely grouped, T-typed and M-typed as previously described (10, 15, 25, 33). For typing and phagocytosis studies, bacteria were grown at 37°C in Todd Hewitt broth (Difco), whereas streptococcal cells to be infected with phage were propagated at 30°C in Z6 broth-proteose peptone (4%), yeast extract (0.2%), NaCl (0.6%), $Na_2HPO_4$ (0.07%), $CaCl_2$ (0.02%), glucose (0.1%), and hyaluronidase (Sigma Chemical Co.) (68 μg/ml). Hyaluronidase was added as a filter-sterilized concentrate after the basic media had been autoclaved. Autoclaved solutions of $CaCl_2$ and glucose were also added separately to the sterile basic media.

**Bacteriophage.** Phage A25, a virulent phage, was originally described and supplied by W. R. Maxted (21). Propagation of phage lysates on strain K56 has been described (30). Plaque-forming units (PFU) were assayed in soft agar overlays of strain K56. Soft agar was composed of Z6 broth with 0.7% agar; bottom agar was the same, except the concentration of agar was 1%. Overlay plates were incubated for 18 h at 37°C prior to counting PFU.

**Phage adsorption.** Overnight 30°C cultures were washed twice with Z6 broth and then heat-killed by incubating suspended cells for 30 min at 56°C. The adsorption mixtures, approximately $4 \times 10^6$ PFU and heat-killed cells equivalent to $9 \times 10^8$ colony-forming units (CFU) in 1 ml of Z6 broth, were incubated at 37°C and samples were removed at timed intervals. Irreversible adsorption was quantitated by diluting the sample 1/100 in Z6 broth; then, residual PFUs were immediately assayed. Reversible adsorption was quantitated in the same manner, except the bacteria were sedimented by centrifugation prior to the 1/100 dilution and only the supernatant was assayed for residual PFU.

**Bacterial survival and infectious centers.** Overnight cultures, grown in Z6 broth with hyaluronidase, were cooled on ice. Chilled cultures were examined microscopically to determine average chain lengths and were also serially diluted for viable counts. Diluted bacterial suspension (0.1 ml) was added to 0.1 ml of diluted phage lysate to achieve the desired multiplicity of infection (MOI). MOI was defined here as the number of PFU per cell. Controls were also performed (separate suspensions of bacteria and phage). After a 15-min adsorption period, the phage-bacterial mixture was diluted to 5 ml with Z6 broth and centrifuged at 4°C. The sedimented bacteria were suspended in 0.5 ml of phage A25 antisera and this mixture was incubated for 10 min at 4°C. Subsequently, the serum suspension was serially diluted and samples were assayed for viable CFU in Z6 overlay plates and for infectious centers in Z6 overlay plates containing 0.1 ml of indicator bacteria. CFU and infectious centers were counted after 18 h of incubation at 37°C.

**Trypsin digestion.** Depending on the experiment, either viable or heat-killed cells were digested with trypsin. Cell suspensions were washed once with phosphate buffer (0.06 M phosphate [pH 8.0]) and

then suspended in the same buffer containing 0.5% trypsin (Difco 1:250). After 30 min of incubation at 37°C, the cells were chilled at 4°C and suspended in cold Z6 broth containing 0.5% trypsin. Control cells were treated in the same manner, however, without trypsin in the buffer or broth.

**Hyaluronic acid assay.** One milliliter of culture supernatant from overnight Todd Hewitt broth cultures was assayed for hyaluronic acid by the method of Tolksdorf et al. (29).

**Streptococcal proteinase.** The protein in culture supernatants obtained from overnight 100-ml Todd Hewitt cultures (Oxoid) was concentrated by ammonium sulfate precipitation, 80% saturation. The enzyme assay was based on the procedure of Teh-Yung Liu et al. (28).

**Resistance to phagocytosis.** Cultures were tested for the ability to grow in human blood by the method previously described (2). Whenever this procedure was performed, control cultures, $M^+$ and $M^-$, were included in the experiment.

## RESULTS

**Isolation and characterization of $A25^R$ mutants.** In search of selective conditions for the manipulation of genes controlling M protein synthesis, a detailed analysis of the mechanism of phage A25 resistance and its relationship to M protein synthesis was carried out. Ten independent, spontaneous phage A25-resistant ($A25^R$) mutants of the group A streptococci, strain K56, were isolated and purified from within phage A25 plaques. To avoid the selection of colonies which were dependent on capsules for resistance, agar plates contained bovine hyaluronidase. Supernatants from uninduced and mitomycin-induced cultures of phage-resistant strains were free of phage particles that would form plaques on the parental K56 strains. Five of these mutants were examined for their ability to adsorb and support growth of phage A25, after exhaustive purification on Z6 agar plates seeded with phage A25 (Table 1). The efficiency of plating of phage A25 on all mutants was less than $10^{-5}$ when compared to the wild-type K56 culture. Two temperate phages, phage 3651 and phage 49, formed plaques efficiently on K56 and the mutant cultures, indicating that the mutations had specifically altered the infectious cycle of phage A25 and had not altered the physiology of the cell with respect to these unrelated phages. Adsorption of phage A25 to K56 cells has been shown to occur in two steps: a reversible interaction between cell wall peptidoglycan and phage particles, and an irreversible reaction, yet undefined (Cleary, Wannamaker, Fisher, and Laible, in press). Mutant cultures were tested for their ability to irreversibly inactivate phage A25 (Table 1). Irreversible adsorption to heat-killed cells were blocked to various degrees depending on the mutant and was much reduced in comparison to the parental K56 culture. Moreover, $A25^R$ cultures were also unable to reversibly adsorb the phage (data not shown). Inability to adsorb the phage could be due to either a direct alteration of the phage receptor or the steric masking of the receptor by a macromolecule. If M protein was masking receptors, its removal by proteolytic enzymes should reverse the adsorption block. Digestion of heat-killed cells with trypsin did expose receptor sites, thus removing the adsorption barrier caused by the mutations (Table 1). Trypsin, however, did not alter the plating efficiency of phage A25. Trypsin digestion also slightly increased the adsorption capacity of the parent K56 culture. The importance of the latter observation will become more apparent at a later time.

To illuminate more fully the relationship of M protein to masked phage A25 receptors, the remaining experiments made use of one $A25^R$ culture, strain CS44. As expected from other studies, this phage-resistant mutant exhibited increased levels of extractable M protein (22). The amount of M-12 antigen extracted from CS44 was eight times greater than that extracted from the parent K56 culture (Table 2). $M^-$ variants of strain K56 and strain CS44, strains CS46 and CS64, respectively, will be described later in this section (also see Fig. 7). Inasmuch as streptococcal proteinase activity could not be detected in culture supernatants or in ammonium sulfate concentrates of culture supernatants, the possibility that this enzyme

TABLE 1. *Plating efficiency and adsorption of phage A25 to phage-resistant mutants of strain K56*

| Bacterial strain | EOP[a] | Fraction of phage particles adsorbed[b] | Fraction of phage particles adsorbed by trypsin-digested cells |
|---|---|---|---|
| K56 | 1.0 | 0.82 | 0.91 |
| CS44 | $3 \times 10^{-6}$ | 0.10 | 0.86 |
| CS59 | $1 \times 10^{-8}$ | 0.08 | 0.88 |
| CS60 | $8 \times 10^{-6}$ | 0.30 | 0.89 |
| CS61 | $1 \times 10^{-6}$ | 0.13 | 0.99 |
| CS63 | $6 \times 10^{-6}$ | 0.00 | 0.92 |

[a] The efficiency of plating (EOP) is equal to the number of PFU on the test culture lawn divided by the total number of PFU added to the lawn which contained the phage-sensitive culture strain K56.

[b] The fraction of phage particles adsorbed is equal to the residual PFU after a 10-min adsorption period subtracted from the total PFU added to the heat-killed bacterials cells; this difference divided by the total PFU.

was responsible for different levels of M antigen in the $A25^R$ and A25-sensitive ($A25^S$) cultures was ruled out. Culture supernatants of strains K56, CS44, and their respective $M^-$ variants had equivalent concentrations of hyaluronic acid. Digestion of $A25^R$ cells with hyaluronidase did not alter the adsorption barrier, additional evidence that hyaluronic acid played no part in determining the $A25^R$ phenotype.

Consistent with other published reports concerning the stability of virulence in group A streptococci (2), storage of stationary-phase cultures of strain CS44 resulted in the segregation of $M^-$ cells. Lancefield acid extracts of the $M^-$ culture (CS44I) lacked type 12 M antigen, and the culture failed to multiply in rotated human blood. This $M^-$ culture retained its original T-type, and paradoxically each CFU proved to be resistant to phage A25. Concurrent with these changes, the $M^-$ culture regained the capacity to adsorb phage A25. Adsorption experiments (Fig. 1) clearly demonstrated that CS44 cells were unable to adsorb phage A25. $M^-$ cells which arose by spontaneous segregation, strain CS44I, or resulted from trypsin digestion of CS44 cells efficiently adsorbed phage particles.

On further storage the CS44I stock culture decreased in viability; only $A25^S$ $M^-$ CFU survived. From the survivors, an $A25^S$ colony, designated strain CS64, was purified on blood agar plates and the $M^-$ nature of this strain was confirmed by precipitin test, phagocytosis assays, and electron microscopy of sectioned cells. Thus, upon storage, strain CS44, $A25^RM^+$, evolved through an unstable intermediate state, $A25^RM^-$ (culture CS44I), to a final, stable $A25^SM^-$ state (strain CS64). The intermediate state would not be expected if phage resistance was directly dependent on the M protein. One plausible explanation for the intermediate state was that this culture consisted of chains composed of both $A25^RM^+$ cells and $A25^SM^-$ cells. This possibility was tested by quantitating both infectious centers and phage-resistant cells in the intermediate culture (Table 3). The original CS44 culture was completely resistant to phage A25, 94% survival and no infectious centers, and trypsin digestion did not alter the phage-resistant characteristic of this culture. In contrast, the intermediate culture CS44I contained CFU which were resistant to phage A25, yet also able to form infectious centers, and the total number of colonies which were $A25^R$ and infectious centers was 1.7 times greater than the total number of chains originally infected. The simplest explanation for this fact was that most chains in the CS44I culture harbored both $A25^SM^-$ cells and $A25^RM^+$ cells. Further segregation yielded a stable culture from which strain CS64 was purified and this culture contained chains which were composed entirely of $A25^S$ cells (Table 3). Strain K56 was included as an $A25^S$ control.

**Influence of curing agents on the segregation of $A25^S$ cells.** The simultaneous change to hyperproduction of M protein and $A25^R$, and the cosegregation of both phenotypes, were circumstantial evidence that genes controlling M pro-

TABLE 2. *M-12 precipitin and hyaluronic acid associated with variants of strain K56*

| Strain | Precipitin dilution end point[a] | Hyaluronic acid ($\mu$g/ml) | Multiplication in human blood |
|---|---|---|---|
| K56 | 1/4 | 60 | +++ |
| CS46 ($M^-$ variant) | ND[b] | 30 | − |
| CS44 | 1/32 | 70 | +++ |
| CS64 ($M^-$ variant) | ND[b] | 40 | − |

[a] Cell concentrations were equalized before the preparation of Lancefield extracts. Equal volumes of diluted extracts and type 12 antisera (Communicable Disease Center, Atlanta, Ga.) were added to capillary tubes, and the presence of precipitate was recorded after 18 h at 4°C. Strain CS46 is an $M^-A25^S$ variant isolated from strain K56 ($M^+A25^S$), and strain CS64 is an $M^-A25^S$ variant isolated from strain CS44 ($M^+A25^R$).

[b] ND, Not detectable.

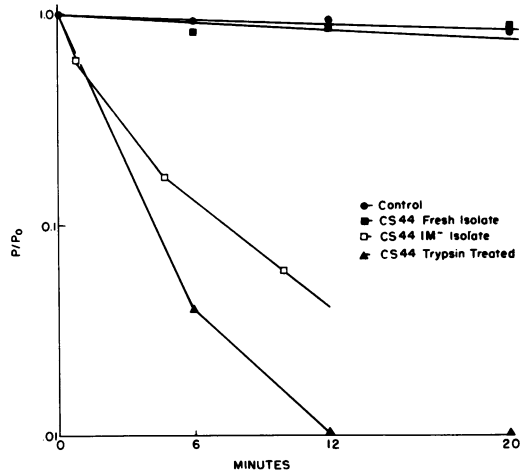


FIG. 1. *Adsorption kinetics of strain CS44 and strain CS44I, an $M^-$ variant of CS44. Phage A25 and heat-killed streptococci were incubated together at 37°C. Samples were removed at the indicated times and diluted 1/100 immediately in fresh Z6 broth to stop adsorption. Residual PFU were assayed on strain K56 lawns. $P_0$ equals the number of PFU initially added to cells and $P_1$ equals the number of PFU remaining at the indicated times. The control contained only phage particles.*

tein synthesis and A25$^R$ were either interdependent for their expression or genetically linked. Previous genetic experiments reported by our laboratory suggested that M protein synthesis was coded by an extrachromosomal element (2). If the mutation creating A25$^R$ was linked to genes controlling M protein synthesis, then cultures of strain CS44 grown in the presence of curing agents may be induced to segregate A25$^S$ cells. Because streptococci grow in chains, the most sensitive method to detect A25$^S$ cells was to assay infectious centers rather than phage-sensitive colonies. With this assay any infected A25$^S$ cell within a chain will cause that chain to become an infectious center, no matter how many A25$^R$ cells were also in the chain. Accordingly, strain CS44 was grown in the presence of rifampin, ethidium bromide, and acridine orange (2). All three agents significantly increased the fraction of A25$^S$ CFU among the total CFU in the culture (Table 4). Acridine orange was by far the most active curing agent. In the control culture, infectious centers were not detectable in numbers above the background of unadsorbed phage particles. From the culture containing acridine orange, 1,000 CFU were replica-plated onto plates seeded with phage; none proved to be A25$^S$. Thus the formation of pure chains, composed of only A25$^S$ cells, was too rare to be detected.

TABLE 3. *Sensitivity of CS44 and M$^-$ variants to phage A25*

| Bacterial culture | M antigen | CFU surviving infection[a] (%) | CFU able to become infectious centers[b] (%) |
|---|---|---|---|
| CS44 | M$^+$ | 94.0 | ($10^{-3}$) |
| CS44 | M$^-$ (trypsin-digested) | 100.6 | ($10^{-3}$) |
| CS44I | M$^-$ | 82.0 | 89.0 |
| CS64 | M$^-$ | 3.0 | 91.0 |
| K56 | M$^+$ | 25.0 | 75.0 |

[a] The percentage of CFU surviving infection is equal to the number of CFU surviving infection divided by the total CFU infected; this fraction multiplied by 100. The MOI in all cases was greater than 5.

[b] The percentage of CFU able to become infectious centers equals the number of infectious centers divided by the total CFU infected; this fraction multiplied by 100. The percentages in parentheses were indistinguishable from the control without streptococcal cells. See Materials and Methods for experimental details.

The spontaneous and chemically induced variation of the A25$^R$M$^+$ phenotypes could be due to either mutations in genes controlling these traits or the segregation of such genes by an unequal distribution of extrachromosomal or chromosomal-linked units of DNA during cell division. If these genes were segregated as a unit by the loss of an extrachromosomal element, then M$^-$A25$^S$ cultures should not revert to the M$^+$A25$^R$ phenotype. To test for reversion, phage resistance was used as a positive selection. M$^-$A25$^S$ cultures were found to revert to A25$^R$; however, none of the revertants tested proved to be M$^+$. Further analysis of these revertants showed that A25$^R$ revertants from M$^-$ cultures became resistant to phage A25 by a different mechanism than did M$^+$ cells. Although not conclusive, this result left open the possibility that genes controlling the A25$^R$M$^+$ phenotype reside on an extrachromosomal element.

**Heterogeneity in wild-type cultures of strain K56.** Experimental data to this point suggested that M protein can mask phage A25 receptors, permitting the bacterial cell to resist viral infection. This conclusion, however, creates a curious paradox, inasmuch as the wild-type culture was also M$^+$, yet sensitive to phage A25. One possible explanation is that the M$^+$ K56 culture was a mixture of M$^+$ and M$^-$ cells and only the latter were susceptible to the phage. Glossy, M$^-$ variants were easily obtained from K56 cultures streaked on blood

TABLE 4. *Induced segregation of A25$^S$ CFU from strain CS44*

| Curing agent | Concn of curing agent ($\mu$g/ml) | Total CFU examined | CFU to become infectious centers (%) |
|---|---|---|---|
| None | 0 | $2.5 \times 10^9$ ($5 \times 10^5$)[a] | 0.04 (0.008)[a] |
| Rifampin | 0.075 | ($8.0 \times 10^5$) | (0.019) |
| | 0.10 | $1.5 \times 10^6$ | 0.026 |
| Ethidium bromide | 0.6 | $4.5 \times 10^4$ | 0.038 |
| | 0.7 | $1.8 \times 10^4$ | 0.242 |
| Acridine orange | 35.0 | $2.1 \times 10^4$ | 0.119 |
| | 45.0 | $2.6 \times 10^4$ | 0.86 |

[a] The rifampin portion of the experiment was performed on a different day than the others. The numbers in parentheses represent the results from that day. The number of infectious centers among cells grown without a curing agent was equivalent to the background PFU in the control without cells.

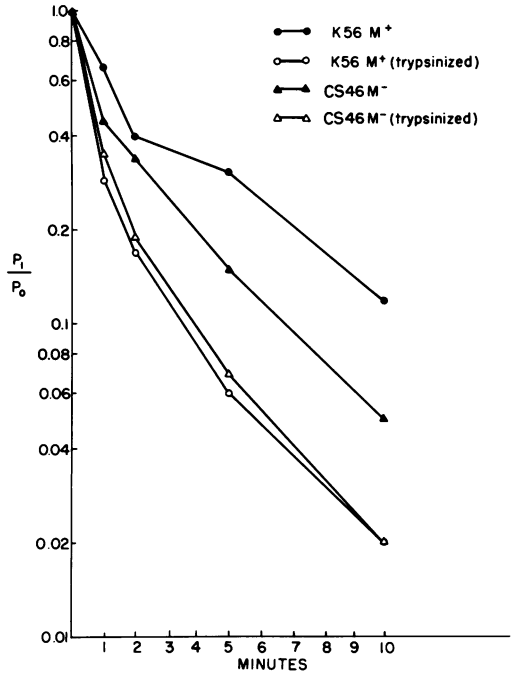


FIG. 2. *Adsorption kinetics of strain K56 M+ and M- cells. (Solid symbols) Adsorption by untreated heat-killed cells; (open symbols) adsorption by trypsin-digested cells.*

agar plates. One such variant, strain CS46, was isolated and exhaustively restreaked on blood agar plates, a necessity to obtain a stable, pure M- variant. The rate of irreversible adsorption to M- cells, strain CS46, was reproducibly greater than the rate of adsorption to M+ cells, strain K56 (Fig. 2). Trypsin digestion, which will degrade M protein, increased the rate of adsorption of phage A25 to both M+ and M- cells. One problem encountered with this experiment was that the rate of adsorption to M+ cells varied somewhat from day to day. This variation could not be controlled and seems to reflect the levels of extractable M antigen.

In support of the adsorption experiments, differences in phage sensitivity between M+ and M- cells could be demonstrated by comparing the survival frequency of the two strains at varied MOIs (Fig. 3). In the absence of antiserum, the successful infection of any cell in a chain will likely result in the subsequent infection of other cells in the chain (7). The survival curve, curve A (M+ culture), was biphasic, indicating that this culture was indeed a mixed population: 60% of the CFUs were highly sensitive to phage A25 and killed according to single-hit kinetics (Fig. 3, curve A; dashed line represents the theoretical kill curve calculated from the Poisson distribution) and approximately 40% of the streptococcal chains were more resistant to phage infection and required higher MOIs for the killing event to occur. Because both portions of the survival curve were linear, the two populations most likely differed with respect to the density or the affinity of available phage receptors. The experiment depicted in curve B differed from that shown in curve A in that phage A25 antiserum was added to the assay plates to prevent subsequent infection on the agar plate. As expected, because CFU represented chains composed of four cells on the average, the shape of the curve was convex which indicated that multiple hits were required to eradicate 1 CFU. The frequency of survival of M- cells, curve C, was different from that of the M+ cells and corresponded more closely to the theoretical survival curve. No antisera were added to assay plates in the experiment shown in curve C. It should be noted that the initial phase of the survival curve of the M+ culture had a slope identical to that of the M- culture. This identity suggested that 60% of the CFUs in the M+ culture were phenotypically M- and able to adsorb the phage more readily.

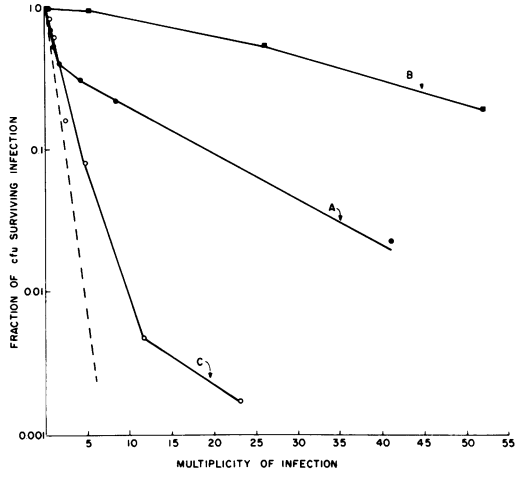


FIG. 3. *Survival of streptococci as a function of multiplicity of phage A25 infection. The experiment was performed as described in Materials and Methods. Controls, uninfected cells of both M+ and M- strains, were manipulated in the same manner as infected cells. The cell number used for the calculation of multiplicity was determined by direct counts under the light microscope. The fraction of CFU surviving infection equalled the number of CFU surviving infection divided by the number of CFU in the uninfected control. (Curve A) Wild-type K56 culture; (curve B) same as curve A except phage A25 antiserum was added to the viable count assay plates; (curve C) the M- variant of K56, strain CS46. The dashed line represents the expected survival frequency calculated from the Poisson distribution.*

TABLE 5. *Phage sensitivity of K56 and variants distinguished by their levels of extractable M protein*

| Strain | Phenotype | MOI | CFU surviving infection[a] (%) | CFU to become infectious centers[a] (%) |
|---|---|---|---|---|
| K56 | $M^+$ | 11 | 10.0 | 82.0 |
| K56 | $M^+$ (trypsin digestion) | 11 | 6.0 | 84.0 |
| CS46 | $M^-$ | 5 | 8.0 | 100.0 |
| CS46 | $M^-$ (trypsin digestion) | 8 | 4.0 | 96.0 |

[a] See Table 3.

Measurements of infectious centers also verified that K56 cultures were mixtures of cells with differing phage sensitivity. On infection with A25 phage particles, any chain with one or more sensitive cells will become an infectious center. As expected from survival data, 82% of the infected CFU became infectious centers and 10% were refractory to phage infection (Table 5). The fraction of CFUs surviving infection varied from 10 to 30% on different days. During the 15-min adsorption period, 90 to 99% of the phage particles had been adsorbed, and longer adsorption periods, up to 1 h, did not influence the number of survivors. Repeated infection of the same cells diminished the number of survivors by 70%, indicating that the original survivors were not absolutely resistant (Cleary, unpublished data). Reproducibly, trypsin digestion of the $M^+$ culture slightly increased the number of infectious centers and decreased the number of survivors. Cultures of strain CS46, the $M^-$ variant of K56, were regularly more sensitive to phage A25. Approximately 100% of the total CFU infected with phage became infectious centers.

**Morphological comparison of $A25^R$, $A25^S$, and $M^-$ variants of strain K56.** Reduced adsorption rates could result from increased deposition of M protein onto the surface of each cell in the $A25^R$ culture or from enrichment of the culture with $M^+$ cells. To distinguish between these two possibilities, thin sections of strain CS44, strain K56, and their $M^-$ derivatives, strains CS64 and CS46, respectively, were examined by electron microscopy. It has been thoroughly established that $M^+$ cells can be differentiated from $M^-$ cells by a prominent layer of "fuzz" on their surface which is composed of M protein (27). Accordingly, the phage-resistant culture, strain CS44, was examined and found to be composed entirely of cells covered with M protein "fuzz" (Fig. 4A). The inset of Fig. 4A shows a pair of cells at higher magnification which were uniformly covered with M protein, whereas the $M^-A25^S$ derivative of this strain was completely devoid of this material (Fig. 4B). No other differences between strains CS44 and CS64 were observed. Cells from the wild-type culture presented a sharp contrast to those in the CS44 culture. Although M precipitin could be extracted from this culture and log-phase cultures resisted phagocytosis, few $M^+$ cells were seen in the electron micrographs (Fig. 5A). Most cells in all fields examined were devoid of M protein. From this experiment it was not possible to determine whether $A25^R$ cells were more uniformly covered with M protein "fuzz" than the $M^+$ cells present in the K56 culture. Consistent with the unstable nature of the K56 culture, chains in the process of segregation were observed. The inset of Fig. 5A shows a chain which appeared to have segregated the potential to synthesize M protein. These two diplococci are the product of two cell divisions; the potential to deposit M protein on the cell surface was lost either at or prior to the first division. One unusual feature of the diplococcus indicated by the arrow was the unilateral distribution of M protein, even though the cell has not completely divided and the cytoplasm of both halves appears continuous. The cells present in the $M^-$ culture purified from strain K56 appeared identical to $M^-$ cells in the mixed K56 culture and to those purified from strain CS44 (Fig. 5B).

**Phage sensitivity and resistance to phagocytosis.** The above experiments lead to the very interesting possibility that bacteriophage may fortuitously select and maintain virulent group A streptococci in our environment. If the degree of phage susceptibility is dependent on the presence or absence of M protein, which also determines the susceptibility of the streptococcal cell to phagocytosis, then overnight, stationary-phase cultures of strain K56 should be a mixture of chains, both sensitive and resistant to phagocytosis. The experiment shown in Fig. 6 tested this possibility. Cells from a stationary-phase, overnight culture were rotated in fresh human blood and samples were removed at 15-min intervals for viable counts. As predicted by the phage survival experiments, approximately 85% of the CFU were rapidly destroyed by the blood. Therefore, by the usual definition, these chains harbored $M^-$ cells. Viable counts remained constant when phagocytosis was impeded (no rotation). Those CFUs able to resist phagocytosis, approximately 15%, began to multiply after a 90-min lag. The correlation between resistance to phagocytosis and phage susceptibility was further substantiated by the fact that rotation of strain K56 cells in human

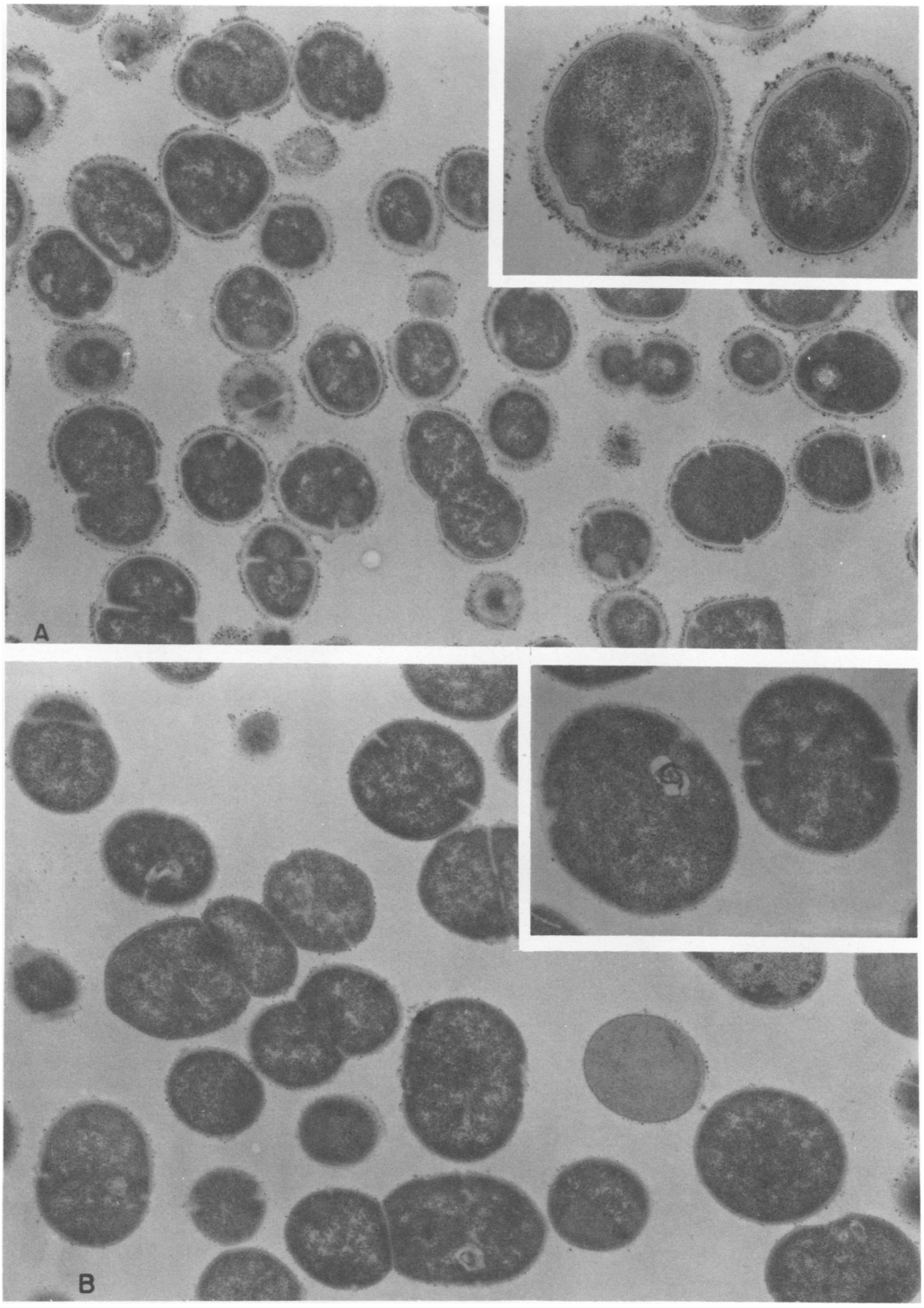


FIG. 4. *Electron micrographs of strain CS44. Overnight cultures in Z6 broth were fixed without washing. (A) Strain CS44, M⁺A25ᴿ; (B) strain CS64, an M⁻ A25ˢ variant of strain CS44. Magnification, ×20,000.*

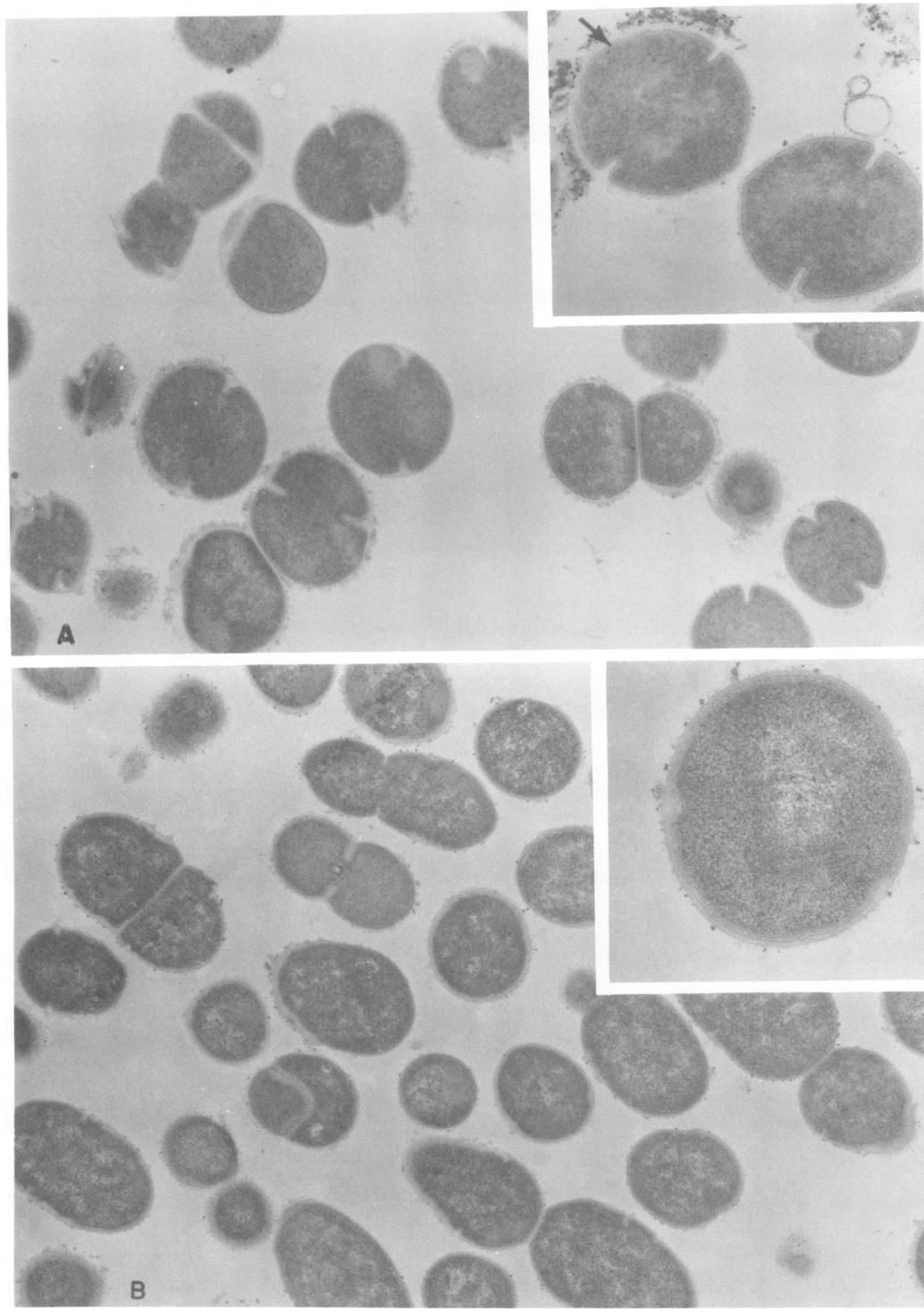


FIG. 5. *Electron micrographs of strain K56. Overnight cultures in Z6 broth were fixed without washing. (A) Strain K56, M⁺A25ˢ; (B) strain CS46, an M⁻A25ˢ glossy variant of strain K56. Magnification, ×20,000.*

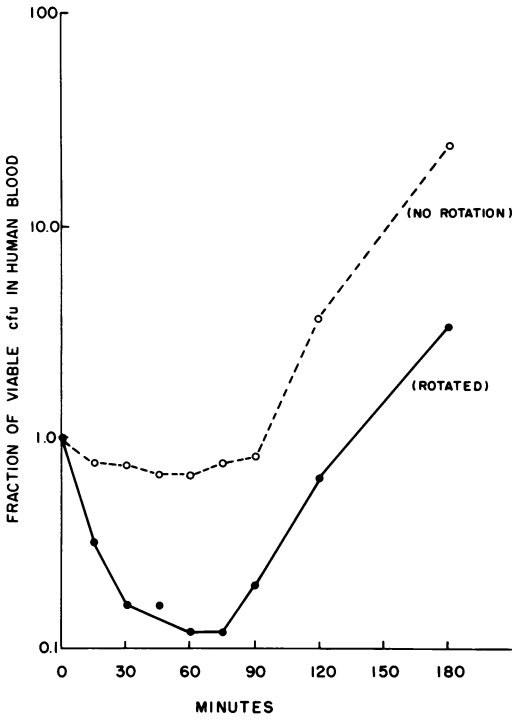


FIG. 6. *Resistance to phagocytosis of strain K56 cells. Diluted 18-h, stationary-phase culture was added to fresh human blood. The number of CFU added to blood was determined by viable counts, and this number was used as the zero time point. Symbols: ----, the control mixture which was not rotated; (therefore, no phagocytosis occurred); ——, the bacterial survival in the rotated mixture. The fraction of viable CFU equals the number of CFU initially added to blood divided by the number of viable CFU at the indicated times.*

blood enriched for chains which were resistant to phage A25 under the conditions described. Cells which survived a 3-h rotation in human blood ($pgc^R$) were grown for 18 h in fresh medium and then tested for infectious centers and phage resistance. Two experiments are shown in Table 6. As in other experiments, the nonrotated cultures were a mixture of $A25^S$ and phenotypically $A25^R$ CFU; the former were the predominant cell type. Cultures originating from cells which had resisted phagocytosis were highly resistant to phage A25; only 4% of all chains harbored a phage-sensitive cell. The enrichment was less pronounced in the second experiment, but clearly evident. Digestion with trypsin reversed the effect of phagocytosis, converting $A25^R$ CFU to infectious centers. Unexplained and contrary to the effect of trypsin on CS44 cells and stock K56 cultures, trypsin greatly decreased the phage survival frequency of cells from cultures enriched for M protein by phagocytosis.

## DISCUSSION

Although it should be obvious to any student of biology that the interaction of a bacterium with its total environment is complex and diverse, the evolution of pathogenic organisms is often only considered in light of the human or animal component of their environment. As is now clearly the case for many bacterial pathogens, their virulence and continued presence in animal populations are either directly or indirectly dependent on bacteriophage. The bacterial pathogen is informed by the genome of a resident prophage and depends on the continued presence of their viral parasite for maximum virulence (1, 3, 5, 11). The group A streptococci are no exception; specific strains have been reported which produce a phage-controlled toxin, responsible for the symptoms of scarlet fever (35). An early report by W. R. Maxted described a different interaction between bacteriophage and group A streptococci that results in the selection of highly virulent bacteria (22). Whereas lysogenic conversion requires residence of the bacteriophage in the bacterial cell, these highly virulent streptococci are mutants which are resistant to the phage, and which hyperproduce M protein and hyaluronic acid capsule, both considered primary virulence factors for this organism. We became interested in this selective process and attempted to gain insight into the relationship of phage resistance to virulence.

Spontaneous mutants of strain K56 are resistant to phage A25, partly because phage particles are unable to attach reversibly or irreversibly to their surface. For convenience, the inability to irreversibly adsorb phage A25 is termed the $IRA^-$ phenotype. The peptidoglycan

TABLE 6. *Selection of phage-resistant streptococci by phagocytosis*

| Strain | Phenotype | MOI | CFU surviving infection[a] (%) | CFU to become infectious centers[a] (%) |
|---|---|---|---|---|
| Expt 1 | | | | |
| K56 | $M^+$ | 0 | 100.0 | 0 |
| K56 | $M^+$ | 8 | 24.0 | 73.0 |
| K56 | $M^+pgc^R$ | 4 | 87.0 | 4.0 |
| Expt 2 | | | | |
| K56 | $M^+pgc^R$ | 7 | 56.0 | 45.0 |
| K56 | $M^+pgc^R$ (trypsin digested) | 10 | 3.0 | 83.0 |

[a] See Table 3.

component of their cell wall, the known receptor for phage A25 (Cleary, submitted for publication) has been either altered or is no longer exposed to the phage. Concurrent with the loss of receptor activity, mutant cells have acquired significant increases in the amount of acid-extractable M protein, whereas no differences in the concentration of hyaluronic acid or streptococcal proteinase are found. To determine whether the adsorption block depends on M protein, two additional kinds of evidence are provided. First, it has been firmly established that streptococcal cells are denuded of M protein by trypsin digestion (9, 17). Accordingly, such cells regain their adsorptive capacity. Alone this result supports the possibility that M protein blocks phage adsorption, but it does not exclude the possibility that other trypsin-sensitive proteins are involved. Secondly, variants of the $A25^R$ mutants, shown to be $M^-$ by electron microscopy, serological tests, and their sensitivity to phagocytosis, regain the ability to adsorb phage particles. Thus, the presence of M protein directly correlates with the lack of phage adsorption. This conclusion, however, is clouded by the simple fact that although proteolysis removes M protein and exposes the buried receptors, mutant $IRA^-$ cells do not become sensitive to the phage. Trypsin-treated cells are not killed by the phage nor do they become productive infectious centers. Therefore, it is necessary to postulate a second block in the phage reproductive cycle, subsequent to the adsorption process. This block is termed the $REP^-$ phenotype. The $REP^-$ phenotype was originally proposed for a group of mutations in *Escherichia coli* which failed to support growth of specific phages (4). We are using this notation here to distinguish between a block in phage development, $REP^-$, as opposed to a block in adsorption, and in no way imply that the $REP^-$ phenotype is similar to that described for *E. coli*.

In many ways our system resembles the interaction of certain bacteriophage and coliform bacteria. The lysogenic state for *Salmonella* phages E15, E34, and P22 depends on cytoplasmic immunity as well as the surface exclusion of super-infecting viruses. Surface exclusion is contingent upon antigenic changes in the somatic O antigen which functions in part as the phage receptors (18, 24, 34, 36). The exclusion of bacteriophage by surface antigens also has been described for *Staphylococcus aureus*. Analogous to the situation described here, protein A, a staphylococcal surface antigen, has also been shown to mask phage receptor, whereas protein A-negative variants efficiently adsorb phage particles (19).

The mutant phenotypes $IRA^-$ and $REP^-$ are more than likely due to a single mutation since both arise and revert simultaneously and spontaneously. Therefore, the genes coding for these traits ($IRA^-$, $REP^-$) and M protein synthesis are either genetically linked, controlled by a common gene, or biochemically interdependent. We have previously suggested that M protein synthesis is controlled by genes residing on plasmid or prophage DNA (2). The $A25^R$ phenotype has also proven to be unstable and, as expected of plasmid-borne properties, curing agents induce segregation of the mutant allele. The mutation to $A25^R$ could reside in either a chromosomal or an extrachromosomal gene. In either case the segregation of genes coding for M protein synthesis and the $REP^-$ phenotypes would result in an $A25^S$ cell.

In contrast to our results, other have reported that M protein plays no part in the phage adsorption process. Friend and Slade compared $M^+$ amd $M^-$ pairs of strains and found little difference in their adsorption capacity (7). Our data suggest that M protein interferes with adsorption of phage A25 and that $M^-$ cells in a wild-type K56 culture are more sensitive to the phage than $M^+$ cells in the same culture. It has been our experience that group A streptococcal cultures are usually a mixture of $M^+$ and $M^-$ cells, and the ratio of one to the other varies considerably. Thus, the contradiction between our data and that of Friend and Slade could be due to the variability of $M^+$ cultures and the difference in methods used to assay phage sensitivity.

The experimental data provided there and elsewhere suggest that group A streptococci assume various phenotypic states (8, 16, 32). Figure 7 depicts the phenotypic variations with respect to M protein synthesis and phage A25 sensitivity that are observed for strain K56. The wild-type culture (strain K56) is a mixture of $M^+$ and $M^-$ cells; the $M^-$ members of the population, symbolized $A25^{SS}$, are more sensitive to phage A25 at a given MOI, i.e., killed more readily than $M^+$ cells which are symbolized $A25^S$. Our experiments are unable to discriminate between two models which explain the differences in phage susceptibility in this mixed culture. In both models the cells which make up CFU differ in the number of exposed receptors: (i) the culture contains chains composed of cells with all possible receptors available to the phage and those composed of cells with fewer receptors exposed, i.e., some fraction of the receptors masked by M protein; (ii) the culture contains chains composed completely of phage-sensitive cells, all receptors uncovered, and chains which are a mixture of

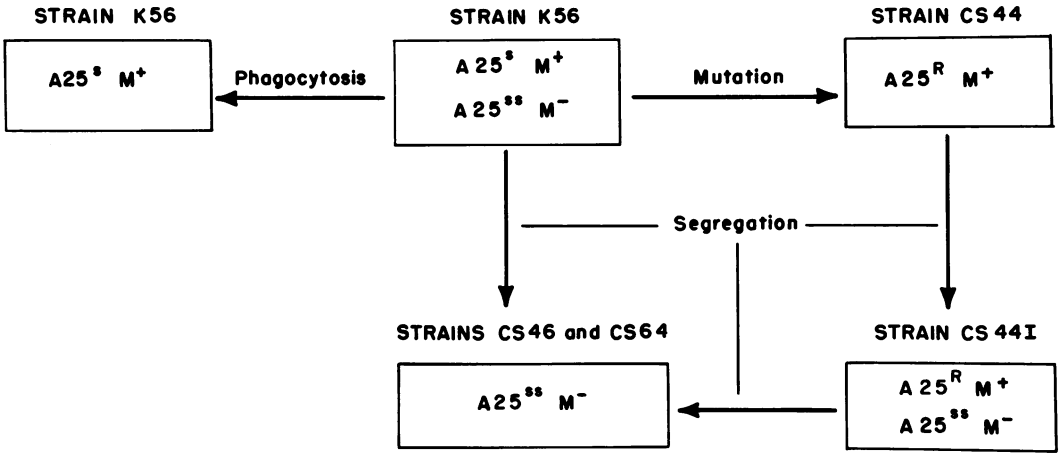


FIG. 7. *Phenotypic states of strain K56 cultures. The boxes represent different populations of cells with respect to M protein synthesis and sensitivity to phage A25. $A25^{SS}$ is meant to symbolize cells which are highly sensitive to phage A25 and $A25^{S}$ symbolizes cells which are less sensitive than $A25^{SS}$ cells. $A25^{R}$ symbolizes cells which are resistant to phage infection. $M^+$ indicates that the cells have M protein on their surface; $M^-$ cells have none.*

cells, those which are $M^+$ phage resistant and those which are $M^-$ phage sensitive. The second hypothesis requires that the $M^+$ cells become phage sensitive at some stage in their life cycle. From this mixed culture (strain K56) both $M^+$ and $M^-$ cells can be purified. Phagocytosis by human leukocytes enrich the culture for $M^+$ cells which are less sensitive to phage A25. Selective purification of glossy (16) or serum opacity factor-negative (2, 31) colonies results in a highly phage-sensitive $M^-$ culture (strain CS46).

At this point it is essential to make a distinction between phage-resistant cells of the wild-type culture, $A25^S$, and those of the mutant culture symbolized by $A25^R$ in Fig. 7. Although a fraction of the CFU in the wild-type culture survive phage infection at high multiplicities, large numbers of survivors are only observed if adsorption occurs in a broth medium for a limited period of time. No cells except rare mutants form colonies on agar plates seeded with phage. In contrast, $A25^RM^+$ cells, strain CS44, form colonies on agar plates seeded with phage after 18 h of incubation. $M^+$ phage-resistant cells surviving phagocytosis also differ from the $A25^R$ mutant cells by their $REP^+$ phenotype. Accordingly, wild-type $M^+$ cells surviving phagocytosis become phage sensitive when M protein is removed by trypsin. Wild-type cultures enriched for $M^+$ cells resemble the mutant culture in that both produce more extractable M precipitin, both grow in broth media as large aggregates which settle to the bottom of the culture vessel, and both cultures spontaneously give rise to $M^-$ phage A25-sensitive cells. The mutant culture can pass through an intermediate state in which chains are a mixture of phage-sensitive and phage-resistant cells. Morphological comparison of thin sections by electron microscopy of $A25^R$ cells from cultures of strain CS44 and cells from cultures of strain K56 confirm the above interpretation. Accordingly, most cells from CS44 cultures are uniformly covered with M protein fuzz, whereas the K56 culture is a mixed population, the majority of cells lacking M protein fuzz. The mutation to $A25^R$ has enriched the culture for $M^+$ cells and thus reduced the adsorption rate.

Although our data establishes that the $IRA^-$, $REP^-$, and M protein hyperproduction are interrelated, it does not conclusively define the nature of the mutation responsible for these characteristics. The possible explanations are numerous, but as a working hypothesis we are proposing that the mutation has enriched the culture for cells that harbor an extrachromosomal factor which controls M protein synthesis. We predict that the enrichment is due in part to the reduced segregation of extrachromosomal genes.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI08724 from the National Institute of Allergy and Infectious Diseases, by a grant-in-aid from the American Heart Association, and with funds contributed in part by the Minnesota Heart Association.

We thank Donna Ritzi for performing the electron microscopy.

## LITERATURE CITED

1. **Barksdale, L., and S. Arden.** 1974. Persisting bacteriophage, infectious lysogeny and phage conversion. Annu. Rev. Microbiol. 28:265-299.

2. **Cleary, P. P., Z. Johnson, and L. Wannamaker.** 1975. Genetic instability of M protein and serum opacity factor of group A streptococci: evidence suggesting extrachromosomal control. Infect. Immun. 12:109–118.
3. **Collier, R. J.** 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54–85.
4. **Denhardt, D. T., M. Iwaya, and L. L. Lanison.** 1972. The rep mutation. II. Its effect on *Escherichia coli* and on the replication of bacteriophage $\phi$X174. Virology 49:486–496.
5. **Eklund, M. W., P. T. Poysky, J. A. Meyers, and G. A. Pelroy.** 1974. Interspecies conversion of *Clostridium botulinum* type C to *Clostridium novyi* type A by bacteriophage. Science 186:456–458.
6. **Fischetti, V. A., E. Gotschlich, G. Siviglia, and J. B. Zabriskie.** 1976. Streptococcal M protein extracted by nonionic detergent. I. Properties of the antiphagocytic and type-specific molecules. J. Exp. Med. 144:32–53.
7. **Friend, P. L., and H. D. Slade.** 1966. Characteristics of group A streptococcal bacteriophages. J. Bacteriol. 92:148–154.
8. **Fox, E.** 1974. M proteins of group A streptococci. Bacteriol. Rev. 38:57–86.
9. **Fox, E. N., and L. D. Krampitz.** 1956. Studies on the biosynthesis of the M protein of group A hemolytic streptococci. J. Bacteriol. 71:454–461.
10. **Griffith, F.** 1934. The serological classification *Streptococcus pyogenes*. J. Hyg. 34:542–584.
11. **Inoue, K., and H. Iida.** 1970. Conversion of toxigenicity in *Clostridium botulinum* type C. Jpn. J. Microbiol. 14:87–89.
12. **Kantor, F. S.** 1965. Fibrinogen precipitation by streptococcal M protein. I. Identity of the reactants, and stoichiometry of the reaction. J. Exp. Med. 121:849–859.
13. **Kjems, E.** 1958. Studies on streptococcal bacteriophages. III. Hyaluronidase produced by the streptococcal phage host cell system. Acta Pathol. Microbiol. Scand. 44:429.
14. **Krause, R. M.** 1963. Symposium on relationship of structure of microorganisms to their immunological properties. IV. Antigenic and biochemical composition of hemolytic streptoccal cell walls. Bacteriol. Rev. 27:369–380.
15. **Lancefield, R. C.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. 57:571–595.
16. **Lancefield, R. C., and E. W. Todd.** 1928. Antigenic differences between matt hemolytic streptococci and their glossy variants. J. Exp. Med. 48:769–790.
17. **Lancefield, R. C.** 1943. Studies on the antigenic composition of group A hemolytic streptococci. I. Effects of proteolytic enzymes on streptococcal cells. J. Exp. Med. 78:465–476.
18. **Lindberg, A. A.** 1973. Bacteriophage receptors. Annu. Rev. Microbiol. 27:205–241.
19. **Nordstrom, K., and A. Forsgren.** 1974. Effect of protein A on adsorption of bacteriophages to *Staphylococcus aureus*. J. Virol. 14:198–202.
20. **McCarty, M.** 1964. The streptococcal cell wall and its biological significance, p. 3–18. *In* J. W. Uhr (ed.), The streptococcus, rheumatic fever, and glomerulonephritis. Williams and Wilkins Co., Baltimore.
21. **Maxted, W. R.** 1952. Enhancement of streptococcal bacteriophage lysis by hyaluronidase. Nature (London) 170:1020–1021.
22. **Maxted, W. R.** 1955. The influence of bacteriophage on *Streptococcus pyogenes*. J. Gen. Microbiol. 12:484–495.
23. **Maxted, W. R., and J. P. Widdowson.** 1972. Protein antigens of group A streptococci, p. 251–226. *In* L. W. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press Inc., New York.
24. **Robbins, P. W., and T. Uchida.** 1965. Chemical and macromolecular structure of O-antigens from *Salmonella anatum* strains carrying mutants of bacteriophage E. J. Biol. Chem. 240:373–383.
25. **Rotta, J. R. M., R. Krause, R. Lancefield, W. Everly, and H. Lackland.** 1971. New approaches for the laboratory recognition of M types of group A streptococci. J. Exp. Med. 134:1298–1315.
26. **Stollerman, G. H., and R. Eksted.** 1960. Factors affecting the chain length of group A streptococci. II. Quantitative M-anti-M relationships in the long chain test. J. Exp. Med. 112:687–698.
27. **Swanson, J., C. Konrad, and E. Gotschlich.** 1969. Electron microscopic studies on streptococci. I. M. antigen. J. Exp. Med. 130:1063–1091.
28. **Teh-Yung Liu, N. P. Neumann, S. D. Elliott, S. Moore, and W. H. Stein.** 1963. Chemical properties of streptococcal proteinase and its zymogen. J. Biol. Chem. 238:251–256.
29. **Tolksdorf, S., M. H. McCready, R. McCullagh, and E. Schwenk.** 1949. The turbidimetric assay of hyaluronidase. J. Lab. Clin. Med. 34:74–89.
30. **Wannamaker, L. W., S. Almquist, and S. Skjold.** 1973. Intergroup phage reactions and transduction between group C and group A streptococci. J. Exp. Med. 137:1338–1353.
31. **Widdowson, J. P., W. R. Maxted, and D. L. Grant.** 1970. The production of opacity in serum by group A streptococci and its relationship with the presence of M antigen. J. Gen. Microbiol. 61:343–353.
32. **Wiley, G., and P. Bruno.** 1972. The M antigen variation and interrelationships, p. 235–249. *In* L. Wannamaker and J. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press Inc., New York.
33. **Williams, R. E. O.** 1958. Laboratory diagnosis of streptococcal infections. Bull. W.H.O. 19:153–176.
34. **Wright, A., and N. Barzilai.** 1971. Isolation and characterization of nonconverting mutants of bacteriophage $E^{34}$. J. Bacteriol. 105:937–939.
35. **Zabriskie, J. B.** 1964. The role of lysogeny in the production of erythrogenic toxin, p. 53–70. *In* J. W. Uhr, Jr. (ed.), The streptococcus, rheumatic fever, and glomerulonephritis. Williams and Wilkins Co., Baltimore.
36. **Zinder, N. D.** 1958. Lysogenization and superinfection immunity in Salmonella. Virology 5:291–326.