0022-538X/78/0025-0202$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 202-206

Vol. 25, No. 1
Printed in U.S.A.

Host Function-Dependent Induction of Defective Interfering Particles of Vesicular Stomatitis Virus

C. YONG KANG* AND RAE ALLEN

Department of Microbiology, The University of Texas Health Science Center, Southwestern Medical School, Dallas, Texas 75235

Received for publication 12 July 1977

Suppression of host cell function by treatment with actinomycin D prior to infection prevented the induction of defective interfering particles of vesicular stomatitis virus, which had been cloned and propagated in cells pretreated with actinomycin D. Replication of defective interfering particles already present in an infecting virus stock, however, was not affected by pretreatment of cells with actinomycin D. Thus, the induction, but not the replication, of defective interfering particles appears to be a host cell function-dependent phenomenon. The implications of this phenomenon for host defense mechanisms against virus infections are discussed.

The existence of defective interfering (DI) particles in a preparation of an animal virus was described first by Von Magnus (13) in influenza virus. Huang and Baltimore (6) postulated a role for DI particles in host defense mechanisms in overcoming the viral infections. The physical and biological properties of DI particles have been reviewed previously (5, 14).

One of the major goals in DI particle-mediated viral interference research is to understand the mechanism of the induction of DI particles during standard virus replication. DI particles are usually generated during serial undiluted high-multiplicity passages of standard infectious virus (5), but little is known about the mechanism of induction of DI particles. Holland et al. (4) suggested that DI particles of vesicular stomatitis virus (VSV) are induced at each passage of virus, including the first one, and then are selectively amplified during successive passages to produce a detectable level of DI particles in the later passages. By using a single, freshly cloned preparation of VSV (VSV_{IND}), we found that serial undiluted high-multiplicity passages of the virus in four different cell lines produced different size classes of DI particles at different passage numbers (C. Y. Kang, T. Glimp, J. P. Clewley, and D. H. L. Bishop, Virology, in press). These data strongly indicated that the induction of DI particles is dependent upon a function of the host cells. Accordingly, we have examined the generation of DI particles of Indiana serotype of VSV (VSV_{IND}) in cells pretreated with actinomycin D. This report describes the evidence for a host cell function involved in the induction of DI particles of VSV_{IND}.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cell clone 21 (BHK_{21}) was obtained from the American Type Culture Collection; BHK_{53} was a kind gift of D. H. L. Bishop, University of Alabama; and B77 strain of avian sarcoma virus transformed rat cells [R(B77)] was obtained from Howard M. Temin, McArdle Laboratory, University of Wisconsin. None of these cell lines produce any detectable amounts of endogenous virus. R(B77) cells were grown as monolayer cultures in Dulbecco modified minimum essential medium (DMEM) containing antibiotics and supplemented with 5% heat-treated fetal calf serum (FCS). BHK_{21} and BHK_{53} cells were grown in the same media supplemented with 10% FCS. All the media, sera, and antibiotics were purchased from Grand Island Biological Co., Grand Island, N.Y. The prototype strain of the VSV_{IND} used in this paper has been described previously (2, 8).

Preparation of stock virus from a single plaque isolate. Virus stock of VSV_{IND} was obtained from five consecutive plaque purifications followed by two additional plaque purifications in cells pretreated with actinomycin D. Confluent monolayer cultures of either R(B77) cells or BHK_{21} cells in 100-mm culture dishes containing approximately 1×10^7 cells were treated with 1 μg of actinomycin D per ml for 24 h. Actinomycin D-pretreated monolayer cultures were infected with diluted virus to give approximately 50 plaques in each culture dish and overlayed with agar. Single, well isolated plaques were picked and used to inoculate other cultures also pretreated with actinomycin D. The viruses from this second plaque isolation from either R(B77) cells or BHK_{21} cells were used to infect cells pretreated with 1 μg of actinomycin D per ml at a multiplicity of infection of 0.1 PFU per cell. Virus was grown for 16 h, at which time the virus fluid was harvested and centrifuged at $600 \times g$ for 15 min to remove the cellular debris. Portions of virus

fluid (3 ml) were stored at −75°C. The titer of the stock virus was approximately 2×10^9 PFU/ml.

Successive undiluted passages of VSV_{IND}. Culture dishes (100 mm) containing approximately 1×10^7 cells in monolayer were treated with 1 μg of actinomycin D per ml for 24 h. A parallel control culture was not treated with actinomycin D. The monolayer cultures were infected with 0.5 ml of the stock virus to give a multiplicity of infection of approximately 100 PFU per cell for the first passage; for the subsequent passages, 0.5 ml per 100-mm dish of undiluted virus from the previous passage was used. The virus was adsorbed to the cells for 45 min at 37°C, and 7 ml of DMEM supplemented with 5% FCS was added followed by incubation at 37°C for 7 h in a CO_2 incubator with saturated humidity. The virus was harvested and centrifuged at $600 \times g$ for 15 min to remove cellular debris, and the virus was analyzed by plaque assay on BHK_{53} cell monolayers in a 60-mm culture dish and/or by sucrose gradient centrifugation as described previously (8, 9).

RESULTS

Serial undiluted passages of VSV_{IND} in cells with and without actinomycin D pretreatment. Serial undiluted high-multiplicity passages of a single clone of VSV_{IND} in four cell lines produced different size classes of DI particles at different passage numbers (Kang et al., in press). These data indicate that the induction of DI particles is dependent on a host cell function. This prompted us to investigate induction of DI particles in cells pretreated with actinomycin D, a known inhibitor for DNA-directed information flow (12) with no known effects on VSV replication (9). The stock virus was plaque purified and prepared in cells pretreated with actinomycin D as described above, and serial undiluted passages were carried out in cells with and without actinomycin D pretreatment. Fig. 1a shows the titer of VSV_{IND} propagated in R(B77) cells with and without actinomycin D pretreatment. The untreated R(B77) cells yielded approximately the same amount of virus up to passage 4 and then showed a rapid reduction in virus titer during the subsequent passages. In contrast, when the same virus was propagated in R(B77) cells pretreated with actinomycin D, the titer of VSV in each passage up to passage 11 remained constant. The number of cells in actinomycin D-pretreated cells was less than that of control cultures at the time of infection; therefore, virus titers in actinomycin D-pretreated cells are lower than in control cells. We carried out similar experiments with BHK_{21} cells to rule out the possibility that the phenomenon is dependent upon a particular cell type. There was no reduction in virus titer up to passage 10 when the virus was propagated in BHK_{21} cells pretreated with actinomycin D,

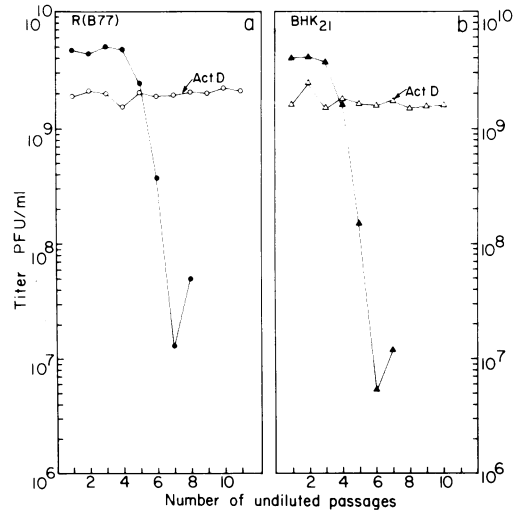

FIG. 1. *Yield of standard infectious virions from cells with and without actinomycin D pretreatment during successive undiluted passages. The initial stock virus was prepared by the consecutive plaque isolation method described in the text. Confluent monolayer cultures containing approximately 1×10^7 cells per 100-mm culture dish were pretreated with 1 μg of actinomycin D per ml for 24 h (Act. D). The cell density in the parallel control cultures was adjusted to give about 1×10^7 cells per 100-mm culture dish. The confluent monolayer cultures were infected with 1×10^9 PFU of the stock virus for the first passage. The subsequent passages were made by inoculating 1×10^7 cells per culture dish with 0.5 ml of undiluted previous passage. At the end of each 7-h passage, the yield of infectious virus was measured by plaque assay on a BHK_{53} cell monolayer in a 60-mm culture dish. Actinomycin D-pretreated cells (Act. D) and the cell types are indicated.*

whereas it took 6 passages to give approximately a 3-log reduction from the original titer in the absence of actinomycin D (Fig. 1b). The rising titer of VSV at passage 8 in R(B77)-untreated cells (Fig. 1a) and passage 7 in BHK_{21}-untreated cells (Fig. 1b) may demonstrate the cyclic production of infectious B virions, as has been reported (10).

To determine whether the reduction of titer after serial undiluted passages of VSV was the result of the generation of DI particles, the viruses from each passage were analyzed by sucrose gradient centrifugation. Figure 2 demonstrates that at least three different size classes of DI particles were produced in R(B77) cells when the virus was propagated in cells without actinomycin D pretreatment, whereas no detectable amounts of DI particles were produced from the cells pretreated with actinomycin D. Two different size classes of DI were produced

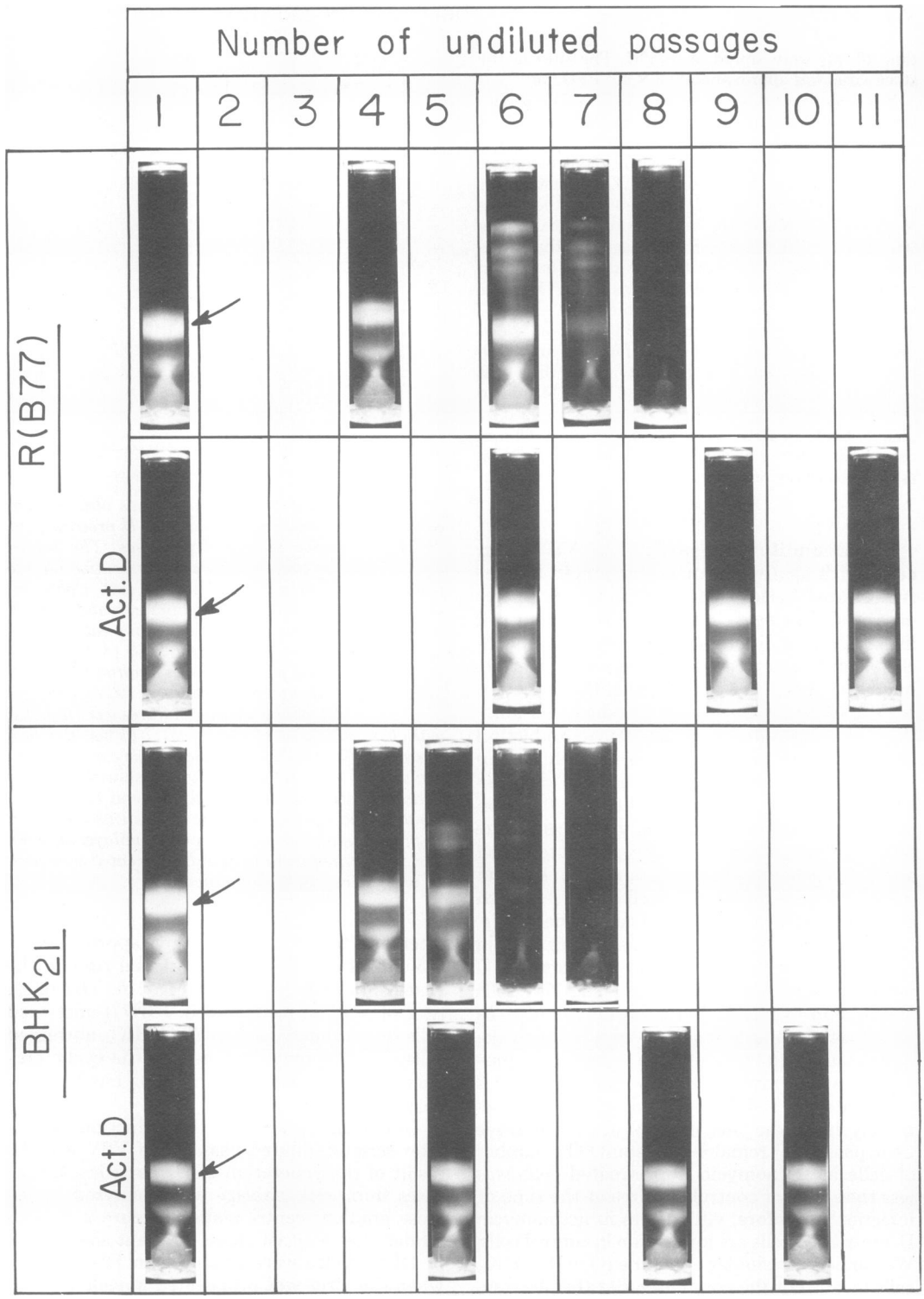

FIG. 2. *Sucrose gradient analysis of total virus particles from successive passages. Samples of lysates from four culture dishes of each passage illustrated in Fig. 1 were centrifuged at 600 × g for 15 min. The virus particles were pelleted from the supernatant by centrifugation at 81,000 × g for 75 min in a Spinco SW27 rotor, suspended in 0.3 ml of phosphate-buffered saline, and layered on a linear 5 to 30% sucrose gradient made in phosphate-buffered saline. After centrifugation at 110,000 × g at 5°C for 35 min in a Spinco SW41 rotor, the gradients were photographed. The arrows indicate the standard infectious particles. The three additional bands above the standard band shown in passage 6 in R(B77) cells and the two additional bands above the standard virion band shown in passage 5 in BHK_{21} cells are DI particles. Electron microscopic examinations of material in bands sedimenting just below the standard virion revealed only clumps of particles identical to standard B virion. The clumped particles were infectious.*

in untreated BHK_{21} cells, and no DI particles were detectable when the virus was propagated in actinomycin D-pretreated cells (Fig. 2).

We have examined the state of the cells after 24 h of treatment with actinomycin D by determining the cellular RNA synthesis. Table 1 shows the [^{3}H]uridine incorporation into R(B77) and BHK_{21} cells after treatment with three different concentrations of actinomycin D. Approximately 95% of cellular RNA synthesis was inhibited by 1 μg of actinomycin D treatment per ml for 24 h. Consequently, most cellular macromolecular syntheses must have been depressed at the time of virus infection, as shown previously (9).

Production of DI particles in cells with and without actinomycin D pretreatment using stock virus known to contain DI particles. We examined the growth of DI particles in cells pretreated with actinomycin D by using virus stocks containing known DI particles to determine whether the production of DI particles is also dependent upon a cellular function. Two stock viruses containing DI particles prepared from cells without actinomycin D pretreatment (that is, the passage 5 virus from R(B77) cells and the passage 4 virus from BHK_{21} cells shown in Fig. 2) were propagated in cells pretreated with actinomycin D and in untreated cells. The data in Fig. 3 demonstrate virtually identical patterns of DI particle production in cells with or without actinomycin D pretreatment. The actinomycin D-pretreated cells produce DI particles and the standard infectious virions (Fig. 3, BHK_{21} passage no. 2) as effectively as untreated cells, demonstrating that actinomycin D-pretreated cells are capable of supporting the growth of not only the standard infectious virions but also DI particles already present in a virus stock. Accordingly, we conclude that the induction of DI particle is host function dependent, but their production does not require the same host function.

DISCUSSION

Although indirect evidence suggested that host function(s) determine the generation of DI particles, we know very little about DI particle induction during serial undiluted passages of standard infectious virions. It has been postulated that DI particles arise as artifacts in viral preparations or by mutational events during the replication of standard virions (3, 13). However, Holland et al. (4) have not been able to generate DI particles by mechanical disruption or UV irradiation of the virus.

We have demonstrated in this paper that the induction of DI particles requires a function of host cells that is sensitive to actinomycin D, since we could not induce DI particles when the virus was plaque purified and propagated in cells pretreated with actinomycin D. It is important to stress the point that one must plaque purify the virus in cells pretreated with actinomycin D to demonstrate the lack of induction of DI particles. We have performed the above experiment with plaque-purified stock virus prepared in the cells without prior actinomycin D treatment and found that DI particles were induced at later passages even though the virus was propagated in actinomycin D-pretreated cells. Our result is consistent with the idea of Holland et al. (4) that DI particles are generated from the first passage and amplified during subsequent passages. Our data suggest that induction of DI particles by virus-specified premature termination of RNA replication, improper cleavage of messenger RNA transcripts, or mutational events of the virus itself during the virus replication are unlikely. We cannot rule out the possibility that there are virus-induced cellular function(s) that may be responsible for induction of DI particles.

Our results define another possible host defense mechanism occurring at the cellular level that protects the host from a highly cytolytic

TABLE 1. *RNA synthesis in cells pretreated with actinomycin D for 24 h*[a]

Concn of actinomycin D (μg/ml of media)	R(B77) cells		BHK_{21} cells	
	[^{3}H]uridine incorporated (cpm)	Residual RNA synthesis (%)	[^{3}H]uridine incorporated (cpm)	Residual RNA synthesis (%)
0	610.700	100.0	533.500	100.0
0.5	37.810	6.2	32.780	6.1
1.0	30.040	4.9	28.200	5.2
2.0	20.680	3.4	22.160	4.2

[a] Approximately 1×10^7 cells per 100-mm culture dish were incubated with 10 ml of DMEM supplemented with 5% FCS and the indicated amounts of actinomycin D for 24 h. The actinomycin D-pretreated cells were labeled with 5 μCi of [^{3}H]uridine (21 Ci/mmol, Schwarz/Mann) per culture dish for 7 h. Trichloroacetic acid-precipitable radioactivity in the cells was determined by the method described previously (9). Counts per minute of [^{3}H]uridine incorporation represent the total RNA synthesis in 1.5×10^6 cells. The values are the average of determinations from duplicate culture dishes.

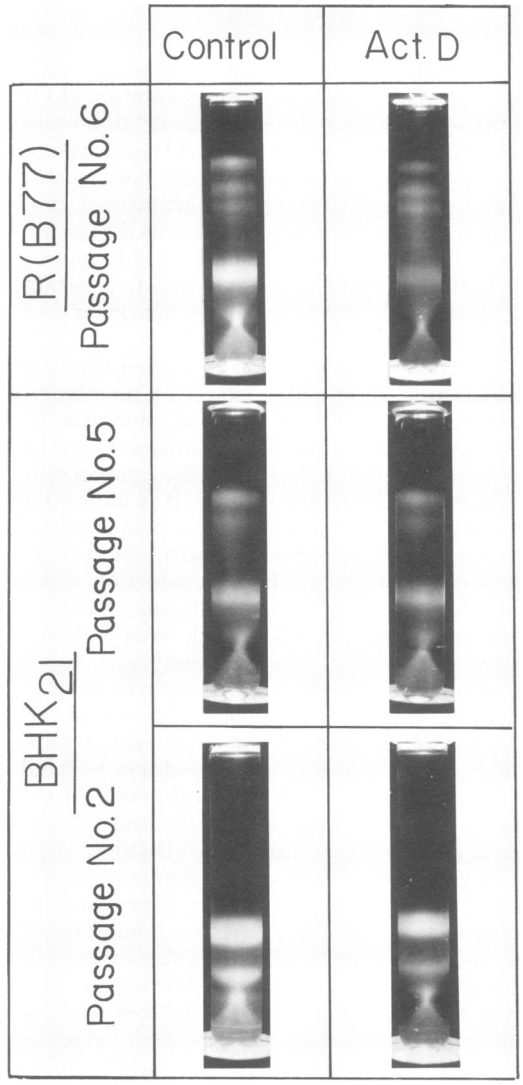

FIG. 3. *Production of DI particles in cells pretreated with actinomycin D. Approximately 1 × 10^7 cells per 100-mm culture dish were pretreated with 1 μg of actinomycin D (Act. D) per ml for 24 h, or left untreated (control), and infected with the virus from passage 5 of the control R(B77) cells (Fig. 2) and the viruses from passages 1 and 4 of the control BHK_{21} cells (Fig. 2). Virus was grown for 7 h and analyzed in sucrose gradients as described in the legend to Fig. 2.*

virus infection. DI particles may play a critical role in host defense during the initial infection of virus, especially with fast-growing cytolytic viruses. In such cases, the host is unable to respond quickly enough to combat the infecting virus early in infection with production of protecting levels of antiviral antibodies. Other possible host defense mechanisms, such as induction of interferon (7) or the generation of cytotoxic T cells (15), may not be as effective early in infection as DI particle-mediated viral interference. We have evidence that host cell function is critical for the induction of DI particles and suggest that the induction of these particles may not be a self-destructive viral mechanism, but rather a host cell function-dependent mechanism that will protect the host from further spread of virus.

ACKNOWLEDGMENTS

We thank B. Ozanne, J. Bednarz-Prashad, and J. Shadduck for their detailed reviews of this manuscript. We are very grateful to T. H. Stoudt, Merck Sharp and Dohme for the generous gift of actinomycin D.

This investigation was supported by Public Health Service grants CA 16479 and CA 20012 from the National Cancer Institute.

LITERATURE CITED

1. **Cartwright, B., C. J. Smale, and F. Brown.** 1970. Dissection of vesicular stomatitis virus into the infective ribonucleoprotein and immunizing components. J. Gen. Virol. **7**:19-32.
2. **Clewley, J. P., D. H. L. Bishop, C.-Y. Kang, J. Coffin, W. M. Schnitzlein, M. E. Reichmann, and R. E. Shope.** 1977. Oligonucleotide fingerprints of RNA species obtained from rhabdoviruses belonging to the vesicular stomatitis virus subgroup. J. Virol. **22**:152-166.
3. **Hackett, A. J.** 1964. The possible morphologic basis for the autointerference phenomenon in vesicular stomatitis virus. Virology **24**:51-59.
4. **Holland, J. J., L. P. Villarreal, and M. Breindl.** 1976. Factors involved in the generation and replication of rhabdovirus defective T particles. J. Virol. **17**:805-815.
5. **Huang, A.** 1973. Defective interfering viruses. Ann. Rev. Microbiol. **27**:101-117.
6. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease processes. Nature (London) **226**:325-327.
7. **Isaacs, A., and J. Lindenmann.** 1957. Virus interference. I. The interferon. Proc. R. Soc. London **147**:258-273.
8. **Kang, C. Y., and L. Prevec.** 1969. Proteins of vesicular stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. J. Virol. **3**:404-413.
9. **Kang, C. Y., and L. Prevec.** 1971. Proteins of vesicular stomatitis virus. III. Intracellular synthesis and extracellular appearance of virus-specific protein. Virology **46**:678-690.
10. **Palma, E. L., and A. Huang.** 1974. Cyclic production of vesicular stomatitis virus caused by defective interfering particles. J. Infect. Dis. **129**:402-410.
11. **Prevec, L., and C. Y. Kang.** 1970. Homotypic and heterotypic interference by defective particles of vesicular stomatitis virus. Nature (London) **228**:25-27.
12. **Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum.** 1962. Action of actinomycin D on animal cells and viruses. Proc. Natl. Acad. Sci. U.S.A. **48**:1238-1244.
13. **Von Magnus, P.** 1954. Incomplete forms of influenza virus. Adv. Virus Res. **2**:59-79.
14. **Wagner, R. R.** 1975. Reproduction of rhabdoviruses, p. 1-93. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 4. Plenum Press, New York.
15. **Zinkernagel, R. M., and P. C. Doherty.** 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature (London) **248**:701-702.