INFECTION AND IMMUNITY, Aug. 1982, p. 439–444
0019-9567/82/080439-06$02.00/0

Vol. 37, No. 2

Defective Interfering Particles of Respiratory Syncytial Virus

MARY W. TREUHAFT[1]* AND MARC O. BEEM[2]

Marshfield Medical Foundation, Inc., Marshfield, Wisconsin 54449,[1] and Department of Pediatrics, University of Chicago, Chicago, Illinois 60637[2]

Received 7 December 1981/Accepted 26 March 1982

A multiplicity-dependent interference was observed in respiratory syncytial virus preparations (Randall strain) grown in HEp-2 cells, and the factor mediating this interference was characterized. Cloned virus did not demonstrate this multiplicity-dependent interference, but its replication was shown to be inhibited by the interfering factor by assays of reduction of infectious center formation and reduction of infectious yield. With the reduction of infectious yield assay, the interfering factor was found to be particulate, to be inactivated by UV irradiation, and not to interfere with the replication of a heterologous virus, vesicular stomatitis virus. These characteristics are compatible with the physical properties and biological behavior of defective interfering particles. Defective interfering particles were generated by four undiluted passages of cloned virus but were not apparent after eight passages at a multiplicity of infection of 0.1.

Defective interfering (DI) particles are noninfectious virions which require the helper function of homologous infectious virus to replicate but which also interfere with the replication of that helper virus (7). DI particles contain normal viral structural proteins but only a part of the functional viral genome. The interference caused by DI particles is not mediated by interferon and is specific for the homologous virus from which the DI particles are derived.

During studies of the structural proteins of respiratory syncytial (RS) virus, we observed a multiplicity-dependent interference phenomenon in virus stocks of the Randall strain which had been passaged at a multiplicity of infection (MOI) of 1 to 5 PFU/cell (M. W. Treuhaft, Ph.D. thesis, University of Chicago, Chicago, Ill. 1972). We present here a characterization of this interference and propose that it is caused by DI particles of RS virus. Although DI particles have been described for almost all major animal virus families (9), to our knowledge this is the first report of DI particles for a pneumovirus.

MATERIALS AND METHODS

Cells. HEp-2 cells (ATCC CCL 23) were grown at 37°C without antibiotics in modified minimal essential medium with Eagle salts supplemented with 5% fetal bovine serum.

Virus. The Randall strain of RS virus (1) and the Indiana strain of vesicular stomatitis virus (VSV) (obtained from Dorothy Hamre, University of Chicago, Chicago, Ill.) were used in this study.

Both viruses were grown in HEp-2 cells at 37°C in viral growth medium (VGM) consisting of minimal essential medium with Eagle salts supplemented with 2.5% chicken serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 μg of amphotericin B (Fungizone; E. R. Squibb & Sons, Princeton, N.J.) per ml. Eight-ounce (ca. 240-ml) glass bottles, seeded with 4×10^6 HEp-2 cells 24 h before infection, were exposed to virus in a 1-ml volume for 2 h at 37°C with gentle rocking. After adsorption, the inoculum was removed, and 15 ml of VGM was added.

RS virus was harvested after 48 to 72 h when >70% of the cells showed syncytia formation, rounding, or detachment. Cellular debris was removed by centrifugation (1,000 × *g* for 10 min), and the supernatant which contained virus was stored at −70°C. RS virus interfering preparations were obtained by successive undiluted passages of stocks which exhibited multiplicity-dependent interference (MOI, 1 to 5 PFU/cell). RS standard virus was obtained from an interfering preparation by three successive plaque isolations and was subsequently propagated at an MOI of 0.1 PFU/cell.

VSV was propagated at an MOI of 0.01 PFU/cell in the same manner as RS virus, but it was harvested at 18 to 24 h, when destruction of the host cell monolayer was complete.

Infectivity assay. Infectious virus was quantitated by plaque assay in 15-mm-diameter plastic multiwell tissue culture plates (Falcon Plastics, Oxnard, Calif.) containing 1.5×10^5 HEp-2 cells. Serial dilutions of virus in 0.1 ml were allowed to adsorb for 2 h at 37°C with gentle rocking. The inoculum was then removed, and wells were filled with a plaquing overlay containing VGM, 1.2% methylcellulose (6,000 centipoise), and either 2.5% fetal bovine serum for RS virus or 2.5% chicken serum for VSV. After 5 days of incubation at 37°C for RS virus and 3 days for VSV, cells were fixed with 4% formaldehyde, washed, and stained with 1.2% crystal violet. RS virus plaques appeared either as holes in the cell monolayer or as giant syncytia and were counted with the aid of a dissecting microscope. Plaques of VSV appeared as clear areas due to cell destruction. The titers given here are the means of triplicate wells.

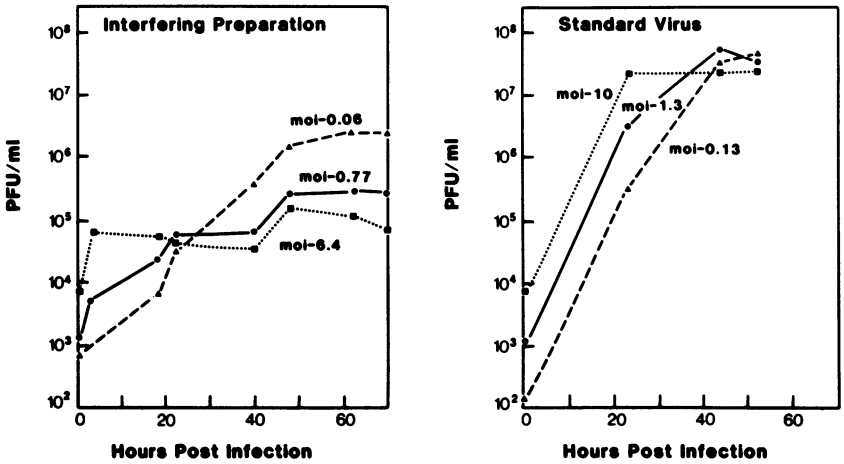

FIG. 1. Effect of MOI on yield of RS virus. Monolayers containing 5×10^6 HEp-2 cells were infected with either virus preparations exhibiting multiplicity-dependent interference or plaque-purified standard virus at the MOIs indicated. Culture fluid was withdrawn at the times indicated and centrifuged at $1,000 \times g$ for 10 min, and supernatants were stored at −70°C until assayed for PFU.

Infectious center assay. HEp-2 cell monolayers were infected with either standard virus at an MOI of 7 PFU/cell or a mixture of standard virus (MOI, 3.5 PFU/cell) and the interfering preparation (MOI, 3.5 PFU/cell). After adsorption, the virus inoculum was removed, and the monolayers were dispersed as single cells with 0.025% trypsin. Viable cell count was determined by trypan blue exclusion. Twofold dilutions of infected cells in VGM were mixed with 1.3×10^6 freshly trypsinized uninfected cells and allowed to attach to 60-mm-diameter plastic culture dishes for 5 h. Culture fluid was then removed, and 10 ml of plaquing overlay was added. After 6 days of incubation at 37°C, monolayers were fixed and stained, and the plaques were counted. Plaques formed in this assay were considered to result from infectious centers because no infectious virus was detected in the culture fluid during the attachment of infected cells, and the addition of virus to freshly trypsinized cells did not result in plaque formation.

Reduction of infectious yield assay. Plastic culture tubes (16 by 125 mm) containing 2×10^5 HEp-2 cells were infected with standard virus alone (MOI, 5) as the control or with a mixture of standard virus and the interfering preparation. After virus adsorption, the tubes were washed three times with VGM and finally fed with 1 ml of VGM. After 48 to 72 h of incubation at 37°C, >70% of the cells in the control tubes showed a characteristic cytopathic effect. Culture fluid was then removed from the tubes and centrifuged at $1,000 \times g$ for 10 min, and supernatants were stored frozen until assayed for infectivity. Yield reduction was determined by comparing titers of tubes coinfected with standard virus and interfering preparation to those infected with standard virus alone. The data were analyzed by using the Student t test for two means (14).

Centrifugation. RS virus interfering preparations (2 ml) were centrifuged at $150,000 \times g_{av}$ for 40 min. The supernatant was removed, and the pellet was suspended with a 21-gauge needle in 2 ml of VGM.

UV irradiation. A 1-ml amount of RS virus interfering preparation in a 60-mm-diameter plastic culture dish was exposed to a 15-W germicidal lamp (General Electric, Cleveland, Ohio) for various times at a distance of 40 cm.

RESULTS

A stock of RS virus which had been passaged undiluted was examined for ability to produce infectious virus at MOIs of 6.4, 0.77, and 0.06 (Fig. 1). When HEp-2 cultures were infected at an MOI of 6.4, little infectious virus was produced over a 70-h period. In contrast, a 100-fold reduction in MOI resulted in a marked increase in infectious virus production over the same time period. Thus, the interference observed was multiplicity dependent and could be reduced by diluting the infecting virus preparation.

The stock of RS virus showing this multiplicity-dependent interference was then plaque purified, and the effect of multiplicity on infectious virus production by the plaque-purified virus was observed over a 52-h period (Fig. 1). A 2-log variation in MOI (0.13 to 10) with the plaque-purified virus resulted in uniformly high titers of infectious virus at 45 to 52 h. Plaque-purified RS virus was subsequently passaged at an MOI of 0.1 and continued to show no evidence of multiplicity-dependent interference. It was therefore used as the standard virus in the studies reported here.

Two assay systems for the detection of the interference factor were examined: reduction of

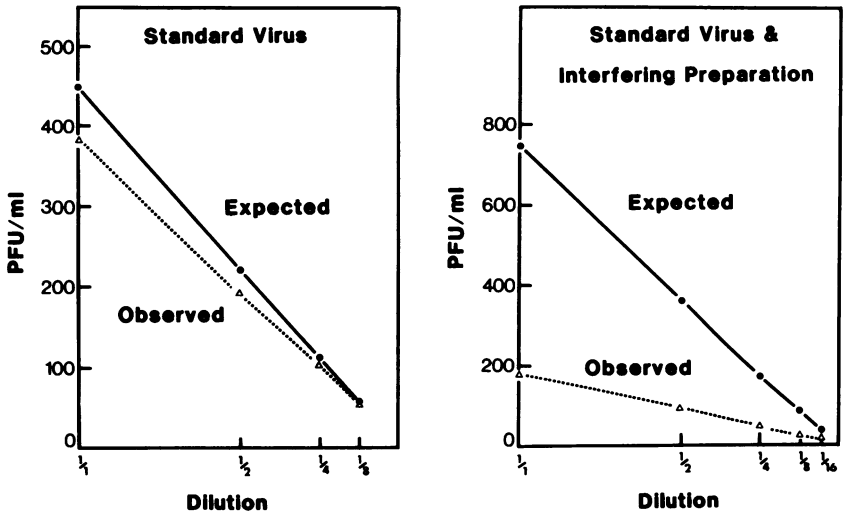

FIG. 2. Effect of interfering factor on infectious center formation.

infectious center formation and reduction of infectious yield of standard virus.

The number of infectious centers observed from infection with standard virus alone corresponded well to the number expected on the basis of cell count over an eightfold dilution of infected cells (Fig. 2). In contrast, the number of infectious centers observed when cells were infected with a mixture of standard virus and interfering preparation was only 25 to 33% of those expected from cell counts over a 16-fold dilution of the infected cells (Fig. 2). The size of plaques produced by infectious centers resulting from coinfection was similar to that of plaques produced by infectious centers infected with standard virus alone.

The ability of an interfering preparation to reduce the infectious yield of standard RS virus in a tube assay is demonstrated in Table 1. When standard virus alone was used to infect tubes, no reduction in infectious yield at 48 h was observed over an MOI range of 0.4 to 80, demonstrating that high MOI alone does not result in interference with standard virus replication. In contrast, when tubes were infected with standard virus at an MOI of 4 and interfering preparation at an MOI of 2, a 1-log reduction in infectious yield at 48 h was observed, in comparison with standard virus alone at an MOI of 4. Interference was also observed at 10-fold higher MOIs of standard virus and interfering preparation. Interference would probably also have occurred with standard virus at an MOI of 0.4 if a larger amount of interfering preparation had been used.

TABLE 1. Reduction of infectious yield assay for interference

Standard virus MOI	Interfering prepn MOI	Titer (log PFU/ml)[a]	Yield reduction (log PFU)
0.4	0.0	7.05 ± 0.14	
4.0	0.0	7.58 ± 0.01	
40.0	0.0	7.71 ± 0.06	
80.0	0.0	7.49 ± 0.12	
0.4	0.2	6.90 ± 0.05	0.15;NS[b]
4.0	2.0	6.57 ± 0.05	1.01[c]
40.0	20.0	5.91 ± 0.02	1.80[c]

[a] Mean ± standard error of the mean.
[b] NS, Not significant; $P > 0.05$.
[c] $P < 0.0005$.

Because of the relative simplicity and the greater sensitivity of the reduction of infectious yield assay as compared with the infectious center assay, the former was used in subsequent studies to characterize the interfering factor. On the basis of the data in Table 1, an MOI of 5 for standard virus was selected to produce the control virus yields. Yields of tubes coinfected with standard virus and various interfering preparations were then compared to these control yields.

A summary of the behavior of the RS interfering factor on centrifugation at 150,000 × g_{av} for 40 min is presented in Table 2. These centrifugation conditions are adequate to sediment over 99% of infectious RS virus (data not shown). The interfering preparation used here (P59) effected a 1-log reduction of infectious virus yield before centrifugation. Little or no interfering activity was recovered in the supernatant,

TABLE 2. Centrifugation of interfering factor

Interfering prepn	Titer (log PFU/ml)[a]	Yield reduction (log PFU)	
None[b]	6.17 ± 0.03	0.00	
P59			
Uncentrifuged	5.08 ± 0.10	1.08[c]	
Supernatant	6.24 ± 0.05	−0.07	NS[d]
	5.80 ± 0.10	0.37	
Pellet	5.10 ± 0.03	1.07	[c]
	5.24 ± 0.06	0.93	

[a] Mean ± standard error of the mean.
[b] Control titer is the mean of triplicate plaquing wells of triplicate control tubes infected with standard virus alone.
[c] $P < 0.0005$.
[d] NS, Not significant; $P > 0.05$.

whereas the resuspended pellet was able to reduce infectious yield to the same extent as the uncentrifuged preparation.

The ability of UV irradiation to inactivate the interfering factor is shown in Table 3. Before irradiation, the interfering preparation (P59) contained 2×10^5 PFU/ml and caused a 1-log reduction in the infectious yield assay. After exposure to UV irradiation for 5 min, infectious virus could no longer be detected, but interfering activity was undiminished. However, after exposure to UV irradiation for 10 min, interfering activity was no longer evident.

The ability of RS virus interfering preparations to interfere with the replication of a heterologous virus, VSV, was also examined (Table 4). Dual infection with VSV and RS virus interfering preparations did result in a small reduction in VSV infectious yield. However, when the RS interfering preparations were centrifuged at $150{,}000 \times g_{av}$ for 40 min, this small amount of interfering activity for VSV was recovered in the supernatant rather than in the pellet, in contrast to the particulate behavior observed for the interference active in the RS virus–HEp-2 cell system.

TABLE 3. UV inactivation of interfering factor

Interfering prepn	UV irradiation (min)	Titer (log PFU/ml)[a]	Yield reduction (log PFU)
None[b]	0	6.17 ± 0.03	0.00
P59	0	5.08 ± 0.10	1.08[c]
	5	5.09 ± 0.16	1.07[c]
	10	6.33 ± 0.04	−0.16; NS[d]
	20	6.28 ± 0.03	−0.11; NS

[a] Mean ± standard error of the mean.
[b] Control with standard virus alone was the same as that used in Table 2.
[c] $P < 0.0005$.
[d] NS, Not significant; $P > 0.05$.

TABLE 4. Effect of RS interfering preparations on VSV infectious yields[a]

Interfering prepn	VSV titer (log PFU/ml)[b]	Yield reduction (log PFU)
None[c]	6.29 ± 0.03	0.00
P59		
Uncentrifuged	5.74 ± 0.26	0.56[d]
Supernatant	6.02 ± 0.06	0.27[d]
Pellet	6.28 ± 0.05	0.02; NS[e]
P60		
Uncentrifuged	5.74 ± 0.26	0.56[d]
Supernatant	6.06 ± 0.02	0.23[f]
Pellet	6.21 ± 0.07	0.08; NS
Standard RS virus	6.16 ± 0.08	0.14[g]

[a] Plastic culture tubes seeded with 1.5×10^5 HEp-2 cells 24 h before use were first infected with RS preparations for 2 h at 37°C and then infected with VSV at an MOI of 5. At 18 h after infection, culture fluid was removed and centrifuged at $1{,}000 \times g$ for 10 min, and the supernatant was stored at −70°C until assayed for infectivity. Yields were compared with control tubes infected with VSV alone.
[b] Mean ± standard error of the mean.
[c] Control titer is the mean of triplicate plaquing wells of triplicate control tubes infected with VSV alone.
[d] $P < 0.001$.
[e] NS, Not significant; $P > 0.05$.
[f] $P < 0.005$.
[g] $P < 0.05$.

To define the conditions under which DI particles are generated in HEp-2 cells, plaque-purified virus was passaged undiluted at an MOI of 1.7 to 4 or diluted at a MOI of 0.1, and the progeny was evaluated for infectious and interfering activities. On undiluted passage, interfering activity appeared by passage 4 and was followed by a decrease in infectious titer at passages 5 to 6 (Fig. 3). In contrast, plaque-purified virus passaged at an MOI of 0.1 maintained an infectious titer of $>6 \times 10^6$ PFU/ml over eight passages and showed no evidence of multiplicity-dependent interference (data not shown) and no interfering activity in the reduction of infectious yield assay (Table 5).

Interfering activity was accompanied by reduced cytopathology. The early undiluted passages of plaque-purified virus caused extensive cytopathic effect, with greater than 90% of the cells detached from the monolayer by 72 h. The drop in infectious titer at passages 5 to 6 was accompanied by a reduction in cytopathic effect. Reduced syncytium formation and cell death were also seen for undiluted passages 4 to 6 in the reduction of infectious yield assay, indicating that in this system, DI particles not only reduced infectious virus production but also

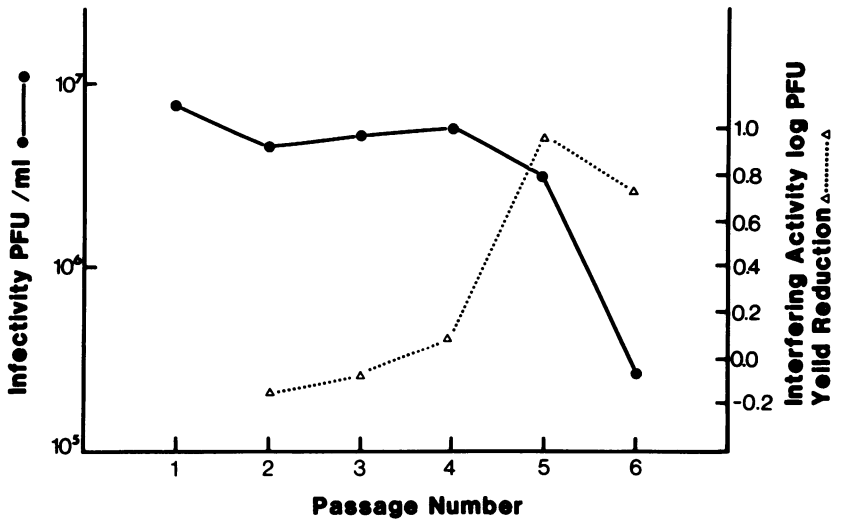

FIG. 3. Generation of DI particles on undiluted passage of plaque-purified virus. Flasks containing 1.8×10^6 HEp-2 cells were infected with 1 ml of undiluted culture fluid supernatant from the preceding passage, and then 10 ml of VGM containing 2.5% fetal bovine serum was added. After 72 h at 37°C, the culture fluid supernatant (centrifuged at 1,000 × *g* for 10 min) was frozen until used in assays for infectivity by plaque formation and for interference by the reduction of infectious yield assay.

provided some protection of the host cell from typical virus-induced cytopathology.

DISCUSSION

The characteristics of the interfering factor active in the RS virus–HEp-2 cell system described here are compatible with the physical properties and biological behavior of DI particles. The interference was dependent on MOI and could be eliminated by plaque purification. The interference was particulate, was inactivated by UV irradiation, and was not active against a heterologous virus, VSV. The interference could be detected both by reduction of infectious centers and by reduction of infectious yield of standard RS virus. Both assays for interference can be used in future studies of RS DI virus.

DI particles were rapidly generated on undiluted passage of standard virus and may contribute to the difficulties experienced by many investigators in producing high-titered RS virus preparations. In contrast, passage of standard virus at an MOI of 0.1 appeared to be adequate to prevent the accumulation of DI particles in the HEp-2 cell system.

TABLE 5. Absence of interfering activity on low MOI (0.1 PFU/cell) passage of plaque-purified standard virus in reduction of infectious yield assay

Interfering prepn	Titer (log PFU/ml)[a]	Yield reduction (log PFU)
None	6.22 ± 0.04	0.00
Passage 8A	6.44 ± 0.03	−0.22
Passage 8B	6.49 ± 0.03	−0.27

[a] Mean of triplicate plaquing wells of four replicates ± standard error of the mean.

The small amount of interference observed in the VSV–HEp-2 cell system may have been due to interferon, because this interference remained in the supernatant after centrifugation under conditions that resulted in the recovery of RS virus infectivity and interference in the pellet. Whether infection with the Randall strain of RS virus results in interferon production by HEp-2 cells is not known. Moehring and Forsyth (10) reported that low levels of interferon were produced by HEp-2 cells on infection with the Long strain of RS virus. However, infection with three other strains produced no detectable interferon. The Randall strain was not examined in this study (10).

DI particles have been proposed as important determinants of the course of natural viral infection (8). Although there have been no reports of the role of DI particles in human viral infections, there is evidence from animal systems that DI particles are generated in vivo and that they can modify the infection. Work with VSV (3, 6) and reovirus (15) indicates that DI particles are generated in vivo and that they can markedly temper the virulence of the infection. Semliki Forest virus DI particles inhibited the production of

infectious virus in mice and prevented the usual lethal outcome of virus infection (2). Influenza virus passaged in eggs and enriched in DI particles markedly attenuated pulmonary infection in 7-week-old Swiss mice, but not in 3- to 4-week-old mice (13). DI particles have been demonstrated in lymphocytic choriomeningitis carrier mice (11), and mixtures of DI and standard VSV have been observed to cause a slowly progressive disease of the central nervous system on intracerebral inoculation into mice (12).

In this study we observed that DI particles protected HEp-2 cells from typical RS virus-induced cytopathology. Observations on the natural history of RS infections suggest that a mechanism like DI particle interference may play a significant role in the outcome of infection. Persistent shedding of RS virus for up to several weeks is characteristic of severe RS infection in children. When Hall et al. (4, 5) examined infants hospitalized with RS lower respiratory tract disease, they observed that younger infants shed higher titers of virus longer than did older infants and that the severity of illness was related to titer and duration of shedding. Perhaps the inability to generate and replicate DI particles contributes to the long duration of virus shedding and the high titers observed in severe RS disease. Analysis of nasal wash specimens from children with RS virus infection for DI particles is currently in progress. The ability of DI particles to interfere with RS virus detection in clinical materials when standard isolation procedures are used is also being investigated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant A 100238 from the National Institutes of Health, the Children's Research Foundation, and the Marshfield Medical Foundation. M.W.T. was a Young Investigator of the American Lung Association.

We thank Corinne Hackbarth for her excellent technical assistance.

LITERATURE CITED

1. **Beem, M., F. H. Wright, D. Hamre, R. Egerer, and M. Oehme.** 1960. Association of the chimpanzee coryza agent with acute respiratory disease in children. N. Engl. J. Med. **263**:523–530.
2. **Dimmock, N. J., and S. I. T. Kennedy.** 1978. Prevention of death in Semliki forest virus-infected mice by administration of defective-interfering Semliki forest virus. J. Gen. Virol. **39**:231–242.
3. **Doyle, M., and J. J. Holland.** 1973. Prophylaxis and immunization in mice by use of virus-free defective T particles to protect against intracerebral infection by vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **70**:2105–2108.
4. **Hall, C. B.** 1977. The shedding and spreading of respiratory syncytial virus. Pediatr. Res. **11**:236–239.
5. **Hall, C. B., R. G. Douglas, Jr., and J. M. Geiman.** 1975. Quantitative shedding patterns of respiratory syncytial virus in infants. J. Infect. Dis. **132**:151–156.
6. **Holland, J. J., and L. P. Villarreal.** 1975. Purification of defective-interfering T particles of vesicular stomatitis and rabies viruses generated *in vivo* in brains of newborn mice. Virology **67**:438–449.
7. **Huang, A. S.** 1973. Defective interfering viruses. Annu. Rev. Microbiol. **27**:101–117.
8. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease processes. Nature (London) **226**:325–327.
9. **Huang, A. S., and D. Baltimore.** 1977. Defective interfering animal viruses, p. 73–116. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10, Regulation and genetics. Viral gene expression and integration. Plenum Publishing Corp., New York.
10. **Moehring, J. M., and B. R. Forsyth.** 1971. The role of the interferon system in respiratory syncytial virus infections. Proc. Soc. Exp. Biol. Med. **138**:1009–1014.
11. **Popescu, M., and F. Lehmann-Grube.** 1977. Defective interfering particles in mice infected with lymphocytic choriomeningitis virus. Virology **77**:78–83.
12. **Rabinowitz, S. G., M. C. Dal Canto, and T. C. Johnson.** 1977. Infection of the central nervous system produced by mixtures of defective-interfering particles and wild-type vesicular stomatitis virus in mice. J. Infect. Dis. **136**:59–74.
13. **Rabinowitz, S. G., and J. Huprikar.** 1979. The influence of defective-interfering particles of the PR-8 strain of influenza A virus on the pathogenesis of pulmonary infection in mice. J. Infect. Dis. **140**:305–315.
14. **Schefler, W. C.** 1980. Statistics for the biological sciences, 2nd ed. Addison-Wesley Publishing Co., Reading, Mass.
15. **Spandidos, D. A., and A. F. Graham.** 1976. Generation of defective virus after infection of newborn rats with reovirus. J. Virol. **20**:234–247.