Studies of Laryngotracheitis Virus in Avian Tissue Cultures

III. Enhancement of Infectivity by Diethylaminoethyl-Dextran

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Diethylaminoethyl-dextran (DEAE-D) enhanced the infectivity of laryngotracheitis virus (LTV) for chicken kidney (CK) cells when cultures were treated before inoculation with virus and when DEAE-D was present in the inoculum. Infectivity was not increased when cultures were treated after virus had adsorbed to cells; since infection was not synchronized, most of the virus had probably already penetrated the plasma membrane by the time DEAE-D was added. Maximal enhancement occurred when DEAE-D was present in the inoculum. Enhancement of a lesser degree occurred when virus and DEAE-D were mixed, diluted, and inoculated onto cultures. Adsorption of LTV at 37 C as compared to that at 5 C usually yields about a threefold greater number of plaques after a 2-hr adsorption period. However, when DEAE-D was incorporated in the inoculum, greater enhancement occurred at 5 C than at 37 C, and the number of plaques produced at both adsorption temperatures was about equal. Results are compatible with the hypothesis that increased adsorption is a factor in enhancement of infectivity of LTV by DEAE-D.

Laryngotracheitis virus (LTV) is a herpesvirus which produces a respiratory infection in chickens. The N71851 strain of LTV, with which this work is concerned, was originally isolated from a chicken with infectious laryngotracheitis. Characteristics of the structure (24) and thermal stability (17) of the virus, the cytopathic effect of the virus (16, 23), and a plaque assay of the virus (17) have been described. Recently, we found that LTV N71851 is deficient in the capacity to adsorb to chicken kidney (CK) cells and that treatment of cultures with diethylaminoethyl-dextran (DEAE-D) prior to infection enhanced the infectivity of the virus (C. R. Rossi and A. M. Watrach, Arch. Gesamte Virusforsch., in press).

Because of the difficulty of obtaining sufficient quantities of virus to produce synchronized infection of cells and to understand better the events involved in infection of cells by LTV N71851, it was decided to investigate the enhancement of infectivity produced by DEAE-D. DEAE-D has been reported to enhance the infectivity of viral ribonucleic acid (RNA; 1, 7, 14, 15), viral deoxyribonucleic acid (DNA; 22), and intact viruses (2, 5, 6, 13, 19-21) by influencing events which occur early in the infective cycle. Although enhancement of the infectivity of virions has usually been attributed to increased adsorption or penetration, enhancement of the infectivity of nucleic acids has usually been thought to be due to stabilization of nucleic acids.

Results presented in this report suggest that increased adsorption of virions to cells is a factor in enhancement of infectivity of LTV N71851 for CK cells.

MATERIALS AND METHODS

Tissue cultures. CK tissue cultures were prepared from 1- to 5-week-old chickens obtained by mating New Hampshire males with Columbian females. Cultures were prepared and grown as chicken embryo kidney cultures (17) except that 4 ml of a 0.5% cell suspension, instead of 4 ml of a 0.3 to 0.4% cell suspension, was seeded per 60-mm plastic petri dish.

Virus. The N71851 strain of LTV was used throughout these investigations (23).

Plaque assay. The plaque assay described previously (17) was used with a few modifications. An inoculum of 0.50 ml was adsorbed on CK monolayers at 37 C for 2 hr. The inoculum was removed by aspiration, and the cultures were washed twice with Hanks balanced salt solution (HBSS). A 5-ml amount of agar overlay medium containing neutral red was added on the fourth day. Plaques were counted 6 to
24 hr later. Five replicate cultures were used for each treatment group.

Reagent. DEAE-D with a mean molecular weight of 2,000,000 was obtained from Pharmacia, Inc., Uppsala, Sweden. A sterile stock solution of 4,000 μg of DEAE-D per ml in phosphate-buffered saline was prepared and stored at 5°C.

Treatment of cultures with DEAE-D. Unless otherwise indicated, when cultures were treated prior to inoculation, they were incubated with 2 ml of DEAE-D in growth medium at 37°C for 1 hr, washed twice with HBSS, and inoculated with virus. When cultures were treated after adsorption of virus and prior to addition of agar overlay medium, they were incubated with DEAE-D in the same manner as when treated before adsorption. DEAE-D was then removed, the cultures were washed with HBSS, and agar overlay medium was added.

Treatment of virus with DEAE-D. Virus was mixed with various concentrations of DEAE-D and held at 5°C for 1 hr. Immediately prior to inoculation of cultures, the mixtures were diluted to a concentration of DEAE-D which did not enhance infection during adsorption (6.4 μg of DEAE-D per ml or less), and the plaque assay was conducted as described above.

RESULTS

Treatment of cultures with DEAE-D before inoculation with virus. Maximal enhancement was obtained with concentrations of DEAE-D of 160 and 320 μg/ml (Fig. 1). Higher concentrations caused less enhancement. All concentrations that enhanced plaque formation were toxic to cells, as evidenced by necrosis of the cells observed on the following day. When plaques were counted on the fourth day, there was no gross difference between DEAE-D-treated and untreated cultures stained with neutral red. In successive experiments in which 160 μg of DEAE-D per ml treatment was employed for 1 hr prior to infection, enhancement ranged from 2.5- to 11-fold.

The duration of treatment of cells which would produce maximal enhancement was determined by using DEAE-D at a concentration of 160 μg/ml. Enhancement developed after 3 min and was substantially complete after 30 min (Table 1).

To determine whether the effect of DEAE-D on cells in producing enhancement was long-lasting, the relationship between the time after which treatment was terminated and cultures were inoculated was investigated. After treatment of cultures with 160 μg of DEAE-D per ml for 1 hr, cultures were washed with HBSS, growth medium was added, and cultures were incubated at 37°C. At specified intervals thereafter, growth medium was removed, cultures were inoculated with virus, and the assay of virus was conducted as previously described. The effect of DEAE-D treatment decreased considerably after 1 hr and was reduced almost completely thereafter (Table 2). Removal of DEAE-D from the surface of cells by passage into the cells, dilution of DEAE-D into the medium, or repair of slightly damaged membranes which might be expected to adsorb virus more readily than undamaged membranes (18) might have been responsible for the reduction of enhancement. On the basis of the present data, we cannot distinguish between these cases.

Incorporation of DEAE-D in inoculum. Enhancement of plaque formation when DEAE-D was present in the inoculum was greatest at concentrations of 160 and 320 μg/ml (Fig. 2). Cellular toxicity, which was usually prominent when cultures were treated with the larger volume of 2 ml either before or after the adsorption period, was not as severe when the smaller volume of

![Graph showing effect of DEAE-D treatment of CK cultures on infectivity of LTV. Cultures were incubated with DEAE-D at 37°C for 1 hr. The cultures were washed with HBSS and infected with LTV.](image-url)
### Table 2. Effect of the interval between diethylaminoethyl-dextran (DEAE-D) treatment of cultures and infection on infectivity of laryngotracheitis virus (LTV)

| Time between end of DEAE-D treatment and infection* | Treatment (1-hr) with 160 μg of DEAE-D per ml | Relative titer |
|---------------------------------------------------|-----------------------------------------------|---------------|
| hr                                                |                                               |               |
| NA                                                                 | No                                             | 1.0           |
| 0                                                 | Yes                                            | 6.0           |
| 1                                                 | Yes                                            | 2.3           |
| 2                                                 | Yes                                            | 1.8           |
| 4                                                 | Yes                                            | 1.6           |

*After removal of DEAE-D, cultures were washed with Hanks balanced salt solution and medium was added. At specified intervals, medium was removed and the cultures were infected with LTV.

*b Not applicable.

### Table 3. Effect of diethylaminoethyl-dextran (DEAE-D) treatment of laryngotracheitis virus (LTV) on infectivity of LTV

| DEAE-D treatment of virus (μg/ml)* | Relative titer |
|-----------------------------------|---------------|
| 0                                 | 1.0           |
| 10                                | 1.2           |
| 20                                | 1.1           |
| 40                                | 1.4           |
| 80                                | 1.1           |
| 160                               | 1.5           |
| 320                               | 1.4           |
| 640                               | 2.0           |
| 1,280                             | 1.9           |

*LTV-DEAE-D mixture was kept at 5 C for 1 hr. Before infection of chicken kidney cultures, the mixture was diluted so that the concentration of DEAE-D in the inoculum was not a factor in enhancing infection.

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**Treatment of virus with DEAE-D.** Results of an experiment with concentrations of DEAE-D from 10 to 1,280 μg/ml are shown in Table 3. Although enhancement was considerably less than that obtained when cultures were treated before infection or when DEAE-D was present in the inoculum, it was, nevertheless, significant, and enhancement up to twofold occurred. Since insufficient DEAE-D was carried over to the assay system to affect enhancement, enhancement must have been due to the effect of DEAE-D on virions during incubation in the cold.

**Treatment of cultures with DEAE-D after adsorption of virus.** When DEAE-D at a concentration of 160 μg/ml was added to cultures immediately or at 0.5 or 1 hr after the adsorption period, no enhancement occurred. Nor did addition of DEAE-D in the agar overlay medium at concentrations of 80 and 160 μg/ml produce any enhancement.

**Adsorption of virus at 37 and 5 C.** Adsorption of virus for 2 hr at 37 C has been shown to yield about a threefold greater number of plaques than adsorption at 5 C (C. R. Rossi and A. M. Watrach, Arch. Gesamte Virusforsch., in press). Addition of DEAE-D to the inoculum and its effect on plaque production showed that enhancement was greater at 5 C than at 37 C, and that the number of plaques produced at 37 and 5 C was about equal (Table 4). Treatment of cultures with DEAE-D prior to inoculation with virus also enhanced plaque formation and abolished the difference between the number of plaques produced at 37 and 5 C. The data are best explained by attributing enhancement to increased adsorption of virus to cells.

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![Fig. 2. Effect of adding DEAE-D to the inoculum on the infectivity of LTV. Virus and DEAE-D were mixed and immediately inoculated onto CK cultures.](image-url)
TABLE 4. Effect of diethylaminoethyl-dextran (DEAE-D) on infectivity of laryngotracheitis virus adsorbed at 37 and 5°C

| Presence of 200 µg of DEAE-D per ml in inoculum | Relative titer at adsorption temp of | 37°C | 5°C |
|-----------------------------------------------|-------------------------------------|------|-----|
| No                                            | 1.0                                 |      |     |
| Yes                                           | 26                                  | 0.35 | 23  |

DISCUSSION

The effect of polycations on the enhancement of infectivity of intact viruses has been the subject of numerous investigations. However, the mechanism(s) of action of polycations has not been adequately defined. Also, all polycations do not have the same effect, i.e., some polycations may enhance whereas others may inhibit the same virus, although viruses which are enhanced by one polycation are generally also enhanced by other polycations. Most studies indicate that enhancement takes place in the early stages of infection. Because polycations stimulate pinocytosis (18), and pinocytosis has been generally considered as the mechanism by which viruses enter cells (3), it is attractive to hypothesize that enhancement is due to stimulation of pinocytosis with a consequent increase in the uptake of virions. Although the hypothesis is appealing, as yet no direct evidence to substantiate it has been produced. Furthermore, recent evidence indicates that pinocytosis does not constitute the mechanism by which virions enter cells to produce infection. Nonenveloped viruses have been shown to pass directly through the plasma membrane (4, 8, 12), and enveloped viruses have been shown to fuse with the plasma membrane. Subsequent to fusion, both membranes appear to undergo dissolution, and the nucleocapsids enter the cytoplasm (9–11). When virions have been found in pinocytotic vesicles, there has been little evidence to substantiate the views that virions in pinocytotic vesicles are being transported to sites of viral synthesis (10–12). It has been suggested also that virions in vesicles are actually isolated from the cytoplasm, and that pinocytosis is a cellular defense mechanism (12). If productive penetration occurs only by nonpinocytotic mechanisms, then the hypothesis that polycations enhance viral infectivity by stimulating pinocytosis must be rejected. Since the more recent views on penetration of the plasma membrane have been gaining support, mechanisms other than increased uptake by pinocytosis should be considered in explaining the enhancing effect of DEAE-D on the infectivity of virions. Although enhancement caused by different polycations may not be the same, conceivable mechanisms of their action may involve (i) disrupting viral aggregates into smaller infectious units, (ii) increasing adsorption of virions to cells by altering ionic charges, (iii) increasing penetration of cells due to damage to cells by DEAE-D, (iv) stabilizing the virion-cell union to prevent elution of virions during penetration, (v) modifying inducer or inhibitor enzymes that may be involved in penetration, and (vi) stabilizing virions or viral nucleic acids against degradation until they reach sites of viral synthesis or sites where degradation is necessary for the continuation of the infective process.

The data presented in this work describe conditions for enhancement of infectivity of LTV N71851 by DEAE-D and provide evidence that increased adsorption of virus to cells is a factor in the enhancement process. It cannot, however, be deduced that increased adsorption is the only or the main process in enhancement in the present system. When DEAE-D was added before but not after infection, considerable enhancement occurred. However, penetration of virus into cells was not synchronized, and herpesviruses have been shown to penetrate rapidly the plasma membrane (11). Therefore, the inability to demonstrate enhancement when DEAE-D was added after the adsorption period may be due to the fact that most of the virions had already penetrated the plasma membrane by the time adsorption was terminated. If DEAE-D acts by enhancing penetration or by affecting early steps other than adsorption, it is unlikely that its effect could be demonstrated by experiments conducted in the absence of synchronized infection. Experiments are presently underway to investigate postadsorption effects under conditions of synchronized penetration. The most effective enhancement occurred when DEAE-D was present in the inoculum. The lesser enhancement obtained when DEAE-D was added prior to infection could be due either to the greater cellular toxicity resulting from the use of a greater volume of DEAE-D or to removal of DEAE-D by washing before infection.

Experiments in which adsorption of virus was carried out at 37 and 5°C yielded results compatible with the hypothesis that increased adsorption of virions to cells is a significant factor in enhancement. Although, as previously reported (C. R. Rossi and A. M. Watrach, Arch. Gesamte Virusforsch., in press), there was a near threefold greater number of plaques at 37 than at 5°C when DEAE-D was not present in the inoculum, when DEAE-D was present in the inoculum there was
an approximate equality in the number of plaques at both adsorption temperatures (Table 4). Enhancement was, therefore, much greater at 5°C than at 37°C. The results can be interpreted to indicate that substantially all of the infectious virus had adsorbed at both temperatures with DEAE-D in the inoculum. A more direct indication that enhancement of viral infectivity involves a reaction between virions and polyions can be seen in Table 3. Treatment of virus with DEAE-D enhanced the infectivity of the virus twofold. Since the concentrations of DEAE-D after dilution for assay were too low to have enhancing activity, the most likely conclusion is that DEAE-D attaches to virions to enhance their infectivity, probably by altering ionic charges on the virions to promote adsorption of virions to cells. If DEAE-D acts only by promoting adsorption of virions to cells, the lesser degree of enhancement under these conditions, compared to that obtained when DEAE-D was present in the inoculum, could be due to removal of DEAE-D from virions by dilution.

On the basis of the latter argument, the possible effect of disruption of viral aggregates may be considered. Since the enhancement caused by DEAE-D being present in the inoculum varied from 12- to 34-fold, then the same degree of enhancement could be expected in the latter experiment. Under these conditions, the possibility of reaggregation of virions after dilution of the virus-DEAE-D mixture could be expected to be insignificant. However, since the enhancement was only twofold, the argument for disruption of viral aggregates cannot be supported by this data.

Although some of the data presented here indicate that adsorption of LTV N71851 to cells is increased by DEAE-D, it is not certain whether other factors may not also be involved. Further studies are needed to determine which other factors may be involved in the enhancement by DEAE-D of the infectivity of LTV N71851 for CK cells.

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