little amniotic fluid as most treated embryos. Therefore, the reduction in amniotic fluid caused by these teratogens is not the effect which is primarily responsible for development of the cleft palates.

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In vitro Production of an Interferon-Like Inhibitor of Viral Multiplication by a Poikilothermic Animal Cell, The Tortoise (*Testudo greca*).* (29918)

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The capacity of animal cells to react to viral infection by synthesizing substances which inhibit viral multiplication, appears to be a general phenomenon in cells derived from warm-blooded animals. Protein factors (interferons) which inhibit intracellular viral synthesis have been previously demonstrated in infected homeothermic animal cells, *i.e.*, chick embryonic cells(1), many different types of normal and malignant human cells (2,3,4), monkey kidney cells(5), rabbit kidney cells, and calf kidney cells(6).

Although all interferon-like substances (ILS) act during the intracellular phase of viral synthesis, important differences concerning physico-chemical properties such as ether sensitivity and heat stability have been reported. Since the synthesis of these substances appears to depend on the cellular and not on the viral genome, such physico-chemical differences may be attributed to the cell type from which they originate. This may also explain the relative species specificity which has been previously observed(7,8).

Since interferon-like substances are liberated by almost all types of virus-infected cells, while antibody is produced only by immunologically competent cells, one may speculate that such factors may be produced by infected cells at different levels of the zoological scale, perhaps even by unicellular organisms. To test the interferon-producing capacity of cells derived from cold-blooded animals, tortoise cells have been chosen because of previous experience with these cells in culture(9).

Material and methods. Cell culture: The technics of propagation of tortoise kidney cells in culture and their viral susceptibility have been described elsewhere(10). Kidney cells were dispersed by 0.25% trypsin in buffered saline solution (BSS) and monolayers were obtained in Roux bottles and Kahn tubes by growing the cells at 30°C in a casein hydrolysate culture medium supplemented by 5% heat inactivated calf serum.

Monolayers of chick, mouse embryo, human amnion, guinea pig and rat kidney cells were grown according to standard methods using the same casein hydrolysate medium with 10% calf serum.

Continuous cell lines of human KB, monkey MA 104 (received from Microbiological Associates, Bethesda, Md.), and hamster BHK 21(11), were maintained in casein or Eagle's medium supplemented with 10% calf serum.

Virus strains: Parainfluenza type 1 (Sendaï) virus was used as inducing agent in production of interferon. The virus was an egg-

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adapted strain. Sindbis virus grown in hamster fibroblasts BHK 21 was used as challenge virus for the demonstration and the titration of interfering substances. Both types of virus were demonstrated to be able to multiply in tortoise kidney cells.

Results. Production of interferon-like substances (ILS). Monolayers of tortoise kidney cells maintained for 8 days were infected by Sendaï virus at a multiplicity of approximately 1. After an adsorption time of 20 minutes at 37°C, cells were twice washed with Hanks' solution and then re-incubated in fresh medium for 48 hours at 37°C. At this time about half of the cell population was destroyed by the virus and the infected cell culture fluid was harvested (ILS). Control tissue culture fluid was harvested at the same time from uninfected cells, ILS and control fluids were centrifuged for 10 minutes at 3000 r.p.m. for clarification and then centrifuged for 2 hours at 40,000 r.p.m. to sediment viral particles. The two upper thirds of each supernatant were removed and stored at 4°C before testing. Both culture fluids were shown to be free of infectious virus.

Assay of ILS on tortoise cells. Since many factors such as pH, cell nutrition etc., may interfere with viral multiplication in long experiments, ILS was first assayed in single cycle experiments as previously described with KB cells(12).

Tortoise tissue cultures were prepared as described in *Material and methods,* in 250 ml flasks. When the cell monolayer was confluent, the growth medium was discarded, and ILS at the final dilution of 1:4 was incubated with the cells for 24 hours at 37°C. A control tissue culture medium from uninoculated cell cultures was employed at the same dilution. Following incubation the tissue culture medium was removed and the cells washed with Hanks' solution. The flasks were then inoculated with 10^5 $IDTC_{50}$ of Sindbis virus. After one hour adsorption time, the medium was replaced with fresh medium, and the amount of unadsorbed virus titrated. The difference between the titer of the inoculated virus and the unadsorbed virus reflected the amount of virus adsorbed to the cells.

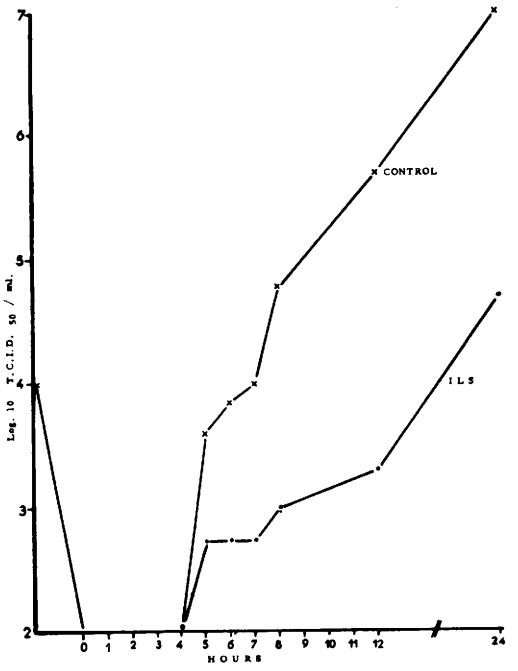

FIG. 1. Action of ILS on multiplication of Sindbis virus in tortoise cells, during the first cycle and during further cycles of viral multiplication, as measured by the production of extracellular virus. Rise of titer between 7-12 hr is probably related to lysis of initially infected cells. (Multiplicity of infection = 0, 25.)

At chosen intervals 0.5 ml samples of supernatant medium were taken and frozen at —80°C until titration.

ILS did not prevent adsorption of the virus to the cell. As shown in Fig. 1, at the end of the first cycle of viral multiplication significantly less infectious viral particles were released in the tissue culture by ILS treated cells when compared with controls. When further cycles were permitted to occur, the difference was equally large for the first 24 hours but decreased thereafter.

These results demonstrated that ILS acts within the cell during the latent period of the viral growth cycle.

The demonstration of anti-viral action in turtle cells enabled us to develop the following standard method for its titration. Serial 2-fold dilutions of ILS (1st tube 1/4) in culture medium, were added to lots of susceptible cells (4 tubes per dilution); after 24 hours, all tubes were inoculated with 1000

TABLE I. Species Specificity of ILS.

Type of cells	Species	Challenge virus	Results*
Amnion	Human	1000 $TCID_{50}$ Sindbis	0
KB	"	100 $TCID_{50}$ poliovirus Sabin 1	0
MA 104	Monkey	*Idem.*	0
Kidney	Guinea pig	1000 $TCID_{50}$ Sindbis	0
"	Rat	*Idem.*	0
Fibroblasts	Chick embryo	"	0
"	Mouse embryo	"	0
Kidney	Tortoise	"	+

* Total protection (+) or no protection (0) in presence of 1/4 dil. ILS.

IDTC of Sindbis virus and were placed at 37°C.

The end-point titer was calculated when infected control cells without ILS were completely destroyed. It is given as the highest dilution of ILS which protects 50% of the infected cells.

Species specificity. The sensitivity of different animal cells to the protective effect of tortoise cell ILS was tested as shown in Table I. Protection was observed only in homologous cells. In addition, tortoise kidney cells treated with interferon-like substances prepared in human amnion cells or leucocytes were not protected against 1000 $IDTC_{50}$ Sindbis virus. Furthermore tortoise cell ILS demonstrated activity in homologous cells with other viruses, *i.e.*, Sendaï, mumps, herpès simplex, and vaccinia. These data show that:

a) ILS protects only cells which derive from homologous species.

b) The cells are protected against a great variety of antigenically unrelated viruses.

Physico-chemical properties of ILS. Ether treatment: One part of ILS was mixed with one part of ether, shaken for 10 minutes at 4°C, and then centrifuged at 3000 r.p.m. for 10 minutes. The aqueous phase was separated and residual ether eliminated by passing nitrogen-gas through this fraction. In addition, the heat stability of ILS was determined by heating in an agitated water bath at 56°C for 30 minutes or at 100°C for 10 minutes.

Ether and heat treated ILS were titrated and results are presented in Table II. ILS was not affected by ether treatment, partly destroyed by heating at 56°C, but almost completely inactivated by heating at 100°C.

Effect of trypsin, RNA-ase, and DNA-ase on ILS. ILS was incubated at 37°C and pH 7, with 250 γ/ml of 2× crystallized trypsin (Laboratory Choay, Paris). After incubation trypsin activity was inhibited with 2500 units of Iniprol (Laboratory Choay, Paris). In parallel experiments ILS was treated with 100 γ per ml deoxyribonuclease (Worthington Biochemical Laboratories), and with 100 γ per ml ribonuclease (Laboratory Choay, Paris), for 2 hours at 37°. Table III shows that ILS was inactivated by trypsin and not by DNA-ase or RNA-ase.

Additional assays of ILS. Dialysis at pH 2 for 24 hours followed by dialysis at pH 7 for 24 hours did not diminish the ILS titer. The ILS titer was not affected by mixing with equal parts of Sendaï antiserum at 37°C for one hour. ILS did not lose its activity after filtration through 100 mμ millipore membrane. Direct incubation of ILS with Sindbis virus for ½ hour at room temperature did not decrease the infectivity of the virus.

Discussion. The data here presented show that virus-infected cells derived from a poikilothermic animal produce a viral inhibitory

TABLE II. Ether and Heat Treatment of ILS.

Treatment	ILS titer*
None	64
56°C 30 m	8
100°C 10 m	4
Ether	64

* Titers are expressed as a reciprocal of the dilution which inhibits completely the multiplication of the challenge virus.

TABLE III. Enzyme Effect on ILS.

Enzyme treatment	ILS titer*
None	16
Trypsin	4
DNA-ase	16
RNA-ase	16

* Titers are expressed as a reciprocal of the dilution which inhibits completely the multiplication of the challenge virus.

substance which blocks the intracellular multiplication of related and unrelated viruses.

This inhibitor was not neutralized by virus specific antisera, and it was readily separated from the inducing viral particles when the latter were sedimented by ultracentrifugation. It was destroyed by trypsin but not by RNA-ase or DNA-ase, consequently the activity can be related to a protein component present in the preparation. Under the conditions of the present experiments the inhibitory activity of the protein can be demonstrated only in cells of the same species and not in heterologous cells. These latter findings support the hypothesis that the information of the production of interferon-like substances is in the cellular and not in the viral genome.

The cellular origin and the site of action of the viral inhibitor here studied resemble interferon or other related viral inhibitory proteins demonstrated in homeothermic cells (1,2,3).

Since most of these products have not yet been obtained in a sufficiently pure form, no comparison can be made concerning their physico-chemical properties. Some of the more general properties such as heat and pH sensitivity are similar to the interferon described by Isaacs and Lindenmann in chick cells, while ether resistance recalls the interferon-like inhibitor produced by human malignant cells and described by Chany(3,12).

The fact that a poikilothermic animal cell produces an interferon-like substance supports the hypothesis that similar substances may be liberated by virus-infected cells lower in the evolutionary scale than birds and mammals; in addition it appears that these substances can be produced by any cell and not only by highly differentiated cells. If such is the case one might speculate that these substances might represent one of the more ancient defense mechanisms against viral parasitism.

Summary. An interferon-like substance liberated by tortoise kidney cells possesses some biological and physico-chemical properties previously described for interferons derived from virus-infected homeothermic animal cells. It is a cell-related protein which inhibits intracellular multiplication of related and unrelated viruses. The hypothesis that such substances might represent an early defense mechanism in phylogenetic evolution is discussed.

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