Demonstration of Double-Stranded Ribonucleic Acid in Concentrates of RNA Viruses (36793)

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Tytell and associates (1) presented evidence in 1967 to indicate that the induction of interferon by animal virus was due to formation of double-stranded RNA (dsRNA) such as found in the virions of reovirus (a double-stranded RNA virus) or as a replicative form produced in the course of replication of single-stranded RNA (ssRNA) viruses. Subsequent observations by Falcoff and Falcoff (2) using Mengo virus, by Inglot (3) using Sindbis virus, and by Colby and Duesberg (4) using vaccinia virus, have shown that of all the nucleic acids known to be involved in viral replication, only the double-stranded RNA's are highly active interferon inducers.

Interferon induction has been observed (5–7) following exposure of cells to concentrates of unpurified single-stranded RNA virus virions under conditions in which no viral RNA synthesis was detectable. These data could be interpreted erroneously to indicate that viral replication and formation of dsRNA is not essential to induction of interferon.

Explanations for this apparent enigma are based on two kinds of observation. First, it has been shown (8, 9) that double-stranded RNA can be synthesized by cells treated with ultraviolet-irradiated Newcastle disease virus (NDV) even though fully infectious virus is not produced. Second, the present studies showed that double-stranded RNA capable of inducing resistance to viral infection was present in nucleic acid extracts of unpurified concentrates of the virions of NDV, Sindbis virus, and Semliki Forest virus. These concentrates were similar to those used by other workers in their study of purported interferon induction by single-stranded RNA (ssRNA) viruses. Thus, the contamination of concentrates of single-stranded RNA virions with dsRNA may account for the induction of interferon and resistance to viruses in the absence of detectable viral replication.

Materials and Methods. Viruses and cell cultures. NDV, Semliki Forest virus and Sindbis virus were propagated in chick embryo cell culture and the preparations were stored frozen at −70° in sealed glass ampules. Primary cell cultures of chick embryo were prepared by standard procedures using 10-day-old embryos from leukosis-free eggs.

Preparation of nucleic acids from virus concentrates. The viruses were harvested from the infected cell cultures following 3 days incubation at 35°, the time of maximal development of cytopathology. The harvested fluids were clarified by low speed centrifugation and were then concentrated by centrifugation at 37,000*g* for 1.5 hr. The resultant pellet containing virus was resuspended in phosphate buffered saline (PBS) (0.006 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.0) to give an approximate 200-fold concentrate. The concentrates were dialyzed overnight at 4° against 100 vol of 0.01 *M* Tris buffer (pH 8.2 and containing 0.1 *M* sodium chloride, 0.001 *M* Na_2 EDTA and 0.05 *M* mercaptoethanol). Extraction of nucleic acids was performed by addition of sodium lauryl sulfate and mercaptoethanol in final concentrations of 1%. The nucleic acid concentrate was extracted 3 times with an equal volume of 88% phenol equilibrated previously with the Tris buffer. The final aqueous layer was dialyzed successively 4 times with 100 vol of PBS. Total nucleic acid content was calculated based on absorption at 260 nm and an

assumed extinction coefficient ($E^{1\%}_{1\,cm}$) of 210.

Preparation of nucleic acid from uninfected chick embryo cells. The uninfected cell monolayers were removed from the glass by trypsinization, washed once with PBS, and resuspended in Tris buffer at pH 8.2. Nucleic acid extraction was carried out as described above for the viral preparation.

Polynucleotides. The single-stranded and double-stranded polynucleotides used in the complement-fixation tests were described in an earlier report (10).

Detection of presence of dsRNA. dsRNA was detected serologically employing the complement-fixation (CF) test. Specific antiserum against poly I:C was prepared in rabbits according to the methods previously described (10). The CF method was that of Osler, Strauss and Mayer (11) employing 50% hemolytic endpoints, 4 units of inactivated sheep erythrocyte-adsorbed antiserum, and 5 units of guinea pig complement. The nucleic acid preparations were also titrated for their capacity to induce protection of primary rabbit kidney cell cultures against vesicular stomatitis virus (VSV) as previously described (12). Protection against VSV can be induced by dsRNA at 0.001 to 0.01 μg/ml but not with ssRNA or DNA at 10 μg/ml or less (13).

Results. Serological detection of dsRNA in the RNA preparations. The findings given in Table I show that poly I:C antiserum prepared in the rabbit was capable of detecting as little as 0.02 to 0.04 μg/ml of the double-stranded polyribonucleotides poly I:C, poly A:U and MU9 coliphage dsRNA (10). By contrast no reaction occurred with the single-stranded polyribonucleotides poly I, poly C, poly A, poly U, and yeast RNA, or with dsDNA of calf thymus at 10 μg/ml or less. Heat denaturation of MU9 dsRNA to produce predominantly single-stranded RNA caused an approximate 60-fold reduction in reactivity with this antiserum.

Detection of antiviral-inducing activity of RNA preparations. Nucleic acid preparations extracted from concentrates of NDV, Semliki Forest virus, and Sindbis virus were all active in inducing resistance against VSV

TABLE I. Reaction of Rabbit Poly I:C Antiserum with Double- and Single-Stranded Nucleic Acids.

Rabbit antiserum prepared against	Minimum concn of nucleic acid detected (μg/ml)										
	dsRNA			Δ dsRNA	ssRNA and DNA						
	Poly I:C	Poly A:U	MU9 dsRNA	Δ MU9 dsRNA[a]	Poly I	Poly C	Poly A	Poly U	Yeast RNA	Calf thymus DNA
Poly I:C	0.02	0.04	0.04	2.5	NA[b]	NA	NA	NA	NA	NA

[a] MU9 dsRNA denatured by heating at 125° for 15 min followed by quenching in an ethanol dry ice bath.
[b] NA, not active when tested at 10 μg/ml or less.

TABLE II. Detection of Double-Stranded RNA in Nucleic Acid Preparations from Concentrates of ssRNA Virus Virions.

Nucleic acid preparation		Induced protection of primary rabbit kidney cells			
Source	Concn (μg/ml)	Antiviral[a] titer	Estimated dsRNA[b] concn (μg/ml)	dsRNA (%) in nucleic acid preparation	Complement-fixation titer using poly I:C antiserum[c]
Newcastle disease virus	66	160	0.16–1.6	0.2–2.4	128
Semliki Forest virus	36	40	0.04–0.4	0.1–1.1	256
Sindbis virus	100	160	0.16–1.6	0.2–1.6	16

[a] Reciprocal of dilution to give protection against VSV infection.

[b] Calculation based on minimum of 0.001 to 0.01 μg/ml required for other dsRNA's to induce resistance in primary rabbit kidney cells.

[c] Reciprocal of serum dilution. Preimmunization serum was unreactive with any dsRNA preparations or with virus nucleic acid preparations.

infection in primary rabbit kidney cell cultures and were also reactive with poly I:C antiserum (Table II). Based on the minimum of 0.001 to 0.01 μg/ml required for other double-stranded RNA's to induce resistance in primary rabbit kidney cells, the nucleic acid preparations extracted from these virus concentrates were calculated to contain about 0.04 μg dsRNA/ml for the Semliki Forest virus preparation and approximately 1.6 μg dsRNA/ml for the NDV and Sindbis preparations. This represented up to 2% of the total nucleic acid extracted. These calculations do not take into account differences in specific activity and the values therefore must be regarded as approximate. Nucleic acid prepared from uninfected chick embryo cells was inactive as an inducer of resistance in cell culture even though this preparation, at a concentration of 120 μg/ml of total nucleic acid, had a CF titer of 1:300 for dsRNA.

Ribonuclease resistance of double-stranded-like RNA. Lampson *et al.* (12) showed that double-stranded viral RNA in PBS was resistant to treatment for 30 min at 25° with 0.2 μg/ml pancreatic ribonuclease (RNase) but was destroyed by treatment for 2 hr with 20 μg/ml RNase at 56°. As shown in Table III, both the capacity of the virus nucleic acid preparations to induce resistance to viral infection and their serological activity were reduced by treatment with 20 μg RNase/ml at 56°, but not by treatment with 0.2 μg RNase/ml at 25°. The chick embryo cell nucleic acid preparation which reacted like dsRNA in CF tests, was not active in inducing interferon and was degraded by treatment with 0.2 μg RNase/ml at 25°, a condition under which the dsRNA from viruses was resistant.

Discussion. The induction of interferon by single-stranded RNA viruses under conditions in which no viral RNA synthesis and, hence, no production of dsRNA could be detected has been reported for NDV and Semliki Forest virus (5–7). In all these studies, unpurified preparations of virus were used at high concentration. The data given in the present report showed that unpurified virus concentrates such as the above-mentioned workers used may contain as much as 2% of their total RNA in double-stranded form and that such contaminating dsRNA is active in inducing resistance to viral infection. Falcoff, Falcoff and Catinot (14) have shown that the interferon-inducing capacity of Mengo virus dsRNA was not destroyed by irradiation with ultraviolet light even though its infectiousness is inactivated.

Contamination with dsRNA is but one means whereby ssRNA virus virions can in-

TABLE III. Ribonuclease Sensitivity of Double-Stranded RNA Present in Phenol-Extracted Preparations of ssRNA Virus Virions and in Uninfected Chick Embryo Cell Concentrates.

Nucleic acid preparation		Pancreatic ribonuclease treatment[a]	Minimum dilution affording protection of primary rabbit kidney cells	Complement-fixation titer using rabbit anti-poly I:C
Source	Concn μg/ml			
Newcastle disease virus	66	None	320	128
		20 μg/ml	<10	<1
		None	320	90
		0.2 μg/ml	320	128
Sindbis virus	48	None	80	32
		20 μg/ml	<10	<1
		None	80	32
		0.2 μg/ml	80	64
Uninfected chick embryo cells	120	None	<5	256
		20 μg/ml	—	<1
		None	<5	512
		0.2 μg/ml	—	1

[a] Pancreatic ribonuclease treatments were with 20 μg RNase/ml for 2 hr at 56°, or with 0.2 μg RNase/ml for 30 min at 25°. Control preparations were incubated under the same conditions without added RNase.

duce interferon in the absence of replication of infectious virus. Thus, dsRNA can be synthesized by cells treated with ultraviolet-irradiated NDV even though infectious NDV is not produced (8, 9). Clavell and Bratt (9) have thrown light on the mechanism for this in their demonstration that the activity of the virion-associated RNA polymerase is more resistant to the destructive effect of ultraviolet light than are the elements needed to produce infectious virus. Hence, the retained polymerase activity may account for the production of dsRNA by ultraviolet-irradiated virus.

Normal chick embryo cell nucleic acid preparations contained "double-stranded-like" RNA, detectable by serological methods. Under the conditions of our assay, however, this nucleic acid was quite different from viral dsRNA. Thus, it was not capable of inducing resistance to VSV infection in primary rabbit kidney cell cultures and its serologic activity was much more sensitive to destruction by RNase than was the dsRNA formed by virus-infected cells. The presence of such dsRNA in extracts of chick embryo fibroblasts has been previously demonstrated by Colby and Duesberg (4), Stern and Friedman (15), and by Kimball and Duesberg (16). The origin and role of this double-stranded RNA in uninfected chick cells have not been explained to the present.

Summary. Nucleic acid preparations from unpurified concentrates of Newcastle disease virus, Sindbis virus and Semliki Forest virus were found to contain up to 2% double-stranded RNA. Double-stranded RNA was identified by reaction with antisera specific for double-stranded RNA, capacity to protect primary rabbit kidney cells against virus infection, and relative ribonuclease resistance. The presence of such double-stranded RNA in virus preparations provides an explanation for how inactivated crude suspensions of single-stranded RNA virus virions may induce interferon in the absence of detectable viral replication.

Serologically active double-stranded RNA was also detected in normal chick embryo cell nucleic acid extracts. This differed greatly from double-stranded viral RNA, however, since the serologic activity was more sensitive to destruction by ribonuclease than the viral dsRNA and it did not stimulate resistance to virus infection in primary rabbit kidney cells.

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1. Tytell, A. A., Lampson, G. P., Field, A. K., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. **58**, 1719 (1967).

2. Falcoff, R., and Falcoff, E. T., Biochim. Biophys. Acta **182**, 501 (1969).

3. Inglot, A. D., Arch. Immunol. Ther. Exp. **18**, 401 (1970).

4. Colby, C., and Duesberg, P. H., Nature (London) **222**, 940 (1969).

5. Dianzani, F., Gagnoni, S., Buckler, C. E., and Baron, S., Proc. Soc. Exp. Biol. Med. **133**, 324 (1970).

6. Goorha, R. M., and Gifford, G. E., Proc. Soc. Exp. Biol. Med. **134**, 1142 (1970).

7. Gandhi, S. S., and Burke, D. C., J. Gen. Virol. **6**, 95 (1970).

8. Huppert, J., Hillova, J., and Gresland, L., Nature (London) **223**, 1015 (1969).

9. Clavell, L. A., and Bratt, M. A., J. Virol. **8**, 500 (1971).

10. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **139**, 1113 (1972).

11. Osler, A. G., Strauss, J. H., and Mayer, M. M., Amer. J. Syph. Gonor. Vener. Dis. **36**, 140 (1952).

12. Lampson, G. P., Tytell, A. A., Field, A. K., Nemes, M. M., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. **58**, 782 (1967).

13. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. **61**, 340 (1968).

14. Falcoff, R., Falcoff, E., and Catinot, L., Biochim. Biophys. Acta **217**, 195 (1970).

15. Stern, R., and Friedman, R. M., Nature (London) **226**, 612 (1970).

16. Kimball, P. C., and Duesberg, P. H., J. Virol. **7**, 697 (1971).

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