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PRODUCTION OF INTERFERON-LIKE INHIBITOR INDUCED BY EXTRACT FROM ROUS SARCOMA

II. PROPERTIES OF THE INDUCER IN THE EXTRACT[1]

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Summary An extract was prepared by the phenol method from Rous sarcoma tumor tissue formed on the chorioallantoic membrane (CAM) of chicken eggs. It contained a substance inducing production by chick embryo cell cultures of an interferon (IF)-like inhibitor of Rous sarcoma virus (RSV) infection. The chemical nature of this substance was studied. It was found in the polysaccharide fraction of the extract and was sensitive to hyaluronidase.

The fractionation and physico-chemical properties of this substance were studied, and it was suggested that it is a hyaluronic acid or a closely related compound.

INTRODUCTION

Since the discovery of an interferon by Isaacs *et al.* (1957), many inducers of interferon production have been reported. Statolon (Kleinschmidt *et al.*, 1962) and bacterial endotoxin (Stinebring *et al.*, 1964), as well as viruses, were also reported to be interferon inducers.

It has recently been demonstrated that double stranded RNA from *Penicillium funiculosum* (Lampson *et al.*, 1967) or from reovirus-3 (Tytell *et al.*, 1967), and multistranded synthetic polynucleotide complexes (Field *et al.*, 1967) induce interferon production *in vivo* and *in vitro*.

On the other hand, Bader (1962) showed that Rous sarcoma virus can also induce interferon in chick embryo cells and that this interferon inhibits both the reproduction of the virus and the characteristic morphological conversion of the chick embryo cells.

One of us has reported that an IF-like inhibitor is produced in the culture fluids of chick embryo cells treated with RNA preparation, which is extracted from Rous sarcoma tissues by the phenol method (Watanabe, 1965). He suggested that the inducer of the inhibitor seemed to be a double stranded RNA, RNA-DNA hybrid or polysaccharide which was present as a contaminant in the phenol extract, since the inducing substance is resistant to the action of pancreatic ribonuclease (RNase). We studied the nature of this IF inducer and obtained evidence that it is hyaluronic acid, or a closely related compound. This paper presents experiments on the biologi-

1 An abstract of this report was presented at the Annual Meeting of Virology of Japan, Chiba, 1967.

cal and biochemical properties of this substance.

MATERIALS AND METHODS

1. *Tissue culture*

RIF free chicken eggs from Kimber Farm, U.S.A., were used for tissue culture and inoculation of CAM with virus. Primary cultures of chick embryo cells were prepared from 10 day old embryos by chopping up the embryos with scissors and treating the mince with 0.25% trypsin (Difco) in tris-buffered saline. The cells (3×10^7) were seeded onto 100 mm petri-dishes in 15 ml of medium containing 8.5 parts of Eagle's basal medium, 1.0 part of tryptose phosphate broth (Difco), and 0.5 part of bovine serum. The cultures were incubated at 38°C in a humidified atmosphere composed of 5% CO_2 in air in a CO_2-chamber. Secondary cultures were made by treating the primary cultures with 0.05% trypsin and then seeding 1.5×10^6 cells in 15 ml of growth medium onto 100 mm petri-dishes.

2. *Virus*

Bryan's high titer strain of RSV was used. Virus suspension was prepared as follows; RSV was inoculated onto the CAM of 12 day old eggs. After incubation at 38°C for 7 days, the tumor tissues formed on the CAM were harvested and homogenized in 5 volumes of Eagle's medium with quartz sand. The supernatant prepared from the homogenate by centrifugation at 3,000 rpm was used for experiments.

3. *Virus titration*

RSV was titrated as focus forming units by RUBIN'S method (1960). In this method, the cells were infected in suspension or in monolayer cultures in 30 mm petri-dishes, and medium containing 0.6% agar (Difco, Noble) in Eagle's growth medium was used as an agar overlayer. The foci formed were counted after 7 days incubation at 38°C in a CO_2-chamber.

4. *Preparation of RNA and polysaccharide from the CAM tumor tissues*

The tumor tissue was homogenized with a glass homogenizer in 5–10 volumes of solution containing 0.5% sodium lauryl sulphate in 0.02 M potassium phosphate buffer, pH 7.0. An equal volume of water-saturated phenol was added to the homogenate and the mixture was stirred with a magnetic stirrer at 0°C for 30 min and then centrifuged at 2,000 rpm for 10 min. The aqueous layers were pooled. The intermediate phase and precipitate were extracted again with an equal volume of water-saturated phenol and then the combined aqueous phase was washed once with the same phenol. A clear extract was obtained by removing the phenol with ether and then bubbling N_2 gas through the extract. In subsequent procedures, we used two methods for preparation of RNA and polysaccharide.

Method (1)

To an aliquot of the extract was added NaCl to 0.14 M and then 2 volumes of 95% ethanol. The mixture was centrifuged at 2,000 rpm for 10 min and the precipitate was washed once with ethanol-water (2:1), dissolved in Dulbecco's PBS (D-PBS) (DULBECCO *et al.*, 1954) and then centrifuged in a Spinco ultracentrifuge at 40,000 rpm for 2 hr. The supernatant was dialyzed against D-PBS overnight. The dialyzate was used as fraction 1 (Fr. 1). Polysaccharide-free RNA preparation was obtained from Fr. 1 by Kirby's method, which included treatment with 2-methoxyethanol (KIRBY, 1956). This preparation did not give a positive carbazol reaction, and was designed as fraction 2 (Fr. 2).

Method (2)

The phenol extract was fractionated by the method of WESTPHAL (1954). To an aliquot of the extract was added potassium acetate to 2%, and then 10 volumes of ethanol. The precipitate formed on standing the mixture overnight in the cold room was dissolved in distilled water and potassium acetate was added to 2%. An equal volume of ethanol was added to this solution, and the mixture was stood at 0°C for 30 min and then centrifuged at 10,000 rpm for 20 min. At this step, two fractions were separated, the precipitate and the supernatant. The precipitate was dissolved in distilled water and then dialyzed against D-PBS overnight. The dialyzate was used as the RNA fraction (Fr. 3) and did not give a typical carbazol reaction. The supernatant was mixed with 6 volumes of ethanol, stood for 1 hr and then centrifuged at 10,000 rpm for 20 min. The precipitate was dissolved in distilled water and dialyzed against distilled water overnight. To remove traces of RNA, the dialyzate was treated with pancreatic RNase (final concentration, 30–40 μg) for 1 hr at 38°C. After incubation, the mixture was made up 2% with respect to potassium acetate and material was again precipitated by addition of 6 volumes of

ethanol. The mixture was centrifuged at 10,000 rpm for 20 min and the precipitate was dissolved in distilled water and centrifuged at 2,000 rpm for 10 min. The supernatant was dialyzed against distilled water overnight. The dialyzate was used as the polysaccharide fraction (Fr. 4).

5. *Preparation of interferon*

The culture media of cells treated with various fractions were harvested after 2 days incubation, and dialyzed successively against 1/20 M KCl-HCl buffer, pH 2.0, D-PBS, and then Eagle's medium for 24 hr each. The dialyzate was centrifuged at 40,000 rpm for 2 hr in a Spinco ultracentrifuge. The supernatant was used as the IF sample.

6. *Biochemical analyses*

Acid mucopolysaccharide was determined by the method of BOLLET (1958). After hydrolysis with 0.5 N NaOH, samples were neutralized and were made up to 5% perchloric acid by addition of 70% solution. Subsequent procedures were similar to those of Bollet. The carbazol method of Dishche modified by BITTER (1961) was used for uronic acid determination.

RNA was determined by the modified orcinol method of MEJBAUM (1939) or from the UV absorption.

For estimation of hexosamine, samples were hydrolyzed in 6 N HCl at 100°C for 8–14 hr. In subsequent procedures, a modification of the method of BOAS (1953) was used.

RESULTS

1. *Effects of treatment of chick embryo cells with various fractions of the phenol extract on their infection by RSV*

A chick embryo cell suspension at a concentration of 6-10×10^6 cells per ml of D-PBS was prepared from primary cultures. It was incubated with various fractions of phenol extracts at concentrations of 180–200 μg of RNA per ml for Fr. 1–3, and 8.0 μg/ml of uronic acid for Fr. 4, respectively, at 38°C for 2 hr. After washing the cells twice with Eagle's medium, they were seeded on 100 mm petri-dishes at a concentration of 3×10^6 cells/15 ml of growth medium and were cultured at 38°C.

After 2 days incubation, a certain amount of RSV was titrated with these treated cells to test their sensitivity to RSV infection. Simultaneously, aliquots of these treated cells were infected with RSV at a multiplicity of infection of 0.1, and the culture fluids 2 days after infection were titrated for RSV to estimate the rate of virus production in the treated cell cultures.

As shown in Table 1, both the sensitivity of cells to focus formation and the rate of RSV production in the cell cultures treated with Fr. 1 were reduced to 50% or less of those in the control culture. This result confirms that reported previously (WATANABE, 1965). None of the other fractions, Fr. 2, Fr. 3, and Fr. 4 had any inhibitory effect on the treated cells.

TABLE 1 *Effect of Treatment of Chick Embryo Cells with Phenol Extracts of Rous Sarcoma on Formation of Foci and Virus Growth*

Exp. No.	Sample used	No. of foci per plate	
		Treated cells	Virus growth
1.	None	142	408
	Fr. 1	54	103
	Fr. 2 (− polysaccharide)	126	418
2.	None	62	86
	Fr. 3 (RNA)	54	90
	Fr. 4 (polysaccharide)	58	97

2. *Interferon production induced by various fractions of phenol extract*

The culture media of the cells after treatment with each of the fractions were harvested after two days incubation and IF samples were prepared as described in Materials and Methods. Monolayer cultures formed by overnight incubation of chick embryo cells in 30 mm petri dishes were treated with 2 ml of these IF samples at 38°C for 4 hr, and then washed three times with Eagle's growth medium. The sensitivity of these cells to RSV and the rate of virus production in them were examined as described in experiment 1.

As shown in Table 2, Fr. 1 and Fr. 4 induced the production of inhibitor in the culture media of treated cells. That is, the inducing capacity of the original extract, Fr. 1, was not present in Fir. 2, which contained no polysaccharide. On the other hand, the polysaccharide fraction, Fr. 4, obtained in the second method of preparation could induce IF

TABLE 2 *Interferon Production Induced by Fractions of Phenol Extract from Rous Sarcoma*

Exp. No.	Sample used	No. of foci per plate	
		Treated cells	Virus growth
1.	None	257	148
	Fr. 1	109	53
	Fr. 2 (− polysaccharide)	263	125
2.	None	52	72
	Fr. 3 (RNA)	63	87
	Fr. 4 (polysaccharide)	25	40

production, while the RNA fraction, Fr. 3, could not.

3. *Physico- chemical properties of the polysaccharide*

The chemical nature of the substance in Fr. 4 which induced production of an IF-like inhibitor in the chick cells was examined as shown in Table 3.

The fraction absorbed to protamine sulfate, which was thought to be an acid mucopolysaccharide, was shown to constitute 91.5% of the total uronic acid. The molar ratio of uronic acid to hexosamine was 1.03. A trace of nucleic acid was detected as a contaminant by UV absorption. No protein was detectable by using phenol reagent (FOLIN-CIOCALTEAU, 1927).

TABLE 3 *Physico-chemical Properties of Polysoccharide*

1. Carbazol reaction for uronic acid	positive
2. Acid mucopolysaccharide	91.5% of Total uronic acid
3. Molar ratio of uronic acid to hexosamine	1.03
4. Metachromasia with toluidine blue or azur A	negative
5. Nucleic acid (UV-absorption)	trace

The metachromasia of the substance was compared with those of hyaluronic acid, chondroitin sulfate, and heparin at the same concentrations. The metachromatic color of the substance in Fr. 4 developed on filter paper with Toluidine Blue was very faint just as that of a commercial preparation of human umbilical cord hyaluronic acid, but the color faded more rapidly than the latter. The metachromatic color of this substance was quite different from the clear and stable color of chondroitin sulfate or heparin.

4. *Effect of testis hyaluronidase on the inducing capacity of the polysaccharide*

When Fr. 4 was pretreated with testis hyaluronidase (130 μg/ml final concentration) at pH 6.0 in D-PBS at 38°C for 1 hr, the capacity to induce IF production was completely lost, as shown in Table 4. Using a fraction precipitated with ethanol after treatment of Fr. 4 with hyaluronidase, the same result was obtained.

TABLE 4 *Effect of Hyaluronidase on IF-inducing Capacity of Polysaccharide*

Sample used	No. of foci per plate	
	Treated cells	Virus growth
None	110	24
Polysaccharide (Fr. 4)	77	9
Hyaluronisase control	103	23
Hyaluronidase-treated Polysaccharide	104	17

DISCUSSION

The experiments with various fractions of the phenol extracts of Rous sarcoma described above, suggest that the polysaccharide in the extract induces IF production in cultures of chick embryo cells, but nucleic acid does not.

As indicated in the previous report (WATANABE, 1965), the decrease in sensitivity to RSV infection of chick embryo cells treated with Fr. 1 seems to be due to the presence of a particular kind of RNA, since this inhibition was RNase sensitive. The RNA fraction in Fr. 1 which inhibits infection by RSV, seems to be different from the materials in Fr. 2 and Fr. 3, but this particular RNA has not yet been isolated.

Fr. 4 induces IF production, but the sensitivity to RSV of chick embryo cells is not affected by treatment with Fr. 4.

This may be explained by supposing that in

this case the IF in the cell is in an inactive form, but the actual reason is still obscure.

The polysaccharide in Fr. 4 was found to consist almost entirely of acid mucopolysaccharide, in which the molar ratio of uronic acid to hexosamine was 1.03, and this substance was sensitive to hyaluronidase. Biochemical analyses and experiments on the effect of its digestion by hyaluronidase suggest that this material is a hyaluronic acid or chondrotin sulfate. The metachromatic activity of acid mucopolysaccharides increase with their sulphur content (WALTON *et al.*, 1954; SYLVÉN *et al.*, 1952). The metachromasia of this material with Toluidine Blue or Azur A is faint or negative, so it seems to be sulphur free and to be a hyaluronic acid or very closely related compound.

The sulphur-free acid mucopolysaccharide prepared from Fr. 4 by the Azur A precipitation method (SYLVÉN *et al.*, 1952) can also induce IF production.

From these results, it seems very likely that the polysaccharide in the phenol extract of Rous sarcoma, which is an IF inducer is a hyaluronic acid or a substance closely related to this.

GOLDÉ *et al.* (1959) showed that incubation of RSV at 37°C with a mucopolysaccharide prepared from Rous sarcoma ascites lowered its infectivity for whole chick embryo cell cultures.

This inhibitor did not seem to be hyaluronic acid, since it had an absorption maximum between 270 and 280 mμ, and the inhibition of virus infectivity caused by hyaluronic acid in our experiments was due to induced IF production, and was not a direct action of hyaluronic acid on the virus.

KIMOTO *et al.* (1965) indicated that the hyaluronic acid of Rous sarcoma might have a relatively small molecular weight, compared with mammalian hyaluronic acid from human umbilical cord or other sources. We failed to demonstrate the ability of hyaluronic acid from human umbilical cord to induce IF production, so that the hyaluronic acid which has the capacity to induce IF production may be a smaller sized molecule.

KLEINSCHMIDT *et al.* (1964) demonstrated that statolon, which is a complex polysaccharide from a mould with a relatively high content of galacturonic acid, induces IF production in mouse cells and also chick cells. Our report shows that another polysaccharide besides statolon, namely hyaluronic acid, can also induce IF production.

On the other hand, it has also been demonstrated that acid mucopolysaccharide is produced in chick embryo cell cultures infected with RSV (ERICHSEN *et al.*, 1961), and TEMIN (1965) suggested that an increased rate of acid mucopolysaccharide production may be associated with the conversion of cell morphology, but the relation between the two is not yet clear.

However, it has been demonstrated that the difference between the variants of the polyoma virus in oncogenic potential might have been due to the greater IF production induced by infection with the poorly oncogenic M variant than by infection with the highly oncogenic S variant (FRIEDMAN *et al.*, 1964).

Moreover, it has been reported that the mouse cell line 3T3, exposed to IF prior to, or immediately after infection with SV40 was protected against the transforming effect of the virus (TODARO *et al.*, 1965) and against the formation of T antigen (OXMAN *et al.*, 1966).

These reports and the results presented in this paper suggest that cellular transformation by virus is inhibited in some way by IF. The relation between IF production induced by the hyaluronic acid of Rous sarcoma and the inhibition of cell conversion by IF is a very interesting problem in carcinogenesis induced by RSV and should be studied further.

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