Cytotoxic Action of Influenza Virus. Failure to Induce Acquired Resistance Phenomenon in Tissue Culture.* (21986)

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A number of substances modify the toxic action of influenza and related viruses in the intact animal. Sublethal doses of homologous or heterologous virus injected 24 hours prior to the challenge toxic inoculum prevent fever and lymphopenia in rabbits(1), convulsions in mice(2), and hemorrhagic encephalitis in chick embryos(3). Pretreatment of animals with *Cholera vibrio* filtrates also inhibits the pyrogenic(4) and neurotoxic(2) action of influenza viruses. Xerosin, a microbial product derived from *Achromobacter xerosis*, has the most profound inhibitory effect on the toxic pneumonitis(5) and encephalitis(6) caused by influenza and Newcastle disease viruses.

Henle, Girardi, and Henle(7) recently demonstrated that 4 strains of influenza virus produce a non-transmissible cytopathogenic effect in tissue cultures of a human epidermoid carcinoma (strain HeLa, Gey). Replication of infectious virus particles could not be demonstrated in this system but an increase in cellular non-infectious hemagglutinins occurred similar to the formation of "incomplete" virus in mouse brain that accompanies the neurotoxic action of influenza virus(8). This *in vitro* response of HeLa cells afforded a convenient method for determining whether biological inhibitors of influenza virus toxicity acted by interfering directly with host cell-virus interaction.

Materials. HeLa cell culture tubes were obtained from Microbiological Associates Inc. and incubated for an additional 24 hours at 35°-36°C before replacing the growth medium. The cells were then washed 3 times with 1 ml volumes of Hanks' balanced salt solution and the medium replaced with 0.8 ml of Scherer's maintenance solution and 0.2 ml of horse serum. After an additional 3 days of incubation a final change of medium was made to 10% horse serum-maintenance solution prior to inoculation of the cultures. Intact sheets of healthy cells usually persisted in control tubes for at least 3-4 days longer, the duration of the experiment. *Virus* preparations with high toxic activity were prepared by allantoic inoculation of 10-11-day-old chick embryos with a 10^{-6} dilution of seed material. After incubation for 48-72 hours at 35.5°C the allantoic fluids were harvested, pooled, tested for hemagglutinin (HA) content and stored at −20°C. It was not necessary to concentrate these preparations but since their cytotoxic action diminished on storage, fresh material was prepared frequently. To obtain virus preparations of lower toxicity, eggs were inoculated with undiluted seed(9) and incubated for 72 hours. Most experiments were carried out with an egg-passage line of the Lee strain of influenza B virus. Certain comparisons were made with the mouse-pathogenic WS strain and the mouse-unadapted A/England/1/51 strain of influenza A virus. *Cholera filtrate* was prepared from the 4Z strain of *V. cholerae* and used both as the crude filtrate and concentrated, purified material with receptor-destroying enzyme (RDE) activity (10) of 5,000 units/ml. *Xerosin* was kindly supplied by Dr. Vincent Groupé. It was

TABLE I. Cytotoxic Effect on HeLa Cell Cultures of Lee Virus Added to the Medium 24 Hr after Cholera Filtrate or Xerosin.

	Controls (NAF)	Lee virus
Distilled water	—	4, 4, 4, 4, 4
RDE, 10 units	0, 1, 0, 0, 0*	4, 4, 4, 4, 4
" , 100 "	0, 0, 0, 0, 0	4, 4, 4, 4, 4
Xerosin, 100 μg	0, 0, 0, 0, 0	2, 4, 4, 4, 4
" , 1000 "	4, ±, 0, 1, 0	4, 4, 4, 4, 3

* 0, no cytopathogenic effect in 48 hr. 4, all cells destroyed. ± to 3, intermediate stages of cellular destruction.

* This study was supported by a research grant from the National Institutes of Health, Public Health Service.

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TABLE II. Influence of Subtoxic Virus, Cholera Filtrate and Xerosin Added to HeLa Cell Cultures 1 Hr before a 60% Cytotoxic Dose of Lee Virus.

	Controls (NAF)	Lee virus
Distilled water	0, 0, 0, 0	3, 2, 4, 2, ± 1, 3, 1, 2, 4
Subtoxic Lee virus	0, 0, 0, 0	3, 2, 4, 4, 4
RDE, 100 units	0, 0, 0, 0, 0	2, 3, 1, 1, 1
Xerosin, 1000 μg	0, 0, 0, 0	3, 3, 3, 4, 3

Read on scale of 0-4+ cytopathogenicity 72 hr after challenge.

dissolved in distilled water to the desired concentration and sterilized by autoclaving.

Results. Inoculation of HeLa cell cultures with 0.1 ml of undiluted "toxic" Lee allantoic fluid produced characteristic cytopathogenic effects. In 24 hours the cells were usually maintained as an intact sheet but islands of rounded cells with granular cytoplasm could be seen. By 48 hours after virus inoculation a considerable amount of destruction and clumping of cells was noted and by 72 hours most of the cells had dropped off the glass and could be seen floating in the medium. A 10-fold dilution of the Lee virus inoculum resulted in delayed and incomplete cytopathogenic effects and with a 1:100 dilution most of the cultures were unaffected. Allantoic fluid preparations of Lee virus made from undiluted seed, but possessing the same hemagglutinating activity as the "toxic" virus, were approximately one-tenth as potent.

Various concentrations of "subtoxic" virus, cholera filtrate and xerosin were incorporated into the maintenance medium, allowed to react with the cells, and the cultures challenged one or 24 hours later with 0.1 ml of toxic Lee virus. "Positive" virus controls consisted of tubes incubated in horse serum-maintenance solution with equivalent amounts of distilled water; "negative" controls, containing the same concentrations of test materials, were "challenged" with 0.1 ml of normal allantoic fluid (NAF). Distilled water, 200 AD of subtoxic virus, cholera filtrate and NAF had no effect on the microscopic appearance of the HeLa cells even after prolonged incubation. Minimal, sporadic cellular changes were observed in tubes containing 1 mg of xerosin, but these could be readily differentiated from typical virus pathogenicity. Ordinarily 5 or more tubes were used for each concentration of test material and "blind" readings made every day for 4 days after virus challenge. The extent of cellular damage was expressed on a scale of 0 to 4+. The numbers of test cultures are not always equal because it was necessary to discard an occasional contaminated tube.

Table I shows that the presence in the media of high concentrations of cholera filtrate and xerosin for 24 hours prior to Lee virus challenge did not inhibit cytotoxicity. In another experiment, a series of HeLa cultures were exposed to 200 AD of sub-toxic virus, 100 RDE units of cholera filtrate or 1 mg of xerosin and challenged one hour later with a preparation of Lee virus that produced only a 60% cytopathogenic effect in 72 hours. Under these conditions, it was again noted that these materials were ineffective as anticytotoxic agents (Table II). Lastly, in order to afford the most favorable conditions for demonstrating potential inhibition of cytotoxic action, a dose of toxic Lee virus was used that was capable of producing only 1+ cytopathogenicity in 72 hours. Cell cultures incubated for 24 hours with subtoxic Lee virus and xerosin were challenged with this minimally effective concentration of Lee virus. As shown in Table III, not only was there no inhibition of cellular damage, but pretreatment of cells with subtoxic virus or as little as 1 μg of xerosin enhanced the cytotoxic action of Lee virus in the 72-hour cultures. Even washing the cells free of xerosin and replacing the maintenance solution prior to virus inoculation rendered the cells more susceptible to the cytotoxic effects.

Many of these experiments were repeated with the WS and A/England/1/51 strains of influenza A virus. Although these viruses had more limited cytopathogenic action than the Lee strain, it was again noted that homologous or heterologous subtoxic virus, 100 RDE units of cholera filtrate (purified and crude) and 100-1000 μg of xerosin were incapable of preventing their *in vitro* cytotoxicity. In contrast, all of these materials effectively counteracted the neurotoxic action of the 3 strains of influenza virus. Mice injected intracerebrally with 200 AD of virus, 50 RDE units of cholera filtrate, or 30 μg of xerosin were highly re-

TABLE III. Apparent Enhancement of Minimally Cytotoxic Lee Virus by Subtoxic Virus and Xerosin.

	Control readings		Lee virus readings	
	72 hr	96 hr	72 hr	96 hr
Distilled water	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 1, 1, 1	4, 3, 3, 4, 3
Subtoxic Lee virus	0, 0, 0, 0, 0	0, 0, 0, 0, 1	3, 1, 1, 3, 2	4, 4, 2, 4, 4
Xerosin, 1 μg	0, 0, 0, 0	0, 0, 0, 0	4, 3, 4, 4, 4	4, 4, 4, 4, 4
" , 1 " (washed)*	—	—	3, 3, 4, 4	4, 4, 4, 4

* Cultures incubated with 1 μg of xerosin for 24 hr and washed 3 times before virus challenge.

sistant to the convulsive and lethal effects of 5 LD_{50} of influenza virus administered 24 hours later.

Discussion. In the original studies(1) on acquired resistance of the intact host to virus toxins, the evidence seemed to support a local alteration in cellular susceptibility as the mechanism of action. This conclusion was based in part on the known capacity of virus and cholera filtrate to destroy the cell membrane receptors for influenza viruses(10). However, it was subsequently demonstrated that the degree of tolerance to the fever-producing(11,12) and neurotoxic(2) effects of influenza and mumps virus was not dependent on the enzymic action or receptor-gradient position of the protective virus. In addition, it is now quite clear that the antitoxic property of cholera filtrate is unrelated to receptor-destroying enzyme. Wagner(2) intimated that partially purified RDE was less effective in producing resistance to virus neurotoxin than crude cholera filtrate and French(11) proved that highly purified, concentrated RDE had no antipyrogenic activity. Finally, Groupé and coworkers(13) conclusively ruled out RDE as the antineurotoxic principle by demonstrating that autoclaved cholera filtrate, devoid of receptor-destroying activity, was almost as effective as unheated cholera filtrate. In spite of these studies, the likelihood persisted that resistance to virus neurotoxicity was a local cellular phenomenon since the protective effects of both subtoxic virus and cholera filtrate were manifested only by intracerebral inoculation(2).

Unlike influenza virus and cholera filtrate, xerosin does not affect cellular virus receptors(5) and induces some tolerance to toxic pneumonitis(5) and encephalitis(6) when administered parenterally subsequent to virus challenge. Nevertheless, it is most effective when injected intracerebrally 24 hours before influenza virus and the duration of tolerance is strikingly similar to that produced by subtoxic virus and cholera filtrate. For these reasons, Groupé and Hermann(6) postulated that xerosin might have two modes of action, one directly on host cell-virus interaction and the other indirectly on the host response. However, studies on xerosin inhibition of toxic pneumonitis led Ginsberg(14) to conclude that its influence was exerted purely by bolstering host resistance. He found that xerosin was effective by the subcutaneous route, and that it did not suppress the formation of "incomplete" virus nor prevent virus destruction of bronchiolar epithelium. Lastly, Ginsberg emphasized the nonspecific nature of xerosin-induced resistance by showing that it protected mice again non-viral, chemical pneumonitis.

The present studies demonstrated that the cytotoxic effects of influenza virus on HeLa cell cultures were not diminished by doses of subtoxic virus, cholera filtrate or xerosin in excess of those required to protect mice against viral neurotoxic action. The tissue culture methods assured direct exposure of susceptible cells to high concentrations of presumed antitoxic substances. If it can be assumed that the neurotoxic and cytopathogenic actions of influenza virus are related phenomena, it would appear that tolerance of the intact host is not due to local alteration in cellular susceptibility. It is apparent that the whole question of the mechanisms of acquired resistance to virus toxic action requires reexamination.

Summary. Subtoxic virus, cholera filtrate

and xerosin failed to protect HeLa cell cultures against the *in vitro* cytopathogenic effects of the Lee strain of influenza B virus and the WS and A/England/1/51 strains of influenza A virus. Smaller doses of these materials effectively rendered mice tolerant to the neurotoxic action of influenza viruses. The possible implications of these studies on the mechanisms by which the intact host acquires resistance to virus toxic action are discussed.

1. Bennett, I. L., Jr., Wagner, R. R., and LeQuire, V. S., *J. Exp. Med.*, 1949, v90, 335.

2. Wagner, R. R., *Brit. J. Exp. Path.*, 1952, v33, 157.

3. Burnet, F. M., and Fraser, K. B., *Aust. J. Exp Biol. M. Sc.*, 1952, v30, 447.

4. Wagner, R. R., and Bennett, I. L., Jr., *J. Exp. Med.*, 1950, v91, 135.

5. Groupé, V., Pugh, L. H., and Levine, A. S., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 710.

6. Groupé, V., and Hermann, E. C., Jr., *J. Immunol.*, 1955, v74, 249.

7. Henle, G., Girardi, A., and Henle, W., *J. Exp. Med.*, 1955, v101, 25.

8. Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 541.

9. McKee, A. P., *J. Immunol.*, 1951, v66, 151.

10. Burnet, F. M., and Stone, J. D., *Aust. J. Exp. Biol. M. Sc.*, 1947, v25, 227.

11. French, E. L., *ibid.*, 1952, v30, 479.

12. Wagner, R. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 612.

13. Groupé, V., Hermann, E. C., Jr., and Daugherty, R. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 636.

14. Ginsberg, H. S., *Fed. Proc.*, 1954, v13, 494.

Received August 24, 1955. P.S.E.B.M., 1955, v90.

Effect of Epinephrine Upon Level of Plasma Amino Acids in the Eviscerate Rat. (21987)

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Some hormones affect the level of plasma amino acids in the eviscerate animal. The effect of epinephrine upon the level of plasma amino acids has not been studied in the eviscerate preparation although it has an effect in the intact animal(1). In the present study of eviscerate rats, doses of epinephrine which had striking effects upon glucose tolerance did not affect the level of plasma amino acids in either the presence or absence of insulin until large doses of epinephrine were administered with glucose and insulin for 18 hours.

Methods. Male rats of the Sprague-Dawley strain were fed Rockland rat diet. At a weight of 250 ± 5 g non-fasted rats were anesthetized with cyclopal sodium and functionally eviscerated. The intestinal tract was removed but the liver remained *in situ* after its arterial and portal vessels were tied. Continuous intravenous infusions of solutions of glucose with and without insulin and with and without epinephrine (Parke-Davis Adrenalin Chloride) were made by a continuous injection machine. The fluid was delivered into the saphenous vein of the right hind leg at the rate of 20 cc/24 hours/rat. Six rats were infused at a time so that the control and experimental animals were studied simultaneously. Temperature was constant at 26° ± 0.50C.

At the end of the infusion period the blood was drained from the abdominal aorta by cannula. Heparin was used as an anticoagulant. The level of plasma amino acids was determined by the method of Hamilton and Van Slyke(2) and that of blood glucose by the method of Miller and Van Slyke(3).

Results. The conditions of each experiment and the results are shown in Table I. In agreement with earlier reports(1) insulin suppresses the rate of rise of plasma amino acids following evisceration in the rat. During a 3-hour period epinephrine failed to affect the level of plasma amino acids in either the presence or absence of insulin although in the presence of insulin and a glucose load of 70 mg/100 g of rat per hour the