Effects of Ionophores and Dicyclohexylcarbodiimide on
Mycoplasma gallisepticum Adherence to Erythrocytes

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To test the influence of the electrochemical ion gradient across mycoplasma membranes on the capacity of organisms to adhere to host cells, Mycoplasma gallisepticum cells were treated with valinomycin, carbonylcyanide m-chlorophenylhydrazone, and N,N'-dicyclohexylcarbodiimide (DCCD) singly or in combination. Uptake of [3H]tetraphenylphosphonium by the treated cells was employed as a measure of the effects of the ionophores on membrane potential. In the absence of K+, valinomycin increased, whereas carbonylcyanide m-chlorophenylhydrazone and DCCD decreased [3H]tetraphenylphosphonium uptake. However, with a high level of K+ or with DCCD, uptake of [3H]tetraphenylphosphonium in the presence of valinomycin decreased below control levels, indicating that, generally, the ionophores affected membrane potential in the expected manner. The treated organisms were tested for their capacity to attach to glutaeraldehyde-fixed human erythrocytes. DCCD was the best inhibitor of mycoplasma attachment, and in combination with valinomycin, attachment capacity decreased by about 40%. The combination of valinomycin plus carbonylcyanide m-chlorophenylhydrazone was less effective; it decreased attachment by about 15 to 25%. It was concluded that the dissipation of ion gradients across cell membranes decreases only partially mycoplasma adherence, in line with previous findings that isolated mycoplasma membranes retain the major part of the attachment capacity of intact cells.

The adherence of the human respiratory pathogen Mycoplasma pneumoniae and the avian pathogen Mycoplasma gallisepticum to eucaryotic cell surfaces both in vivo and in vitro and to inert surfaces, such as glass and plastic, are well established phenomena (1, 4, 9, 10, 11, 14, 18, 25, 27, 30). Sialic acid residues serve as major receptor sites in the adherence of these mycoplasmas to eucaryotic cells (3–5, 25). Obviously, the absence of these receptors from glass and plastic suggest that the mechanism of mycoplasma attachment to cells and inert surfaces may be different. Nevertheless, our recent finding that cell energization plays an important role in the attachment of M. pneumoniae cells to glass (12, 13) prompted us to investigate the effects of the physiological state of mycoplasmas on their ability to adhere to eucaryotic cells. Our results (3) showed that viability of the mycoplasmas per se was not essential for M. gallisepticum attachment to erythrocytes (RBC), as cells killed by UV irradiation and membranes isolated by lysing M. gallisepticum cells by various means retained attachment capacity. However, treatment of the mycoplasmas by protein-denaturing agents, such as heat, glutaeraldehyde, or prolonged exposure to low pH, drastically affected or even abolished attachment, supporting the protein nature of the M. gallisepticum membrane components responsible for specific binding to the sialoglycoprotein receptors on RBC. Treatment of mycoplasmas with ionophores (2, 12) or limiting their energy sources (8) induced changes in the disposition of membrane components, including the decreased exposure of membrane proteins on the cell surface (2, 16a). This led us to the idea that agents which dissipate the electrochemical gradient of ions across cell membranes may decrease the attachment capacity of mycoplasmas by decreasing the exposure of the mycoplasma binding components on their cell surfaces. Our finding that carbonylcyanide m-chlorophenylhydrazone (CCCP) alone, and more effectively, in combination with valinomycin, as well as N,N'-dicyclohexylcarbodiimide (DCCD) markedly decreased the energy-dependent attachment of M. pneumoniae cells to glass (12, 13) indicated that it is worthwhile to investigate the effects of ionophores on mycoplasma attachment to eucaryotic
cells.

We recently showed that attachment of isolated *M. gallisepticum* membranes to RBC was markedly decreased by neuraminidase pretreatment of the RBC (3), suggesting a similar mechanism of attachment of intact cells and membranes of this mycoplasma to RBC. Moreover, the finding that the isolated membranes retained most of the attachment capacity shown by washed intact cells indicated that the effects of ionophores, if any, could not be an all-or-none effect, but may only be expressed as a small decrease in attachment capacity. To be able to distinguish with a satisfactory degree of reproducibility small differences in adherence values, an organism with high attachment capacity was sought. Strain S6 of *M. gallisepticum* was found to fulfill this requirement. The data reported in the present communication indicate that treatment of *M. gallisepticum* cells with ionophores decreases their capacity to adhere to RBC.

**MATERIALS AND METHODS**

**Organism and growth conditions.** *M. gallisepticum* S6 obtained from Sharon Levisohn (The Veterinary Institute, Beit Dagan, Israel) was grown in a modified Edward medium containing 8% (vol/vol) horse serum and 150 μCi of [9,10-3H]palmitic acid (The Radiochemical Centre, Amersham, England) per liter. The organisms were harvested after 18 to 20 h at 37°C, at which point the absorbance of the culture at 640 nm reached 0.17 to 0.2 and the pH decreased to about 7.3. The organisms were washed once by centrifugation at 6,000 × g for 15 min at 4°C with buffer A (0.1 M NaCl containing 1 mM CaCl2 plus 0.05 M Tris, pH 7.2 (4)) either alone or supplemented with 10 mM glucose. For determination of [3H]traphenylphosphonium (TPP+) uptake, the pellet was resuspended in buffer A supplemented with 10 mM glucose to about 1.3 mg of cell protein per ml. For attachment experiments, the pellet was resuspended in buffer A either alone or supplemented with 10 mM glucose to about 0.7 mg of cell protein per ml. To remove cell aggregates (3), the suspensions were dispensed in 5-ml samples and centrifuged at 400 × g for 3 min in a Sorvall GLC-2 centrifuge, using a swinging-out bucket rotor. The upper 4 ml of each tube were collected carefully, brought to a final cell protein concentration of 120 μg/ml, and used for attachment experiments.

**Chemicals.** CCCP, valinomycin (Sigma Chemical Co., St. Louis, Mo.), or DCCD (Fluka AG, Buchs, Switzerland) was dissolved in dimethyl sulfoxide. Unlabeled TPP+ (K&K, ICN Pharmaceuticals, Inc., Plainview, N.Y.) was dissolved in ethanol at a final concentration of 10-2 M. [3H]TPP+ (1 mCi/ml, 2,500 Ci/mmol; Nuclear Research Center, Negev, Israel) was diluted 10-fold with double-distilled water. All solutions were stored at −20°C until used.

**RBC.** Fixed human RBC were prepared by adding 0.5% glutaraldehyde to washed 10% packed RBC in phosphate-buffered saline as described previously (5). After the final washing, the fixed RBC were stored at 4°C with 0.05% NaN3 in buffer A. For each use, samples of fixed RBC suspension were washed once and resuspended at a final concentration of 2% (vol/vol) in buffer A.

**Ionophores and DCCD treatment.** Stock solutions of the ionophores (5 × 10-3 M) or DCCD (1 × 10-2 M) in dimethyl sulfoxide and KCl solution in water were added to mycoplasma cell suspensions in buffer A supplemented with 10 mM glucose to give the final concentrations shown in Table 1. In case of a mixture of two or three substances, the order of addition was KCl, CCCP, and valinomycin. For the combination of DCCD and valinomycin, DCCD was added 5 min before the addition of valinomycin. Total incubation time in all cases was 15 min at 37°C. Samples of the treated suspensions (0.1 ml) in tetrapiolate were used for assessment of mycoplasma attachment capacity. The marked tendency of *M. gallisepticum* cells to lyse in an NaCl-containing buffer devoid of glucose (22) hampered efforts to test the effects of ionophores on *M. gallisepticum* attachment in the absence of glucose. To overcome this difficulty, we lowered the concentration of glucose to 1 mM and shortened the preincubation time with the inhibitors listed in Table 2 to 5 min at 37°C before assessment of attachment capacity.

**Assessment of mycoplasma attachment.** Attachment was assessed with a procedure slightly modified from that previously described by Banai et al. (4). In short, 0.1 ml of mycoplasma suspension was added to 0.1 ml of 2% glutaraldehyde-fixed RBC in Nunc plastic tubes (11 by 70 mm), which replaced the siliconized glass tubes. The reaction was stopped after 15 min of incubation at 37°C by adding 0.5 ml of cold buffer A (supplemented with 10 mM glucose in experiments in which glucose was included), and the RBC were washed three times, using the swinging-out bucket rotor of the Sorvall GLC-2 centrifuge at 370 × g to remove free mycoplasmas. Radioactivity of mycoplasmas attached to the RBC was determined as described previously (4).

**Assessment of [3H]TPP+ uptake.** Samples (0.5 ml) of mycoplasma cell suspensions (1.3 mg of cell protein in buffer A supplemented with 10 mM glucose) were preincubated at 37°C for 5 min with 100 μM unlabeled TPP+ to saturate membrane sites which bind nonspecifically this cation. Then, KCl or dimethyl sulfoxide stock solutions of the ionophores (5 × 10-2 M) or DCCD (10-2 M) were added. A mixture of two or three substances was added in the order KCl, CCCP, and valinomycin. In the combination of DCCD and valinomycin, DCCD was added 5 min before the addition of valinomycin (the dimethyl sulfoxide concentration never exceeded 1%). After 1 min of treatment at 37°C, [3H]TPP+ was added to a final concentration of 1.06 × 106 cpm/ml, and incubation was continued at 37°C for 15 min. The reaction was stopped by diluting 0.2 ml of the treated mycoplasma suspensions with 2 ml of cold buffer A supplemented with 10 mM glucose and immediate filtration (by negative pressure) through a 500-nm (micropore size), 25-mm (diameter) cellulose acetate filter (EH type; Millipore Corp., Bedford, Mass.). The filter was washed once with 2 ml of cold buffer A and thoroughly dried for radioactivity counting. Each reaction mixture was sampled in duplicate.

**Analytical procedures.** Protein was determined by the method of Lowry et al. (17). Radioactivity was determined by scintillation spectrometry with a Triton X-100 toluene liquor (15).
TABLE 1. Effect of ionophores and DCCD on *M. gallisepticum* uptake of [3H]TPP* and attachment to fixed RBC

<table>
<thead>
<tr>
<th>Substances tested</th>
<th>Net [3H]TPP* uptake( \pm \text{SD} ) (cpm)</th>
<th>% Mycoplasma attachment# of total in binding mixture ( \pm \text{SD} )</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer alone</td>
<td>4,688 ± 518</td>
<td>35.7 ± 1.0</td>
<td>100</td>
</tr>
<tr>
<td>KCl (10 mM)</td>
<td>4,013 ± 573</td>
<td>34.9 ± 1.1</td>
<td>97.7</td>
</tr>
<tr>
<td>Valinomycin (0.01 mM)</td>
<td>8,712 ± 603</td>
<td>36.5 ± 1.2</td>
<td>102.2</td>
</tr>
<tr>
<td>Valinomycin (0.01 mM) + KCl (10 mM)</td>
<td>5,939 ± 567</td>
<td>35.0 ± 1.1</td>
<td>98.0</td>
</tr>
<tr>
<td>Valinomycin (0.01 mM) + KCl (100 mM)</td>
<td>2,879 ± 447</td>
<td>ND#</td>
<td>ND</td>
</tr>
<tr>
<td>CCCP (0.01 mM)</td>
<td>3,377 ± 538</td>
<td>34.9 ± 1.1</td>
<td>97.7</td>
</tr>
<tr>
<td>CCCP (0.01 mM) + valinomycin (0.01 mM)</td>
<td>8,019 ± 531</td>
<td>30.4 ± 1.5</td>
<td>85.1</td>
</tr>
<tr>
<td>CCCP (0.01 mM) + valinomycin (0.01 mM) + KCl (10 mM)</td>
<td>4,930 ± 475</td>
<td>34.8 ± 1.0</td>
<td>97.5</td>
</tr>
<tr>
<td>DCCD(#) (0.05 mM)</td>
<td>3,307 ± 498</td>
<td>27.7 ± 2.0</td>
<td>77.6</td>
</tr>
<tr>
<td>DCCD (0.05 mM) + valinomycin (0.01 mM)</td>
<td>2,687 ± 483</td>
<td>20.7 ± 1.1</td>
<td>58.0</td>
</tr>
</tbody>
</table>

\* Mycoplasmas (1.3 mg of cell protein per ml of buffer A supplemented with 10 mM glucose) were treated with the indicated substances as described in the text. Data represent the mean values of at least six experiments. Net uptake was calculated by subtracting the value obtained at zero time incubation (3,874 ± 437 cpm).

\# Mycoplasmas (120 μg of cell protein per ml of buffer A containing 10 mM glucose) were preincubated at 37°C for 15 min in the presence of the indicated substances and tested for their attachment capacity (see text). Data represent the mean values of at least eight experiments.

\# ND, Not done.

\# Mycoplasmas were treated for 5 min at 37°C with DCCD, and then valinomycin was added for another 10 min of preincubation.

**RESULTS**

Growth, cultivation, and attachment capacity of *M. gallisepticum* S6. *M. gallisepticum* S6 was chosen for use in these experiments because this strain, unlike strain A5969, which was used in our previous studies, is pathogenic to chickens (S. Levisohn, personal communication) and when compared with *M. gallisepticum* A5969, attachment capacity of *M. gallisepticum* S6 was five- to sixfold higher, so that minor changes in its attachment capacity are easier to detect. Attachment capacity of the organisms to fresh RBC was difficult to assess, as the RBC lysed during the attachment process, probably owing to the strong hemolytic activity of strain S6 (29). Thus, we chose to work with glutaraldehyde-fixed RBC, which resisted hemolysis activity. Preliminary experiments showed that attachment of the organisms to fixed erythrocytes resembled attachment to fresh RBC in both attachment values and sensitivity to neuraminidase treatment of the RBC.

Effect of ionophores and DCCD on [3H]TPP* uptake. Measurement of the distribution of the tritium-labeled lipophilic cation [3H]TPP* across cell membranes (28) has been introduced as a reliable method for measuring membrane potential in *M. gallisepticum* (22). We applied this method to assess the effects induced by the ionophores and DCCD on *M. gallisepticum* membrane potential. Our preliminary data showed that this lipophilic cation tended to bind nonspecifically to the mycoplasmas. Therefore, to overcome this problem, the mycoplasmas were pretreated with an excess of unlabeled TPP*. Treating the mycoplasmas with valinomycin in the absence of potassium ions increased [3H]TPP* uptake (Table 1). The enhanced uptake by valinomycin was reversed in the presence of a high concentration of potassium ions. Thus, in buffer containing 100 mM KCl, [3H]TPP* uptake in the presence of valinomycin was lower by 39% than in buffer alone. CCCP and, more effectively, DCCD decreased [3H]TPP* uptake. Nevertheless, in combination with valinomycin, CCCP did not overcome the enhancing effect in [3H]TPP* uptake induced by valinomycin alone, whereas DCCD affected it in the same manner that 100 mM KCl did. Phase-contrast microscopy did not reveal any evidence of cell lysis under any of the conditions described above.

Effect of ionophores and DCCD on attachment. Attachment capacity of the mycoplasmas was almost unchanged in the presence of CCCP and valinomycin, even though they were used in a relatively high concentration (Table 1). Lower concentrations of these ionophores did not affect...
attachment either (data not shown). However, by using a combination of both and omitting potassium, the attachment capacity of the mycoplasmas was reduced by about 15%. This reduction did not occur when a combination of CCCP and valinomycin was used in the presence of 10 mM KCl, although KCl alone at this concentration did not affect attachment.

Pretreatment of the mycoplasma suspension with DCCD resulted in a reduction of about 25% in attachment values (Table 1). This effect could be reproduced by using half of the concentration of DCCD mentioned in Table 1, but not with less (data not shown). An increase in DCCD concentration to 0.1 mM caused rapid lysis of the mycoplasmas at 37°C, even in the presence of glucose. Treatment of the mycoplasmas by DCCD followed by valinomycin decreased attachment capacity by more than 40% (Table 1).

**Effect of glucose on attachment.** The effect of glucose on the attachment capacity of *M. gallisepticum* cells was tested in the following two ways: (i) the mycoplasmas were suspended in glucose-containing buffer for 1 h at 37°C and preincubated in the presence of 10^{-4} M iodoacetate, and (ii) the cells were harvested and washed in a glucose-free buffer, and the attachment experiment was carried out in the total absence of glucose or in the presence of less than 0.5 mM glucose. Preincubation of the mycoplasmas at 37°C for 1 h with iodoacetate did not affect attachment (data not shown). Neither was attachment capacity of the cells affected by drastically reducing the glucose content of the buffer in the reaction mixture of the attachment assays (compare attachment values of untreated mycoplasmas in buffer only in Tables 1 and 2). On the other hand, preincubation of the mycoplasmas with ionophores in low-glucose buffer decreased attachment as long as KCl was not added (Table 2). Thus, valinomycin decreased attachment by 15%, whereas in combination with CCCP, attachment decreased by 25%. However, addition of 10 mM KCl to the treated mycoplasma suspensions eliminated or decreased these effects.

**DISCUSSION**

*M. gallisepticum* S6 was used in this study because this strain has remained pathogenic and possesses high attachment capacity. Because the mycoplasmas lysed fresh RBC (29), we used glutaraldehyde-fixed RBC (5). This also prevented the drugs used in the attachment experiments from affecting the RBC. In addition, it should be stressed that glutaraldehyde treatment of the RBC did not affect their capacity to attach *M. gallisepticum* specifically through sialic acid receptors on the RBC.

Several treatments were employed to manipulate the energized state of *M. gallisepticum* cells, including incubation in buffer depleted of energy sources (7, 11, 16), inhibition of metabolic pathways (7, 12, 14), and blocking the Mg^{2+}-ATPase activity with DCCD (6, 16, 22, 26). LeBlanc and LeGrimelloc (16) obtained energy-depleted *Mycoplasma mycoides* subsp. *capri* cells by incubating the mycoplasma in a glucose-free buffer for at least 2 h. Such a treatment was not possible in our case, as *M. gallisepticum* cells tended to lyse in an Na^{+}-containing, glucose-free buffer (22). To rapidly induce selective changes in the *M. gallisepticum* energized membrane state, we treated it with valinomycin, a K^{+} selective ionophore; CCCP, a proton conductor; and DCCD, a specific inhibitor of the H^{+}-translocating Mg^{2+}-ATPase. [^{3}H]TPP^{+} accumulation in the cells was affected by the ionophores in the manner expected. Thus,
whereas treatment of the mycoplasmas with valinomycin in a KCl-free buffer increased their \(^{3}H\)TPP\(^+\) uptake apparently by inducing hyperpolarization of their membrane potential (6, 23, 24), the same treatment in the presence of a high concentration (100 mM) of KCl reduced their \(^{3}H\)TPP\(^+\) uptake to a value below that of the untreated cells (22). A similar decrease in \(^{3}H\)TPP\(^+\) uptake by the cells was achieved when they were treated with a combination of both DCCD and valinomycin (Table 1), in accordance with the results of LeBlanc and LeGrimelloc (16), who showed that such a treatment had dissipated the membrane potential in M. mycoides subsp. capri. According to LeBlanc and LeGrimelloc (16), treatment of M. gallisepticum cells with the combination of CCCP and valinomycin in a K\(^+\)-free buffer should have dissipated the cell membrane potential; in our experiments, however, it only slightly reduced the hyperpolarization effect which was induced by valinomycin alone. This probably means that the efflux of K\(^+\) ions induced by valinomycin (26) is faster under these conditions than the dissipation of the H\(^+\) gradient by CCCP (16).

Our data do not show a straightforward correlation between changes in membrane potential as measured by \(^{3}H\)TPP\(^+\) uptake and the attachment capacity of M. gallisepticum cells to RBC. Hence, it is impossible on the basis of these results to conclude that attachment of M. gallisepticum cells to RBC depends on membrane potential. Comparison of the present data with those obtained in testing the effects of ionophores and DCCD on attachment of M. pneumoniae cells to glass (12), though showing some similarities such as decreased attachment by DCCD, also reveal dissimilarities; i.e., in the glass attachment studies, CCCP decreased attachment, whereas valinomycin increased attachment. It should be stressed, however, that attachment to glass involves apparently different receptors than attachment to RBC, and the glass attachment studies were carried out with M. pneumoniae and not with M. gallisepticum cells. Despite these inconsistencies, the data obtained in both the glass attachment and the present studies show that ionophores and DCCD can influence mycoplasma attachment. The mechanism by which the ionophores and DCCD decrease the ability of M. gallisepticum cells to attach to RBC is not clear and requires further studies. It is possible that the changes in the ion gradients across the mycoplasma membranes, brought about by the ionophores and DCCD, affect the vertical disposition and, consequently, the exposure of the binding components on the cell surface. Furthermore, it is also possible that the lateral distribution of the binding components over the mycoplasma cell surface is influenced by the changes in the ion gradients. Both M. gallisepticum and M. pneumoniae cells possess specialized tip organelles that appear to play a role in attachment (21). Hence, the binding components may not be randomly distributed over the entire surface of the mycoplasma cell, but rather concentrate at the tip area to ensure maximum adherence capacity. Another related factor to be considered concerns the possible effects of the electrochemical ion gradient and energized membrane state on the proper alignment and function of the contractile elements apparently present in motile M. pneumoniae and M. gallisepticum cells (19, 20). The function of these elements may depend on ATP and on their proper association with the membrane. It can thus be speculated that any agent damaging these elements may influence cell shape and motility and in this way affect indirectly the mycoplasma capacity to adhere.

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LITERATURE CITED


