Adsorption of the Tailed Mycoplasma Virus L3 to Cell Membranes

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The adsorption properties of the tailed bacteriophage L3 to *Acholeplasma laidlawii* cells were studied. Adsorption followed a biphasic curve. Reversibility and virus heterogeneity were not sufficient to explain the break in the adsorption curve. Binding studies showed that each colony-forming unit could bind about 350 virions. The electrostatic nature of L3 adsorption was indicated by the effect of cations, pH, and temperature on the adsorption rate constant. L3 adsorption appeared to have a requirement for Ca^{2+} , which could not be replaced by the mono- and divalent cations examined. Ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid inhibition of adsorption was totally reversed by added Ca^{2+} . The effects of EDTA, proteases, and lectins on adsorption indicated that membrane proteins are the L3 receptors. The model for L3 adsorption is a multivalent one involving lateral diffusion of adsorbed virions and receptor proteins.

The infection of mycoplasmas by morphologically complex bacteriophages presents unique problems in virus-cell surface interactions. Recent phylogenetic studies have shown that the mycoplasmas arose as a degenerate subline within the gram-positive eubacteria (11). This evolution apparently involved the loss of a significant amount of genetic information and the capacity for making cell wall. However, these wall-less procaryotes are still capable of being infected by tailed bacteriophages.

Acholeplasma laidlawii can be infected by the morphologically complex mycoplasma virus L3. The virion has a polyhedral head (about 60 nm in diameter), a collar, a short tail (about 10 nm wide and 20 nm long), and tail fibers. L3 contains linear double-stranded DNA with a molecular weight of about 26×10^6 (3).

Replication of L3 in *A. laidlawii* cells involves a unique nonlytic cytocidal type of infection (3). After a 90-min latent period, infected cells release progeny virus continuously for 10 to 15 h. Nonlytic virus release is a complex process, apparently including a transient extracellular vesicular structure containing progeny virus (4).

Adsorption kinetics studies showed that the L3-cell membrane interaction is specialized, since the experimental adsorption rate is slower than the rate theoretically possible based on virus-cell collisions (3). Adsorbed L3 virions have lateral mobility on the cell surface; as seen by electron microscopy, randomly adsorbed virions redistribute and form caps or clusters on the cell membrane (4). This capping process is

temperature sensitive (4) and is reduced by metabolic inhibitors (K. Haberer and J. Mani-loff, *Rev. Infect. Dis.*, in press); hence, adsorbed L3 particles behave as polyvalent ligands, with the tail fibers presumably cross-linking laterally mobile cell surface receptors.

We report here studies to describe the biophysical and biochemical parameters of the adsorption of mycoplasma virus L3 on the *A. laidlawii* cell surface.

MATERIALS AND METHODS

Cells and virus. The *A. laidlawii* K2 clone (the host cell used in these studies) and mycoplasma virus L3 were described previously (3). K2 cells were assayed as colony-forming units (CFU), and L3 was assayed as PFU on lawns of K2. For each experiment, virus was prepared by dilution of a high-titer stock (5×10^{12} PFU/ml).

Medium and buffers. Tryptose broth and tryptose-agar plates were prepared as described previously (3). All plating was done by the soft agar overlay technique: the sample to be plated was added to 1 ml of tryptose broth and briefly incubated at 42°C. When we plated for CFU, the sample was 0.01 or 0.1 ml of K2 cells; and when we plated for PFU, the sample was 0.01 or 0.1 ml of virus plus 0.1 ml of K2 cells. Soft agar (containing, per liter, 9 g of NaCl, 6 g of Tris-hydrochloride and 7 g of agar [Difco Laboratories], pH 8.0) was kept at 42°C, and 2 ml was added to the plating mixture. The 3 ml was poured onto a tryptose-agar plate and incubated at 37°C for 24 h (for PFU) or for 3 days (for CFU).

TS buffer (containing 0.001 M Tris-hydrochloride and 0.145 M NaCl, pH 8.0) was used to wash cells and as an adsorption buffer. For experiments in which the

effect of cations was examined, TS buffer was replaced by Tris-sucrose (0.001 M Tris-hydrochloride adjusted to 400 mosmol with sucrose). The osmolality of tryptose broth had been experimentally determined to be about 400 mosmol; therefore, this buffer was used to protect cells from osmotic lysis.

Adsorption procedure. Freshly growing cultures of K2 cells were used for all experiments. These were prepared by diluting overnight cultures 1:5 into fresh medium 2 to 4 h before infection. The cultures were either used directly or washed in TS buffer according to the required conditions. Treatment of the cultures for every experiment is described in the appropriate figure legend. For every experiment the titers of the cell suspension were determined just before infection with L3. The adsorption mixture was mixed by vigorous shaking and then incubated at 37°C without agitation. Adsorption was terminated by centrifugation to remove cells and adsorbed virus. Centrifugation for 4 min in an Eppendorf Microfuge 5412 (Brinkmann Instruments Inc., Westbury, N.Y.) was found to be sufficient to remove more than 95% of the cells. To minimize the loss of the high-titer virus stock, adsorption at a high multiplicity of infection (MOI) was done in small volumes (50 μ l), and a Beckman (Palo Alto, Calif.) Airfuge was used for centrifugation; position 1 for 25 s was found to give satisfactory results. These procedures allowed a number of samples to be treated simultaneously.

From the first-order kinetics curves, the rate constant k was calculated from: $d \ln N/dt = -kB$, where N is the free virus concentration, t is time, and B is the cell concentration. A least-squares data analysis was used.

Lectins and proteases. Concanavalin A and *Ricinus communis* type I and II lectins and trypsin (type III) were obtained from Sigma Chemical Co. (St. Louis, Mo.), and pronase B was obtained from Calbiochem (La Jolla, Calif.). Lectin biological activity was confirmed by erythrocyte agglutination. Both proteases were active as determined by the release of incorporated [3 H]leucine from overnight prelabeled *A. laidlawii* cells (data not shown).

RESULTS

Adsorption kinetics. Previous experiments on the initial adsorption of L3 in medium showed first-order kinetics, with a rate constant of 3×10^{-10} cm³/min (3). The more detailed experiments reported here are in good agreement with those results, showing a first-order rate constant of $k = 2.7 \times 10^{-10}$ cm³/min for the initial slope of the reaction in the medium (Fig. 1). However, after about 10 to 15 min, there was a break in the adsorption curve, and adsorption continued at a slower rate. At the time of this rate change, between 50 and 95% of the total virus had adsorbed, depending on the concentrations of virus and cells and on the experimental conditions (cf. Fig. 1, 2, and 8). It should be noted that there was some variation in the measured rate constant due to experimental conditions, as will be discussed below.

A departure from strict first-order kinetics can

FIG. 1. Adsorption kinetics of mycoplasma virus L3. A growing *A. laidlawii* K2 culture (6.7×10^8 CFU/ml) was infected at an MOI of 0.35. Samples (0.3 ml) were removed at various times and assayed for PFU.

be caused by several factors (1, 10): a reversible step in virus-cell interactions, heterogeneity with respect to adsorption in the virus population, and heterogeneity in cell surface virus attachment sites. Each of these possibilities was considered.

The L3 latent period is about 90 min (3). Therefore, to avoid possible interference from the appearance of progeny virus, we did all adsorption experiments for 45 min or less. This restriction means that the studies reported here examined only the first 90 to 95% of the adsorbing virus population (Fig. 1).

Reversibility of adsorption. To investigate the extent of reversible adsorption, cells (4.9×10^8 CFU/ml) and mycoplasma virus L3 (2.4×10^8 PFU/ml) were mixed. After 20 min of adsorption, cells were removed by centrifugation, and the supernatant was assayed for PFU to determine the number of free virus. The free virus concentration was found to be 3.0×10^7 PFU/ml; hence, 2.1×10^8 PFU/ml had adsorbed. The cell pellet was suspended in fresh medium and incubated. Samples were removed at several times, centrifuged to remove cells, and assayed for PFU to determine the desorbed virus concentration. The free virus concentration rose to 1.3×10^6 PFU/ml by 20 min and did not increase further. Hence, about 1% of the bound virus desorbed, indicating that some fraction of the adsorbed viruses was reversibly attached. The reversible fraction cannot be calculated without a detailed model for adsorption and rate constant data.

Virus heterogeneity. The slow adsorption kinetics observed after 10 to 15 min might be due to "slow adsorbers" in the virus population. The fraction of such a hypothetical slow compo-

FIG. 2. Adsorption kinetics of mycoplasma virus L3 stock treated to enrich for possible slow adsorbers and of original L3 stock. To enrich for slow adsorbers, a K2 culture (5 ml) was infected at an MOI of 5, and virus was allowed to adsorb for 20 min. After cells and adsorbed virus were removed by filtration through a 0.2- μ m membrane filter, 3 ml of the resulting free virus suspension was used to resuspend a cell pellet from 3 ml of an uninfected culture. After the adsorption was repeated, the resulting virus suspension (from two adsorption cycles) should have been enriched for slow adsorbers as described in the text. Parallel K2 cultures (4.6×10^8 CFU/ml) were infected with this virus suspension at an MOI of 0.09 (O) and with the original unselected L3 at an MOI of 0.03 (●), and adsorption kinetics were measured as described in the legend to Fig. 1.

nent in the population would be given by the intercept of the slow part of the adsorption curve extrapolated back to the y-axis. From Fig. 1, 20% of the virus would be the hypothesized slow-adsorbing component.

To try to enrich for slow-adsorbing virus, a virus stock was carried through two cycles of adsorption; each cycle was for 20 min. Based on typical adsorption kinetics (Fig. 2), after a 20-min adsorption, the 23% unadsorbed virus would be due to about 6.5% "fast adsorbers" and 16.5% slow adsorbers. Thus, after a single adsorption cycle, about 28% of the remaining free virus would be fast adsorbers, and after a second adsorption cycle, only 8% would be fast adsorbers and the remaining 92% would be slow adsorbers.

Figure 2 shows adsorption kinetics of a typical virus stock and of that preparation after two cycles of adsorption enrichment for slow adsorbers. The initial rate constant for the adsorption enrichment sample, calculated from the initial slope, was slightly less than that of the original virus stock, 2.0×10^{-10} cm³/min as compared with 2.9×10^{-10} cm³/min, in this experiment. However, from the y-intercepts of

the slow component of each curve, the hypothetical slow adsorbers comprised 33% of the original virus stock used in this experiment and 47% of the adsorption enrichment preparation. These data suggest that there is some virus heterogeneity, but that it is insufficient to account for the break in the adsorption curve.

Attachment site heterogeneity. Since adsorption experiments, as in Fig. 1 and 2, were done at low MOIs, it seemed improbable that heterogeneity in virus attachment sites on the cell surface would have been affecting these adsorption kinetics. Equilibrium binding experiments to investigate site heterogeneity could not be done, because it was not possible to inhibit viral penetration and, thereby, study reversible attachment.

To obtain an estimate of the number of virus attachment sites per cell, we measured the number of PFU bound per CFU as a function of MOI (Fig. 3). These data show a linear relationship between the number of PFU of bound virus and MOI (slope = 0.92 for the linear part of the log-log curve in Fig. 3), reaching saturation at about 350 PFU bound per CFU.

Effect of cations. Electrostatic interactions apparently play a greater role in the attachment of phages to bacterial cell walls (reviewed in reference 10) than in the attachment of animal viruses to cell membranes (reviewed in reference 7). To study the importance of electrostatic interactions in the adsorption of L3 to the *A. laidlawii* cell membrane, L3 adsorption was compared in tryptose medium (the medium used in all the above experiments) and in TS buffer. The rate constant decreased significantly; the value in TS

FIG. 3. Number of PFU of L3 bound per CFU as a function of MOI. Samples of a cell culture (5.8×10^7 CFU/ml) were mixed with various L3 concentrations. After a 20-min incubation at 37°C, cells and adsorbed virus were removed by centrifugation, and the supernatant was assayed for PFU. Since some cell clumping was possible, the data are expressed as PFU bound per CFU at each MOI.

buffer was fivefold lower than that in medium (data not shown). Therefore, the effects of mono- and divalent cations and of chelating agents on L3 adsorption in TS buffer were studied.

It should be noted that, throughout this study, the absolute value of the rate constant was found to vary from experiment to experiment by as much as a factor of 5. This appeared to be a function of two interdependent variations, in the physiological condition of the cells and in the composition of different medium lots. Neither variation could be readily controlled over the duration of this study; therefore, experiments on the effects of chemical and physical agents on adsorption were done as comparative studies, and rate constants are expressed as relative values.

The effects of various concentrations of monovalent (Na^+ , K^+ , and NH_4^+) and divalent (Ca^{2+} and Mg^{2+}) cations on the L3 adsorption rate constant are shown in Fig. 4. Relative to the adsorption rate constant without added cations, monovalent cations increased the rate constant

FIG. 4. Relative L3 adsorption rate constants in the presence of various cations. Cells were washed twice and suspended in Tris-sucrose buffer (pH 8.0). L3 was added for an MOI of 0.53, and samples (0.15 ml) were immediately diluted 1:1 into Tris-sucrose buffer containing added cations. The added monovalent cations (a) were NaCl ($\blacktriangle$), KCl ($\triangle$), or NH_4Cl ($\square$), and divalent cations (b) were CaCl_2 ($\bullet$) or MgCl_2 ($\circ$). After 10 min, cells and adsorbed virus were removed by centrifugation, and the supernatant was assayed for PFU. The data are expressed as the ratio of the adsorption rate constant at each cation concentration to the rate constant in a control Tris-sucrose sample containing no added cations.

TABLE 1. Stability of mycoplasma virus L3 under conditions used in these studies^a

Treatment	% Surviving PFU
Trypsin (20 $\mu\text{g}/\text{ml}$)	94
Pronase (20 $\mu\text{g}/\text{ml}$)	97
1 mM EDTA	86
1 mM EGTA	89
5 mM CaCl_2	108
pH 8.0	100
pH 6.0	92
pH 5.0	64
pH 4.0	46

^a The titers of mycoplasma virus L3 were determined before and 15 min after each treatment at 37°C.

moderately (perhaps with an optimum at about 40 mM), Mg^{2+} reduced the rate constant slightly (at all concentrations examined), and Ca^{2+} increased the rate constant by about a factor of 1.6 (at all concentrations examined). To examine the divalent cation effect further, we determined L3 adsorption rate constants in TS buffer containing chelating agents (data not shown). The rate constants in 1 mM EDTA and 1 mM ethylene glycol-bis(β -aminoethyl ether)- N,N -tetraacetic acid (EGTA) were each reduced to about 0.2 that of a control sample (in buffer alone). However, the chelator effects appeared to be complex. The EDTA effect could be only partially reversed; after EDTA treatment and Ca^{2+} addition, the rate constant was the same as that for samples in buffer alone. But, EGTA inhibition could be totally reversed; after EGTA treatment and Ca^{2+} addition, the rate constant was the same as that for samples in buffer plus added Ca^{2+} (i.e., about 1.6 times greater than for buffer alone). EDTA, EGTA, and Ca^{2+} did not significantly affect L3 viability (Table 1).

To try to determine the Ca^{2+} requirement for L3 adsorption, we measured rate constants for cells suspended in TS buffer containing 1 mM EDTA and various Ca^{2+} concentrations (Fig. 5). Below 1.5 mM Ca^{2+} , the rate constant increased with increasing Ca^{2+} concentration. At $[\text{Ca}^{2+}] = [\text{EDTA}]$ (i.e., at 1 mM Ca^{2+}), the rate constant was the same as that in the control sample without Ca^{2+} or EDTA. In these studies, no $[\text{Ca}^{2+}]$ threshold could be observed (even in what should be excess EDTA) below which there was little L3 binding. Hence, cell membrane-bound Ca^{2+} may have been affecting L3 adsorption, as will be discussed below.

Effect of temperature. Temperature had only a slight effect on the L3 adsorption rate constant (Fig. 6). The rate constant at 42°C was only 1.5 times greater than that at 4°C.

Effect of pH. The effect of pH on adsorption was difficult to determine, since L3 was unstable at pH's lower than 6 (Table 1). Therefore, at

FIG. 5. L3 adsorption rate constant in 1 mM EGTA and added Ca^{2+} . Cells were prepared as described for Fig. 4 and suspended in TS buffer (pH 8.0) containing 1 mM EGTA and various concentrations of Ca^{2+} . Samples were taken at 0, 5, and 10 min, cells were removed by centrifugation, supernatants were plated for PFU, and rate constants were calculated. The data are expressed as the ratio of the adsorption rate constant at each Ca^{2+} concentration to that in a control sample in TS buffer alone (i.e., no Ca^{2+} and no EGTA).

each pH, adsorption data were corrected for inactivation due to pH. The L3 adsorption rate constant was independent of pH between 7 and 9, but decreased below pH 6 (Fig. 7). At pH 4, the rate constant was 0.14 that at pH 7 to 9.

Effects of proteases and lectins. To investigate the nature of L3 receptors on the *A. laidlawii* surface, the effects of proteases and lectins on the adsorption rate constant were measured. The proteases used, trypsin and pronase, did not inactivate L3 under these experimental conditions (Table 1).

Trypsin and pronase both reduced L3 adsorption (Fig. 8a), indicating involvement of cell surface proteins in L3 attachment. The observation that trypsin was somewhat more effective than pronase in reducing L3 adsorption was surprising, since under these experimental conditions it was found that pronase released more

FIG. 6. L3 adsorption rate constant k as a function of temperature. At each temperature, cells (4.2×10^8 /ml) in tryptose broth were infected at an MOI of 0.58. After 10 min, cells and adsorbed virus were removed by centrifugation, and the supernatant was assayed for PFU. The data are expressed relative to the rate constant at 37°C.

FIG. 7. L3 adsorption rate constant k as a function of pH. Cells were washed once, suspended in TS buffer (3.9×10^8 CFU/ml) adjusted to various pH values, and infected at an MOI of 0.62. Parallel samples without cells were used to determine the surviving virus fraction at each pH, and this was used to correct the adsorption data. After 10 min, cells and virus were removed by centrifugation, and the supernatant was assayed for PFU. The data are expressed relative to the rate constants for the pH range of 6 to 9.

A. laidlawii membrane protein than trypsin did (data not shown).

Although they do not agglutinate *A. laidlawii* cells, concanavalin A and *R. communis* lectins bind to *A. laidlawii* membranes (6, 9). Therefore, pretreatment with lectins was used to block exposed sugar groups on the cell surface before adsorption: concanavalin A binds glucose and mannose, *R. communis* type I lectin binds *N*-acetylgalactosamine, and *R. communis* type II lectin binds galactose. The pretreatment of cells with these lectins had no effect on L3 adsorption (Fig. 8b).

DISCUSSION

For viruses of the eubacteria, the phage tail functions to attach the virion to a cell enclosed by a wall structure. Mycoplasma virus L3 is a system in which the phage tail functions to attach the virion to a cell enclosed only by a membrane. The studies reported here describe some of the adsorption properties of this system and provide data on the nature of the virus-cell interaction.

Mycoplasma virus L3 adsorption kinetics (Fig. 1) is similar to that reported for the bacteriophages. Unfortunately, no adequate model is available to explain the form of the phage adsorption curve (1, 10). Like biphasic bacteriophage adsorption curves, L3 adsorption may involve the superposition of several factors: some degree of reversibility in the L3 attachment reaction, some degree of virus heterogeneity with respect to adsorption, and some degree of heterogeneity in cell surface receptors. The data above show that reversibility and virus

FIG. 8. Effect of proteases and lectins on L3 adsorption. Cells were washed once and suspended in TS buffer (pH 8.0) containing (a) proteases or (b) lectins. The protease experiment samples contained either MgCl_2 (●), trypsin and MgCl_2 (▲), or pronase and MgCl_2 (△), and the lectin experiment samples contained either no addition (●), concanavalin A (○), *R. communis* type I lectin (▲), or *R. communis* type II lectin (△). The MgCl_2 concentration was 4 mM, and all proteases and lectins were 25 $\mu\text{g}/\text{ml}$. Since the pronase required MgCl_2 for activity, MgCl_2 was included in the trypsin and control samples to allow comparison of the data. For the proteases, cells (8.0×10^8 CFU/ml) were infected at an MOI of 0.3, and for the lectins, cells (1.2×10^8 CFU/ml) were infected at an MOI of 0.5. Adsorption was measured as described in the legend to Fig. 1.

heterogeneity are present, but are almost certainly not sufficient to explain the L3 adsorption curve. Virus heterogeneity may be expected simply due to differences in numbers of functional tail fibers on individual virions. No evidence of membrane receptor heterogeneity or of cooperativity (positive or negative) in the adsorption reaction could be seen in binding studies (Fig. 3).

The binding studies did show that each CFU can bind a maximum of about 350 L3 virions. This may not necessarily be the number of binding sites per cell, because (due to clumping) each CFU might represent, on the average, more than one cell. It should be noted that it would take about 1,100 closely packed L3 viruses to cover the surface of the 1- μm -diameter *A. laidlawii* cell.

The electrostatic nature of L3 adsorption is indicated by the effect of cations (Fig. 4) on and the pH dependence (Fig. 7) of the adsorption rate constant. These effects on the L3 rate constant are much less pronounced than those on bacterial virus rate constants (10), as expected since L3 must attach to a cell membrane which has significantly less negative surface charge density than the bacterial cell walls to which bacterial viruses must bind.

The small temperature dependence of the L3 adsorption rate constant (Fig. 6) means that there is little activation energy needed for adsorption, as expected for electrostatic interactions. Parenthetically, this indicates that cell metabolism is probably not required for adsorption. As noted by Garen and Puck (2), small

changes in the adsorption rate with temperature can be explained by temperature dependence of the solvent viscosity. Hence, the 1.5-fold change in the L3 adsorption rate constant between 4 and 42°C was probably due to the approximate 2-fold change in water viscosity over this temperature range.

L3 adsorption appears to have a requirement for Ca^{2+} , which cannot be replaced by Mg^{2+} , Na^+ , K^+ , or NH_4^+ (Fig. 4). This indicates that the role of Ca^{2+} in L3 binding is not simply an electrostatic one. However, added Ca^{2+} could not increase the adsorption rate constant in buffer to that in tryptose medium. This may be due to one or both of the following factors: other, still unknown, components in tryptose medium may be required for L3 adsorption; and washing and resuspension in buffer may have a physiological effect, thereby reducing the maximum possible adsorption rate.

L3 adsorption in Ca^{2+} -free buffer was inhibited by EGTA and EDTA. Excess Ca^{2+} could totally reverse the EGTA effect. However, the Ca^{2+} effect appeared to be a complex one and, even when the added Ca^{2+} concentration was less than the EGTA concentration, adsorption was found to be dependent on the Ca^{2+} concentration (Fig. 5). Since *A. laidlawii* membranes have been shown to bind medium Ca^{2+} (5), the Ca^{2+} dependence of adsorption in excess EGTA and the adsorption measured in Ca^{2+} -free buffer probably reflect the involvement of cellular Ca^{2+} in adsorption. The role of cell-bound Ca^{2+} in adsorption may also explain the observation that Mg^{2+} slightly inhibited adsorption (Fig. 4b),

since Mg^{2+} has been reported to be a weak competitor for cell membrane Ca^{2+} -binding sites (5).

Added Ca^{2+} only partially reversed the inhibition of adsorption by EDTA. EDTA has been reported to cause the loss of some *A. laidlawii* membrane proteins (8). This might explain why EDTA inhibition of L3 adsorption was not completely reversed by added Ca^{2+} and suggests that cell membrane proteins might function in L3 binding.

In agreement with this indication that cell membrane proteins are the L3 receptors, protease treatment inhibited L3 adsorption (Fig. 8a). Although lectins did not inhibit L3 adsorption (Fig. 8b), the possibility that the receptor may be a glycoprotein cannot be excluded from these data. The lectins used in this study are the only ones reported to bind to *A. laidlawii* and, based on proteolytic digestion and lipid extraction studies, are believed to bind to glycolipid or lipopolysaccharide (6, 9). Since glycoproteins have not been detected in *A. laidlawii* membranes (K.-E. Johansson, personal communication), it is probable that membrane proteins are the L3 receptors.

The data presented here, together with those demonstrating capping of adsorbed L3 virions (4; Haberer and Maniloff, in press), indicate that the model for L3 adsorption on the *A. laidlawii* cell membrane resembles the multivalent attachment mechanism proposed for the adsorption of animal viruses to animal cell membranes (7). The multivalent nature of the attachment probably requires lateral diffusion of both adsorbed virions along the surface and receptor proteins within the membrane.

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