INFECTION AND IMMUNITY, June 1973, p. 911-917

Vol. 7, No. 6
Printed in U.S.A.

Interferon Sensitivity of Venezuelan Equine Encephalomyelitis Virus

GEORGE W. JORDAN

U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Received for publication 11 January 1973

Two strains of Venezuelan equine encephalomyelitis virus, which differ in virulence for mice, have been studied for their production of and sensitivity to chick and mouse interferon. Little interferon was produced by chick cells in response to the virulent Trinidad strain or the attenuated TC-83 strain without either aging or priming the cultures. Consistent differences in the production of chick interferon were not found between the two strains. Plaque variants of the Trinidad strain produced higher titers of mouse interferon than the TC-83 strain in both primed and control L-cell cultures. The TC-83 strain was found to be more sensitive than the Trinidad strain to the inhibitory effects of interferon. The greater sensitivity of the TC-83 strain was observed at both high and low multiplicities and for both chick and mouse interferons. These results are consistent with the hypothesis that interferon sensitivity may have a role as a determinant of virulence in some virus-host systems.

Since its discovery, interferon has been considered as a major factor determining the outcome of virus infections and as a possible determinant of virus virulence (2). Evidence supporting this interpretation has been obtained with strains of Newcastle disease virus (16), vesicular stomatitis virus (22), foot-and-mouth disease virus (17, 18), and Semliki Forest virus (5). On the contrary, no correlation between the production of interferon and virulence was found in studies of Sindbis virus (21), influenza virus (8, 10), and vaccinia virus (11).

To investigate further the possible relationship of virulence and the interferon mechanism, virulent and attenuated strains of Venezuelan equine encephalomyelitis (VEE) virus have been compared for their sensitivity to interferon and their ability to stimulate its production.

MATERIALS AND METHODS

Cell culture. Chicken embryo cells (CEC) and mouse L cells were prepared as described (9). Vero cells obtained from the American Type Culture Collection were grown in medium 199 with 5% fetal calf serum in a humidified atmosphere of 5% CO_2 in air. Maintenance medium consisted of medium 199, 2% fetal calf serum, and antibiotics.

Virus strains. The virulent Trinidad strain (14, 19) and the attenuated TC-83 strain (3, 13) of VEE virus were obtained from the Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md. Virus preparations were made from CEC and 10% suckling mouse brain.

Production of interferon by aged CEC. Monolayers of CEC were prepared in 250-ml Falcon flasks. The medium was replaced with maintenance medium on the 1st, 4th, and 7th days, and the monolayers were used on the 10th day after planting. Two flasks each containing approximately 3.5×10^7 cells were inoculated with 1 ml of each virus dilution. After 1 h, the monolayers were washed and 30 ml of maintenance medium was added. After 18 h of incubation at 37 C, the medium from each pair of flasks was pooled and assayed for virus and interferon.

Growth curves. CEC monolayers and L-cell monolayers each containing approximately 4.5×10^6 cells were prepared in 60-mm petri dishes. The day after seeding, 4 ml of maintenance medium containing a specified dilution of chick or mouse interferon was added. The CEC cultures were incubated with interferon for 4 h and the L-cell cultures were incubated for 18 h before inoculation with virus. Virus used for the inoculation of CEC was grown in suckling mouse brain, and virus used for the inoculation of L cells was grown in CEC to avoid the presence of homologous interferon in the inoculum. After 1 h the inoculum was removed and the monolayers were washed twice with fresh medium. Medium (4 ml) was added and the cultures were incubated at 37 C for various periods of time. Growth was stopped by placing the petri dishes at −70 C. After thawing, the medium from three replicate plates was pooled and assayed for virus and interferon content. Interferon-treated cultures are called "primed cultures" in discussions of interferon production.

Virus assay. Infective virus was assayed on monolayers of CEC in 60-mm petri dishes. An overlay of medium 199 with 2% fetal bovine serum, 0.6% Ionagar, and antibiotics was used. After 24 h a second overlay containing neutral red at a dilution of 1:12,000 was added and the plaques were counted 4 h later. Three replicate cultures were used at each virus dilution.

Interferon and interferon assay. Chick and mouse interferons were produced and characterized as described (9). Samples to be assayed for interferon were centrifuged at 100,000 × *g* for 90 min, dialyzed against HCl-KCl buffer at pH 2 for 3 days, and brought to pH 7.2 by dialysis against phosphate-buffered saline. Samples to be assayed were diluted in maintenance medium, and 0.4-ml amounts of each dilution were added in triplicate to cell monolayers in petri dishes containing 3.6 ml of maintenance medium. Six to twelve plates, unexposed to interferon, were retained for virus challenge as controls. L-cell monolayers were incubated with interferon for 18 h and CEC monolayers for 4 h prior to challenge with approximately 100 plaque-forming units (PFU) of vesicular stomatitis virus. After a 1-h adsorption period with intermittent rocking, the plates were overlaid with 4 ml of maintenance medium containing 0.6% Ionagar without removal of the inoculum. L-cell monolayers were incubated for 44 h and CEC monolayers were incubated for 24 h prior to the addition of a second overlay containing neutral red at a dilution of 1:12,000. After another 4 h of incubation, the petri dishes were inverted on an X-ray viewbox and all plaques visible under these conditions were counted regardless of size. The plaque reduction data were analyzed by the probit method (9). Laboratory standards of chick or mouse interferon were included in each assay; the standard titers did not show greater than a twofold variation during the course of these experiments.

Serum neutralization experiments. Antisera to the various VEE virus strains were made in rabbits. Sera were inactivated at 56 C for 30 min, and twofold dilutions were made in Hanks balanced salt solution containing *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid buffer (10 mmol/liter), 0.25% human serum albumin, and antibiotics. The serum dilutions were mixed with equal volumes of virus diluted to yield 100 PFU. The serum-virus mixtures and controls were incubated at 37 C for 1 h. Each serum-virus mixture was added in triplicate to monolayers of Vero cells in 35-mm wells and allowed to adsorb for 1 h. An agar overlay was added and the monolayers were stained with neutral red after plaques had formed. The reciprocal of the serum dilution expected to reduce the number of plaques by 50% was taken as the titer.

Animals. Suckling (1–3 day) and weanling (18–21 day) random-bred Swiss-Webster mice were obtained from Microbiological Associates, Inc., Walkersville, Md. Each mouse received 0.03 ml by intracerebral (i.e.) or 0.1 ml by intraperitoneal (i.p.) injection of the virus dilutions. The mean lethal dose (LD_{50}) per ml was calculated (15) from the number of mice dying between the 2nd and 14th day after inoculation. Survivors were challenged with 100 LD_{50} to test for immunity.

RESULTS

VEE plaque variants. The passage history for the VEE virus strains is shown in Fig. 1. Stable large (1.5 mm) and small (0.5 mm) plaque types of the Trinidad strain were selected during three serial passages in CEC. The attenuated TC-83 strain used in the following experiments had been selected from a single plaque in its derivation (13), and only one 2.0-mm plaque type was observed.

To demonstrate that the large (Trin-L) and small (Trin-S) plaque types were variants of VEE virus, cross-neutralization tests were performed (Table 1). The results were analyzed for evidence of antigenic similarity by using the geometric mean (r) of each pair of titer ratios (1). A value for 1/r between 1/1 and 1/5 has been taken to indicate that virus strains are antigenically indistinguishable (5). From the titer ratios in Table 1 the following values for 1/r are obtained: Trin L-Trin S, 1/3.2; Trin L-TC 83, 1/4.2; and Trin S-TC 83, 1/5.8. The data indicate that Trin L, Trin S, and TC-83 are all closely related VEE virus variants.

Virulence for mice. Weanling mice (18–21 days old) were inoculated by the i.c. and i.p. routes. Animals that survived for 14 days were challenged i.c. with 100 LD_{50} of virulent virus. Both the small and the large Trinidad plaque variants were virulent by either route. The TC-83 strain caused no deaths, and the animals were protected against challenge with the virulent strains (Table 2).

For experiments with chick cells, virus stocks were prepared in mouse brain to avoid the presence of chick interferon in the inoculum. The TC-83 strain has been shown to regain virulence after repeated passage in mouse brain (13); however, the TC-83 strain remained avirulent after a single mouse brain passage (Table 2).

Effect of multiplicity on interferon production. These experiments were performed to determine the effect of the multiplicity of infection (MOI) on the production of interferon before undertaking a direct comparison of VEE virus strains. Aged cells were used to maximize interferon production (4). As the multiplicity was increased, more interferon and less virus were produced (Fig. 2). Although the TC-83 strain grew to slightly higher titers than the Trinidad strain, each produced about the same amount of interferon at comparable multiplicities. The only suggestion of a difference in

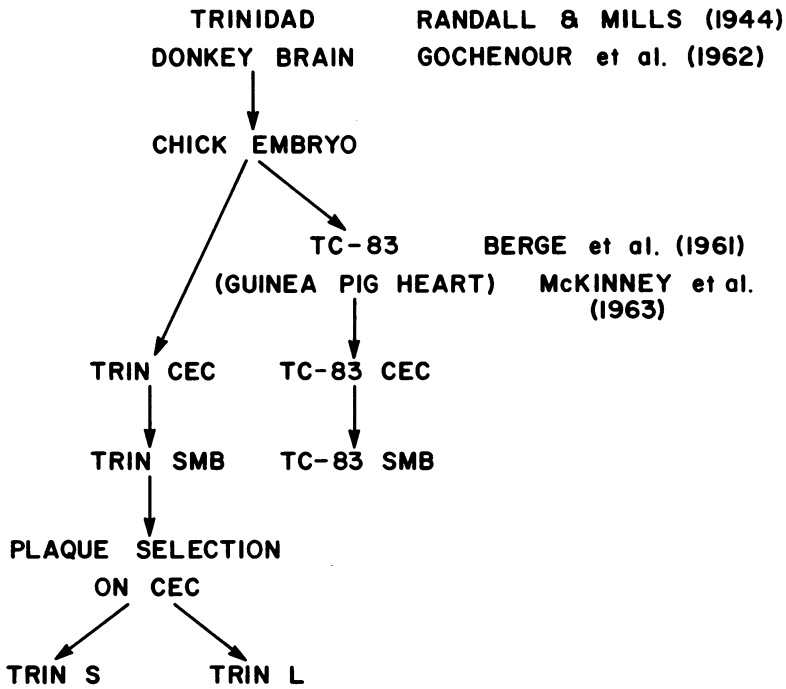

FIG. 1. *Passage history of VEE virus strains. CEC, virus grown in chicken embryo cells; SMB, virus grown in suckling mouse brain. Plaque diameters on CEC: Trin S, 0.5 mm; Trin L, 1.5 mm; TC-83, 2.0 mm.*

interferon production between the two strains occurred in Fig. 2B, where a progressive increase in interferon titer was seen with the Trinidad strain.

The source of the inoculum appeared to influence the amount of interferon produced and, again, an inverse relationship between virus and interferon production was observed. When the inoculum was prepared from a stock of 10% mouse brain (Fig. 2B, D), more virus and less interferon were produced than with input virus grown in CEC (Fig. 2A, C). Samples of the working stock used for the inoculum in these experiments were processed for interferon assay, and the results are shown in Table 3. Interferon of the species corresponding to the source of the virus stock was present in each case, and the different results from the two inocula may be an example of the priming phenomenon. Similar results regarding the source of the inoculum have been reported by Giron (6) for the growth of MM virus in L cells.

Effect of chick interferon. CEC 48 h after planting were exposed to interferon for 4 h and inoculated with VEE virus strains which were prepared from mouse brain. When the input virus was added at a high multiplicity (20 PFU/cell), the maximum titers were reached by 12 h of incubation; at low multiplicity (0.01 PFU/cell), maximum titers were reached at 18 h. For comparison, the maximum infectivity produced by interferon-treated cultures has been expressed as a percentage of the control value (Fig. 3). At both high and low multiplici-

TABLE 1. *Cross-neutralization reactions with VEE virus*

Serum	50% Plaque neutralization titer[a]				
	Trin L virus	Trin S virus	TC-83 virus	WEE[b] virus	EEE[c] virus
Trin L	47,800 (1)[d]	53,400 (1.1)	7,720 (1/6.2)	<10	<10
Trin S	10,000 (1/11)	115,000 (1)	10,800 (1/11)	<10	<10
TC-83	8,140 (1/2.8)	7,290 (1/3.1)	22,500 (1)	<10	<10
WEE	<10	<10	<10	4,160	<10
EEE[e]	<10	<10	<10	<10	190
NRS[f]	<10	<10	<10	<10	<10

[a] Titers reported for the VEE viruses against VEE antisera are the geometric mean of two determinations.
[b] WEE, Western equine encephalitis.
[c] EEE, Eastern equine encephalitis.
[d] Numbers in parentheses represent titer ratio.
[e] Human immune serum.
[f] NRS, Nonimmune rabbit serum.

TABLE 2. *Virulence of VEE virus strains for mice*[a]

Virus	Route of inoculation	Virus dilution $\log_{10}$							LD_{50}	PD_{50}	Type of response
		-3	-4	-5	-6	-7	-8	-9			
Trin S CEC	i.c.[b]	10:0:0	10:0:0	10:0:0	10:0:0	10:0:0	4:0:6	3:0:7	4.3×10^9		Lethal, no immunity[d]
	i.p.		10:0:0	10:0:0	10:0:0	10:0:0	2:0:9	1:0:9	4.9×10^8		Lethal, no immunity
Trin L CEC	i.c.	10:0:0	10:0:0	10:0:0	10:0:0	9:0:1	5:0:5	1:0:9	3.3×10^9		Lethal, no immunity
	i.p.		9:1:0	8:2:0	8:2:0	5:1:4	0:1:9	0:1:9	3.7×10^7		Lethal, occasional protection
TC-83 CEC	i.c.	0:6:0	0:6:0	0:6:0	0:6:0	0:6:0	0:6:0			$>10^9$	Protection to challenge with Trin S
	i.p.	0:10:0	0:8:2	0:7:3	1:7:2	0:5:5	0:1:9			3.0×10^7	Protection to challenge with Trin S
	i.p.	0:10:0	0:9:1	0:9:1	0:7:3	0:6:4	0:5:5			1.5×10^8	Protection to challenge with Trin L
TC-83 SMB	i.p.		0:6[e]	0:6	0:6	0:6	0:6				Not lethal for mice after a single passage in mouse brain

[a] Abbreviations: LD_{50}, mean lethal dose; PD_{50}, mean protective dose; SMB, suckling mouse brain.

[b] Mice 18 to 21 days of age were inoculated with 0.03 ml i.c. or 0.1 ml i.p.

[c] Results are shown as numbers of mice dying within 14 days of primary inoculation and before challenge, survivors of lethal challenge (protected), and dying within 14 days of challenge. The sum of these numbers is the number of mice inoculated at that dose level.

[d] Fourteen days after primary inoculation survivors were challenged by the i.c. route with 100 LD_{50} of the same virus unless otherwise indicated.

[e] Results are shown as number dead and number inoculated.

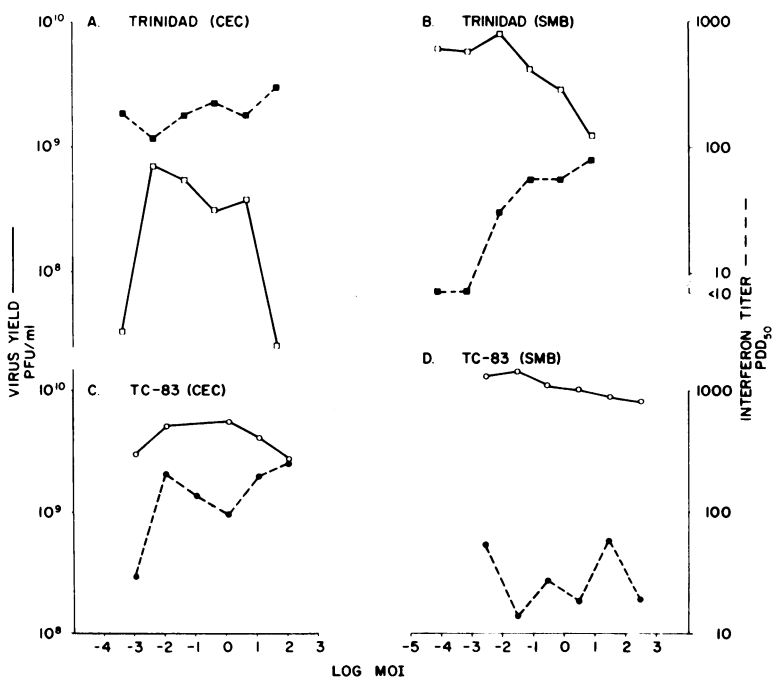

FIG. 2. *Effect of multiplicity on the production of VEE virus and interferon in aged chicken embryo cells (CEC). A, Inoculated with Trinidad strain grown in CEC; B, Trinidad strain grown in suckling mouse brain (SMB); C, TC-83 strain grown in CEC; D, TC-83 strain grown in SMB. Symbols: squares, Trinidad; circles, TC-83; open, virus yield; solid, interferon titer.*

TABLE 3. *Interferon present in VEE virus inoculum*[a]

Virus strain	Grown in	Interferon titer (PDD_{50})	
		Chick	Mouse
Trin	CEC	16	<20
TC-83	CEC	13	<20
Trin	SMB	<10	16,900
TC-83	SMB	<10	6,360

[a] Abbreviations: PDD_{50}, reciprocal of dilution expected to cause 50% reduction of vesicular stomatitis virus; SMB, suckling mouse brain.

ties, the growth of the TC-83 strain was inhibited to a progressively greater extent by interferon than growth of the Trinidad strain.

The production of interferon during the growth of the two virus strains is shown in Table 4. Very little interferon was produced by either strain in unprimed cells. In interferon-treated cultures, i.e., primed cells, interferon was detected earlier and in greater amounts. In primed cells, the TC-83 strain produced higher titers than the Trinidad strain.

Effect of mouse interferon. The growth of the Trin S, Trin L, and TC-83 strains in interferon-treated L cells is compared in Fig. 4. Input virus grown in CEC was added at multiplicities of approximately 5 and 0.01 PFU/cell; maximum titers were reached at 24 and 30 h, respectively. No difference was observed in the sensitivity of the two virulent strains. However, at both high and low multiplicities the growth of the TC-83 strain was inhibited to a greater extent than that of either virulent strain.

In contrast to CEC, unprimed L cells produced significant quantities of interferon after inoculation with each strain, although higher titers were produced by primed cells (Table 5). In contrast to the results in chick cells, the virulent strains produced more interferon than the TC-83 strain in interferon-treated and control cultures. In the case of both CEC (Table 4) and L cells (Table 5), less interferon was produced as the priming dose was increased, in agreement with the findings of Lockart (12).

DISCUSSION

The purpose of this study was to compare closely related virus strains with different virulence properties to see if a relationship existed between virulence and interferon production or sensitivity. In chick cells little interferon was produced by young, unprimed cultures in response to either the Trinidad or TC-83 strain. In young, primed cultures interferon titers were higher in response to the TC-83 strain. This is in contrast to the suggestion in Fig. 2D that less interferon was obtained with the TC-83 strain in aged CEC. The data may suggest a difference in the mechanism of enhanced interferon production due to aging and priming.

In L cells the Trinidad plaque variants produced more interferon than the TC-83 strain regardless of priming, multiplicity, or time after infection. This is similar to results in animals showing greater amounts of interferon produced by virulent strains (8, 10, 11) or severe infections (20, 21). These findings are contrary to what might be expected if the inability to produce interferon were an important determinant of virulence.

When interferon sensitivity was examined, the TC-83 strain was inhibited to a greater extent than the Trinidad strains. The greater sensitivity of the TC-83 strain was observed at both high and low multiplicities and for both chick and mouse interferons. The greater production of interferon by the TC-83 strain on interferon-treated CEC may be part of the mechanism which resulted in the observed greater sensitivity of this strain to chick interferon. However, this mechanism could not ac-

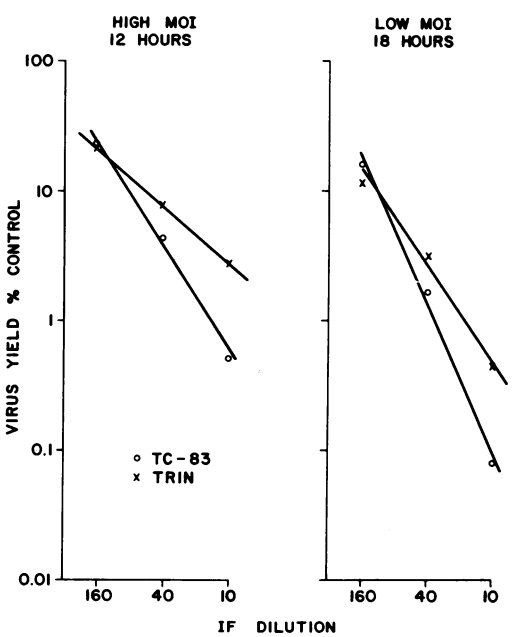

FIG. 3. *Inhibition of VEE virus strains by chick interferon. Symbols: ×, Trinidad; O, TC-83. Maximum titers from control cultures (PFU/ml)—high MOI Trinidad, 8.4 × 10⁸; TC-83, 6.5 × 10⁹. Low MOI—Trinidad, 2.5 × 10⁹. TC-83, 1.5 × 10¹⁰. The difference in percent inhibition between the two strains at the 1:10 interferon dilution is significant at the 0.05 level for both the high and low multiplicity (Student's t test).*

TABLE 4. *Production of chick interferon during the growth of VEE virus*

Interferon priming dilution	Interferon titer (PDD_{50})[a]							
	6 h		12 h		24 h		36 h	
	Trin	TC-83	Trin	TC-83	Trin	TC-83	Trin	TC-83
High MOI[b]								
Control	<10	<10	<10	<10	9	10	15	10
160					*76*	*199*[c]		
40	*62*	*21*	*139*	*496*	*129*	*412*	*154*	*363*
10					207	317		
Low MOI								
Control	<10	<10	<10	<10	8	16	10	13
160					*75*	*178*		
40	<10	<10	6	13	*123*	*345*	*122*	*251*
10					114	191		

[a] The interferon titer is the reciprocal of the dilution expected to give 50% reduction of vesicular stomatitis virus plaques.

[b] MOI, multiplicity of infection.

[c] Italicized pairs indicate nonoverlapping 95% confidence intervals.

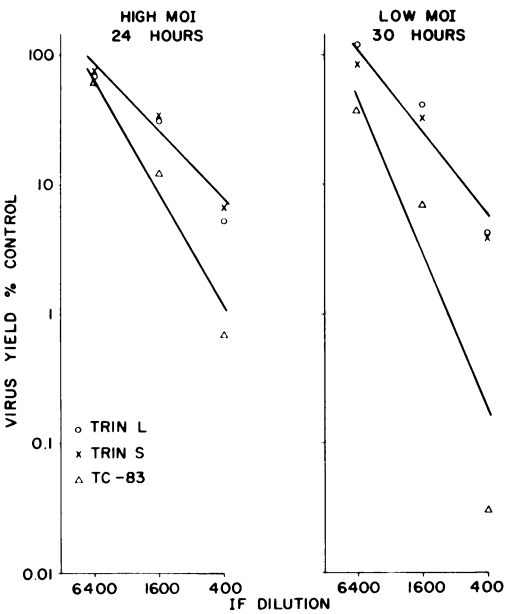

FIG. 4. *Inhibition of VEE virus strains by mouse interferon. Symbols: ×, Trin S; O, Trin L; Δ, TC-83. Maximum titers from control cultures (PFU/ml): High MOI—Trin S, 3.2 × 10^8; Trin L, 1.9 × 10^9; TC-83, 9.8 × 10^8. Low MOI—Trin S, 1.1 × 10^9; Trin L, 3.7 × 10^9; TC-83, 5.4 × 10^8. The difference in percent inhibition at both the 1:400 and 1:1,600 dilution is significant at the 0.05 level for the following pairs: High MOI, S-1 vs. TC-83; Low MOI, S-1 vs. TC-83 and L-1 vs. TC-83 (Student's t test).*

TABLE 5. *Production of mouse interferon during the growth of VEE virus*

Virus strain	Interferon priming dilution	Interferon titer (PDD_{50})[b]		
		High MOI[a]		Low MOI[a]
		12 h	24 h	24 h
Trin S	Control	2,140[b]	21,300	1,730
	64,000	2,470	17,700	3,310
	16,000	2,650	16,400	5,340
	4,000	2,130	8,520	682
Trin L	Control	272	12,400	1,950
	64,000	308	10,400	2,830
	16,000	1,060	19,200	3,610
	4,000	485	18,700	453
TC-83	Control	<20	5,570	235
	64,000	30	6,520	70
	16,000	<20	2,570	27
	4,000	<20	43	<20

[a] MOI, multiplicity of infection.

[b] The interferon titer is the reciprocal of the dilution expected to give 50% reduction of vesicular stomatitis virus plaques.

count for the greater sensitivity of the TC-83 strain to mouse interferon. Greater interferon sensitivity of attenuated strains has also been observed with foot-and-mouth disease virus (17) and Semliki Forest virus (5). Further evidence for interferon sensitivity as a possible determinant of virulence comes from studies by Hanson et al. (7) on the natural resistance of inbred strains of mice. Cells from resistant animals were shown to be more responsive to the antiviral effects of interferon than cells from susceptible animals; differences in interferon production were not observed. The finding that the attenuated TC-83 strain of VEE virus is inhibited to a greater extent by interferon than the

virulent Trinidad strains is consistent with the hypothesis that virus virulence may be in part determined by a relative lack of interferon sensitivity in some virus-host systems.

ACKNOWLEDGMENTS

I am grateful to Earl Dendy, Darrell Parker, and Helen Ramsburg for technical assistance.

LITERATURE CITED

1. Archetti, I., and F. L. Horsfall, Jr. 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization *in ovo* with heterologous immune serum. J. Exp. Med. **92**:441-462.
2. Baron, S. 1966. The biological significance of the interferon system, p. 268-292. *In* N. B. Finter (ed.), Interferons. W. B. Saunders Co., Philadelphia.
3. Berge, T. O., I. S. Banks, and W. D. Tigertt. 1961. Attenuation of Venezuelan equine encephalomyelitis virus by *in vitro* cultivation in guinea-pig heart cells. Amer. J. Hyg. **73**:209-218.
4. Carver, D. H., and P. I. Marcus. 1967. Enhanced interferon production from chick embryo cells aged in *in vitro*. Virology **32**:247-257.
5. Finter, N. B. 1964. Interferon production and sensitivity of Semliki Forest virus variants. J. Hyg. **62**:337-349.
6. Giron, D. J. 1969. Role of interferon in the propagation of MM virus in L cells. Appl. Microbiol. **18**:584-588.
7. Hanson, B., H. Koprowski, S. Baron, and C. E. Buckler. 1969. Interferon-mediated natural resistance of mice to arboB virus infection. Microbios **1B**:51-68.
8. Inglot, A. D., M. Lobrodzinska, I. Biernacka, and E. Niedzwiedzka. 1966. Changes in the production of interferon in the process of adaptation of influenza type A2 virus to the mouse lung. Arch. Immunol. Ther. Exp. **14**:333-338.
9. Jordan, G. W. 1972. Quantitative aspects of interferon-induced plaque reduction: kinetics of interferon action. Virology **48**:425-432.
10. Link, F., D. Blaskovic, and J. Raus. 1965. Relationship between virus multiplication and interferon production in mouse lungs after infection with adapted and unadapted influenza viruses. Acta Virol. **9**:95.
11. Link, F., D. Blaskovic, J. Raus, and P. Albrecht. 1965. Investigations into the adaptation of a neurovaccinia virus to mouse lungs. Acta Virol. **9**:323-331.
12. Lockart, R. Z., Jr. 1963. Production of an interferon by L cells infected with Western equine encephalomyelitis virus. J. Bacteriol. **85**:556-566.
13. McKinney, R. W., T. O. Berge, W. D. Sawyer, W. D. Tigertt, and D. Crozier. 1963. Use of an attenuated strain of Venezuelan equine encephalomyelitis virus for immunization in man. Amer. J. Trop. Med. Hyg. **12**:597-603.
14. Randall, R., and J. W. Mills. 1944. Fatal encephalitis in man due to the Venezuelan virus of equine encephalomyelitis in Trinidad. Science **99**:225-226.
15. Reed, L. J., and H. Muench. 1938. A simple method for estimating fifty per cent endpoints. Amer. J. Hyg. **27**:493-497.
16. Ruiz-Gomez, J., and A. Isaacs. 1963. Interferon production by different viruses. Virology **19**:8-12.
17. Sellers, R. F. 1963. Multiplication, interferon production and sensitivity of virulent and attenuated strains of the virus of foot-and-mouth disease. Nature (London) **198**:1228-1229.
18. Sellers, R. F. 1964. Virulence and interferon production of strains of foot-and-mouth disease virus. J. Immunol. **93**:6-12.
19. Tigertt, W. D., and W. G. Downs. 1962. Studies on the virus of Venezuelan equine encephalomyelitis in Trinidad, W.I. I. The 1943-1944 epizootic. Amer. J. Trop. Med. Hyg. **11**:822-834.
20. Vainio, T., R. Gwatkin, and H. Koprowski. 1961. Production of interferon by brains of genetically resistant and susceptible mice infected with West Nile virus. Virology **14**:385-387.
21. Vilcek, J. 1964. Production of interferon by newborn and adult mice infected with Sindbis virus. Virology **22**:651-652.
22. Wagner, R. R., A. H. Levy, R. M. Snyder, G. A. Ratcliff, Jr., and D. F. Hyatt. 1963. Biologic properties of two plaque variants of vesicular stromatitis virus (Indiana serotype). J. Immunol. **91**:112-121.