Lack of Uptake of Intact Polyriboinosinic-Polyribocytidylic Acid by Cells (37630)

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In 1967 Field *et al.* (9) and Lampson *et al.* (10) demonstrated that the double-stranded synthetic RNA, polyriboinosinic-polyribocytidylic acid (In·Cn) was capable of inducing interferon formation in mammalian cells. Since that time, the mechanism of action of In·Cn has been under investigation—one of the questions being whether or not there is cellular uptake of the inducer. Using radioactive In·Cn several groups (2, 6, 8, 11, 14) have all reported the uptake of a small percentage of the applied In·Cn. More recently, Schell (15) has reported that In·Cn, does not act upon the cell as an entity; Cn remains at the cell surface, while part of the In enters the cell. [For more complete discussion see review by Colby (7)]. The radioactive techniques employed by these investigators, however, do not definitively answer the question of whether or not there is any double-stranded, biologically active (capable of inducing interferon-type resistance) In·Cn found in association with the cell. It is the object of this paper to approach this question by means of a sensitive biological assay. In these experiments rabbit kidney cells were exposed to In·Cn, the cellular RNA was extracted, and tested biologically in rat embryo cells for the presence of In·Cn.

Materials and Methods. 1. Tissue culture. Primary rabbit kidney and secondary rat embryo cells were grown and maintained by standard methods in both 150 × 16 mm tubes and 32 oz prescription bottles, using Eagle's minimal essential medium (MEM) with 7% fetal bovine serum.

2. Virus. The Sindbis virus used for hemagglutination (HA) tests was obtained through the courtesy of Dr. Samuel Baron, National Institutes of Health.

3. Hemagglutination assay (HA). Virus yields in control and In·Cn treated cells were determined by the hemagglutination assay as described by Clarke and Casals (5).

4. Polynucleotides. Single-stranded In and Cn were purchased from P-L Biochemicals, Inc., Milwaukee, WI. Cn^3H (11.81 mCi/mmole) was purchased from Miles Laboratories, Elkart, IN. Double-stranded $In{\cdot}Cn^3H$ was prepared by annealing equi-molar amounts of In and Cn^3H in equal volumes of pyrogen-free saline at 44° for 10 min. The resultant specific radioactivity of $In{\cdot}Cn^3H$ was 0.028 μCi/μg. The efficiency of complexing was tested by the ability of $In{\cdot}Cn^3H$ to induce interferon. In all cases 300 μg/ml neomycin sulfate (Mycifradin, Upjohn Co., Kalmazoo, MI) was used as an enhancing adjunct for In·Cn in the experimental medium (3).

5. Test for the presence of In·Cn. Eight to ten 32 oz bottles of rabbit kidney cells were exposed to In·Cn as indicated. The cells were washed rapidly 4 times with 30 ml of cold phosphate buffered saline (PBS). RNA was extracted from the cells by the method of Scherrer and Darnell (16). A yield of RNA of about 100 μg/bottle was usually obtained. It was tested for the presence of biologically active In·Cn as follows. Rat embryo cells in test tube culture were exposed to 50 μg (in 1 ml medium) of the cell RNA overnight, washed and then challenged with Sindbis virus. Virus yields after 24 hr were determined by the hemagglutination assay. Known concentrations of In·Cn were used with each assay to establish a standard re-

sponse curve for that batch of cells.

Results. Control experiments were performed in order to determine the amounts of In·Cn needed to develop the virus-resistant state in rabbit kidney cells. This served as a guide for subsequent experiments. It was demonstrated that as little as 0.015 μg/ml of In·Cn was adequate to induce detectable resistance (>0.5 log inhibition) to Sindbis virus in rabbit kidney cells.

The efficacy of recovery by phenol extraction of In·Cn from cells was determined by mixing rabbit cells with 50 μg of In·Cn immediately prior to the addition of phenol in one case; in the other just after the addition of phenol. A titration in rat embryo cells of both samples of extracted RNA indicated complete recovery of the added In·Cn (Fig. 1). It can also be seen that 0.015 μg of In·Cn induces significant resistance to Sindbis virus in rat embryo cells.

Detection of In·Cn in RNA from rabbit cells exposed to In·Cn for each of the dose–time schedules indicated in Table I was attempted by means of the biological assay. In no case was In·Cn detectable in the extracted rabbit cell RNA. In addition, all the added In·Cn could be recovered from the medium. A bottle of rabbit kidney cells exposed to 20 μg/ml of In·Cn contained a total of 1000 μg of the compound. Since 100 μg of the extracted cell RNA (the yield from one bottle) contains less than 0.03 μg of biologically active In·Cn, less than 3×10^{-5} of the total added In·Cn was removered from the cells.

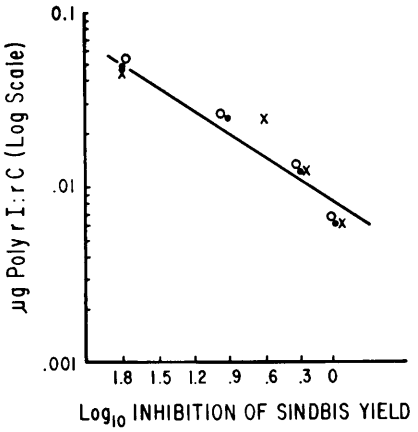

FIG. 1. Titration in rat embryo cells of (●) In·Cn; (○) titration of RNA isolated from 0.3 ml rabbit kidney cells exposed to 50 μg In·Cn immediately after the addition of phenol for extraction; (×) titration of RNA isolated from 0.3 ml rabbit kidney cells exposed to 50 μg In·Cn, then immediately extracted with phenol. About 500 μg of RNA was recovered from the rabbit kidney cells.

TABLE I. Schedules of Exposure of Rabbit Kidney Cells to In·Cn Prior to RNA Extraction.[a]

Dose In·Cn (μg/ml)	Incubation times employed (hr)
5	0, ¼, ½, 1, 2, 3, 4
20	6
50	1, 2
200	¼, ½, 1, 2
500	2

[a] For each of the indicated doses and times, eight to ten 32 oz bottles of rabbit kidney cells were used, each with 50 ml of medium, RNA was extracted and assayed for In·Cn in rat embryo cells as described in Materials and Methods.

In other experiments rabbit kidney cells were treated with 20 μg/ml In·Cn^{3}H for 6 hr (both in the presence and absence of fetal calf serum). The cells were extracted with cold 10% trichloroacetic acid (TCA); the pellet was washed with 0.1% TCA, and subsequently dissolved in NaOH. The acid-soluble and acid-insoluble radioactivity was determined. Table II shows the presence of acid-precipitable radioactive material in both groups, but to a greater extent in the group that had received serum. Figure 2 presents the results of a (2.2%) polyacrylamide gel electrophoretic analysis of phenol extracted RNA from rabbit cells exposed to In·Cn^{3}H. The simultaneous plot of both disintegrations per minute (dpm) and optical density (OD_{260nm}) versus slice number, shows a diffuse pattern of radioactivity corresponding somewhat to the peaks of ultraviolet absorption. In·Cn not exposed to cells, migrates mostly to the position shown on the graph by the arrows (Fig. 2). These data suggest that the bulk of the acid-insoluble radioactivity recovered from the cells is attributable to reincorporation into cellular RNA of the hydrolysis products of the radioactive In·Cn^{3}H. The fact that there were larger amounts of radioactivity in both soluble

TABLE II. Uptake of In • Cn ^{3}H into Rabbit Kidney Cells.

Fractions[a]	Uptake[b]	
	% of applied	μg per 100 μg cell RNA
Cells exposed in presence of serum		
Acid-insoluble fraction	0.022	0.045
Acid-soluble fraction	0.005	0.010
Cells exposed in absence of serum		
Acid-insoluble fraction	0.005	0.011
Acid-soluble fraction	0.001	0.002

[a] Rabbit kidney cells in 100 mm petri dishes were exposed to 20 μg/ml In • Cn^{3}H for 6 hr (both in the presence and absence of serum). The cells were fractionated into acid-soluble and -insoluble parts as described.

[b] Uptake was determined from the amount of cell-associated label and the known specific activity of the applied material.

and insoluble fractions from experiments where serum was present further supports this idea, since serum is known to hydrolyze In•Cn (13).

Discussion. The preceding data reveal that the RNA from cells exposed even to very large doses of In•Cn does not contain amounts of intact In•Cn that are detectable by a sensitive biological assay. Amounts up to 0.03 μg of In•Cn/100 μg cell RNA may have been cell-associated without being detected by the biological assay. In cells exposed to radioactive In•Cn in the presence of serum, acid-insoluble radioactivity was found in the cell which calculated to be the equivalent of 0.045 μg of In•Cn/100 μg cell RNA. That the greatest part of this radioactivity is not intact In•Cn is indicated by the electrophoretograms. The data are in accord with the data obtained with nonradioactive material, that less than 3×10^{-5} of the added In•Cn is present in intact form and probably a good deal less. However, this could still allow for the presence of 1000 molecules/cell. Among the possibilities to explain these observations are (a) either intact In•Cn does not enter the cell, or (b) very little enters, and is then broken down very rapidly, or (c) In•Cn is broken down on the cell surface, and then may enter. If In•Cn does not enter the cell, then it may be acting through effects on membranes.

The fact that no intact In•Cn was found, even though the radioactivity of labeled In•Cn was, suggests that comparative uptake studies of different synthetic RNA's by means of isotope tracing, may not be interpretable as measuring uptake of the nondegraded RNA.

Summary. Rabbit kidney (RK) cells were exposed to polyriboinosinic-polyribocytidylic acid (In•Cn) in an attempt to see if biologically active In•Cn (able to induce interferon) could be found in association with the cells. RNA was then extracted from the rabbit

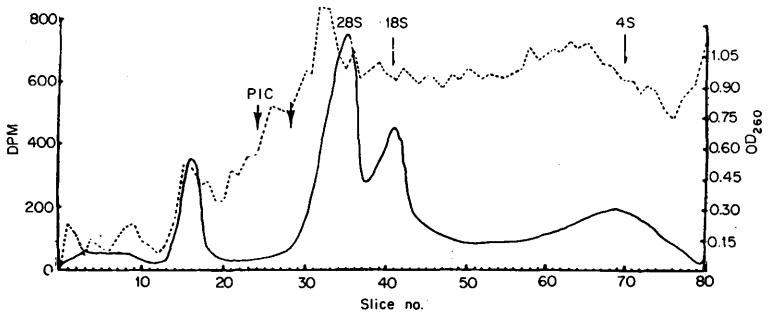

FIG. 2. Polyacrylamide gel electrophoresis of RNA isolated from rabbit kidney cells treated with 20 μg/ml In•Cn^{3}H for 6 hr; OD_{260}; disintegrations per minute (- - -).

kidney cells and tested in rat embryo (RE) cells to see if resistance to virus could be induced. Although suitable controls demonstrated that the RE cells could detect very small amounts of In·Cn none could be found in the RNA extracted from the RE cells. Re cells exposed to radioactive In·Cn yielded radioactive RNA but the radioactivity was not attributable to In·Cn. Some implications of these findings are presented.

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