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Bovine Respiratory Syncytial Virus Infection of Bovine Embryonic Lung Cultures: Enhancement of Infectivity With Diethylaminoethyl-Dextran and Virus-Infected Cells

By

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Summary

The effects of incorporating diethylaminoethyl-dextran (DEAE-D) in the inoculum with bovine respiratory syncytial virus (BRSV) on the infectivity of BRSV was evaluated. A concentration of 40 μg DEAE-D/ml provided maximal enhancement of infection as determined by the time of onset of cytopathic effect (CPE), the percentage of cells infected by the inoculum, and the amount of virus produced. When DEAE-D was used in the inoculum, the CPE appeared a day earlier, the percentage of cells infected by the inoculum, as determined by the fluorescent antibody test, was increased 11 times, and the viral titer was increased 2 times as compared to results obtained without DEAE-D. Bovine respiratory syncytial virus-infected cultures contained much cell-associated virus which could be liberated by sonication to increase the titer of virus stocks. The use of BRSV-infected cells rather than supernates from BRSV-infected cells increased the rate at which a cytopathic effect developed, although it did not substantially increase the titer of virus which was harvested. The use of DEAE-D in the inoculum and the passage of BRSV-infected cells instead of viral suspensions was found to be the quickest and most effective method of consistently obtaining BRSV with a titer of about $10^{5.5}$ $TCID_{50}$/ml.

Introduction

Bovine respiratory syncytial virus (BRSV) is closely related to respiratory syncytial virus (RSV) (1), and although the two viruses cross-react considerably in serum neutralizing tests, they are antigenically distinct (13, 21). Respiratory syncytial virus is considered to be the most important virus in initiating severe respiratory disease in infants (8). Its counterpart in the bovine, BRSV, has been

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found to be associated with respiratory disease in cattle (7, 13, 15, 19); however, its role and importance in this syndrome remain to be elucidated. Under experimental conditions with viruses in cattle, it often has been difficult to produce the severe type of respiratory disease which occurs naturally. In the case of BRSV, this has been equally true (7, 11, 21). Isolation of BRSV has been reported in numerous countries: 1970 from Switzerland (13), Japan (6), and Belgium (23); 1971 from England (9); 1974 from the United States (15, 21). Since reports on the isolation of BRSV, there have been few reports dealing with virus-host interactions. To a considerable extent, this probably has been due to the difficulty of i) obtaining high-titered virus and ii) storing it without considerable loss of infectivity. Bovine respiratory syncytial virus, as RSV (5), has been found to lose considerable activity on storage at −80° C (Rossi and Kiesel, unpublished observation).

The object of this study was to investigate the infectivity of different preparations of BRSV and to develop methods of increasing the infectivity of BRSV stocks. Both the use of diethylaminoethyl-dextran (DEAE-D) in the inoculum and the use of BRSV-infected cells for passage of virus, instead of virus-containing supernates, was found to be effective in increasing infectivity.

Materials and Methods

Cells and Medium

Bovine embryonic lung (BEL) cultures were initiated from the lungs of embryos obtained at a nearby abattoir and were used between the 3rd and 8th passages. Second passage BEL cultures were stored in liquid nitrogen. Cultures were passaged a month in serum free of bovine viral diarrhea (BVD) virus and examined by the fluorescent antibody (FA) technique to be certain they were free of BVD virus and, therefore, satisfactory for use. Medium consisted of minimal essential medium of Eagle (MEM) supplemented with different concentrations of fetal bovine serum (FBS) for different conditions and containing penicillin, streptomycin, and neomycin at concentrations of 200 units, 200 μg, and 100 μg per ml, respectively. Medium for microtiter titrations was supplemented with organic buffers as described (18).

Virus

Bovine respiratory syncytial virus (BRSV) originally isolated by Smith *et al.* and kindly provided by Dr. R. M. Phillips was purified by terminal dilution for use in these experiments.

Preparation of Fluorescein Isothiocyanate-Conjugated Bovine-Anti BRSV

Stock BRSV was prepared on BEL cultures. Calves were inoculated intratracheally and intranasally with $10^{7.4}$ median tissue culture infective doses ($TCID_{50}$) BRSV. Three months later 5 intravenous injections containing $10^{6.9}$ $TCID_{50}$ each of BRSV were given at three-week intervals. Two weeks after the last injection the calves were bled and the immunoglobulin G (IgG) fraction obtained and conjugated with fluorescein isothiocyanate (FITC) at a concentration of 25 μg FITC/mg IgG (20).

Microtitration

Viral suspensions and cells which were titrated were diluted with pipetes in tubes with microtiter medium as diluent. Eight replicates of two-fold dilutions were placed in microtiter plates with microtiter pipets which delivered 0.025 ml per drop. A drop of medium and a drop of BEL cells at a concentration of 300,000 cells per ml were added. Cultures were incubated at 37° C as previously described (18), and the titer of the virus was calculated by the method of Reed and Muench (14).

Toxicity of Diethylaminoethyl-Dextran for Bovine Embryonic Lung Cells

Diethylaminoethyl-dextran (DEAE-D) was prepared as a stock solution in saline at a concentration of 4000 μg/ml, filtered, and diluted in appropriate medium before use. It was tested for its toxicity for BEL cells at different concentrations, different volumes of inocula, with different media used subsequent to treatment with DEAE-D, and with cells in different stages of growth.

Enhancement of BRSV Infection of BEL Cultures With DEAE-D

Three methods were used to evaluate the effectiveness of DEAE-D added to the inoculum in enhancing the infectivity of BRSV for BEL cells. These included i) the percentage of cells infected with BRSV as determined by the FA technique, ii) the extent of cytopathic effect (CPE) produced, and iii) the amount of virus produced.

In the fluorescent antibody technique, BEL coverslip cultures were inoculated with BRSV and different concentrations of DEAE-D. Twenty-four hours later, a time at which fluorescence was maximal and no fluorescence due to a second cycle of infection was present, the cultures were fixed, stained, and examined for fluorescence along with uninoculated cultures and cultures inoculated with heat-inactivated BRSV. Cells infected with BRSV were identified by their typical fluorescence (20). Evans blue at a final concentration of 0.04 per cent was added to the FITC-conjugate to eliminate background fluorescence.

Infectious Center and Cell-Associated Virus Assays

Bovine embryonic lung cultures in 25 cm^2 flasks were inoculated with BRSV containing 40 μg DEAE-D/ml and allowed to absorb at 37° C for two hours. Cultures were washed four times with Hanks' balanced salt solution (HBSS) to remove unattached virus and replaced with MEM containing 5 per cent FBS. At appropriate times the supernate was removed and centrifuged to remove unattached cells and a sample of supernate was used for titration. Attached cells were removed with trypsin-versene and dooled with the cells from the supernate, resuspended in medium equal to the volume of supernate, and a sample was used for titration. To compare titers of infectious virus obtained from the i) supernate, ii) cells, and iii) a mixture of supernate and sonicated cells, the procedure was varied so that after suspending the cells, they were centrifuged, suspended in a small volume of supernate, sonicated, and resuspended in the entire volume of supernate for titration. Titrations of infected cells and viral suspensions were carried out by microtitration.

Results

Toxicity of Diethylaminoethyl-Dextran for Bovine Embryonic Lung Cells

Toxicity at a given concentration of DEAE-D was found to be dependent upon the maturity of BEL cells, the volume of the inoculum, and the presence or absence of serum in the medium subsequent to treatment with DEAE-D. Using confluent BEL tube cultures, inocula of 0.2 or 2.0 ml at concentrations of 2000, 1000, 500 and 250 μg DEAE-D/ml were absorbed on cells for an hour at room temperature. After removing the DEAE-D and washing the cultures 4 times with HBSS, MEM with 5 per cent FBS was added. A slight degree of toxicity at 1000 and 2000 μg DEAE-D/ml with a 2.0 ml inoculum was noted. No toxicity was evident at lower concentrations of DEAE-D, or with a 0.2 ml inoculum. Preliminary experiments indicated that rapidly growing cultures were much more susceptible than confluent cultures to the toxic effect of DEAE-D. Consequently, tubes which were only half covered with BEL cells were inoculated with 1.0 ml of different concentrations of DEAE-D. After absorption for an hour at room temperature, the cultures were washed with HBSS. One group of cultures was

placed under serum-free medium and another group placed under MEM with 10 per cent FBS. Cytotoxicity, evaluated one and three days later, consisted of rounding of cells, cytoplasmic granularity and the loss of cells from the monolayer. Results showed that growing cultures that did not receive serum after treatment with DEAE-D were much more susceptible to the toxic effects than cultures which received serum. Furthermore, cultures which received serum often recovered from the toxic effect of DEAE-D whereas those which did not receive serum did not recover. Cultures which exhibited toxicity and subsequently recovered due to the presence of serum usually had fewer cells than cultures which exhibited no initial toxicity. When serum was not used, concentrations of DEAE-D as low as 30 μg/ml produced some cytotoxicity. When serum was used, concentrations of 60 to 120 μg DEAE-D/ml were usually satisfactory, whereas higher concentrations were toxic. Confluent monolayers of BEL cells in 25 cm^2 flasks were also tested for susceptibility to DEAE-D. Repeated trials showed that inocula of 0.2 ml were not toxic up to concentrations of 2000 μg/ml when 5 per cent or more serum was added subsequent to treatment, whereas cultures inoculated with 1.0 ml or more sometimes exhibited a slight degree of toxicity at concentrations of 1000 and 2000 μg DEAE-D/ml; in some experiments no toxicity at these concentrations was evident.

Effect of DEAE-D on Infectivity of BRSV

Results of FA tests to determine the effect of BRSV on infection of BEL cells are shown in Table 1. Infection was enhanced as much as 10.9 times at a concentration of 40 μg DEAE-D per ml. Considerable enhancement was also present at concentrations of 20 and 80 μg DEAE-D/ml. Using young, rapidly growing cells, higher concentrations of DEAE-D were toxic.

Table 1. *Detection of BRSV-infected cells by FA after using different concentrations of DEAE-D in the inoculum*

DEAE-D (μg/ml)	I	II	Mean increase over cultures not treated with DEAE-D
0	5.5[a]	4.0	0
20	46	38	8.8×
40	63.5	40	10.9×
80	51.5	25	8.0×
160	toxic	19.4	4.9×

[a] Percentage of cells exhibiting BRSV-specific fluorescence 24 hours after inoculation

To determine whether DEAE-D would enhance infectivity of BRSV of very low titer as well as virus of a relatively higher titer, BRSV stock was used undiluted and at different dilutions with varying concentrations of DEAE-D. Bovine respiratory syncytial virus mixed with DEAE-D in a volume of 1.0 ml was inoculated onto BEL flask cultures and allowed to absorb for 2 hours at 37° C. After the absorption period, the inoculum was removed, the cultures were washed 4 times with HBSS, and MEM with 5 per cent FBS was added to the cultures.

The cultures were incubated at 37° C and the CPE recorded daily. Results are shown in Table 2. At 40 μg DEAE-D/ml infectivity was increased compared to untreated cultures at all dilutions of BRSV. High concentrations of DEAE-D may have had a slight enhancing effect at low concentrations of BRSV; there was no indication that high concentrations of DEAE-D inhibited BRSV.

Table 2. *Cytopathogenicity*[a] *of BRSV with DEAE-D on confluent BEL cultures*

Virus dilution	DEAE-D (μg/ml)	Days post-inoculation					
		1	2	3	4	5	6
Undiluted	0	—	1+	2—3+	4+		
	40	1+	2—3+	4+			
	2000	tx[b]	—	±	3+	4+	
1/10	0	—	—	tr[c]	2+	2—3+	4+
	40	tr	tr	1+	3+	4+	
	2000	tx	—	±	2+	3+	3—4+
1/50	0	—	—	—	1+	1+	2+
	40	—	—	tr	2+	2—3+	3—4+
	2000	tx	—	—	2+	3+	3+
Undiluted	0	—	tr	2+	3+	4+	
	40	tr	1—2+	3+	4+		
	200	—	tr	2+	3+	4+	
	1000	tx	—	1—2+	2+	4+	
1/200	0	—	—	tr	1+	1—2+	2—3+
	40	—	—	1+	2+	3+	4+
	1000	—	—	1+	1+	2+	3+

[a] Graded on a scale of 1+ to 4+
[b] A temporary toxicity which consisted of rounding of 10 to 20 per cent of the cells and disappeared by the next day
[c] Trace CPE

The effect of DEAE-D on virus production was determined by inoculating confluent BEL flask cultures with an inoculum of 1.0 ml containing BRSV and different concentrations of DEAE-D. At intervals of 1, 2, 3, and 4 days the CPE was recorded and the flask frozen and kept at −80° C. After all cultures had been frozen, they were thawed and titrated for BRSV in microtiter plates. Higher titers of BRSV were obtained with 40 μg DEAE-D/ml than with other concentrations. Furthermore, the CPE appeared earlier in cultures with 40 μg DEAE-D/ml than in cultures with 0, 200, or 1000 μg DEAE-D/ml (Table 3).

Comparison of Infectious-Center Assay With Virus in Supernate

On the second, third, and fourth day after inoculation of confluent BEL culture flasks with BRSV, supernate and cells were titrated separately. The distribution of infectious BRSV between supernate and cells is shown in Table 4. During the early stages of infection when there was a 1 and 2+ CPE, the majority of BRSV was found associated with cells, whereas when a 3+ CPE was present, even though most of the cells were still attached to the glass, the majority of BRSV was found in the supernate.

Table 3. *Effect of DEAE-D in the inoculum on the production of BRSV in BEL cells*

DEAE-D (μg/ml)	Days after inoculation							
	1		2		3		4	
	CPE[a]	Titer[b]	CPE	Titer	CPE	Titer	CPE	Titer
0	—	360	2+	6,400	3+	7,600	3+	4,600
40	—	5,300	3+	16,000	3—4+	15,000	4+	320
200	—	1,200	2—3+	7,300	3—4+	6,400	3—4+	9,400
1000	—	320	2+	3,800	3+	5,000	3+	400

[a] Graded on a scale of 1+ to 4+
[b] $TCID_{50}$/ml

Table 4. *Titer of BRSV in cells and supernate after infection with BRSV*

	Days after inoculation		
	2 (1+)[a]	3 (2+)	4 (3+)
Supernate	1.8×10^3	2.8×10^4	3.5×10^5
Infectious centers	2.4×10^5	3.2×10^5	3.3×10^4

[a] Cytopathic effect graded on a scale of 1+ to 4+

Release of BRSV From Cells by Sonication

When a 3+ CPE was present, the amount of virus present in the supernate, infected cells, and the virus in the supernate combined with virus from sonicated cells was determined. Titers obtained by microtitration indicate sonication of cells liberated considerable virus (Table 5).

Table 5. *Titer of BRSV and effect of sonication of BRSV-infected BEL cells*

Supernate	3.2×10^{5a}
Infectious centers	1.0×10^5
Supernate and sonicated cells	7.5×10^5

[a] $TCID_{50}$/ml
Bovine embryonic lung cells were inoculated with BRSV. When a 3+ CPE had developed, supernate, cells suspended to the volume of the supernate, and sonicated cells mixed with supernate were titrated for BRSV

Passage of BRSV-Infected Cells Compared With BRSV Suspensions

The capacity to transmit infection to BEL cells from BRSV-infected cells and viral suspensions was compared. From BRSV-infected BEL cultures with a 3+ CPE, supernate and cells were passed to fresh cells. To virus-containing supernate, 40 μg DEAE-D/ml was added and the virus was absorbed at 37° C on BEL cultures for three hours, then replaced with MEM with 5 per cent FBS. The BRSV-infected

cells from the supernate were combined with cells removed from the flask with trypsin-versene, diluted in MEM with 5 per cent FBS, and placed on BEL cells. Viral suspensions with a titer from 10^5 to $10^{5.8}$ produced a 3+ CPE on the third day, whereas the cells from these suspensions produced a 3+ CPE on the second day. Titers of virus which have been obtained by using BRSV-infected cells have been consistently as high, or slightly higher, than maximal titers in which viral suspensions have been used as the inoculum.

Discussion

The toxicity of DEAE-D for BEL cultures primarily depended upon the condition of the cells. Rapidly growing cells were more susceptible than resting, confluent, cultures to the toxic effects of DEAE-D. Concentrations above 60 or 80 μg/ml were usually toxic to rapidly growing BEL cells and prevented the use of higher concentrations in other experiments, but concentrations up to 1000 and 2000 μg/ml were often tolerated by resting cultures. The effect of different concentrations of DEAE-D on the infectivity of BRSV for BEL cells was evaluated by several criteria: number of cells infected by the inoculum, degree of CPE, and amount of infectious virus produced. Because of the ease of counting cells in semi-confluent cultures compared to the difficulty of counting cells in a confluent culture, semi-confluent cultures were used in FA tests to determine the number of cells infected with BRSV. Under these conditions, enhancement was maximal with 40 μg DEAE-D/ml and infection was enhanced almost 11 times as compared with BEL cultures in which DEAE-D was not added to the inoculum (Table 1).

The CPE produced by BRSV occurred a day earlier when DEAE-D was present in the inoculum than when it was absent. This effect occurred over a large range of concentrations of BRSV (Tables 2 and 3). The principal object of determining the capacity of DEAE-D to enhance dilute solutions of BRSV was to evaluate the feasibility of using DEAE-D in isolating BRSV from the respiratory tract of cattle. In both naturally and experimentally infected cattle, the isolation of BRSV has posed problems in that a CPE has not been identified until after subculture, and/or because of the short period of time over which the virus was recoverable (6, 11, 13, 21). Difficulties have been reported in the isolation of RSV from humans (4), which to some extent have been due to the loss of infectivity which occurs on freezing and thawing of the samples. However, even when nasal washings and nasal swabs are placed directly on susceptible cultures, BRSV has been difficult to isolate (Rossi and Kiesel, unpublished observations). Using confluent cultures, DEAE-D enhanced infection with BRSV to the extent that the amount of virus harvested was about twice the amount harvested when DEAE-D was not used (Table 3). The reason for the greater amount of virus harvested with DEAE-D in the inoculum than when it was not present probably reflects the greater infectivity of the inoculum. Since virus is produced more quickly with higher than with lower concentrations of BRSV, the sooner the virus can be harvested, the greater is the probability that the virus will not be inactivated.

The use of polyions, especially DEAE-D, to enhance infection has been investigated with a variety of viruses, including RSV (10, 12, 16, 22). Although the mechanism of action of DEAE-D in enhancing BRSV infection was not investigated, work with laryngotracheitis virus (LTV), an avian herpesvirus, is

probably applicable in explaining enhancement of other viruses with DEAE-D. Studies with LTV have conclusively shown that enhancement of infection with DEAE-D is due to increasing the attachment of virus to cells by the action of DEAE-D on cells, and that DEAE-D has no effect on virus particles themselves (16, 17). Furthermore, work with LTV has shown that there is not a subpopulation of virus particles which lacks the capacity to attach to cells, but that the entire population is deficient in its ability to attach to cells. This by no means rules this out as a possibility for BRSV. The efficacy of using DEAE-D in the inoculum of BRSV in order to experimentally infect cattle is open to question, since DEAE-D probably has little affinity for the virus and might be diluted to insignificant concentrations in the respiratory tract of cattle. One should note, however, that in mice its use with encephalomyocarditis virus has been shown to have a considerable enhancing effect (3).

Since BRSV is an RNA virus which completes its development at the cell membrane (1), disruption of BRSV-infected cells cannot liberate infectious intracellular virus but can only remove virus adherent to the cell membrane. Sonication of BRSV-infected cells was found to be an effective method for liberating BRSV from infected cells in order to obtain higher titered virus stocks (Table 5). The use of virus-infected cells was found to be more efficient in infecting cells than viral suspensions. Viral suspensions are limited in their infectivity by the capacity of one virus particle to infect only one cell, and by the fact that the percentage of virus absorbed from a large inoculum is much less than that absorbed from a small inoculum (2). As a consequence of the latter limitation, increasing the volume of inoculum is not especially effective in increasing infection. However, virus-infected cells all settle to the monolayer and are probably capable of infecting more than one cell.

Since virus stocks of BRSV lose considerable virus because of freezing and due to storage, we have found the following method to be the most suitable to get stock virus preparations to maximal titers of around $10^{5.9}$ $TCID_{50}$/ml. Virus taken from the freezer is mixed with DEAE-D to a final concentration of 40 μg/ml. When a 3+ CPE has been reached, cells from the supernate and those attached to the glass are used to inoculate a culture of BEL cells which cover $^3/_4$ the surface of the flask. When the CPE again reaches a 3+ CPE, the procedure is repeated until a sufficient number of flasks have been inoculated. Passage of cells is made at a 1:1 ratio to begin with and at a 1:2 ratio thereafter. When a sufficient number of flasks have been inoculated and the CPE reaches 3+ on the second to third day, the supernate is harvested and the cells sonicated in a small volume of supernate. This type preparation has been found to give the most consistently high titers of $10^{5.3}$ to $10^{5.9}$ $TCID_{50}$, although it is considerably lower than titers of $10^{7.9}$ which we have obtained with RSV on HEp-2 cells. However, the amount of BRSV produced can be estimated to be at least 11-fold greater than that determined in microtiter assays, or in any assay in which DEAE-D is not used in the inoculum. Since DEAE-D enhances RSV-infection, titers of RSV can also be considered to be low estimates of the infectivity which might be obtained under different conditions. It is possible that further studies using BRSV-infected cells as inocula on BEL cells will reveal methods of obtaining higher titers of BRSV than have been achieved to date.

When using BRSV in serum neutralization tests, one should be aware of the large amount of uninfective and inactivated virus in the suspensions and be cognizant of the reduction this introduces in the sensitivity of the test to detect antibody. Because of this, one can predict that the prevalence of BRSV infection of cattle is greater than that based upon results of serum neutralization tests.

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References

1. Berthiaume, L., Joncas, J., Pavilanis, V.: Comparative structure, morphogenesis and biological characteristics of the respiratory syncytial (RS) virus and the pneumonia virus of mice (PVM). Arch. ges. Virusforsch. **45**, 39—51 (1974).
2. Cooper, P. D.: The plaque assay of animal viruses. Adv. Virus Res. **10**, 319—378 (1961).
3. Craighead, J. E.: Effect of polycations on growth and dissemination of the encephalomyocarditis virus in mice. J. Virol. **1**, 988—995 (1967).
4. Hall, C. B., Douglas, R. G.: Clinically useful method for the isolation of respiratory syncytial virus. J. inf. Dis. **131**, 1—5 (1975).
5. Hambling, M. H.: Survival of the respiratory syncytial virus during storage under various conditions. Brit. J. exp. Pathol. **45**, 647—655 (1964).
6. Inaba, Y., Tanaka, Y., Omori, R., Matumoto, M.: Isolation of bovine respiratory syncytial virus. Japan. J. exp. Med. **40**, 473—474 (1970).
7. Inaba, Y., Tanaka, Y., Sato, K., Omori, T., Matumoto, M.: Bovine respiratory syncytial virus studies on an outbreak in Japan, 1968—1969. Japan. J. Microbiol. **16**, 373—383 (1972).
8. Jackson, G. G., Muldoon, R. L.: Viruses causing common respiratory infections in man. III. Respiratory syncytial viruses and coronaviruses. J. inf. Dis. **128**, 674—702 (1973).
9. Jacobs, J. W., Edington, N.: Isolation of respiratory syncytial virus from cattle in Great Britain. Vet. Rec. **88**, 694 (1971).
10. Kaplan, M. M., Wiktor, T. J., Maes, R. F., Campbell, J. B., Koprowski, H.: Effect of polyions on the infectivity of rabies virus in tissue culture: construction of a single-cycle growth curve. J. Virol. **1**, 145—151 (1967).
11. Mohanty, S. B., Ingling, A. L., Lillie, M. G.: Experimentally induced respiratory syncytial viral infection in calves. Amer. J. vet. Res. **36**, 417—419 (1975).
12. Nomura, S.: Interaction of respiratory syncytial virus with polyions: Enhancement of infectivity with diethylaminoethyl dextran. Proc. Soc. exp. Biol. Med. **128**, 163—166 (1968).
13. Paccaud, M. F., Jacquier, C.: A respiratory syncytial virus of bovine origin. Arch. ges. Virusforsch. **30**, 327—342 (1970).
14. Reed, L. J., Muench, H.: A simple method of estimating fifty percent endpoints. Amer. J. Hyg. **27**, 493—497 (1938).
15. Rosenquist, B. D.: Isolation of respiratory syncytial virus from calves with acute respiratory disease. J. inf. Dis. **130**, 177—182 (1974).
16. Rossi, C. R.: Studies of laryngotracheitis virus in avian tissue cultures. IV. Effect of diethylaminoethyl-dextran on adsorption and elution. Arch. ges. Virusforsch. **35**, 223—231 (1971).
17. Rossi, C. R., Watrach, A. M.: Studies of laryngotracheitis virus in avian tissue cultures. III. Enhancement of infectivity by diethylaminoethyl-dextran. Appl. Microbiol. **19**, 932—936 (1970).

18. Rossi, C. R., Kiesel, G. K.: Antibody to viruses affecting cattle in commercial tissue culture grade fetal calf serum. Appl. Microbiol. **27**, 114—117 (1974).
19. Rossi, C. R., Kiesel, G. K.: Serological evidence for the association of bovine respiratory syncytial virus with respiratory tract disease in Alabama cattle. Infect. Immun. **10**, 293—298 (1974).
20. Rossi, C. R., Kiesel, G. K.: Bovine respiratory syncytial virus infection of bovine embryonic lung cultures: A kinetic study by the fluorescent antibody technique. Amer. J. vet. Res. (Accepted for publication.)
21. Smith, M. H., Frey, M. L., Dierks, R. E.: Isolation, characterization, and pathogenicity studies of a bovine respiratory syncytial virus. Arch. Virol. **47**, 237—247 (1975).
22. Somers, K. D., Kirsten, W. H.: Focus formation by murine sarcoma virus: enhancement by DEAE-D. Virology **36**, 155—157 (1968).
23. Wellemans, G., Leunen, J., Luchsinger, E.: Isolement d'un virus (220/69) serologiquement semblable au virus respiratoire syncytial (RS) humain. Ann. Méd. Vét. **114**, 89—93 (1970).

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