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Interferon Production *in vitro* by Cells Infected with the Murine Salivary Gland Virus.* (29625)

DONALD HENSON AND ROGER D. SMITH (Introduced by G. M. Hass)
Division of Pathology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Infection with the murine salivary gland virus (MSGV) characteristically results in a chronic inapparent infection evidenced by persistent demonstrable virus and intranuclear inclusions in the salivary glands of mice(1,2). On this basis it seems, therefore, that MSGV may serve as an ideal experimental tool for investigation of viral latency. Initial attempts to define the host-virus relationship have led to demonstration of an interfering substance produced *in vitro*.

In this report, the effect of this interfering substance on a heterologous virus as well as some of its properties are described. The results indicate that this interfering substance has many properties similar to those of interferon(3,4).

Materials and methods. Cell cultures. "L" cells, Strain 929, were serially propagated in 1-liter bottles using a growth medium composed of 95% Earle's balanced salt solution, amino acids and vitamins specified by Eagle (5), 5% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml Kantrex, and 10 μg/ml Fungizone. Monolayers were prepared by versenating the cells, resuspending in medium, and adding 1×10^6 cells to each 60 mm plastic Petri dish. Monolayers were used after 24 hours of incubation in an atmosphere of 5% CO_2 in air at 37°C.

Mouse embryonic fibroblasts, prepared by the method of Youngner(6) using 13-15 day-old embryos from SWR/J mice, were grown in 1-liter bottles using the same medium as for the "L" cells. Monolayer cultures in 60 mm Petri dishes were prepared by trypsinizing densely populated bottle cultures, resuspending the cells in medium, and then adding 1.5×10^6 cells to each dish. Cultures were used 24 to 48 hours later.

Viruses. Mice chronically infected with MSGV were obtained through the courtesy of Dr. Henry Pinkerton. Using a procedure similar to that reported by Smith(7), bottle cultures of mouse embryonic fibroblasts were

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readily infected and the virus serially passed as described(8). In tissue culture, MSGV was readily identified by its unique cytopathology(7,8). Viral interference studies were undertaken after the second passage.

Encephalomyocarditis virus (EMC, r^+) was kindly supplied by Dr. K. K. Takemoto. Stocks were prepared in "L" cells, divided into small aliquots, and stored at —20°C.

Virus assay. MSGV was assayed by the plaque method(8) except that the cultures were maintained in an atmosphere of 5% CO_2 in air and the *Tris* buffer was omitted. After 3 days, 2 ml of agar overlay medium was added on top of the initial overlay and, after 5 days, the cultures were stained with neutral red (1/20,000). Plaques were counted on the following day.

EMC was plaque assayed in "L" cell monolayers using 100 μg diethylaminoethyl (DEAE) dextran/ml (Pharmacia, Uppsala, Sweden) to facilitate plaque development (9). Monolayers were stained with neutral red (1/20,000) at 36 hours and the plaques counted at 48 hours.

Preparation of interferon. Bottle cultures of primary or secondary mouse embryonic fibroblasts were infected with approximately 1.0×10^5 plaque-forming units/ml of MSGV and incubated until all cells showed a cytopathic effect, usually 3-5 days. At this time, the culture medium was collected, centrifuged at 1500 rpm for 10 minutes and then used for interference studies, subsequently described. Control medium was obtained from uninfected bottle cultures of mouse embryonic fibroblasts carried in parallel and treated in a similar manner as the infected cultures.

Interferon assay. "L" cell monolayers were inoculated with approximately 0.001 to 0.0001 plaque-forming units of EMC per cell for 15 minutes after which time the cultures were washed once with Hanks' balanced salt solution. Then, 5 ml of medium from the MSGV infected bottle cultures (hereafter referred to as MSGV medium) were added to each plate. After 24 hours of incubation, the cultures were checked for cytopathic effect (CPE), frozen and thawed, and then titrated for EMC. Control cultures received 5 ml of the control medium from the uninfected bottle cultures. Additional controls consisting of fresh growth medium were also used in some experiments.

TABLE I. Result of MSGV Medium, Control Medium, and Fresh Growth Medium on 24 Hour EMC Titer and CPE.

	CPE*	PFUs/ml†
MSGV culture medium	1+	2.5×10^5
Control "	3+	5.0×10^6
Fresh growth "	3+	7.5×10^6

* Expressed as both size and number of infected foci, 1+ approximately 25% cells infected, 3+, about 75%.

† Plaque-forming units/ml.

Results. The effect of MSGV medium, control medium from uninfected bottle cultures, and fresh growth medium on the 24-hour EMC titer and CPE is shown in Table I. In all experiments, a consistent reduction in EMC titer was obtained using the medium from two separately isolated strains of MSGV.

Serial 2-fold dilutions of the MSGV medium in fresh growth medium resulted in a proportional increase in the titer of EMC, suggesting that its inhibitory effect was not the result of a missing growth factor but due to the serial dilution of an interfering substance. Significant interference was still detected even after the MSGV medium was diluted 1/16. In most of the subsequent experiments the MSGV medium was diluted with equal volumes of fresh growth medium prior to interference studies.

To rule out the possibility of MSGV infection and multiplication in "L" cells as the interfering mechanism, "L" cells were frozen at various times following inoculation with high titers of MSGV. After 24, 48, and 72 hours of incubation, no CPE was observed nor could MSGV replication be demonstrated by back titration in mouse embryonic fibroblasts. To test for possible interferon production by "L" cells when exposed to MSGV, "L" cell monolayers were incubated with high titer MSGV medium for 24 hours. Then, after freeze-thawing, the medium was collected and assayed for interferon as described. The results indicated that "L" cells

TABLE II. Effect of Ultracentrifugation, Antibody, and Acid on MSGV and MSGV-Induced Interferon.

	EMC titer*	MSGV titer*
Control medium	5.0×10^6	—
MSGV medium	4.7×10^5	1.0×10^5
MSGV medium 90,000 × *g*	2.0×10^5	8.6×10^4
Idem + MSGV antiserum	2.0×10^5	0
Control medium 90,000 × *g* + MSGV antiserum	1.3×10^6	—
MSGV medium dialyzed at pH 2	5.0×10^5	0
Control medium dialyzed at pH 2	3.2×10^6	—
MSGV medium + .1 g% trypsin	7.5×10^6	0
Control medium + .1 g% trypsin	3.0×10^6	—

* Plaque-forming units/ml.

after 24 hours exposure to high titer MSGV medium did not produce any detectable increase in interferon over and above that initially present in the medium. Thus the decrease in EMC titer was not the result of MSGV replication nor detectable interferon production by the "L" cells.

Effect of pretreatment. "L" cell monolayers pretreated with control medium or MSGV medium for 18 hours were washed once and infected with EMC as described. After washing with Hanks' solution, 5 ml of fresh medium was added to each of the cultures and the EMC titered after 24 hours. The reduction in the EMC titer was of the same order of magnitude as in cultures which received MSGV medium immediately after infection.

Effects of ultracentrifugation and MSGV antibody. Aliquots of MSGV medium and control medium were centrifuged for 2 periods of 60 minutes each at 90,000 × *g* in the model L Spinco ultracentrifuge. Samples of the supernatant were then treated with MSGV neutralizing antiserum obtained from chronically infected mice. Titration of residual MSGV in the supernatant demonstrated marked reduction of the virus following centrifugation and complete elimination following addition of the antiserum. The results (Table II) indicated that the interfering material when tested as described was not sedimented in the ultracentrifuge nor affected with anti-MSGV serum.

Effects of acid, trypsin and heat. Additional evidence for the non-viral identity of the interfering substance was obtained following treatment at pH 2. Aliquots of MSGV medium and control medium were dialyzed for 16 hours against 0.01 N HCl in isotonic saline at 4°C and then against 2 changes of phosphate-buffered saline adjusted to pH 7.2. Titration of the MSGV before and after acidification revealed complete inactivation of the virus following dialysis at pH 2. Addition of this acid treated medium to "L" cells immediately after the 15-minute EMC adsorption period produced approximately the same reduction in the 24-hour EMC yield as untreated MSGV medium (Table II).

Treatment of the MSGV medium with 0.1 gram% trypsin for 8 hours at 37°C not only eliminated MSGV but also inactivated the interfering substance (Table II).

Heating the MSGV medium at 60°C in a water bath for one hour resulted in a marked reduction in interferon activity and complete inactivation of MSGV.

Course of interferon production. Monolayer cultures of mouse embryonic fibroblasts in 60 mm Petri dishes were inoculated with 1.5×10^4 plaque-forming units of MSGV. Following a 60-minute adsorption period the cultures were washed once and then 5 ml of fresh medium added to each dish. Control plates were treated in a similar manner, but not infected with MSGV. At various times after infection, both MSGV infected and control plates were frozen and the medium from the infected plates subsequently assayed for MSGV and interferon activity in the usual manner, using medium from the uninfected monolayers as control medium. Fig. 1 shows the corresponding increase in MSGV and interferon activity.

Interferon titration by plaque reduction. The following experiments indicate that a plaque reduction method(10) can be utilized to titrate the interferon. "L" cell monolayers were incubated with either MSGV medium or control medium, 5 ml being used in each

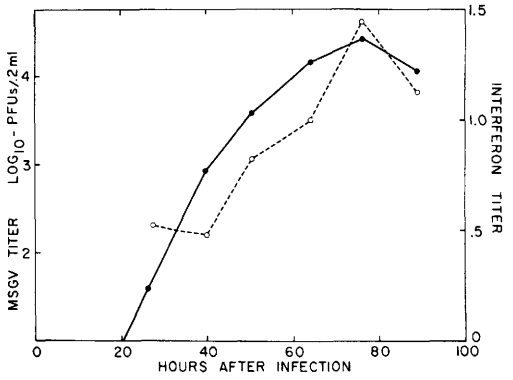

FIG. 1. Increase of interferon activity with increasing titer of MSGV. Solid line—MSGV titer, dashed line—interferon titer. Interferon was assayed as described in Materials and Methods. Interferon titer is expressed as the $\log_{10}$ of the ratio of the EMC titer obtained on control medium to the EMC titer obtained on MSGV medium.

plate. After 18 hours, the media were removed and the plates infected with approximately 40 plaque-forming units of EMC in 0.2 ml of Hanks' balanced salt solution. After 20 minutes of adsorption, the monolayers were covered with 5 ml of agar overlay medium containing 100 μg/ml DEAE, stained 36 hours later, and the plaques counted at 48 hours. The results are presented in Table III. In addition to the marked reduction in count, plaques in the experimental group were approximately half the diameter of those in control plates.

TABLE III. Reduction in Number of EMC Plaques in "L" Cell Monolayers Pretreated for 18 Hours with MSGV Culture Medium.

	Plaques per plate		
Plate No.	I	II	III
Control medium	35	44	40
MSGV "	12	10	12

Summary. Using a heterologous virus-cell system, an EMC virus interfering substance having many properties similar to those of interferon(3,4) was detected in the growth medium from mouse embryo cell cultures infected with the murine salivary gland virus. The interfering material was non-dialyzable, heat labile at 60°C for one hour, stable at pH 2, not affected by MSGV antiserum, not sedimented at 90,000 × *g*, and was active in a cell system unable to support MSGV replication. In infected cultures, the rise in MSGV titer was associated with a corresponding rise in the interferon activity. Efforts to relate interferon production to latency *in vivo* are in progress.

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Evidence for a Non-Calorigenic Effect of Thyroxin on Erythropoiesis as Judged by Radioiron Utilization.* (29626)

HOWARD A. MEINEKE AND ROGER C. CRAFTS†
Department of Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio

It seems certain that the thyroid plays a role in the process of erythropoiesis. Most workers have correlated this effect with the well known role of the thyroid gland in regu-

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