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The compound DATEM inhibits respiratory syncytial virus fusion activity with epithelial cells

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Abstract

The effect of diacetyltartaric acid esters of mono and diglycerides (DATEM) on fusion of respiratory syncytial virus (RSV) with HEp-2 cells was studied using the R18 fluorescence dequenching fusion assay. At DATEM concentrations less than 2.0 μg/ml, the inhibition of fusion increased with the concentration of DATEM. At 2 μg/ml of DATEM, the fusion was suppressed by 80–90%. Studies examining possible mechanism of fusion-inhibition indicated that DATEM was likely adsorbed onto lipid membranes of both viral envelope and target cell membranes. Quantitative measurements of DATEM adsorption onto membranes were also performed using lipid monolayers and vesicles. The surface pressure of lipid monolayer formed at the air/aqueous interface increased as the concentration of DATEM in the monolayer subphase increased, suggesting that DATEM was inserted into the monolayer. As the concentration of DATEM in vesicle suspensions increased, electrophoretic mobility of initially uncharged lipid vesicles also increased, reflective of increased negative charge at vesicle surfaces. These results strongly suggest that the insertion of DATEM onto membranes inhibited viral fusion. DATEM may prove to be effective in limiting the infectivity of RSV by interference with the fusion of the viral envelope with target cell membranes.

Keywords: Respiratory syncytial virus; Membrane fusion; Antiviral agents

1. Introduction

Respiratory syncytial virus (RSV) is the most important viral respiratory pathogen in early human life (McIntosh and Chanock, 1990). RSV infection frequently results in severe lower respiratory disease in infants, and with mortality rates of 3–5% in infants with underlying respiratory or cardiac disorders (Navas et al., 1992). New forms of therapy for this infection are needed.

The infection of respiratory epithelial cells by RSV is dependent on fusion of virus with host cell membranes (Lamb and Kolakovsky, 1996). Infection likely begins with the interaction of the attachment (G) protein of the virus with an as yet unidentified target cell membrane receptor (Levine et al., 1987), and subsequent interaction between viral fusion protein (*F*) and target cell membranes (Elango et al., 1985). Mixing of viral envelope lipids and target cell membrane lipids must occur during fusion of membranes. This process might be affected by the characteristics of the lipids themselves (Hoekstra, 1990; Chernomordik et al., 1995). It is possible, therefore, that compounds which interfere with the fusion and lipid mixing of membranes might limit virus infectivity. The fusion of RSV with target cells can be monitored qualitatively and quantitatively by methods which determine the degree of transfer of fluorescent probes from the virus membrane to that of target cells (Srinivasakumar et al., 1991). These assays use a fluorophore, which combines with membrane lipids but not membrane proteins, therefore, reflecting the flow of membrane lipids during fusion events (Hoekstra et al., 1984; Hoekstra and Duzgunes, 1993).

In this study, we evaluated the capacity of di-acetyl tartaric acid esters of mono and diglycerides (DATEM) to inhibit RSV fusion with human epithelial cells. We found that, at concentrations of DATEM from 0.1 to 2.0 μg/ml, fusion of the RSV envelope with cell membranes could be inhibited by 80–90%. This inhibition appeared to be a result of adsorption and insertion of DATEM into the viral as well

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as target cell membranes, with alteration of membrane lipid properties. These findings suggest that DATEM might be effective in restricting the infectivity of RSV within the human respiratory epithelium.

2. Materials and methods

2.1. Chemicals

A fluorescence probe, octadecyl rhodamine B (R18), was purchased from Molecular Probe Inc., Eugene, OR. Phospholipids (phosphatidylcholine (egg) and phosphatidylserine (bovine brain)) were obtained from Avanti Polar Lipids Inc., Alabaster, AL. Both lipids showed a single spot on silica gel thin-layer chromatographic plates. The antiviral compounds, diacetyl tartaric acid esters of mono- and di-glycerides (DATEM) were provided by Ross Products Division of Abbott Laboratories, Columbus, OH. DATEM C16 and C18 were the diacetyl tartaric acid esters of saturated 16 and 18 carbon mono-glycerides, respectively. Ribavirin was a generous gift from ICN Pharmaceuticals, Costa Mesa, CA. Hank's balanced salt solution without divalent cations (HBSS) was from Gibco BRL, Life Technologies, Grand Island, NY. Hexane (Baker Instra-Analyzed grade, Baker Chemical Co., Phillipsburg, NJ) was used as the solvent for a lipid monolayer spreading solution. All other chemicals used were of reagent grade (Fisher Scientific Co., Fairlawn, NJ).

2.2. Virus and target cells

A laboratory stock strain of RSV was grown to 10^7 plaque-forming units (pfu)/ml in human tracheal epithelial cells (HEp-2), purchased from BioWhitaker, Walkersville, MD. The Long strain of RSV was used, as this strain is capable of causing severe disease in human infants. HEp-2 cells were seeded into flasks and permitted to grow until nearly confluent monolayers were formed. Cells were then harvested by trypsinization and suspended in Eagle's minimum essential medium (Gibco).

2.3. Fluorescent probe labeling of RSV

The virus envelope was labeled with the fluorescent lipid probe, R18, as described by Hoekstra et al. (1984) with slight modification. Briefly, an aliquot (5 μl) of the R18 solution (1 mg/ml ethanol) was added to a vortexing virus suspension of RSV containing 5×10^6 plaque forming units (pfu) in 500 μl of HBSS) and incubated for 0.5 h at room temperature. Unincorporated fluorescent probe R18 was removed by gel filtration on Sephadex G-75 using HBSS as the eluting solution. Thus, excess probe and solvent (ethanol) were removed before the labeled virus was used in the fusion-inhibition assay. The labeled virus was kept on ice and used for experiments within the day.

2.4. Fusion assay

Fusion of fluorescent probe-labeled RSV with target HEp-2 cells was measured by the R18 dequenching fusion assay (Hoekstra et al., 1984). R18 can be inserted into viral membranes at self-quenching concentrations, achieving nearly 90% quenching of R18 fluorescence. The fusion assay is based on relief of R18 self-quenching as it diffuses into target membranes following fusion of the virus envelope with target cell membranes. Dequenching of the R18 probe results in an increase in fluorescence emission signal. R18-labeled virus (10^5 pfu) was suspended in 2 ml of HBSS at 37 °C. After equilibration for approximately in 2 min, HEp-2 cells (4×10^4 cells) were added to the virus suspension. Therefore, the multiplicity on infection (moi) was 2.5. Increased fluorescence resulting from dequenching was monitored over time using a spectrofluorimeter (LS-5, Perkin-Elmer, Oak Brook, IL) with an excitation wavelength of 550 nm and an emission wavelength of 585 nm. The spectrofluorimeter was equipped with a temperature control device for controlling sample temperature. The fluorescence intensity (at 585 nm) of the self-quenched state of virus in the suspension was recorded first as I_0, and then the time-dependent fluorescence intensity (I) as recorded for about 30 min as fusion proceeded. At the end of each experiment, 0.2% of Triton X-100 was added to the reaction mixture, and the maximum value (I_{max}) of fluorescence was obtained. This value was considered equivalent to 100% fusion of RSV with target cells. The extent of fusion (F) is proportional to % fluorescence dequenching maximum and defined as

$$F = \frac{I - I_0}{I_{max} - I_0} \times 100.$$

In examining the effect of antiviral compounds (DATEM) on virus fusion, an aliquot of the compound solution (either 1 mg or 0.1 mg DATEM/ml of HBSS) was added to the virus suspension and held for 3 min to permit equilibration prior to addition of target cells. The fluorescence signal value immediately after target cells were added, was recorded as I_0. The time-dependent fluorescence signal after addition of cells to the virus-DATEM suspension was recorded as I. The monitoring time of the dequenching signal was 20–30 min.

As a positive control, ribavirin (an antiviral agent used clinically to treat RSV infection) was used. Either ribavirin or DATEM was added to R18-labeled RSV, and the fusion assay was repeated as described above. In these experiments, the RSV had been purified by sucrose-gradient centrifugation, in contrast to PEG precipitation described previously. The moi in these experiments was 1.25 (5×10^4 pfu of virus and 4×10^4 HEp-2 cells).

2.5. Antiviral activity of DATEM

The effect of DATEM on the growth of RSV was determined in standard cell culture assays. In one set of

experiments, DATEM was incubated with RSV (100 pfu) for 30 min before inoculation into monolayers of HEp-2 cells. Cell cultures were held for 7 days, and the number of plaques formed was compared to that in control cultures in which only RSV was added. Preincubation of RSV with DATEM for 60 min did not alter the results. In other experiments, RSV was first added to cell culture for 2 h before the addition of DATEM. Cultures were then maintained and read as described above.

2.6. Adsorption studies of antiviral compounds

2.6.1. Surface tension measurements

Phospholipid monolayers of either phosphatidylcholine (PC) or phosphatidylserine (PS) were formed by placing an aliquot of lipid spreading solution (approximately 1 mM lipid in hexane) on an aqueous (HBSS) surface of a constant area (64 cm^2 in a glass dish) using a micro-syringe (Hamilton, Reno, NE). After complete evaporation of hexane from the monolayer film, the surface tension of the monolayer was measured with an electronic balance (Model L, Beckman Instruments, Fullerton, CA) using a microscopic cover glass (18 mm × 18 mm × 0.2 mm) as a Wilhelmy plate (accuracy of ±0.1 dyn/cm). All replicate monolayers yielded approximately the same surface tension (50 dyn/cm). The procedure for measuring the surface tension was detailed (Ohki and Duzgunes, 1979). After monolayers of either PC or PS were formed, surface tension measurements were taken at equilibrium, and then, an aliquot of DATEM solution of either 1 or 0.1 mg/ml was introduced into the subphase of the monolayer. Prior to surface tension measurements, the subphase solution was stirred well using magnetic stirrer. Monolayer surface tension was continuously measured with time. Addition of DATEM into the subphase solution reduced the surface tension with time, (or increased the surface pressure), likely due to the adsorption of DATEM molecules onto the monolayer. When the concentration of DATEM in the subphase was relatively low (e.g. less than 1 μg/ml), the surface tension reached a stationary value within a few minutes, but when DATEM concentrations were greater than a few micrograms per milliliter, decreases in monolayer surface tension were of longer duration (up to several minutes) before reaching equilibrium. Experiments were performed with various concentrations of DATEM, using a single monolayer for each DATEM concentration.

2.6.2. Electrophoretic mobility measurements

Multilamellar lipid vesicles were prepared as follows: phosphatidylcholine was suspended at a concentration of about 1 μmol/ml in HBSS and vortexed for 20 min, forming multilamellar vesicles (MLV). MLV suspensions were maintained as a stock MLV at 4 °C. An aliquot (about 20 μl) of stock MLV solution was added to 3 ml of each experimental preparation of DATEM at various concentrations. Negatively charged DATEM molecules adsorbed onto MLV particles will impart negative surface charge density to those vesicles.

The electrophoretic mobility of lipid vesicles was measured using microelectrophoresis instrument (Mark II, Rank Bros, Bottisham, Cambridge, UK). Detailed procedures were as described elsewhere (Ohki, 1984). Great care was taken to focus the microscope on the stationary layer for the measurements of vesicle mobility.

Unless otherwise mentioned, all experiments were performed at room temperature, 24 ± 1 °C.

3. Results

3.1. Inhibition of fusion

The time course of dequenching fluorescence for R18 incorporated into the RSV envelope after interaction with HEp-2 cells is shown in Fig. 1. Addition of R18-labelled virus (10^5 pfu) in 2 ml HBSS at 37 °C was made at time, t_1, followed by the addition of 4×10^4 HEp-2 cells to the virus suspension at time, t_2, followed by stirring (Fig. 1a). The increase in dequenching signal with time is an indicator of increasing viral fusion with target cell membranes. The dequenching signal reached saturation (to approximately 40% of maximum fusion) after approximately 1 h.

For measurements of the effect of DATEM on virus fusion, an aliquot of the DATEM solution was added to the virus suspension. An immediate increase in fluorescence signal followed by a gradual increase occurred but stabilized within a few minutes after the addition of smaller amounts of DATEM to the virus (less than 2 μg/ml) as seen in Fig. 1b (addition of 0.5 μg/ml at time t_2). With addition of the target cells at time t_3, a sudden decrease in fluorescence (lower dequenching signal) then occurred. These sharp increases and decreases in fluorescence dequenching signal seemed to be due mainly to adsorption and desorption of DATEM onto and from the viral envelope membranes, respectively. After this immediate decrease upon addition of the target cells, the fluorescence dequenching signal again increased with time, which was due to fusion of virus with target cells.

The fusion of RSV with target HEp-2 cells was suppressed by DATEM. The extent of fusion measured at 10 min (Wagner et al., 1998) after addition of target cells is shown in Fig. 2 as a function of DATEM concentration. Suppression of the RSV fusion by DATEM was clearly observed at concentrations as low as 0.1 μg/ml. The extent of fusion decreased as the concentration of DATEM increased. At 2 μg DATEM/ml (C16 and C18), the extent of fusion was suppressed approximately by 80–90%. C16 was slightly more effective in suppressing the fusion of RSV with HEp-2 cells.

The dequenching signal again increased at DATEM concentrations greater than 5 μg/ml (Fig. 2). This increase in dequenching is due in part to a large amount of DATEM adsorption onto the viral envelope (as determined below), which would dilute the probe concentration in the envelope

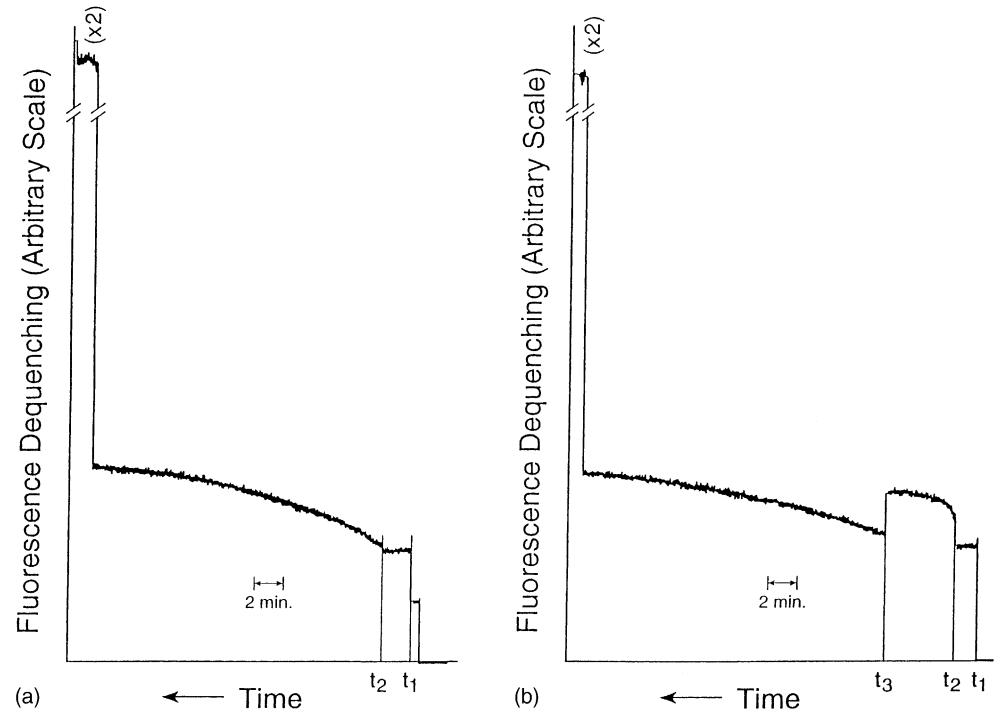

Fig. 1. (a) A time course of R18 fluorescence dequenching (% dequenching maximum) observed for the suspension containing R18-labelled RSV (10^5 pfu) and HEp-2 cells (4×10^4) in 2 ml HBSS. An aliquot of virus suspension was added to the HBSS medium at t_1 and then, the target cells (HEp-2 cells) were added to the virus suspension at t_2; (b) A time course of R18 fluorescence dequenching (% dequenching maximum) observed for the suspension containing R18-labelled RSV and HEp-2 cells in 2 ml HBSS in the presence of 0.5 μg/ml DATEM. An aliquot of the virus suspension was added to HBSS at t_1, an aliquot of DATEM solution was added to the virus solution at t_2, and lastly, target cells were added to the suspension at t_3.

membrane resulting in dequenching. With these higher concentrations of DATEM, dequenching due to dilution of R18 in the envelope prior to the addition of HEp-2 cells appeared to have exceeded the inhibitory effect of suppression of fusion by DATEM on fluorescent signaling. Another possibility is that DATEM (because of its detergent properties) at concentrations above 10 μg/ml may have extracted R18 from the virus membrane. Some of the increased dequenching may also be related to the presence of impurities in the partially-purified virus suspension (see below).

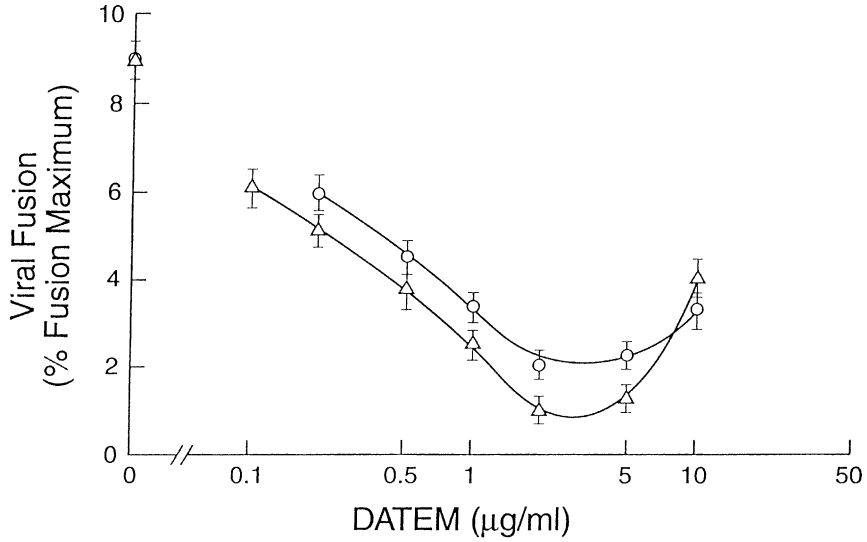

Fig. 2. Extent of fusion, *F*, of RSV (2×10^5 pfu) with HEp-2 cells (4×10^4 cells) in 2 ml HBSS at 37 °C. Dequenching signals were recorded at 10 min after virus and target cells were mixed. Open triangles: DATEM C16; Open circles: DATEM C18. All data points are an average value of at least three experiments and the bars represent the S.D.

In order to determine whether DATEM could inhibit greater degrees of fusion, a separate experiment was completed in which fusion was measured at 1 h, when fusion is known to reach a maximum (Wagner et al., 1998). In this experiment, fusion without DATEM was 30% of the maximum, while DATEM (2μg/ml) reduced fusion to approximately 3%, again a 90% reduction in fusion.

3.2. Penetration of DATEM into the virus membrane

Further experiments were performed to evaluate the possibility that DATEM entered the virus membrane, or extracted R18 from the membrane. As with fusion experiments, similar amounts of R18 labeled RSV and DATEM were mixed, equilibrated for 30 min and centrifuged in a Spinco ultracentrifuge (Beckman, Model L) at 15,000 × *g* for 30 min to collect the RSV pellet. Pelleted RSV was resuspended in 2 ml of HBSS, and the fluorescence dequenching signal of the pellet suspension was measured with (Fig. 3, hatched bars) and without 0.5% (w/w) Triton X-100 treatment (Fig. 3, open bars). The observed dequenching signal for the pellet suspension without Triton X-100 was of approximately the same magnitude as that observed for the fusion experiments when DATEM was added to the virus suspension before the addition of HEp-2 cells, suggesting that the dequenching signal increase observed in fusion experiments after addition of DATEM was mainly due to the adsorption of DATEM onto the viral envelope. The amount of dequenching increased as the concentration of DATEM in the virus suspension increased, resulting in an approximate 3–4% dequenching at DATEM levels of 4–10 μg/ml (open bars, Fig. 3). In addition, DATEM appeared to extract R18 from the virus pellet. That is, the total amount of fluorescent signal recoverable from the pellet after Triton X-100 exposure (hatched bars, Fig. 3) was reduced after incubation of the pellet with 10–30 μg/ml concentrations of DATEM. This suggested that R18 had been lost from the pellet upon incubation with DATEM at high concentrations.

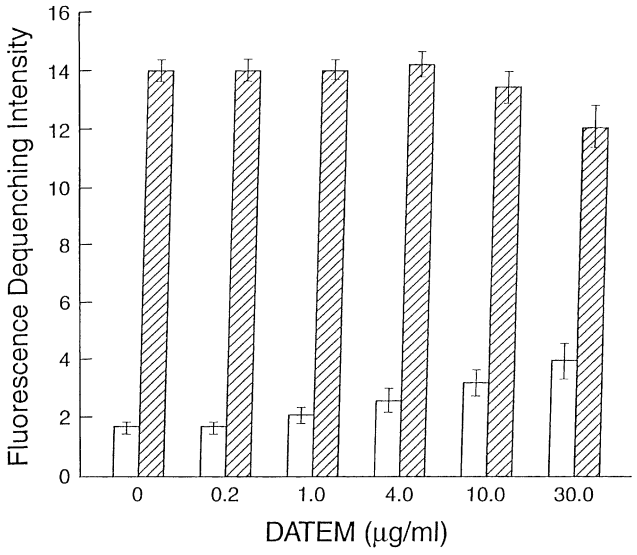

Fig. 3. Relative intensity of fluorescence dequenching for pelleted R18-lebeled RSV suspension after centrifuging virus suspensions containing various amounts of DATEM (C16) at 15,000 × *g* for 30 min. Non-shaded: without Triton X-100; shaded: with 0.5% (w/w) Triton X-100. Each experimental value is an average of at least three experimental data and the bars represent the S.D. The vertical axis is an arbitrary scale reflecting multiples of the fluorescence intensity signal of the labeled virus pellet.

3.3. Membrane adsorption

To further examine the likelihood that adsorption of DATEM onto the membranes had occurred, two types of experiments were performed: (1) monolayer surface pressure measurements following addition of DATEM into the monolayer subphase solution and (2) electrophoretic mobility measurements of lipid vesicles with and without the presence of DATEM in the vesicle suspension. The change in surface pressure of phospholipid monolayer following exposure to different DATEM concentrations is shown in Fig. 4. With increasing DATEM concentrations, the monolayer surface tension, γ, decreased, reflecting a net increase in surface pressure, π ($\pi = \gamma_0 - \gamma$), where γ_0 is the surface tension of the aqueous solution. The surface tension of water, γ_0, of 72 dyn/cm was used to represent that of the aqueous solution. When the concentration of DATEM was less than 1 μg/ml, the increase in surface pressure reached a stationary state within a few minutes and, more importantly, the increase in surface tension was rather small. The increase in monolayer surface pressure occurring at DATEM concentrations above 0.5 μg/ml suggests that DATEM molecules are likely adsorbed onto the monolayer exerting a lateral pressure. This finding is reasonable given the chemical nature of DATEM as a surface active substance. As shown in Fig. 4, C16 DATEM was more readily adsorbed onto the lipid monolayer than C18. DATEM was more adsorbable onto the phosphatidylcholine (PC) monolayer than the phosphatidylserine (PS) monolayer (data for the PS monolayer are not shown).

The initial electrophoretic mobility of the phosphatidylcholine (PC) vesicle alone was nearly zero since the PC vesicle does not have a net charge on its membrane surface. With increasing DATEM concentration, the electrophoretic mobility of the PC vesicle increased reflecting a more negative surface charge on the PC vesicles. This result indicates that as DATEM molecules are inserted into the lipid membrane, the polar head group of the DATEM molecule is oriented at the PC membrane surface. The mobility was very small when the concentration of DATEM was below 0.3 μg/ml, however, above 0.3 μg/ml, mobility increased, suggesting greater adsorption of DATEM onto the membrane. With the measured value of vesicle mobility, the zeta (ζ)-potential of the lipid vesicle surface was calculated from the Helmholtz–Smoluchowski Eq. (A.1) in the Appendix A). The experiments similar to the above but using the PS vesicles resulted in less adsorption of DATEM

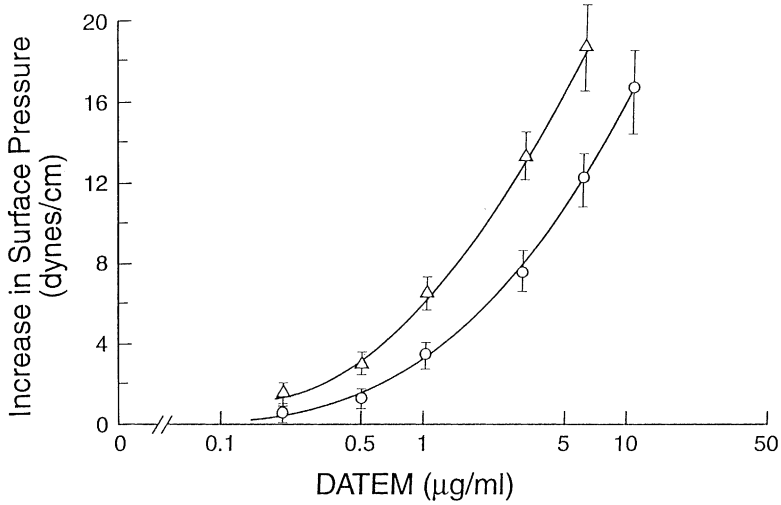

Fig. 4. The increase in surface pressure of the phosphatidylcholine monolayer formed at the air/aqueous (HBSS) interface by addition of various amounts of DATEM in the subphase solutions. The initial monolayer surface tension was 50 dyn/cm. Surface pressure, π, is calculated with use of the following relation: $\pi = \gamma_0 - \gamma$, where γ is the surface tension of the monolayer and γ_0 is the tension of aqueous phase (HBSS), which we assumed to be the same as that of 72 dyn/cm. The bars represent the S.D. of the experimental points. Open triangles: DATEM C16; open circles: DATEM C18.

onto the PS vesicle surface than the PC vesicle (data for the PS vesicles are not shown). The zeta-potentials obtained for phosphatidylcholine vesicles at various concentrations of DATEM in vesicle suspension solution are shown in Fig. 5. Because ζ-potential is related to the surface potential of the membrane, ψ_0, the surface charge density can be calculated from the equations given in the appendix. The ζ-potential of vesicle membrane, its surface charge density and the ratio of a DATEM molecule adsorbed onto lipid membrane per number of lipid molecules are presented in Table 1.

3.4. Comparative fusion inhibition activity of DATEM and ribavirin

The ability of DATEM and ribavirin to inhibit fusion of RSV and cell membranes was evaluated in separate experiments. In these studies, sucrose gradient-purified RSV was labeled and incubated with various concentrations of either DATEM or ribavirin before testing in the fusion inhibition assay. The moi of the purified virus (1.25) was somewhat lower than that used in the

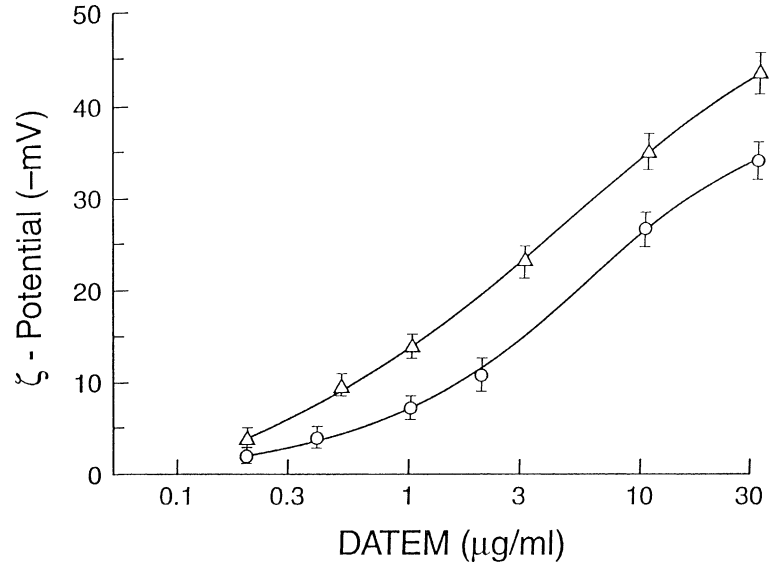

Fig. 5. The ζ-potential of MLV suspended in HBSS is plotted as a function of DATEM concentration in the vesicle suspension solution. The vesicle electrophoretic mobility, u, was measured in the experiments but the electrophoretic mobility was converted to the ζ-potential with the Helmholtz–Smoluchowski equation (see Appendix A). Each experimental point was an average of at least 10 measurements and each bar represents its standard deviation. Open triangles: DATEM C16; open circles: DATEM C18.

Table 1
Adsorption of DATEM (C16 and C18) onto phosphatidylcholine (PC) membranes

Zeta (ζ)-potential (mV)	Surface charge density (e/Å^2)	Lipid/DATEM (molar ratio)	DATEM (C16) concentration (μg/ml)	DATEM (C18) concentration (μg/ml)
−4.0	-2.3×10^{-4}	62	0.2	0.5
−10	-5.9×10^{-4}	24	0.6	1.6
−20	-1.3×10^{-3}	12	2.0	5.4
−30	-2.0×10^{-3}	7.4	6.0*	14.5*
−40	-2.9×10^{-3}	5.0	17.0*	Not done

* Values for DATEM concentrations corresponding to zeta-potentials of −30 and −40 mV may actually be lower than those illustrated values as the experimental systems may not have reached equilibrium.

experiments described above (2.5). The results are shown in Fig. 6.

Ribavirin resulted in approximately a 43% inhibition of fusion (9.3 ± 0.7% fusion at baseline reduced to 5.3 ± 0.4% at 10 μg/ml of ribavirin). In this experiment, DATEM inhibited fusion by 89.6% (10.6 ± 0.7% at baseline versus 1.1 ± 0.4% at 10 μg/ml of DATEM). Thus, DATEM was approximately twice as effective as ribavirin in the fusion inhibition assay.

In the experiments described earlier (using relatively less-purified virus), increased dequenching occurred when higher concentrations of DATEM were used to inhibit fusion. As is illustrated in Fig. 6, increased dequenching did not occur at higher DATEM concentrations in these experiments using sucrose gradient-purified preparations of RSV.

3.5. Antiviral effect of DATEM

It is important to know if DATEM specifically inhibits fusion or, alternatively, reduces fusion nonspecifically by destroying RSV directly. We, therefore, undertook two separate experiments to measure the antiviral activity of DATEM.

In the first of these experiments, we incubated 100 pfu of RSV with various concentrations of DATEM (1–1000 μg/ml) for 30 min before adding the mixture to cell monolayers of RSV. Cultures were maintained for 7 days before being analyzed for plaque formation. As is illustrated in Figs. 7 and 8, inhibition of growth was first noticeable when RSV was incubated with 10 μg/ml of DATEM and increased progressively. Growth of RSV was completely inhibited at 500 μg/ml of DATEM. Toxicity to the cell sheet was noted at 1000 μg/ml concentrations of DATEM. This pattern of

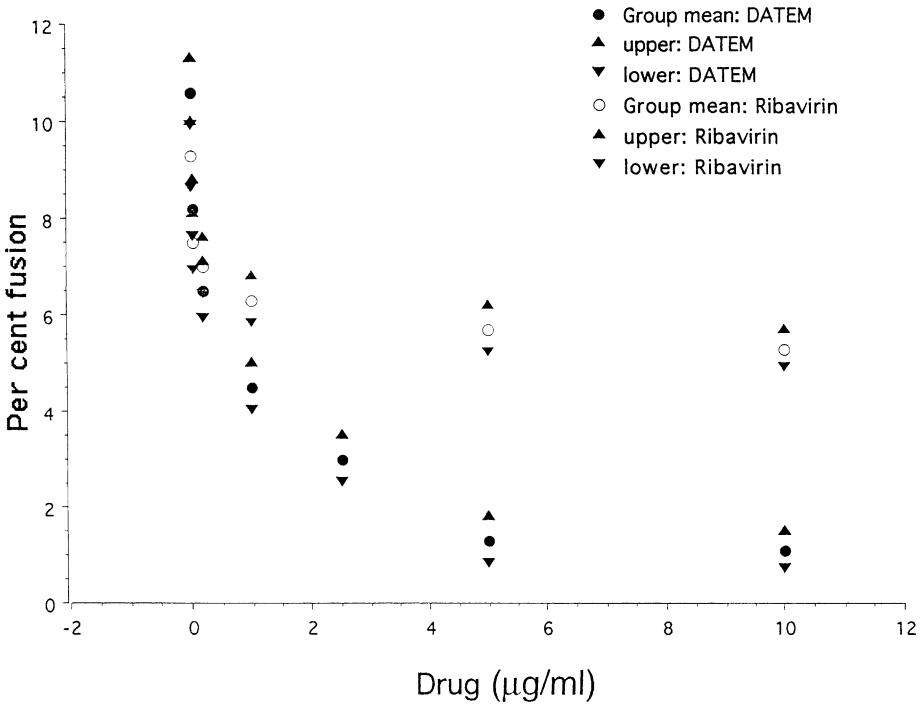

Fig. 6. Extent of fusion of RSV (5×10^4 pfu) with HEp-2 cells (4×10^4 cells) in 2 ml HBSS at 37 °C in the presence of DATEM or ribavirin. Dequenching signals were recorded at 10 min after virus and target cells were mixed. Hatched bars: ribavirin. Filled bars: DATEM C18. All data points are an average value of at least three experiments and the triangles represent the standard deviation.

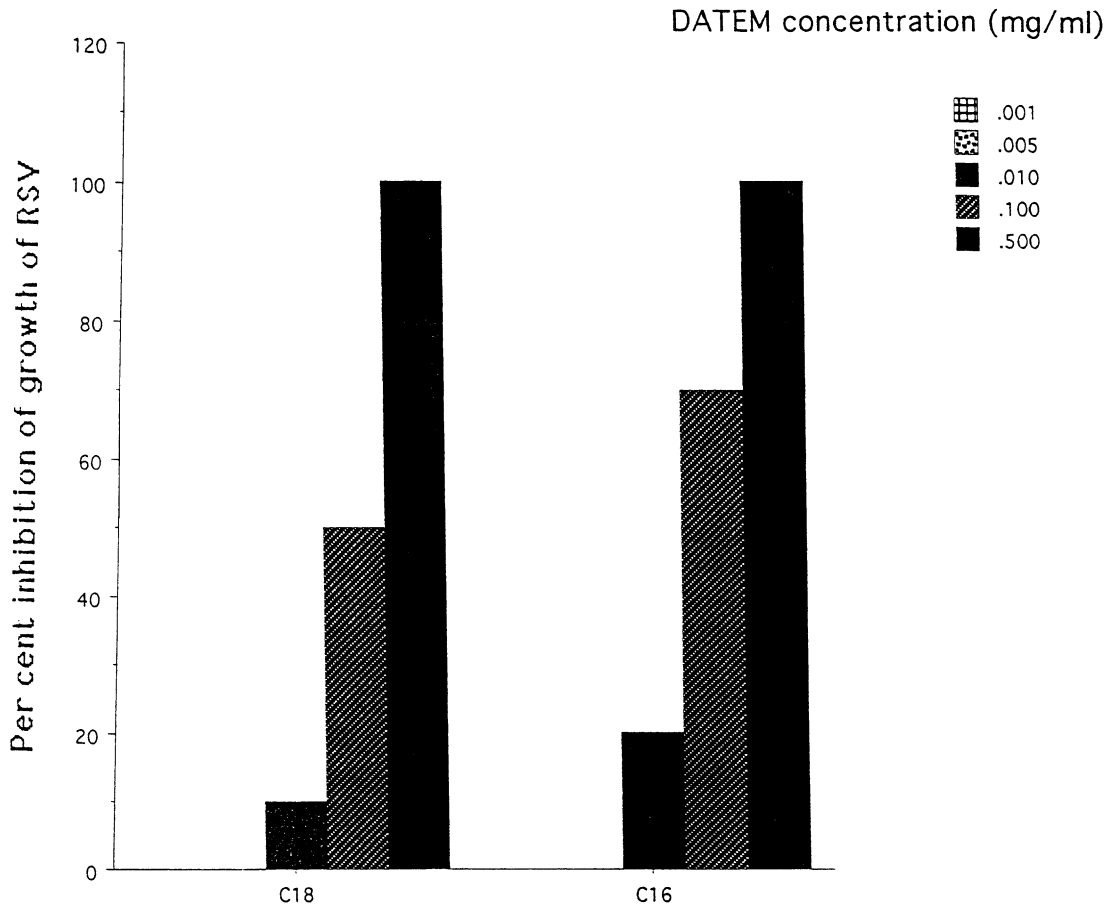

Fig. 7. Antiviral effect of DATEM in cell culture. DATEM was incubated with 100 pfu of RSV for 30 or 60 min before inoculation onto monolayers of HEp-2 cells. Cultures were maintained for 7 days. The percent reduction in plaque formation in the presence of various concentrations of DATEM was calculated versus an equal aliquot of RSV incubated with an equal volume of saline. DATEM had no effect on the growth of RSV when it was added to cell monolayers and subsequently removed by washing before RSV was inoculated. DATEM also had no effect on growth of RSV when RSV was incubated with cell monolayers first, followed by addition of DATEM 2 h later.

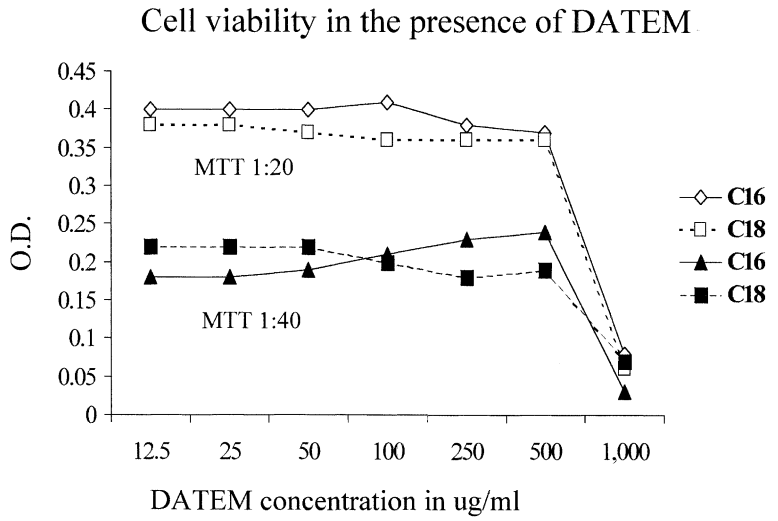

Fig. 8. Cell viability in the presence of DATEM measured by the MTT assay. The ordinate, O.D., refers to the optical density.

toxicity of DATEM was confirmed by incubating cell suspensions with identical concentrations of DATEM and then staining with trypan blue, according to standard methods.

The toxic effect of DATEM on HEp-2 cells was reanalyzed using the MTT assay as an indicator of cytotoxicity (Carmichael et al., 1987). Increasing the concentration of C16 or C18 DATEM from 12.5 through 500 μg/ml had no effect on optical density of the formazan-DMSO solution, indicating no cell death. At 1000 μg/ml the optical density fell sharply, indicating cell toxicity. Thus, toxicity of DATEM was identical when analyzed either by the MTT assay or by visual inspection of the cell sheet.

In the second set of experiments, 100 pfu of RSV were inoculated onto monolayers and allowed to incubate for 2 h. DATEM was then added, and cultures were maintained for 7 days. In these experiments, DATEM had no effect on growth of RSV (data not shown). DATEM also had no effect on the growth of RSV when it was added to cell monolayers and subsequently removed by washing before RSV was inoculated.

4. Discussion

We have shown that fusion of RSV with target cells was suppressed by the application of DATEM at concentrations of a few micrograms per milliliter. The R18 dequenching method used in the study of membrane fusion has been used to examine this event in several other enveloped virus systems (Hoekstra et al., 1984; Srinivasakumar et al., 1991; Ohki et al., 1998; Arbuzova et al., 1994). When dequenching was measured within a few minutes after R18-labelled virus and target cells are mixed, the dequenching was found to be due solely to membrane fusion (Ohki et al., 1998). Non-specific transfer of fluorescence probe to the target cell, unrelated to virus-cell fusion, was found to occur at a much slower rate.

DATEM appears to be adsorbed or inserted readily into membrane surfaces, probably by introducing its hydrophobic chain into the membrane phase and leaving its polar head group at the membrane surface. This hypothesis was confirmed by surface tension studies of lipid monolayers formed at the air/aqueous interface following introduction of DATEM into the subphase solution. Further evidence of insertion was established by the measurement of changes in electrophoretic mobility of lipid vesicles at various concentrations of DATEM. The surface pressure study showed an increase in lateral pressure in the presence of DATEM in the subphase of the surface film. The vesicle electrophoretic mobility study indicated more negative surface charges at increasing concentration of DATEM in vesicle suspensions. Since the polar group of DATEM possesses a negative charge, its adsorption onto membrane surface should result in more negatively charged surface. The ζ-potential of -4 mV corresponds to the membrane with a surface charge density of -2.3×10^{-4} e/Å^2, the surface of which is, then, considered to be composed of one DATEM molecule per 62 phosphatidylcholine molecules. This result occurred at either 0.2 μg/ml of DATEM-C16 and 0.5 μg/ml of DATEM-C18. Similarly, the ζ-potential of -10 mV corresponds with a ratio of one DATEM to 25 PC molecules, which occurred at 0.6 μg/ml of DATEM-C16 or 1.6 μg/ml of DATEM-C18. Since the calculation was done by neglecting the effect of ion-binding to the negatively charged site of the membrane, we believe the actual values are underestimated; more adsorption probably occurs. Therefore, it is conceivable that when high concentrations of DATEM are applied, considerable amounts of DATEM would be adsorbed on both the viral envelope and the target membranes. However, at a DATEM concentration below 1 μg/ml, approximately, one DATEM molecule per 20 lipid molecules would be adsorbed on the membrane. At this adsorption level for a molecule similar to DATEM, no effect on membrane function or membrane stability would be anticipated. Following adsorption of DATEM, membrane stability might instead increase, judging from inhibition of membrane fusion results.

These studies support the concept that the antiviral effect of DATEM is specifically related to inhibition of fusion. This apparently occurs when DATEM is inserted into the membrane of RSV before the mixture is tested in the fusion inhibition assay. DATEM does not falsely appear to inhibit fusion by nonspecific antiviral effects, since DATEM does not inhibit the growth of RSV once the cell sheet has been infected with the virus.

We have not examined the effect of DATEM on attachment of the virus to target cells. DATEM molecules adsorbed onto the membrane may affect virus attachment to target cell membranes, which would in turn affect the extent of the viral fusion. However, we suspect that, because of the relatively small size of the DATEM molecule, effects on the target cell membrane would occur only within a short distance (e.g. approximately 10 Å) from membrane surfaces. The viral attachment protein is of a much larger size. Therefore, binding of the viral protein to target cell receptors may not be affected by the adsorption of the smaller DATEM molecules to the surface.

In experiments using RSV which had been only partially purified, low concentrations of DATEM reduced the observable degree of dequenching, while higher concentrations tended to reverse this and cause an increase in dequenching. As was demonstrated herein, some of this nonfusion-related dequenching was probably due to the extraction of R18 from the labeled virus by DATEM. However, this could not be the sole cause, because nonfusion-related dequenching did not occur in experiments involving more purified preparations of RSV. This suggests that membrane fragments retained in less-purified preparations might have been involved in nonfusion-related dequenching.

We conclude that DATEM inhibits fusion of RSV with epithelial cell membranes, probably by altering membrane surfaces (e.g. creating a more hydrophilic nature) as a result

of DATEM insertion into the lipid membrane of the virus and the target cell. DATEM inhibited the specific step of membrane fusion approximately twice as effectively as ribavirin, although DATEM does not exhibit the overall inhibitory effect on virus growth that ribavirin manifests. DATEM may prove to be an effective agent in limiting the infectivity of RSV and other fusion-dependent viruses in humans. A major limitation of the use of DATEM as an antiviral is the low margin between the maximum antiviral effect of the compound at 500 μg/ml and the threshold of cytotoxicity at 1000 μg/ml. Less toxic derivatives of DATEM may need to be developed.

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Appendix A

The ζ-potential is related to the electrophoretic mobility in the Helmholtz–Smoluchowski equation:

$$\zeta = \frac{u\eta}{\varepsilon_0 \varepsilon_r} \tag{A.1}$$

where u is the electrophoretic mobility, η the viscosity of aqueous suspension, and ε_0, ε_r are the permittivities of free space (vacuum) and aqueous medium, respectively.

The surface potential of the Gouy–Chapman diffused layer is expressed as

$$\psi(x) = \left(\frac{2kT}{e}\right) \ln\left\{\frac{1+\alpha\exp(-\kappa x)}{1-\alpha\exp(-\kappa x)}\right\} \tag{A.2}$$

where

$$\alpha = \frac{\exp(e\psi_0/2kT)-1}{\exp(e\psi_0/2kT)+1} \tag{A.3}$$

and

$$\kappa = \left(\frac{e^2 N \Sigma C_i z_i^2}{\varepsilon_r\, \varepsilon_0\, kT}\right)^{1/2} \tag{A.4}$$

where e is the electronic charge, z_i the valence of the ith ion, k the Boltzmann constant, T the absolute temperature, C_i the bulk concentration (in molar) of the ith monovalent ion, x the distance from the surface, and N is the Avogadro number.

The ζ-potential was obtained with the measured electrophoretic mobility, u, from (A.1). Then, the surface potential ψ_0 at the membrane surface was calculated using Eqs. (A.2) and (A.3), where the plane of shear related to the ζ-potential was chosen to be 2 Å from the membrane charged surface (Ohki, 1984). In this calculation, the effect of ion binding to the surface charge sites was neglected.

Once the surface potential, ψ_0, at the membrane surface is obtained, the surface charge density of the membrane can be calculated from the relation between the surface charge density and the surface potential, ψ_0, which is

$$\sigma = \frac{1}{273}\left\{\Sigma C_i\left[\exp\left(\frac{-z_i e\psi_0}{kT}\right)-1\right]\right\}^{1/2} \tag{A.5}$$

where Σ is expressed in electronic charge per Å^2, and C_i the magnitude of the molar concentration of the ith ionic species in the bulk solution. Temperature 24 °C was used in our calculation.

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