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EFFECT OF DEOXYCHOLATE, AMPHOTERICIN B AND FONGIZONE ON TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS

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SUMMARY

At a concentration of 2 μg/ml, neither amphotericin B nor deoxycholate had an inactivating effect upon transmissible gastroenteritis coronavirus infectivity. However, amphothericin B stimulated plaque formatin in agarose and facilitated the entry of viral RNA into swine testis cells. The combination of amphotericin B + deoxycholate inactivated virus infectivity and induced a decrease in plaque diameter. Finally, in the presence of these agents, the production of infectious virus and interferon was unchanged.

KEY-WORDS: TGE virus, Coronavirus, Fongizone, Amphotericin B, Deoxycholate, Gastroenteritis; Swine, *In vitro*.

INTRODUCTION

Fongizone is a clinical formulation of amphotericin B (AmB) combined with deoxycholate (DOC) as a stabilizer, and has been widely used as an antifungal agent. The antiviral effects of AmB result not only from an alteration of the viral envelope by its combining with sterol componants [9] but also from enhancement of interferon (IFN) production in host cells [2]. Human coronavirus has been found to be inactivated by AmB [6]. On the other hand, AmB reportedly enhances viral RNA infectivity [3]. Since cell cultures for virological studies very often require antibiotics for prevention of fungus contamination, our aim was to further clarify the effect of these compounds, namely fongizione, AmB and DOC, upon virus infectivity using the transmissible gastroenteritis coronavirus (TGEV).

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MATERIALS AND METHODS

Chemicals. — Fongizone (Biopro, Mulhouse, France) was used as indicated by the supplier in the state of its delivery. AmB (Sigma, Mo, USA) was dissolved in ethanol and used immediately. DOC was from Sigma. DEAE-dextran was purchased from Pharmacia (MW 500,000) and proteinase K (Boehringer, Mannheim, Western Germany) was dissolved (1 mg/ml) in distilled water and kept at −70°C.

Cells and virus. — TGEV strains Purdue-115, D-52 and 188-SG, as well as swine testis (ST) and pig kidney (RPD) cell lines have been described elsewhere [1]. Virus stock was prepared by growing TGEV in RPD cells. The plaque assay of TGEV carried out on confluent monolayer ST cells in 6-well plastic plates (Costar) has also been described [1].

Effects of fongizone, AmB and DOC. — TGEV was prepared (about 5×10^2 PFU per ml) in appropriate concentrations of these compounds. After the desired times of incubation at 37°C, 0.2-ml volumes of the preparation were removed and inoculated onto confluent monolayer cells. Following virus-cell incubation for 1 h at 37°C, the inocula were removed by washes with minimal essential medium (MEM), and agarose-overlay medium without serum was replaced for plaque formation. Virus suspensions treated with MEM and/or ethanol (at the same concentration as used in the AmB-treated virus suspension) served as controls. Plaque size and numbers were also recorded when these compounds were added into agarose-overlay medium without serum to study their effects on virus after attachment.

IFN titration. — Confluent monolayer cells in 6-well plates were infected with TGEV at a m.o.i. of 5 to 8 PFU per cell. The inocula were removed after 1 h of incubation at 37°C; fongizone and/or AmB at different concentrations in MEM with or without 2 % bovine fœtal serum were replaced. Virus was harvested at 18 h post-inoculation and titrated. IFN in the virus suspensions was also titrated [11].

TGEV-RNA infectivity. — Virus purification and phenol extraction of TGEV-RNA were performed according to the method described by Brian *et al.* [4]. Precipitated viral RNA was diluted in TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 7.2) to obtain the initial virus volume (1 ml, virus titre: 10^9 PFU/ml). To test infectivity, the RNA solution was serially two-fold diluted in the following solutions: MEM, MEM + DEAE-dextran (100 μg/ml), MEM + DEAE-dextran + AmB (2 μg/ml), MEM + DEAE-dextran + fongizone (2 μg/ml), MEM + AmB and MEM + fongizone. The RNA dilutions were inoculated into the cells grown to confluent monolayers in 24-well plastic plates (Costar). The cells were observed daily during the following 4 days, then frozen and thawed twice; the presence of TGEV was sought and identified by a seroneutralization test using specific antibody from previous studies [1, 14].

RESULTS

In preliminary studies, no effects upon TGEV infectivity of either AmB or DOC separately were found at concentrations up to 5 μg/ml. In the experiment using a concentration of 2 μg/ml of AmB or DOC alone, TGEV

AmB = amphotericin B.
DOC = deoxycholate.
IFN = interferon.
MEM = minimal essential medium.
m.o.i. = multiplicity of infection.
PFU = plaque-forming unit.
TGEV = transmissible gastroenteritis virus.

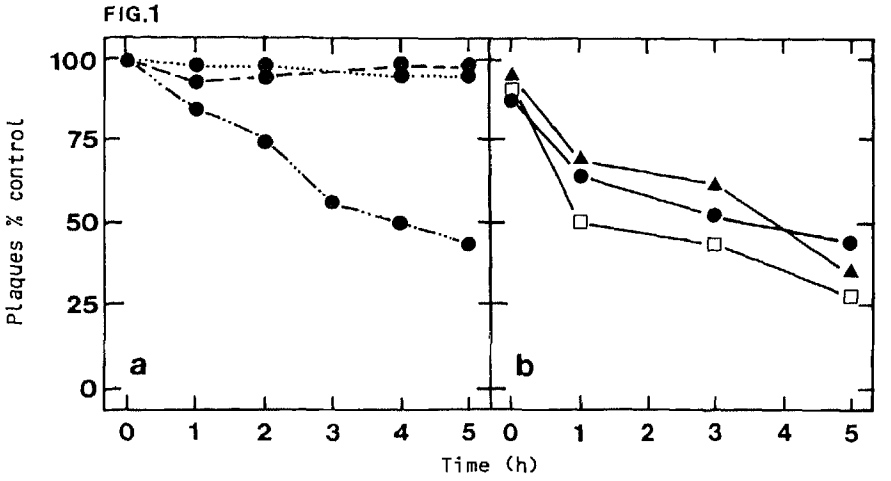
infectivity decreased as a function of incubation time, similar to controls. However, in the presence of fongizone, or of AmB plus DOC, the decrease in TGEV infectivity was clearly different from that of the controls (fig. 1), suggesting that AmB and DOC have a virus-inactivating effect only when they are combined.

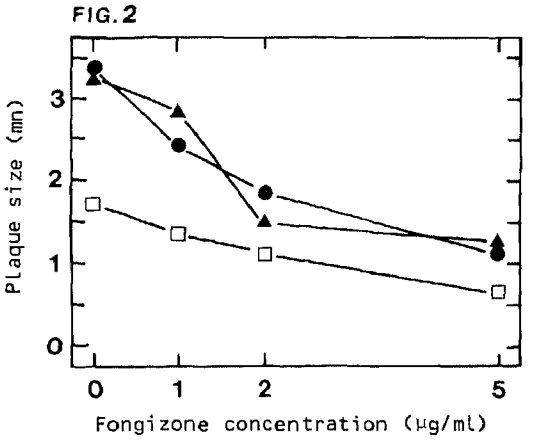
Addition of fongizone, or of a combination of AmB plus DOC in agar-overlay medium, apparently did not affect the plaque number (data not shown), but the plaque size was found to be reduced proportionally to their concentrations (fig. 2). No difference in plaque formation was observed between the control and the experiment using DOC. However, AmB alone added in agar-overlay medium enhanced plaque formation. This enhancement was observed only in ST cells (fig. 3), not in RPD cells. As ethanol had been used to dissolve AmB, a control with ethanol added to the agar-overlay medium was performed which resulted in no variation in plaque numbers (data not shown).

AmB or fongizone in the medium used for virus growth had no effect on either viral yield or on IFN production, as indicated by the similarity of virus and IFN titres between the experiment and the control (data not shown). However, AmB, when added with DEAE-dextran, induced virus replication from viral RNA, although the ratio of RNA titre *vs.* virus titre was about 10^{-8}. However, no virus replication resulted when the RNA was diluted in other solutions (data not shown).

DISCUSSION AND CONCLUSION

TGEV (an enveloped virus) has been reported to be inactivated by DOC at a concentration of 0.05 % **[5, 13]**. At that concentration, we also found that DOC inactivated TGEV by more than 99.99 % (data not shown). At lower concentrations (< 5 μg/ml), DOC did not inactivate TGEV infectivity. The concentration of chemicals used in the present study (2 μg/ml) was chosen because it is usually used to prevent fungal contamination in cell cultures. AmB (at a lower concentration than 5 μg/ml) also did not inactivate TGEV infectivity. However, when AmB and DOC were combined, they were found to have an effect similar to that of fongizone. These results are consistent with those observed by DeVald *et al.* **[6]** and Kessler *et al.* **[10]**, but not with results obtained by Jordan and Seet (1978), who claimed that fongizone at a concentration of 2 μg/ml did not affect enveloped virus infectivity. However, Kessler *et al.* **[10]** claimed that AmB inactivated hepatitis B virus, when they used fongizone, a compound comprising AmB and DOC. It can be assumed that AmB alters the viral envelope by creating pores, similar to treated eukaryote cell membranes **[7, 8, 15]**. However, this alteration is not sufficient to inactivate virus infectivity; DOC is required to complete inactivation once these pores have been produced, or vice-versa. A synergic effect may also be the mechanism of action of the two substances.





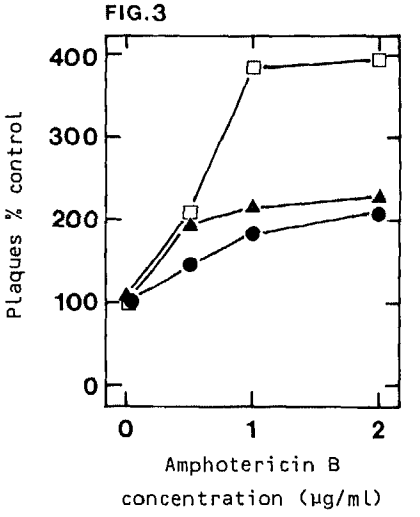


FIG. 1. — *Effect of (a) AmB (···), DOC (---) or fongizone alone (·····) and (b) AmB combined with deoxycholate, upon infectivity of TGE virus.*

Concentration: AmB (2 µg/ml), DOC (2 µg/ml), fongizone (2 µg/ml).
TGEV strains = Purdue-115 (●), D-52 (▲) and 188-SG (□).

FIG. 2. — *Effects of fongizone added to agarose overlay medium upon the size of plaques produced by TGE virus in ST cells.*

TGEV strains as in figure 1. Same results (not shown) were obtained using AmB combined with DOC.

FIG. 3. — *Effects of AmB, added alone to agarose overlay medium, upon plaque formation by TGE virus in ST cells.*

TGEV strains as in figure 1. This effect was not observed in RPD cells.

TGEV is known to induce IFN [11, 12]; in our study, IFN production was found to be unchanged by the addition of fongizone or AmB (table I); therefore, virus inactivation cannot be explained by an enhanced production of IFN which, in turn, inactivated virus multiplication.

Added alone to agar-overlay, AmB enhanced plaque formation in ST cells but did not enhance virus multiplication, as indicated by similar virus titres in both the presence and absence of AmB (table I). Along with the results of viral RNA infectivity in the presence of AmB, this suggests a facilitating action of AmB for intracellular penetration of viral RNA rather than an enhancement of synthesis of viral components. These results are in agreement with observations by Borden *et al.* [3].

Finally, plaque formation was enhanced by AmB in ST but not in RPD cells. In recent studies (to be published), we found that with similar inocula, TGEV induced a plaque number nearly three-fold higher in ST cells than in RPD cells, independently of the virus strain used. In the present study, viral RNA penetration was found to be facilitated by AmB, and therefore more plaques were observed in ST cells. It is possible that a similar reaction occurred in RPD cells, but virus replication remained limited in these cells. However, there is no satisfactory explanation for the enhancing effect of AmB on plaque formation when DOC was jointly added, nor can we explain why, in contrast, AmB plus DOC (fongizone) in agar-overlay medium inactivated TGEV and therefore limited plaque size development, but did not do so in the liquid MEM used for virus growth.

In conclusion, AmB and DOC were found to inactivate TGEV infectivity at a concentration of 2 μg/ml each, while alone at that concentration, neither had such an effect. Moreover, AmB enhanced viral RNA penetration.

RÉSUMÉ

EFFET DU DÉSOXYCHOLATE, DE L'AMPHOTÉRICINE B ET DE LA FONGIZONE SUR LE CORONAVIRUS DE LA GASTROENTÉRITE TRANSMISSIBLE

A la concentration de 2 μg/ml, l'amphotéricine B et le désoxycholate n'ont pas d'effet inactivant sur le pouvoir infectant du coronavirus de la gastro-entérite transmissible. En revanche, l'amphotéricine B stimule la formation des plages sous agarose et facilite l'entrée de l'ARN viral dans les cellules ST. L'association amphotéricine B + désoxycholate inactive le pouvoir infectant du virus et induit une diminution du diamètre des plages. Enfin, en présence de ces agents la production de virus infectieux et d'interféron n'est pas modifiée.

MOTS-CLÉS: Virus TGE, Fongizone, Amphotéricine B, Désoxycholate, Coronavirus, Gastroentérite; Porc, *In vitro*.

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