INDUCERS OF INTERFERON AND HOST RESISTANCE, III. DOUBLE-STRANDED RNA FROM REOVIRUS TYPE 3 VIRIONS (REO 3-RNA)

By A. A. Tytell, G. P. Lampson, A. K. Field, and M. R. Hilleman

DIVISION OF VIRUS AND CELL BIOLOGY RESEARCH, MERCK INSTITUTE FOR THERAPEUTIC RESEARCH, WEST POINT, PENNSYLVANIA

Communicated by Max Tishler, June 5, 1967

Studies in our laboratories have shown that the essential quality of RNA necessary for induction of interferon and of resistance to viruses *in vivo* and *in vitro* is double- or multistranding of the individual polynucleotides and, in certain instances, freedom from inhibitory proteins.[1, 2] The present paper shows that the unique double-stranded RNA isolated from virions of type 3 reovirus is a highly active inducer of interferon in rabbits and of resistance to virus infection *in vitro*. This RNA is referred to as Reo 3-RNA.

Materials and Methods.—(1) *Reovirus:* Dearing strain reovirus type 3 was grown in primary cell cultures of grivet monkey kidney and harvested after three to four days' incubation at 35°C. (2) *Preparation of reovirus RNA (Reo 3-RNA):* The reovirus type 3 in the cell culture fluid was concentrated by the acid precipitation method described by Charney *et al.*[3] The precipitate was collected and resuspended in 0.1 *M* sodium phosphate buffer, pH 8, equivalent to a 50-fold concentrate of the original virus pool and was clarified by centrifuging for 10 minutes at 3000 rpm. The supernate was then centrifuged at 78,000 × *g* for 3 hours. The pellet which contained the virus was resuspended in sodium phosphate buffered saline, pH 7.0, containing 0.005 *M* magnesium chloride to give a 500-fold concentrate of starting material. Further purification of the virus and extraction and purification of the Reo 3-RNA was by the procedure of Gomatos and Tamm.[4] (3) *Assays:* Assays for interferon induction in rabbits, for interferon, and for induction of resistance in cell culture have been described previously.[1, 2] (4) *Characterization* of the interferon induced by Reo 3-RNA, viz., specificity, trypsin sensitivity, molecular weight, and isoelectric point, was carried out as previously presented.[1] (5) The methods employed in characterizing RNA were outlined earlier.[1]

Results.—(1) *Induction of interferon in rabbits:* (*a*) In a dose as small as 0.5 μg per rabbit, Reo 3-RNA was highly active in inducing interferon in rabbits. (*b*) Figure 1 shows the *kinetics* for interferon induction in rabbits by Reo 3-RNA and by whole infectious reovirus type 3. The whole virus dose was 8×10^{10} virions per 0.5 ml based on particle counts by electron microscopy. The 8-μg Reo 3-RNA dose was the equivalent amount of RNA obtained from 8×10^{10} virions. The Reo 3-RNA was not infectious. The RNA was noninfectious based on tests for infectivity in susceptible monkey renal cells and in L cells. Lack of infectiousness of such RNA has been repeatedly shown.[5] A high level of interferon appeared within one hour after injection of the Reo 3-RNA, reached a peak by two hours, and declined slowly to less than $^1/_{16}$ the peak level four hours later. Infectious reovirus 3 virions did not induce a significant amount of interferon before five hours and the maximum level was no more than $^1/_{16}$ that induced by the RNA.

(2) *Characterization of interferon induced in rabbits by Reo 3-RNA:* Identifica-

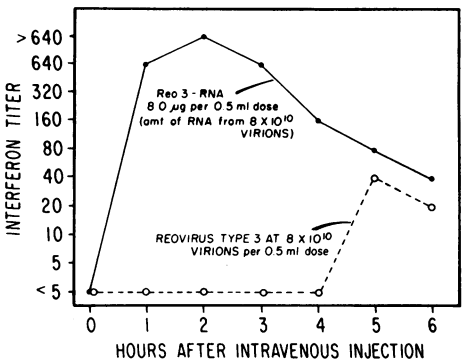

FIG. 1.—Comparison of kinetics for induction of interferon in rabbits by intravenous injection of Reo 3-RNA or reovirus type 3 virions.

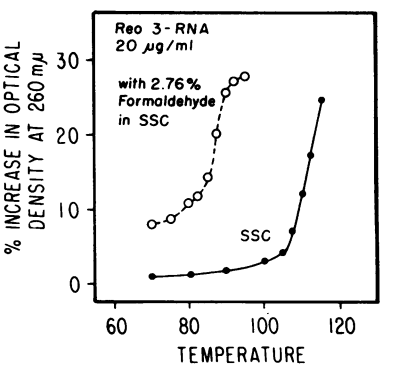

FIG. 2.—Thermal transition of Reo 3-RNA in SSC in absence or presence of formaldehyde.

tion of the viral inhibitory substance in the sera of rabbits injected with Reo 3-RNA was based on the following biological and biochemical properties: (a) *Host species-specificity* showing inhibitory titers of 128–256 in homologous rabbit kidney cells and <32 in heterologous mouse embryo and chick embryo cells in culture; (b) reduction in titer from 32 to <2 by treatment for four hours at 35°C with 50 μg *trypsin*/ml; (c) *molecular weight* of 40,000 based on Sephadex G-200 gel filtration; and (d) *isoelectric point* 7.0 measured by chromatography on CM-Sephadex.

(3) *Induction of host resistance in vitro by Reo 3-RNA:* Tests performed in monolayer primary cell cultures of rabbit kidney carried out as described earlier[2] showed that <0.04 μg Reo 3-RNA was required to prevent formation of plaques by vesicular stomatitis virus.

(4) *Physical and chemical properties of Reo 3-RNA:* (a) *Identification:* The ultraviolet (UV) absorption spectrum was typical for nucleic acid with minimum at 232 and maximum at 260 mμ. The 260:230 ratio was 2.08 and the 260:280 ratio was 2.30. These values were similar to those reported for reovirus 3 virion RNA by Gomatos and Tamm.[4] (b) The *thermal transition midpoint* for Reo 3-RNA was measured in a solution of 0.15 *M* NaCl-0.015 *M* sodium citrate, pH 7.0 (SSC). Figure 2 shows that the *Tm* was about 110°C. Heating the Reo 3-RNA in the presence of 2.76 per cent formaldehyde depressed the *Tm* to 86°C as expected for hydrogen-bonded helical polynucleotides.[6] The capacity to induce interferon in rabbits was destroyed. (c) *Treatment with RNase:* Figure 3 shows that the Reo 3-RNA was not degraded by RNase under conditions which readily destroyed single-stranded yeast RNA, viz., 0.2 μg RNase/ml at 25°C. Reo 3-RNA was degraded very slowly on treatment with RNase at 10 μg/ml and at 56°C tempera-

TABLE 1

RIBONUCLEASE SENSITIVITY OF REO 3-RNA INDUCER

Concentration of RNase*	Interferon titer in individual rabbit sera
10 μg/ml	<5, <5
None (control)	320, 640

* 24 μg/ml of Reo 3-RNA incubated with RNase at 56°C for 2 hr. Each rabbit received 0.5 ml intravenously.

ture. The capacity to induce interferon was not impaired by treatment at 25°C with the lower concentration of RNase. It was, however, rendered inactive by treatment at the high RNase level at elevated temperature and with longer incubation, as shown in Table 1.

Discussion.—The present report shows that noninfectious RNA derived from reovirus type 3 virions was highly active in inducing interferon in rabbits and resistance to viral infection *in vitro*. Induction of interferon occurred within one hour following injection into rabbits whereas five to six hours were required for whole infectious virus. This suggests that the whole virus does not become effective as an interferon inducer until double-stranded RNA has been released. It is worthy of note, also, that the naked RNA was far more efficient as an inducer than was the whole virus. The short induction period shown by the Reo 3-RNA corresponds to that for induction by endotoxin[7] and complexed synthetic polynucleotides.[2] These data suggest that time requirement for viral uncoating is an essential element in the relatively long induction period required by whole virus in contrast to substances such as endotoxin or complexed polynucleotides which have a short induction period.

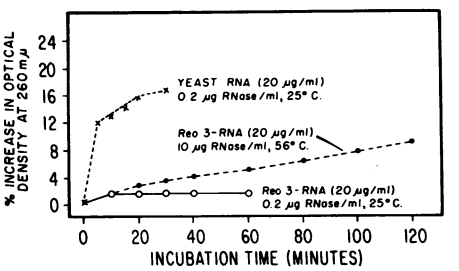

FIG. 3.—RNase susceptibility of Reo 3-RNA under various conditions.

The viral inhibitory substance induced by Reo 3-RNA was interferon based on its physical and chemical properties. The Reo 3-RNA was identified by its UV spectrum and was shown to be double-stranded as evidenced by its relative resistance to destruction by RNase, high thermal transition temperature (*Tm* 110°C),[4] and depression of the *Tm* by heating in the presence of formaldehyde.[6]

The requirement for interferon induction was double- or multistranded RNA shown previously for *Penicillium funiculosum* RNA (HeI-RNA),[1] and for complexed polynucleotides derived from polyinosinic acid and polycytidylic acid,[2] and was clearly supported in the present work with Reo 3-RNA. Our prior demonstration[1, 2] of the failure of the noninfectious, nonreplicative forms of single-stranded ribonucleic acids of viral origin including those of Newcastle disease virus, influenza A virus, and tobacco mosaic virus as well as the single-stranded RNA of nonviral origin and single-stranded synthetic polynucleotides further supports the concept. It may be speculated that the double-stranded replicative form of RNA produced in cells by RNA viruses is the active principle for interferon induction. In reovirus type 3 the replicative form and the virus RNA may be synonymous.[8] Further, the occurrence of such double-stranded RNA in unexpected sources, such as the mycelium of *Penicillium funiculosum*, might reasonably be due to infection of the mold by a virus. The phenomenon of inhibition of the inducer by protein as shown for HeI-RNA may also account for the relatively poor efficiency of induction by intact reovirus.

Summary.—It was discovered that a purified double-stranded RNA obtained from reovirus 3 virions and designated Reo 3-RNA was active in microgram amount in inducing interferon *in vivo* and resistance to viral infection *in vitro*. The induc-

tion period of Reo 3-RNA was only one hour or less in contrast to five hours for whole infectious virus and this may be related to the time requirement for removal of capsid from the RNA before induction can take place. Data relating to the biological, chemical, and physical properties of the Reo 3-RNA and the interferon induced by it are presented. The finding of the requirement for double-stranding of viral RNA for induction of interferon and host resistance to viral infection is consistent with the previous demonstration of the necessity of double-stranding for the similar activities of the RNA from *Penicillium funiculosum* (HeI-RNA) and for active complexes of polynucleotides.

Valuable technical assistance was given by J. Liedtke, K. Young, and J. Armstrong.

[1] Lampson, G. P., A. A. Tytell, A. K. Field, M. M. Nemes, and M. R. Hilleman, these PROCEEDINGS, **58,** 782 (1967).

[2] Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman, these PROCEEDINGS, **58,** 1004 (1967).

[3] Charney, J., R. Machlowitz, A. A. Tytell, J. F. Sagin, and D. S. Spicer, *Virology*, **15,** 269 (1961).

[4] Gomatos, P. J., and I. Tamm, these PROCEEDINGS, **49,** 707 (1963).

[5] Gomatos, P. J., and W. Stoeckenius, these PROCEEDINGS, **52,** 1449 (1964).

[6] Haselkorn, R., and P. Doty, *J. Biol. Chem.*, **236,** 2738 (1961).

[7] Ho, M., *Science*, **146,** 1472 (1964).

[8] Shatkin, A. J., and B. Rada, *J. Virol.*, **1,** 24 (1967).