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Effect of Diethylaminoethyl-Dextran on the Replication of a Murine Sarcoma (Moloney)-Leukemia Virus Complex in Mouse Embryo Cultures (38577)

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The polycation diethylaminoethyl-dextran (DEAE-D) enhances the cellular uptake of infectious picornavirus RNA (1–3) and increases the infectivity of rabies virus in BHK/21 cells (4). Focus formation in chick embryo cells by avian sarcoma viruses of subgroups B and C, but not of subgroup A, also has been found to be enhanced by DEAE-D (5, 6). Duc-Nguyen (7) showed an enhancing effect of DEAE-D on the focus forming titers of the Harvey and Moloney strains of murine sarcoma virus (MuSV) both in rat and mouse cells. Enhancement of focus formation by another strain of MuSV in 3T3 cells pretreated with DEAE-D was demonstrated by Somers and Kirsten (8). Enhancing effects of DEAE-D on infection in vivo with murine sarcoma (9) and leukemia viruses (10) also have been noted. Since these reports, pretreatment of host cells in culture with DEAE-D prior to infection with sarcoma or leukemia viruses has been widely practiced. In attempts to conserve viral stocks by potentiating focus formation of MuSV in secondary mouse embryo cultures, we found that DEAE-D did not enhance focus formation by Moloney preparations of murine sarcoma–leukemia virus complex (11). This paper presents the results of our investigation of this finding and describes the relationship between passage in cell culture and responsiveness of both the sarcoma and leukemia moieties of the Moloney murine sarcoma (MuSV(M))-leukemia (MuLV(M)) virus complex to potentiation by DEAE-D.

Materials and Methods. 1. *Media.* DEAE-D, molecular weights 5×10^5 and 2×10^6, was purchased from Pharmacia, Inc., Piscataway, N. J. Modified McCoy's 5a medium, and minimal essential medium with Earle's salts (MEM), fetal calf serum (FCS), agamma fetal calf serum, phosphate buffered saline (PBS) and glutamine were purchased from Grand Island Biologicals (GIBCO), Grand Island, N. Y.

2. *Viruses.* The sarcoma (Moloney)-leukemia virus complex studied in these experiments (Lots 36 and 42) was obtained from Dr. Robert Holdenreid of the National Cancer Institute, and was prepared from mouse rhabdomyosarcomas by the method given by Moloney (11). The isolate of Friend leukemia virus (FLV), provided by Dr. Charlotte Friend, was prepared from the spleens of infected mice (12).

3. *Viral assays.* The methods for the preparation of primary and secondary N.I.H. Swiss mouse embryo (MEF) cultures have been previously described (13, 14). The titration pattern of the sarcoma virus moiety of the complex (MuSV(M)) was determined by methods previously reported (14). Generally 4×10^5 secondary MEF cells in 60 mm plastic Falcon plates were infected with 0.2 cc of increasing dilutions of virus. After allowing 60 min for viral adsorption at 37°, the plates were fed with 5 cc media and foci were counted after 6 days incubation at 37° in 7% CO_2.

The leukemia moiety of the complex (MuLV(M)) was titered, using dilutions past the focus forming activity, by the syncytium forming XC cell assay given by Klement *et al.* (15). The helper activity of this complex also was determined (16). Friend leukemia virus was titered by the XC assay (15).

The results of the XC assay were expressed as the 50% tissue culture infectious dose ($TCID_{50}$) determined by the method of Reed and Meunch (17).

4. *Passage of virus in secondary mouse embryo cultures.* Moloney preparations of sarcoma–leukemia virus complex (11) were diluted 1:25 with MEM containing 10% FCS and 0.2 ml added to 4×10^5 secondary

MEF cells in 60 mm plastic culture plates. After allowing 60 min for viral adsorption, 4 ml MEM (10% FCS) were added and the plates were incubated at 37° for 5 days. Plates were scraped on the third and fifth days of incubation with a rubber policeman and the suspension of cells in the feeding medium was dispensed in 1 ml aliquots for quick freezing. This first viral passage was titered for the presence of MuSV(M) and MuLV(M) with and without DEAE-D pretreatment (vide infra). Subsequent viral passage was performed by diluting aliquots of the first passage 1:2.5 with MEM (10% FCS) and infecting 2° MEF cells as described above. The infected cultures were processed for further passage as detailed for the first passage. In other experiments, MuSV(M)-MuLV(M) was passaged in 2° MEF cells pretreated with DEAE-D.

5. *Studies with DEAE-D in mouse embryo cultures.* Toxicity studies were performed using both DEAE-D of molecular weights 5×10^5 and 2×10^6. In these experiments 2° MEF cells were treated with 2 cc media containing increasing concentrations of DEAE-D for various periods of time. After the desired period of incubation, the cells were examined for evidence of toxicity and counts of viable cells were performed using trypan blue. Secondary mouse embryo cultures were pretreated with varying concentrations of DEAE-D, not toxic to the cells, for differing periods of time, washed twice and infected with virus. In the experiments whose results are given here, cells were pretreated with DEAE-D at a concentration of 25 μg/ml for 45 min.

6. *Studies with DEAE-D and viral infection in Vivo.* Groups of 2- to 3-day-old Swiss mice were inoculated intramuscularly in the right thigh with 0.05 ml MEM containing 25 μg DEAE-D followed in 10 min by 0.1 ml of a 1:500 dilution of a Moloney preparation of sarcoma–leukemia virus complex, or 1:50 dilutions of aliquots of first or second passage virus. Control mice first were inoculated with 0.05 ml MEM containing no DEAE-D. Other control mice received MEM with DEAE-D but no virus. In other experiments mice were inoculated with DEAE-D in the left thigh and with virus in the right thigh. The mice were examined daily and both the time of first appearance of tumors and tumor size (18) were charted.

Results. 1. *Effects of DEAE-D on titrations of MuSV(M) and MuLV(M) of Moloney preparations of sarcoma–leukemia virus complex.* It was observed that the number of foci developing when 2° MEF cultures were infected with serial dilutions of the Moloney preparations of sarcoma–leukemia virus complex was proportional to the dilution (Fig. 1). Thus the titration pattern was that of "competent" MuSV(M) showing "one-hit" kinetics (13, 14). Interestingly, the number of foci obtained in MEM was consistently greater than that obtained in modified McCoy's 5a medium. The minimal essential medium contained 10% FCS and the McCoy's medium contained 5% agamma FCS. The foci in McCoy's medium were more spread out and not as readily visible as they were in MEM.

Pretreatment of secondary mouse cells with DEAE-D did not significantly increase the focus forming titer of MuSV(M) either in McCoy's medium or MEM (Table I). The experiments given in Table I were performed by pretreating secondary mouse embryo cells with 2 cc media containing DEAE-D at a concentration of 25 μg/ml for 45 min. There was no observable cell toxicty or decrease in viable cell count with this treatment. The titration pattern of the virus did not change by pretreating cells with DEAE-D. The effects of varying the concentration of fetal calf serum and glutamine in MEM on the titer of MuSV(M) are shown in Table II. Pretreatment of mouse cells with DEAE-D did not enhance the focus forming titer of MuSV(M) in any of these experiments. The titer of MuSV(M) was the same when 10 or 5% fetal calf serum was added to the medium (Table II, A and B) even when the concentration of glutamine was decreased by 90% Table II, D and E). Indeed, the titer of MuSV(M) did not decrease when no glutamine was present in the medium. However, in the absence of glutamine or when glutamine was present at a concentration of 2.92 mg/100 ml and the concentration of FCS in MEM was only 2%, even low doses of DEAE-D such as 10 μg/ml for 15

min were toxic to the mouse embryo cells. Varying the number of cells infected from 2×10^5 to 5×10^6 did not produce an enhancing effect of DEAE-D.

DEAE-D did not enhance the titer of MuLV(M) present in the sarcoma–leukemia virus complex (Table III, A). When the complex was diluted past the focus forming end point and the dilutions added to the original complex in the focus forming assay, or when FLV was added as helper to the plates in the focus forming assay, there was a 14% increase in the number of foci. However, there was no further increase when the cells were pretreated with DEAE-D. On the other hand, pretreatment with DEAE-D (25 μg/ml for 45) min consistently yielded a 10- to 20-fold enhancement in the titer of FLV (Table III, B).

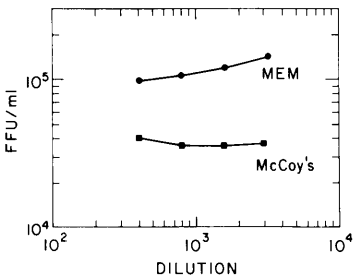

FIG. 1. Titration pattern of MuSV(M) in secondary mouse embryo cells. Virus stock of Moloney preparations was diluted in MEM containing 10% FCS or modified McCoy's 5a medium containing 5% agamma FCS and 0.2 ml aliquots of the dilutions were inoculated into plates containing 4×10^5 Swiss 2° MEF cells. After 60 min at 37° to allow for viral adsorption, the plates were fed with 5 cc of the appropriate medium. The number of foci was counted 6 days later and the results expressed as focus forming units per ml (FFU/ml) of the original virus stock.

TABLE I. TITRATION OF Mu/SV(M) IN MEF CELLS PRETREATED WITH DEAE-D.

Control (FFU/ml)	DEAE-D (FFU/ml)
A. McCoy's medium	
$4.15 \pm 0.34 \times 10^4$	$4.07 \pm 0.42 \times 10^4$
B. MEM	
$2.71 \pm 0.38 \times 10^5$	$2.94 \pm 0.42 \times 10^5$

2. *Enhancement by DEAE-D of infectivity of tissue culture passaged MuSV(M)-MuLV(M).* There was a progressive increase in the enhancement by DEAE-D of the focus forming activity of MuSV(M)–MuLV(M) as virus was passaged in 2° MEF cells. First and fifth passage virus showed 3.4X and 17.1X enhancement of focus formation by DEAE-D, respectively (Fig. 2). The titrations of the passaged MuSV(M) showed "one-hit" patterns and the patterns were not altered by pretreatment with DEAE-D.

The infectivity of first passage MuLV(M) was not potentiated by DEAE-D (Table IV). However, there was a 10- to 457-fold enhancement by DEAE-D of the titer of second through fifth passage MuLV(M) (Table IV).

There were no differences in the effects of DEAE-D of MW 5×10^5 or 2×10^6 in the tissue culture experiments cited. MuSV(M)–MuLV(M) passaged in 2° MEF cells pretreated with DEAE-D also showed progressive enhancement by DEAE-D.

3. *Effect of DEAE-D in Vivo on MuSV-(M)–MuLV(M) infection.* Inoculation of DEAE-D followed by Moloney preparations of MuSV(M)–MuLV(M) showed no decrease in time of appearance or increase in size of tumors (Table V). No potentiation effect of DEAE-D was found even with higher dilutions of virus. However, prior injection of DEAE-D at the site of viral inoculation did increase both the number of mice

TABLE II. TITRATION OF MuSV(M) IN MEM WITH VARYING CONCENTRATIONS OF FCS AND GLUTAMINE.

Control (FFU/ml)	DEAE-D (FFU/ml)	Control (FFU/ml)	DEAE-D (FFU/ml)
A. 10% FCS + 29.2 mg/100 ml Glutamine		D. 10% FCS + 2.92 mg/100 ml Glutamine	
$6.03 \pm 0.4 \times 10^4$	$7.03 \pm 0.32 \times 10^4$	$8.7 \pm 0.38 \times 10^4$	$1.09 \pm 0.44 \times 10^5$
B. 5% FCS + 29.2 mg/100 ml Glutamine		E. 5% FCS + 2.92 mg/100 ml Glutamine	
$7.8 \pm 0.37 \times 10^4$	$6.33 \pm 0.7 \times 10^4$	$6.9 \pm 0.27 \times 10^4$	$6.5 \pm 0.22 \times 10^4$
C. 2% FCS + 29.2 mg/100 ml Glutamine		F. 2% FCS + 2.92 mg/100 ml Glutamine	
$4.13 \pm 0.8 \times 10^4$	$2.8 \pm 0.4 \times 10^4$	$2.1 \pm 0.7 \times 10^4$	—

TABLE III. TITRATION OF MuLV(M) AND FLV BY XC CELL ASSAY IN MEF CELLS PRETREATED WITH DEAE-D.

	Control	DEAE-D
A. MuLV(M) ($TCID_{50}$/0.2 ml)		
	10^9	7.94×10^8
B. FLV($TCID_{50}$/0.2 ml)		
	1.58×10^7	3.16×10^8

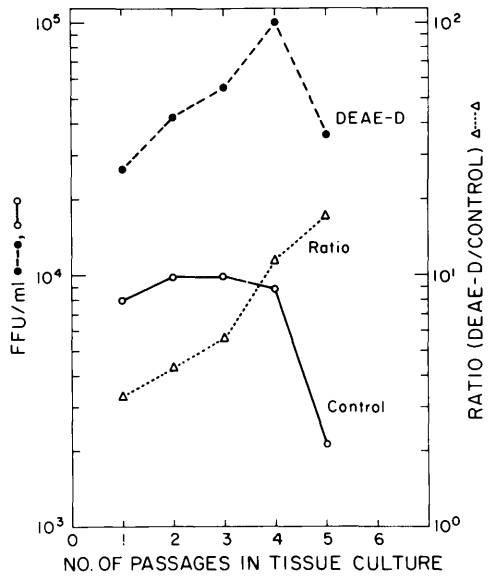

FIG. 2. Enhancement by DEAE-D of focus formation by MuSV(M) repetitively passaged in 2° MEF cells. See text for experimental details.

TABLE IV. EFFECT OF DEAE-D ON INFECTIVITY OF MuLV(M) PASSAGED IN 2° MEF CELLS.

Passage No.	Control ($TCID_{50}$/0.2 ml)	DEAE-D ($TCID_{50}$/0.2 ml)
1	10^6	10^6
2	10^6	10^7
3	10^6	4.57×10^8
4	10^5	10^7
5	10^6	10^7

developing tumors and the size of the tumors when first or second passage virus was inoculated (Table V). No enhancing effect was noted when DEAE-D was injected into the alternate thigh not inoculated with virus.

Discussion. The enhancing effect of DEAE-D on the replication of oncornaviruses in cell culture is not universal. Vogt (5) found that avian sarcoma viruses of subgroups B and C, but not of subgroup A, were enhanced by DEAE-D. Using a semimicro XC cell assay, Bass and Turner (19) found no potentiating effect of DEAE-D on their strains of Rauscher, Friend and Gross leukemia viruses. Thus pretreatment of cells in culture with DEAE-D should not be practiced a priori without first testing the sensitivity of the particular isolate of oncornavirus under the conditions of the infection.

Neither the MuSV nor MuLV moieties of our isolates of Moloney murine sarcoma–leukemia virus complex prepared as cell-free extracts from mouse tumors (11) were enhanced by DEAE-D. The absence of DEAE-D effect did not depend on the medium or the concentrations of glutamine and FCS. The MuSV titrations of the isolates showed "one-hit" patterns. Enhancing effects of DEAE-D on isolates of the Harvey and Moloney strains of MuSV prepared as cell-free extracts from mouse tumors was reported by Duc-Nguyen (7); however, the titration patterns of the MuSV isolates were not given. O'Connor and Fischinger (20) and Gazdar *et al.* (9) found enhancing effects of DEAE-D on MuSV(M) isolates showing "two-hit" titration patterns. However, the "two-hit" titration pattern was not altered by DEAE-D (20).

There was a progressive increase in the enhancing effects of DEAE-D as the Moloney isolates were passaged serially in MEF cultures. The titrations of the passaged MuSV(M) continued to show 'one-hit" patterns. It has been shown that there is attenuation of oncogenicity as oncornaviruses are passaged in tissue culture (21–25). Indeed, DEAE-D enhanced the tumor producing effects of the tissue culture passaged virus, but not the original Moloney virus preparations in mice. Vogt (5) showed that the effect of DEAE-D was on the host cell rather than on virus. It was speculated that DEAE-D may facilitate adsorption of defective virions to host cells (6). Our results indicate that as the Moloney preparation of virus was passaged in cell culture, virions lost both the ability to infect cells in culture and to cause tumors in animals and could be

TABLE V. EFFECT OF DEAE-D *In Vivo* ON INFECTIVITY OF MuSV(M)-MuLV(M).

Virus	DEAE-D			Control		
	No. mice	No. tumors	Tumor size[a] (mm, avg ± sd)	No. mice	No. tumors	Tumor size[a] (mm, avg. ± SD)
Moloney preparation	5	5	7.356 ± 0.448	5	5	7.213 ± 0.582
First passage	6	5	6.854 ± 0.767	5	0	—
Second passage	6	5	7.744 ± 0.478	5	2	6.635 ± 0.318

[a] Tumor size was compared by measuring midthigh the diameters of tumors with a micrometer.

enhanced in these two activities by DEAE-D. Although the patterns of passaged MuSV(M) remained "one-hit", increasing amounts of defective virus seemed to be present in each subsequent tissue culture passage. Thus a "one-hit" MuSV(M) titration pattern (13) is in itself not an indicator of absence of susceptibility to enhancement by DEAE-D. Indeed, passage of MuSV(M)–MuLV(M) in 2° MEF cells pretreated with DEAE-D did not prevent the emergence of virions sensitive to enhancement by DEAE-D.

The comparison of tumor extract virus and virus passaged in tissue culture may offer a system for the investigation of the early events of MuSV replication.

Summary. Isolates of a sarcoma (Moloney)-leukemia virus complex prepared as cell-free extracts from mouse tumors showed no enhancement in infectivity by DEAE-D either in the sarcoma moiety measured by focus formation or in the leukemia moiety measured by XC cell assay. The sarcoma moiety was not enhanced by DEAE-D in MEM and modified McCoy's 5a media or when varying amounts of FCS and glutamine were included in the media. Progressive enhancement of viral infectivity by DEAE-D was found when the viral preparations were passaged serially in MEF cells. DEAE-D also enhanced tumor formation in vivo by tissue culture passaged virus.

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