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Homologous Interference by Incomplete Sendai Virus Particles: Changes in Virus-Specific Ribonucleic Acid Synthesis

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Incomplete Sendai virus particles (I particles) interfered with the replication of several strains of infectious Sendai virions (standard virus) but not with the replication of Newcastle disease virus, mumps virus, or Sindbis virus. I particles did not induce interferon, and ultraviolet irradiation of I particles abolished their ability to interfere. Protein synthesis was not necessary to establish interference. The degree of interference depended on the interval between exposure of cells to the I particles and challenge by standard virus, and this was reflected in the degree of inhibition of virus-specific ribonucleic acid (RNA) synthesis in infected cells. The most dramatic change was decreased accumulation of 50*S* virus-specific RNA in infected cells. RNA species sedimenting slower than 50*S* were not as markedly reduced in total amount, but hybridization experiments showed that a substantial portion of these slowly sedimenting RNA species were plus strands, presumably representing replicas of the RNA species in I particles. When I particles in insufficient numbers to interfere were added to cells as late as 8 hr after standard virus, there were no obvious changes in virus-specific RNA species in the cells; however, significant amounts of 19 and 25*S* RNA species, representing progeny of the I particles, appeared in the culture medium. It was concluded that interference was an intracellular event affecting an early step in virus replication. Competition by I particles for cell sites or substrates needed by standard virus seemed a less likely mechanism of interference than competition for enzymes specified by standard virus.

Among the various types of interference that have been described for animal viruses, homologous interference by defective virus particles may provide a particularly useful tool for studying virus replication and for understanding some virus disease states (10). Defectiveness in this context refers to virus particles which have less than an adequate amount of genetic material to cause a productive infection, as with the slowly sedimenting, noninfectious Sendai virus particles previously called "incomplete" (14, 15) and now termed "I particles." These particles interfered with infectious virus production when they were added to cells together with standard virus; they sedimented at 400*S*, whereas infectious Sendai virions sedimented at 1,000*S* or greater; the 50*S* ribonucleic acid (RNA) present in infectious Sendai virions was absent from the I particles: they contained only 19 and 25*S* "subgenomic" RNA species (14, 15). I particles were not infectious, even at very high multiplicities, and they did not induce measurable virus-specific RNA synthesis in cells (14). To learn more about the mechanism of interference, we have now examined some variables affecting interference in this system, and we have examined virus-specific RNA synthesis in cells manifesting the interfered state, since previous data indicated that the interfering components of I particles were their RNA species (14).

MATERIALS AND METHODS

Viruses and cell cultures. The strains of Sendai virus used in this work and the methods for propagating and isolating Sendai virus I particles and standard infectious virions were as described previously (14, 15). The Enders strain of mumps virus (9) and the C strain of Newcastle disease virus (NDV; reference 13) were propagated in embryonated eggs. The plaque assay for Sendai virus in chick embryo lung (CEL) cells (7) was modified by changing the temperature of incubation to 30 C. This resulted in a more rapid development of plaques and larger plaque sizes. Mumps virus was assayed by plaque formation in chick embryo fibro-

blasts (9), as was NDV. Sindbis virus was grown and titrated in chick embryo fibroblasts (17).

Labeling of RNA species in virions and infected cells. To label virion RNA species, 50 μCi of uridine-5-^3H per ml (specific activity, 20 Ci/mmole, Schwarz Bio-Research) was added 24 hr after infection with standard virus, and virus particles were isolated from the cell culture fluids 24 hr later (14). To label intracellular virus-specific RNA species, cells infected 48 hr previously with standard virus were treated for 1 hr with 50 μg of actinomycin D per ml and exposed to 50 μCi of ^{3}H-uridine per ml for 1 hr in the continued presence of the drug. RNA was released from virions with sodium dodecyl sulfate (SDS) and isolated from cells by phenol extraction as previously described (13).

Sedimentation analysis and RNA hybridization. RNA species were centrifuged in 28-ml linear 15 to 30% (w/w) sucrose in 0.005 M, tris(hydroxymethyl)-aminomethane-hydrochloride, 0.001 M ethylenediaminetetraacetic acid, 0.1 M NaCl, 0.5% SDS (*p*H 7.4), at 20,000 rev/min and 20 C in a Spinco SW 25.1 swinging-bucket rotor. Fractions were collected, and radioactivity was analyzed as described earlier (15). All RNA species used for hybridization were isolated from sucrose gradients. RNA species from cells were thus selected to exclude labeled 50*S* viral genomes. The method for RNA hybridization has already been described in detail (13).

RESULTS

Time dependence of the interference. We showed previously that when I particles were mixed with standard virus they partially interfered with virus replication (14, 15). Pretreating cells with I particles markedly enhanced the interference: with equal multiplicities of I particles and standard virus (10 per cell, as measured by hemagglutination; reference 14) added to cells simultaneously, the virus yield was 40% of that from cells infected with standard virus alone; the same input of I particles 2 hr before standard virus reduced virus yields by more than 99% (Table 1). Other experiments showed that pretreatment with I particles for periods from 30 min to 24 hr gave about as much interference as 2 hr of pretreatment, indicating that the interfering particles must reach some site in the cell or participate in some metabolic function to interfere efficiently. Without a head start, I particles lost a great deal of interfering ability but not all of it. In fact, I particles could still interfere when added 30 min after standard virus, although this interference reached 99% only when the input multiplicity of standard virus was decreased to 1 plaque-forming unit (PFU) per cell and the multiplicity of I particles was made 10-fold greater. Apparently, I particles could block virus replication even after attachment and penetration of standard virus.

Specificity of the interference. Sendai virus I particles interfered as efficiently with replication of two other Sendai virus strains as with the homologous strain. However, replication of two other paramyxoviruses, NDV and mumps virus, and another enveloped RNA virus, Sindbis virus, was not inhibited, even after 2 hr of pretreatment with 10 I particles per cell (Table 2).

Noninvolvement of interferon or protein synthesis. We did not find that I particles induced interferon. Cell culture fluids were harvested 24 hr after inoculation with I particles alone (10 per cell) or with I particles followed 2 hr later by standard virus (10 PFU per cell). The culture fluids were ultraviolet (UV)-irradiated to inactivate infectious virus and added to primary chick embryo fibroblast monolayer cultures. After 16 hr, the fibroblasts were challenged with Sindbis virus. In neither case did we observe a reduction in Sindbis virus plaques in the treated cultures, even when undiluted fluids from the cells exposed to I particles were used.

Protein synthesis did not appear to be involved in establishing interference by I particles. We found that 100 μg of cycloheximide per ml inhibited protein synthesis by 95%, as measured by ^{3}H-amino acid incorporation. The inhibitor was added to cells 30 min before inoculating them

TABLE 1. *Homologous interference by Sendai virus I particles*

Time I particles added (hr before standard virus)[a]	Virus yields at 48 hr	
	PFU/ml	Per cent of control
None added	2.5×10^6	(100)
0	1.0×10^6	40
2	2.0×10^3	0.08

[a] Cells were infected with 10 PFU/cell. I particles were added at a multiplicity of 10 per cell.

TABLE 2. *Effects of Sendai virus I particles on replication of heterotypic enveloped RNA viruses*

Virus inoculated[a]	Virus yields at 48 hr[b] (PFU/ml)
Newcastle disease virus	2.0×10^7
Newcastle disease virus plus I particles	2.2×10^7
Mumps virus	1.8×10^5
Mumps virus plus I particles	3.8×10^5
Sindbis virus	2.1×10^7
Sindbis virus plus I particles	2.2×10^7

[a] Each standard virus preparation was added at a multiplicity of 10 PFU/cell. I particles (10 per cell) were added 2 hr before standard virus. Cells were washed three times to remove unadsorbed inocula 30 min after addition of standard virus.

[b] Only released virus was assayed.

with 10 I particles per cell in the continued presence of cycloheximide. Two hours later, challenge virus (10 PFU per cell), still in the presence of cycloheximide, was added and allowed to remain in contact with the cells for 30 min. The cycloheximide was then washed out, and cells were incubated in medium free from the drug. Control cultures received identical cycloheximide treatments and standard virus but no I particles. Normal yields of virus were obtained from the controls, and interference in the cells treated with I particles was 99%.

Effects of I particles on virus-specific RNA synthesis. The evidence presented above taken with previous data indicating that the RNA species in I particles were the interfering principles suggested that RNA synthesis in cells manifesting interference was a logical target of inquiry. Examination of the RNA species in released virions provided useful supplementary information.

The intracellular RNA species induced by standard virus sedimented as others have described (2); the most abundant component sedimented at 18*S*, there was a prominent 50*S* peak, and a number of species too numerous to be resolved sedimented from 18 to 50*S* (Fig. 1A). Virus particles released from cells contained chiefly 50*S* viral genomes, with relatively little slower sedimenting heterogeneous material (Fig. 1B). Virion-associated RNA sedimenting slower than 18*S* in this and later figures has not been characterized but is presumably contaminating cellular RNA (15).

Virions produced by cells infected with mixtures of I particles and standard virions showed reduced amounts of 50*S* RNA and equal or greater amounts of 19 and 25*S* subgenomic RNA species, depending on the input multiplicity of I particles (Fig. 1B), as previously described (14). Labeling of 50*S* RNA was more markedly affected in cells than in virions (Fig. 1A), indicating that ordinarily much more 50*S* RNA is made than is ever assembled into progeny virus. This is consistent with other results showing marked nucleocapsid accumulation in Sendai virus-infected cells (3, 7). I particles also severely depressed accumulation of RNA species sedimenting between 18 and 50*S*, but the 18*S* peak was less affected (Fig. 1A).

As pointed out above, I particles did not interfere with standard virus replication when added after standard virus, unless the time interval was short and the multiplicity of standard virus was low. However, some changes in viral RNA species could be seen under conditions where no interference occurred. When I particles (10 per cell) were added to cells 2 or 8 hr after infection with 10 PFU per cell of standard virus, intracellular virus-specific RNA species did not appear to be affected (Fig. 2A), and normal amounts of 50*S* RNA appeared in progeny virions. However, 19 and 25*S* RNA species appeared in virus progeny (Fig. 2B). The amounts of these subgenomic RNA

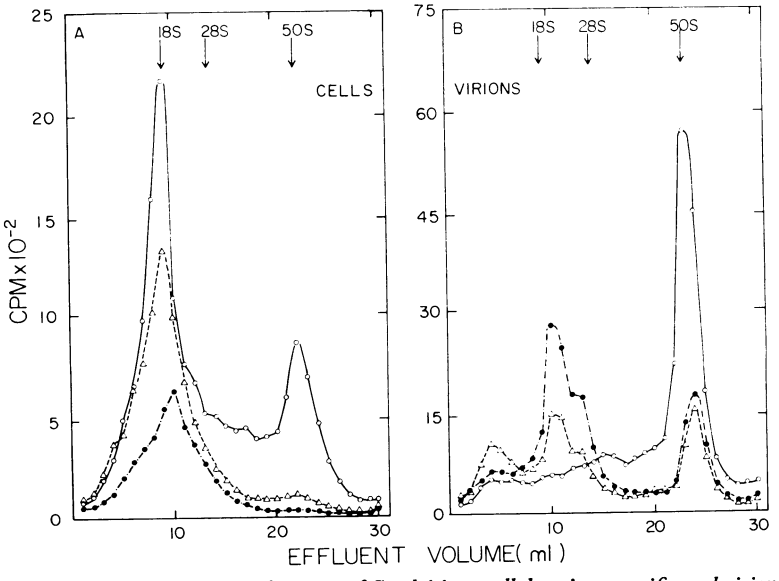

FIG. 1. *Sucrose gradient rate zonal centrifugation of Sendai intracellular virus-specific and virion RNA species from cells infected with mixtures of I particles and standard virus. (A) RNA species from infected cells; (B) RNA species from virions. Cells were infected with 10 PFU per cell of standard virus (○) alone; (△) with an equal number of I particles; (●) with a 10-fold excess of I particles. Incubation, labeling, extraction, and centrifugation of RNA species were described in Materials and Methods.*

species were relatively small, but their presence indicated that even long after initiation of infection by standard virus the RNA species in I particles could enter cells and be replicated with moderate efficiency.

The most severe interference, and thus the most marked changes in intracellular and virus particle RNA species, occurred when cells were treated with I particles before challenge with standard virus. Depending on the duration of pretreatment with I particles, little or no 50S RNA was seen in released virions (Fig. 3B), but some 19 and 25S RNA species were released from cells pretreated for 2 hr. The cells did not accumulate measurable amounts of 50S RNA, and slower sedimenting species were not only reduced about 20-fold to 100-fold (depending on duration of pretreatment) but sedimented more heterogeneously (Fig. 3A).

We previously showed that UV irradiation destroyed the ability of I particles to interfere in simultaneous infection (14). This held true in the more sensitive test of interference, treating cells with irradiated I particles 2 hr before challenge. Near normal amounts of 50S RNA-containing virions were produced by such cells along with some 19S RNA, probably representing replication of the genetic material in a few virus particles escaping UV inactivation (Fig. 3B). Surprisingly, radioactivity incorporated into virus-specific RNA species in these cells was about 10-fold less than in controls (Fig. 3A). The distribution of sedimenting species appeared normal, however, with perhaps a slight accentuation of a 32S peak (Fig. 3A). Here, as in experiments in which I particles were added to cells simultaneously with standard virions, there was a discrepancy between amounts of 50S RNA appearing in cells and amounts appearing in released virions (Fig. 1), supporting the idea that virus-specific RNA species are overproduced in normal infections (3) and that if smaller amounts are made these are still adequate to provide for essentially normal yields of infectious virus.

Changes in hybridization properties of virus-specific RNA species. Blair and Robinson (2) showed that the Sendai virus-specific RNA species from infected cells sedimenting slower than 50S hybridized to 50S RNA from virions. Therefore, as in NDV infections, relatively large amounts of RNA complementary in base sequences to viral genomes accumulated in infected cells. We obtained similar results when we annealed unlabeled virion 50S RNA with virus-specific RNA species sedimenting slower that 50S (Table 3). The labeled RNA self-annealed 23% at saturation (Table 3). RNA from cells simultaneously infected with mixtures of I particles and standard virions also hybridized efficiently with genomes, but self-annealing values were significantly higher reaching 45% at saturation (Table 3). This increased self-annealing indicates that these cells contained increased amounts of plus-type base

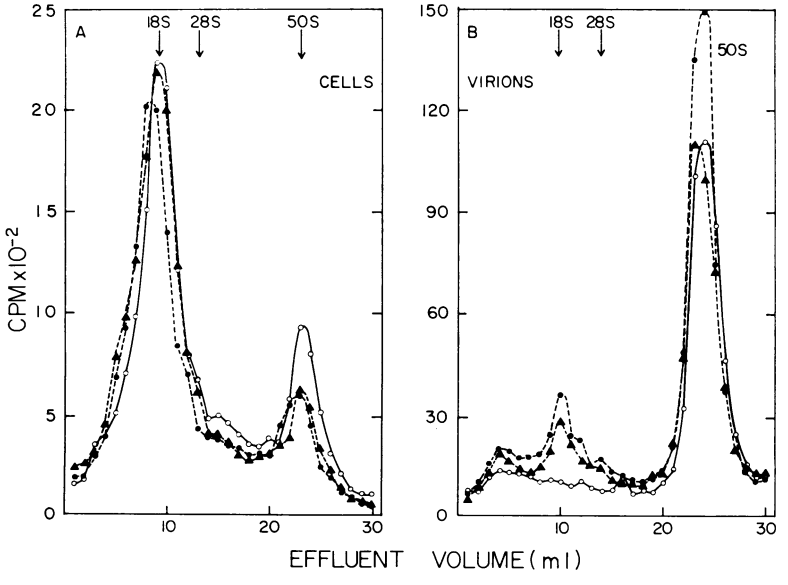

FIG. 2. *Sucrose gradient rate zonal centrifugation of (A) virus-specific and (B) virion RNA species from cells given I particles at intervals after infection with standard virus. Cells were infected with 10 PFU per cell. (○) No I particles were added; (●) I particles (10 per cell) were added 2 hr later; (▲) I particles (10 per cell) were added 8 hr later. Other conditions as in Fig. 1.*

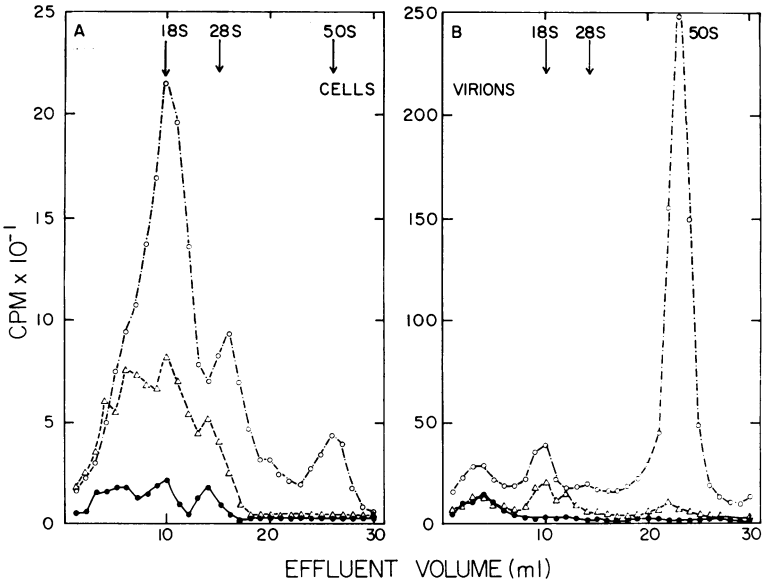

FIG. 3. *Sucrose gradient rate zonal centrifugation of (A) virus-specific and (B) virion RNA species from cells treated with I particles at intervals before challenge with 10 PFU per cell. I particles (10 per cell) were added to cells (△) 2 hr or (●) 8 hr before standard virus. (○) UV-irradiated I particles (10 per cell) were added 2 hr before standard virus.*

sequences, smaller than 50*S* genomes, compared to those from cells with a normal infection. Since these cells produced virus particles containing 19 and 25*S* subgenomic RNA species (Fig 1B), it is likely that the small plus strands detected by self-annealing are intracellular subgenomic RNA species. Proof of this point would require a method for separating plus and minus strands of sedimentation properties so that they could be annealed separately. Nevertheless, it is clear that I particles reduced accumulation of virus-specific minus strands even more than the decreased labeling of these RNA species (Fig. 1A) would indicate. On the other hand, cells showing partial interference by I particles still contained disproportionately large amounts of minus strands relative to 50*S* RNA as compared to controls (Fig. 1A). It should be noted that, when I particles were added to cells 2 hr after infection with standard virus, self-annealing of the less than 50*S* RNA species was only 26% (Table 3), indicating that inhibition of minus strand accumulation and inhibition of 50*S* RNA accumulation were not necessary for subgenomic RNA replication (Fig. 1A, Fig. 2A).

DISCUSSION

Although I particles have been described for a number of animal viruses, the T particle of vesicular stomatitis virus (VSV) has received the most scrutiny with respect to mechanisms of interference (10, 12). Huang and Wagner (12) proposed that a structural protein encoded in the RNA of T particles disrupts the replicative machinery directed by standard virus. More recently, Stampfer et al. (20) suggested that the RNA in T particles competes with standard virus genomes for RNA replicase.

Other systems have been studied in which inter-

TABLE 3. *Hybridization of RNA species from infected cells*[a]

Time I particles added (hr after standard virus)[b]	Per cent ribonuclease resistance	
	Self annealed[c]	Annealed with 50*S* plus strands[d]
None added	23	89
0	45	88
2	26	93

[a] RNA species from infected cells were labeled in the presence of actinomycin D and isolated as described in Materials and Methods. Two repetitions of this experiment gave values within 5% of those shown here.

[b] Cells were infected with 10 PFU/cell, and the I particles were added at equal multiplicity.

[c] Each self-annealed sample contained 750 to 1,100 μg of RNA per ml with specific activity of 200 to 300 counts per min per μg.

[d] Each labeled sample, containing at least 2,000 counts/min, was annealed with 100 μg of 50*S* RNA per ml from Sendai virions.

ference is caused by a heterologous virus (5, 6, 16), a nonlytic homologous virus (4, 21), a homologous virus inactivated by UV (1), and a homologous virus rendered incapable of replicating by a metabolic inhibitor (5), or, if a conditionally lethal virus, by nonpermissive conditions (18, 19). Such systems may not be directly relevant to interference by I particles but do present a number of additional hypotheses which we may consider in attempts to explain I particle interference. These hypotheses include: (i) induction of interferon by the interfering virus (4, 18, 19); (ii) competition for or destruction of cell receptors by the interfering virus (1, 4, 6, 21); (iii) production of a protein coded by interfering virus which destroys heterologous polyribosomes (18), makes "nonfunctional aggregates" with proteins specified by challenge virus (18, 19), or interacts with and blocks the function of the RNA of the challenge virus (16); (iv) competition by the interfering virus for substrate (5), "replicating sites" (5), or a "site used by challenge virus before replication" (18).

Many of these ideas appear to be inapplicable to the Sendai virus I particle interference we have studied. (i) Sendai virus I particles induce no measurable interferon. (ii) UV-irradiated I particles do not interfere and I particles added after standard virus can interfere, although with decreased efficiency. In the first instance, destruction or blockage of receptors by components of the I particle envelope is ruled out, and, in the second instance, a metabolic event induced by I particles which secondarily renders cells refractory to infection is rendered improbable. (iii) Failure of cycloheximide treatment to alter the interference argues against the need for a protein to be synthesized under direction of the RNA in I particles. Despite the long latent period of Sendai virus replication in CEL cells (7), the critical dependence of the degree of interference on the time interval between addition of I particles and standard virus challenge indicates that the interfering event occurs very early. Thus, effects of cycloheximide treatment during this interval are relevant. (iv) If I particle RNA interfered by occupying "replicating sites" or by binding to cell enzymes or substrates, interference by the RNA of UV-irradiated I particles might have been expected. Moreover, such hypothetical cellular entities would have to be peculiarly specific for Sendai virus, since NDV and mumps virus were not interfered with. (v) The RNA species in I particles might interfere by competing for an enzyme specified by standard virus. This could be a replicase or a transcriptase (20). Certainly, if the information for neither of these enzymes were encoded in I particles, their RNA species could neither replicate nor be expressed except by enzymes supplied by standard virus. Although we favor this idea, it does not seem to explain all of our observations, in particular the time dependence of the interference. It is not clear why I particles should be so much less capable of interference when added together with or later than standard virus. Moreover, I particles added as late as 8 hr after standard virus were replicated moderately well (Fig. 2B) but with no sign of interference with infectious virus production.

The time dependence of the interference might reflect the performance of a function by I particles. The RNA species in I particles might be transcribed if the particles contain an RNA transcriptase (11) or if a cell enzyme performs this function. Such transcription might not be detected (14) unless very high multiplicities of I particles were added to cells. It is difficult to understand how I particle transcripts could interfere, if they were messenger RNA species, since protein synthesis did not appear necessary to establish interference. On the other hand, I particle RNA transcripts might be nonfunctional but still able to sequester viral RNA replicase.

Viruses like Sendai virus and VSV may be particularly fruitful models for further investigation of interference phenomena, since they seem to require genome transcription as part of their replication processes. Genome replication was inhibited more than transcription in certain circumstances (Fig. 1), indicating that these functions can be dissociated by I particles. In this connection, we have found that cycloheximide, added late in infection, similarly dissociates genome replication and transcription of mumps virus and NDV (8; C. Pridgen and D. W. Kingsbury, *unpublished data*).

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LITERATURE CITED

1. Baluda, M. A. 1959. Loss of viral receptors in homologous interference by ultraviolet-irradiated Newcastle disease virus. Virology 7:315–327.
2. Blair, C. D., and W. S. Robinson. 1968. Replication of Sendai virus. I. Comparison of the viral RNA and virus-specific RNA synthesis with Newcastle disease virus. Virology 35:537–549.
3. Blair, C. D., and W. S. Robinson. 1970. Replication of Sendai virus. II. Steps in virus assembly. J. Virol. 5:639–650.
4. Bratt, M. A., and H. Rubin. 1968. Specific interference

among strains of Newcastle disease virus. III. Mechanisms of interference. Virology 35:395–407.

5. Cords, C. E., and J. J. Holland. 1964. Interference between enteroviruses and conditions effecting its reversal. Virology 22:226–234
6. Crowell, R. L. 1966. Specific cell-surface alteration by enteroviruses as reflected by viral-attachment interference. J. Bacteriol. 91:198–204.
7. Darlington, R. W., A. Portner, and D. W. Kingsbury. 1970. Sendai virus replication: an ultrastructural comparison of productive and abortive infections in avian cells. J. Gen. Virol. 9:169–177.
8. East, J. L., and D. W. Kingsbury. 1971. Mumps virus replication in chick embryo lung cells: properties of ribonucleic acids in virions and infected cells. J. Virol. 8:161–173.
9. Frothingham, T. E., and A. Granoff. 1961. Plaque formation with mumps virus. Virology 15:213–214.
10. Huang, A. S., and D. Baltimore. 1970. Defective viral particles and viral disease processes. Nature (London) 226:325–327.
11. Huang, A. S., D. Baltimore, and M. A. Bratt. 1971. Ribonucleic acid polymerase in virions of Newcastle disease virus: comparison with the vesicular stomatitis virus polymerase. J. Virol. 7:389–394.
12. Huang, A. S., and R. R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. II. Biologic role in homologous interference. Virology 30:173–181.
13. Kingsbury, D. W. 1966. Newcastle disease virus RNA. II. Preferential synthesis of RNA complementary to parental viral RNA by chick embryo cells J. Mol. Biol. 18:204–214.
14. Kingsbury, D. W., and A. Portner. 1970. On the genesis of incomplete Sendai virions. Virology 42:872–879.
15. Kingsbury, D. W., A. Portner, and R. W. Darlington. 1970. Properties of incomplete Sendai virions and subgenomic viral RNAs. Virology 42:857–871.
16. Marcus, P. I., and H. L. Zuckerbraun. 1970. Viral polymerase proteins as antiviral agents. Ann. N.Y. Acad. Sci. 173:185–198.
17. Pfefferkorn, E. R., and H. S. Hunter. 1963. Purification and partial chemical analysis of Sindbis virus. Virology 20: 433–445.
18. Pohjanpelto, P., and P. D. Cooper. 1965. Interference between polioviruses induced by strains that cannot multiply. Virology 25:350–357.
19. Roizman, B. 1965. Abortive infection of canine cells by herpes simplex virus. III. The interference of conditional lethal virus with an extended host range mutant. Virology 27:113–117.
20. Stampfer, M., D. Baltimore, and A. S. Huang. 1969. Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. J. Virol. 4:154–161.
21. Steck, F. T., and H. Rubin. 1966. The mechanism of interference between an avian leukosis virus and Rous sarcoma virus. II. Early steps of infection by RSV of cells under conditions of interference. Virology 29:642–653.