Interferon Induction by Heat-Inactivated Semliki Forest Virus[1] (34960)

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Several investigators have suggested that RNA viruses induce interferon production by synthesizing double-stranded replicative form of RNA which then acts as the inducing agent (1–3). The suggestion is based on the observation that double-stranded RNA of natural as well as synthetic origin is capable of inducing interferon production while single-stranded polynucleotides induce little, if any, interferon (1, 4–8). The numerous observations showing that inactivated viruses induce interferon synthesis would seem to indicate that the replicative form is not a prerequisite for interferon synthesis. However, inactivated viruses can perform some functions and it is possible that the replicative form may be produced (9). Our results indicate that heat-inactivated Semliki Forest virus induces interferon production in chick embryo cell cultures under conditions when neither double-stranded viral RNA nor any other form of viral specific RNA could be detected in the induced cells.

Materials and Methods. Cells. Primary cultures from 10- to 11-day old, eviscerated and decapitated chick embryos, were prepared according to the method of Lindenmann and Gifford (10). Confluent cell monolayers were usually found within 2 days and contained approximately 4×10^6 cells on an 18-cm^2 surface.

Virus. Semliki Forest virus, a group A arbovirus, was supplied by Dr. J. Porterfield, National Institute for Medical Research, London, England. Stock virus preparations consisted of 10% infected mouse brain suspension in maintenance medium. Semliki Forest virus was assayed on chick embryo cell monolayers employing an overlay consisting of 1% methyl cellulose in Eagle's minimum essential medium with 10% calf serum. Inactivated Semliki Forest virus was prepared by incubating ampules of virus at 37°. The virus was found to inactivate at a rate of one log/3.2 hr. The inactivated virus employed in these studies was incubated for 24 hr at 37° which reduced the titer to less than 5 PFU/ml.

Media. The growth medium consisted of Gey's balanced salt solution (BSS) with 0.1% lactalbumin hydrolysate, 0.1% proteose peptone, and 5% calf serum. The maintenance medium consisted of BSS with 0.1% lactalbumin hydrolysate, 0.1% proteose peptone, and 0.1% yeast extract. A modified maintenance medium, in which 2.5% calf serum replaced the yeast extract, was employed for the determination of RNA synthesis.

Cell infection and uridine incorporation. The cells were exposed to Semliki Forest virus at a multiplicity of 10, or equivalent amount of the heat-inactivated virus, in 0.2-ml volume. Actinomycin D, 4 μg in 0.1-ml volume, was also added to each cell culture and the virus was allowed to adsorb for 60 min at room temperature. The cultures were then washed twice with 2 ml of modified maintenance medium. Each culture was supplied with 2 ml of the same medium and incubated at 37°. At different time intervals, 0.3 ml of solution containing 20 μCi of uridine-5-^{3}H and 3.75×10^{-5} *M* each of thymidine and deoxycytidine, was added to each culture and incubated for 45 min at 37°.

Viral specific RNA synthesis in the infected cells: Pulse labeling method. ^{3}H-uridine

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incorporation was stopped by the addition of 0.1 ml of unlabeled uridine (5×10^{-3} *M*) and immediately placing the cultures into an ice bath. The medium was drained from the cultures and cell monolayers were extracted four times with ice cold, 5% perchloric acid (PCA). The RNA was hydrolyzed in 2 ml of 5% PCA for 30 min at 80°. A 0.2-ml portion was used for determination of ^{3}H-uridine incorporation by measuring radioactivity with a Packard "Tri-Carb" liquid scintillation spectrometer.

Sucrose gradient analysis. For isolation and characterization of the replicative forms of Semliki Forest virus RNA, the method of Friedman (11) was followed with some modifications. After the exposure of the cultures to uridine-5-^{3}H for 45 min, the cell monolayers were washed three times with 2 ml of ice-cold phosphate buffered saline and twice with 2 ml of cold 0.1 *M* sodium chloride, 0.01 *M* sodium acetate buffer pH 5.1. To each bottle was added 1 ml of acetate buffer and cells were scraped off the glass with a rubber policeman. Usually, cells from 8 cultures of each group were pooled and 0.2 ml (15 mg) of coarse bentonite in acetate buffer, prepared according to the method of Petermann and Pavlovec (12), and 0.5 ml of 10% sodium dodecyl sulfate were added. The RNA was extracted by the addition of equal volumes of phenol saturated with the sodium chloride–sodium acetate buffer, pH 5.1, and shaking the mixture for 3 to 5 min at room temperature. The emulsion was broken by centrifugation at 800*g* for 15 min and the aqueous layer was re-extracted as before. RNA was precipitated by the addition of 2 vol of cold absolute ethanol containing 2% potassium acetate and kept overnight at —20°. The RNA was then collected by centrifugation at 15,000*g* for 15 min. The precipitate was dissolved in 2.5 ml of 0.1 *M* KCl, 0.01 *M* Tris, and 0.001 *M* ethylenediaminetetracetate (EDTA), buffered at pH 7.1, and clarified by centrifugation at 10,000*g* for 15 min. One ml of the extracted RNA was layered on a linear sucrose gradient (15 to 30% in 0.1 *M* KCl, 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.1) and centrifuged at 22,000 rpm for 20 hr in SW 25.1 swinging bucket rotor. An additional 1-ml portion of RNA was treated with 2 μg of ribonuclease at 37° for 10 min before centrifugation. Hydrolysis was stopped by the addition of phenol and the nonhydrolyzed RNA was extracted as previously described. One-ml fractions were collected and optical density at 260 mμ was determined. From each fraction, a 0.1-ml portion was used for the determination of ^{3}H-uridine in the liquid scintillation spectrometer.

Interferon. Cell cultures were exposed to heat-inactivated or live Semliki Forest virus in 2 ml of maintenance medium and incubated at 37° for 15 hr. After incubation, supernatant fluids from the cultures were collected and heated at 65° for 30 min. The interferon assay and calculation of PDD_{50} units were performed according to the procedure of Lindenmann and Gifford (13). One PDD_{50} unit is equal to 1.4 units of the research standard for chicken interferon.

Reagents. Uridine-5-^{3}H (7 or 28.1 Ci/mmole) was purchased from New England Nuclear Corporation, Boston, Mass.; pancreatic ribonuclease from Worthington Biochemical Corp., Freehold, N. J.; and ribonuclease-free sucrose from Mann Research Laboratories, New York. Actinomycin D was a gift from Merck, Sharpe, and Dohme, Rahway, N. J.

Results. Interferon production by Semliki Forest virus. Chick embryo cells were exposed to live Semliki Forest virus at a multiplicity of 10 or an equivalent amount of inactivated virus. Considerably more interferon was consistently found with cultures exposed to inactive virus. For example, 65 PDD_{50} units/ml were obtained with inactive virus and 11 PDD_{50} units/ml with live virus. Since it is possible that the inactivated virus was able to undergo a partial replication cycle, we investigated whether heat-inactivated Semliki Forest virus produces a double-stranded replicative form. If the double-stranded replicative form is the interferon inducer, the yield of interferon should be proportional to its concentration and would be found in the cells exposed to inactive virus.

Virus specific RNA synthesis in the induced cells. Live virus infection at a multipli-

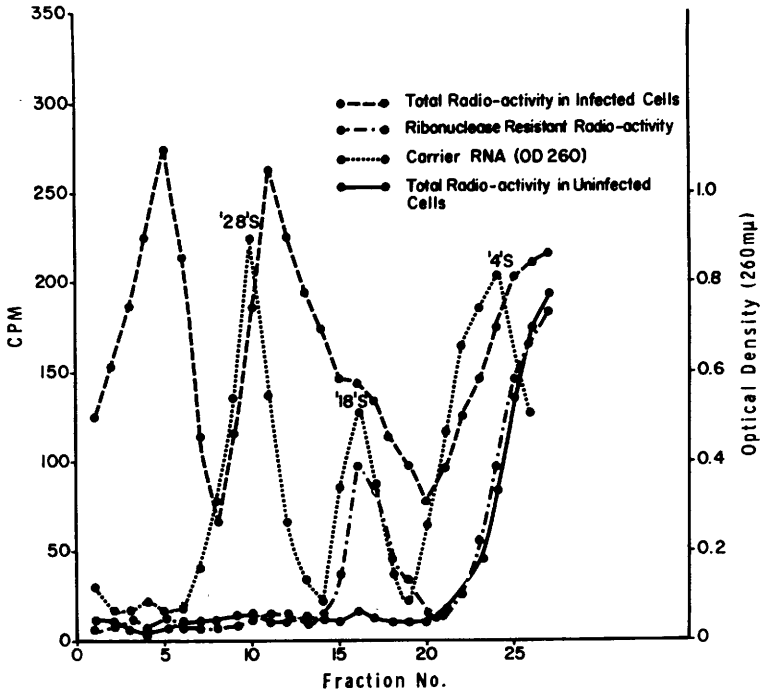

FIG. 1. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures infected for 4 hr with Semliki Forest virus. ^{3}H-Uridine (20 μCi) was added for 45 min, and RNA was then extracted. L cell RNA was employed as carrier RNA.

city of 10 PFU/cell was first employed as a control to detect the double-stranded, replicative form of RNA in the infected cells. Chick embryo cell monolayers were infected with Semliki Forest virus in the presence of 4 μg of actinomycin D. After various periods of incubation at 37°, 20 μCi of ^{3}H-uridine was added for an additional 45 min and the RNA was extracted with phenol as described. Sucrose gradient analysis of the RNA extracted at 4 hr after infection showed a peak of ^{3}H-uridine sedimenting at the 45S region. Another peak of ^{3}H-uridine was also seen in the 26S region with counts extending into regions of lower S values. When a 1-ml aliquot of extracted RNA was incubated with 2 μg of ribonuclease for 10 min at 37° before sedimentation, another peak of radioactivity sedimenting in the 20S region was revealed (Fig. 1). Similar findings were obtained when RNA was extracted at 2 or 6 hr after infection except that the 45S peak was not well defined at 2 hr. These results are similar to those of Friedman *et al.* (14) and Sonnabend *et al.* (15), who reported that viral specific RNA from the infected cells can be resolved into these three components. The 45S peak was infectious and corresponded to the RNA that can be extracted from purified virus. The 26S peak is not infectious, but the base composition of this component was similar to that of 45S RNA. The 20S RNA is ribonuclease resistant and presumably the double-stranded form of RNA. RNA was extracted at 2, 4, and 6 hr after exposure of the cells to heat-inactivated Semliki Forest virus equivalent to 10 PFU/cell. Sucrose gradient analysis of these extracts did not reveal any peak of radioactivity at any of the three regions (Fig. 2). These results indicated that viral specific RNA was not synthesized in the cells exposed to the heat-inactivated virus.

Pulse-labeling method. Viral specific RNA synthesis in chick embryo cells exposed to live as well as heat-inactivated virus was also studied by pulse labeling with ^{3}H-uridine and extracting the RNA with PCA. The induced cells, at various periods after induction

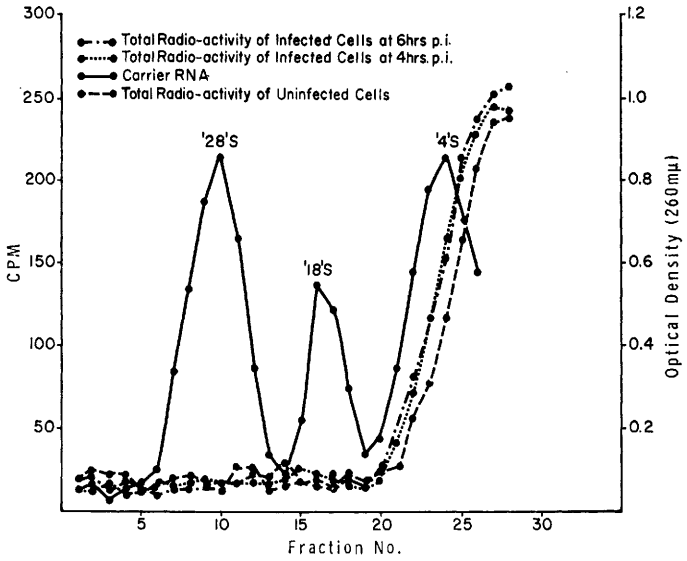

FIG. 2. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures exposed to heat-inactivated Semliki Forest virus for 4 or 6 hr. ^{3}H-Uridine (20 μCi) was added for 45 min and RNA was then extracted. L cell RNA was employed as carrier RNA.

were exposed to 20 μCi of ^{3}H-uridine for 45 min, and RNA was extracted with PCA as described. Chick embryo cells infected with live virus, at a multiplicty of 10, showed the synthesis of viral specific RNA which progressively increased with time. Most of the RNA synthesized in the infected cells from 9 hr onward was viral specific (Fig. 3). However, no viral specific RNA synthesis was detected when cells were exposed to the same

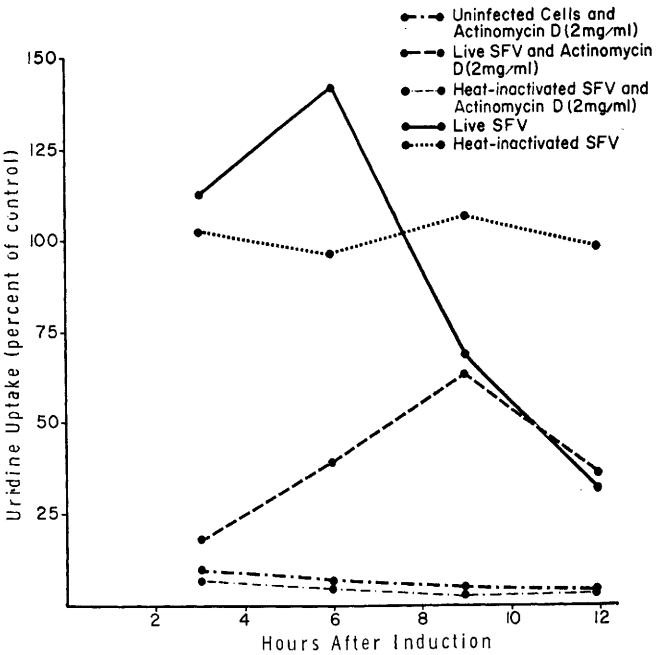

FIG. 3. RNA synthesis in chick embryo cell cultures exposed to heat-inactivated or live Semliki Forest virus in presence or absence of actinomycin D.

amount of heat-inactivated virus. In another experiment, virus directed RNA synthesis could not be detected when cells were exposed to the heat-inactivated virus in the presence of 20 μCi of ^{3}H-uridine for 7 hr.

Discussion. Several workers (1–3) have suggested that RNA viruses induce interferon by synthesizing the double-stranded form of RNA which then acts as the inducing agent. However, it is becoming increasingly apparent that the replicative form is not necessarily a prerequisite for interferon induction. We have shown in this study that viral specific RNA synthesis is not detectable in cells exposed to heat-inactivated virus although these cells were able to produce considerably more interferon than cells exposed to the same amount of live virus. Burke (16) recently reported that a temperature-sensitive mutant of Semliki Forest virus induced interferon under conditions when virus specific RNA synthesis was not detected. Other studies have been recently reported that also indicate that virus RNA synthesis is not required for the induction of interferon (17, 18). It is concluded that the input virus, containing single-stranded RNA, is able to induce interferon without inducing viral specific RNA synthesis. Some other mechanism must be responsible for the induction event.

It has been reported by De Clercq and Merigan (5) that some single stranded homopolymers can induce small amounts of interferon. The inducing characteristic was related to the stability of the secondary structure of the homopolymers as indicated by their high temperature of melting (T_m) values. The RNA of Group A arboviruses such as Sindbis (19) and Western equine encephalomyelitis (20) have been shown to have a high degree of secondary structure as indicated by their melting profiles. In this regard, it is interesting to note that poliovirus, which is not a good inducer of interferon, does not apparently have this same degree of secondary structure (19). Recent studies on the sequence of viral RNA has shown that some viral RNA molecules may consist of double-stranded hairpin regions linked together by single-stranded regions (21, 22). This secondary structure may well be responsible for interferon induction and the replicative form would not be necessary in the cases where double-stranded regions occur naturally in the virion.

Summary. Inactivated Semliki Forest virus, although unable to replicate, was capable of inducing interferon production in chick embryo cell cultures. The amount of interferon induced with inactivated virus was greater than that induced by the same amount of live virus. Viral specific RNA synthesis could not be detected in cells exposed to inactivated virus. It was concluded that synthesis of viral RNA was not necessary for the induction of interferon by this virus.

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