Effect of Sialidase Antiserum on Toxicity of Influenza Virus.* (30040)

J. T. SETO (Introduced by A. F. Rasmussen)
Department of Microbiology, California State College, Los Angeles

During an investigation of the possible relation between toxicity of influenza A virus and sialidase (neuraminidase) activity(1), the effect of sialidase antiserum on cytotoxicity and neurotoxicity was determined. Our resultant discovery that sialidase antiserum neutralized viral neurotoxicity but enhanced toxicity of virus for cell cultures is reported here.

Materials and methods. Viruses. A2/Japan 305/57, A/PR8/34, and two A2 substrains (RI/5+ and RI/5− obtained from Dr. P. W. Choppin) of influenza virus were used. Virus preparations, hemagglutination inhibition (HAI), toxicity tests, and enzyme analyses were carried out as previously described (1,2,3,4). Two cell culture lines, HeLa and BS-C1 obtained from Dr. D. T. Imagawa and H. Hopps respectively, were used. HeLa cells were maintained in calf serum media(5) and BS-C1 cells in the presence of fetal bovine serum(6).

Antisera. Antisera were prepared in rabbits by immunizing them with soluble sialidase from A2 virus as described previously (3). Rabbits were immunized with purified enzyme plus incomplete Freund's adjuvant; enzyme was purified in Sephadex G75 collumns or in polyacrylamide gel columns(3). For the latter, gels were segmented(3) and homogenized in saline (5-20 ml) in a Ten Brock tissue grinder. This material was given intraperitoneally. Antiegg protein antiserum was prepared by injecting rabbits with normal egg protein from 10-12-day eggs; the egg protein was dialyzed, lyophilized, and resuspended in saline prior to immunization. Antisialidase activities of the antisera were measured by adding 0.2 ml of serial 10-fold dilutions of serum to 0.2 ml of soluble enzyme, containing approximately 0.01 of a unit per ml. The reaction mixtures were allowed to stand at room temperature for 30 minutes in the reaction tubes, after which 0.1 ml of 2 mg per ml urine mucoprotein substrate was added to the tubes. Substrate was prepared as described(7). The enzyme reaction was allowed to proceed for 16-18 hours and analyzed for free sialic acid by the thiobarbituric acid method(8).

Results. Three antisialidase sera, designated as AS10, AS614, and AS624, and an egg protein antiserum (AS3) are listed in Table I along with their HAI and enzyme inhibition activities. All but AS3 had HAI activity thus indicating that the sialidase used for immunization contained some residual V antigen. The three antisialidase sera (10^{-3} dilution) inhibited enzyme activity 80-95%. These antisera also contained egg protein antibodies. One of the undiluted antisera (AS10) inhibited 78% of the enzyme activity of 0.1 ml of concentrated virus, the amount of virus used in cytotoxicity assays. Further characterization of sialidase antisera will be published elsewhere.

Results representative of repeated experiments on the influence of antisialidase antiserum on the toxicity of influenza virus are given in Table II. It can be seen that A2 sialidase antiserum enhanced the cytotoxicity of A2 virus in HeLa cells but inhibited the neurotoxicity of A2 virus in mice. That the cytotoxicity is the result of the interaction of sialidase and sialidase antisera is shown by: (1) antiegg protein serum (AS3) was

TABLE I. Hemagglutination Inhibition Titers and Enzyme Inhibition Titers of 3 Antisialidase Antisera and Egg Protein Antiserum.

Serum	HAI	Enzyme inhibition	
		Inhibition	Serum dilution
		(%)	
AS3*	1-10	0	undiluted
AS10	1-320	95	10-3
AS614†	1-160	80	"
AS624	"	95	"

* Egg protein antiserum, the others are sialidase antisera.

† Prepared with enzyme purified in polyacrylamide gel columns.

* This investigation was supported by USPHS Research Grant AI-05929-01 from Nat. Inst. of Allergy & Infect. Dis.

TABLE II. Cytotoxicity, Neurotoxicity of A2/Japan 305/57 and A/PR8/34 of Influenza in the Presence of Antisera.

Virus and serum*	Cytotoxicity†	Neurotoxicity
A2	2+	10/10‡
A2 + AS10	4+	0/10
A2 + AS614	4+	0/5
A2 + AS624	4+	0/5
A2 + AS3	—	5/5
PR8	2+	0§
PR8 + AS10	2+	0
PR8 + AS624	2+	0
PR8 + AS3	2+	0

* One part undiluted serum added to one part virus, allowed to stand 30 min at room temperature prior to injection of mice or inoculation of cell cultures. AS3 is egg protein antiserum and the others are sialidase antisera.

† + positive response. – no response.

‡ Deaths over total mice injected.

§ 0 Not done.

without effect on the toxicity of the virus; (2) the cytotoxicity of a classical A prototype strain, PR8, was not affected in any way by either AS3 or by AS10 and AS614 sialidase antisera; (3) of 2 substrains of subtype A2 virus tested, RI/5+ and RI/5—, the cytotoxicity of RI/5— was slightly enhanced while the toxicity of RI/5+ was not changed by AS10 antiserum and (4) purified enzyme alone was not toxic to HeLa cells (or to mice by intracerebral injection) at the concentrations tested, which were between 0.1 and 1.0 unit.

Further studies carried out with antiserum AS10 indicate that the enhancing factor of cytotoxicity in this serum was not removed by absorbing it with normal egg protein or by heating at 56°C for 30 minutes. Cytotoxicity of suboptimal toxic concentration of virus was also enhanced by undiluted serum as well as serum diluted 10-fold. Normally, virus was added to antiserum and incubated for 30 minutes then introduced into cell cultures. If virus was added first and followed by antiserum, enhanced toxicity also resulted. The enhanced cytotoxicity conferred by sialidase antisera was not restricted to HeLa cells; similar effects were noted in BS-C1 cells, a continuous cell line of vervet monkey kidney cells(6).

Representative photomicrographs of the enhanced cytotoxicity of A2 virus plus sialidase antiserum in HeLa cell tube cultures are shown in Fig. 1. This series of photographs shows the effect of a subtoxic dose of virus and undiluted AS10 antiserum. The nature of the cellular damage resembles that described previously(9). In relation to the time course of the cellular destruction, slight cellular changes were observed after 24 hours' incubation and pronounced changes after 48 hours' incubation.

Discussion. The phenomenon of enhanced cytotoxicity to cell cultures by A2 virus and sialidase antiserum may be in a general sense analogous to the recent observations of cellular damage in tissue cultures by soluble antigen-antibody complexes(10) and enhancement of the cell fusion reaction by other myxoviruses and their antisera(11). However, our results differ in the following respects: 1) the time course of the reaction was not as rapid as shown with soluble complexes(10); 2) the effect noted was specific for A2 virus and its homologous high titer antiserum, whereas the cell fusion reaction was enhanced only by "low titer" and not "high titer" antiserum and by non-specific as well as specific serum(11); 3) gross cytological changes in cell cultures were distinctively different from the above observations (10,11).

Since the sialidase antisera contained V antibodies (HAI activity), the possible influence of V antigen-antibody complexes was considered. The lack of effect of AS10 on the toxicity of RI/5+ and the slight enhancement of the toxicity of RI/5— would lend support in ruling out the role of V antigen-antibody complexes in enhancing cytotoxicity. As RI/5+ substrain is highly sensitive to antibodies and RI/5— substrain is relatively insensitive to antibodies(4), it would be of vital interest to know if the difference in sensitivities to virus neutralizing antibodies of these substrains applies as well to antibodies prepared against their sialidases. In addition, in view of the presence of egg proteins in virus concentrates and egg protein antibodies in the sialidase antisera, soluble antigen-antibody complexes could form and cause increased cellular damage (11). However, this seems unlikely since sialidase antiserum with the egg protein antibodies absorbed out still retained their cytotoxic enhancing factor. Likewise, sialidase

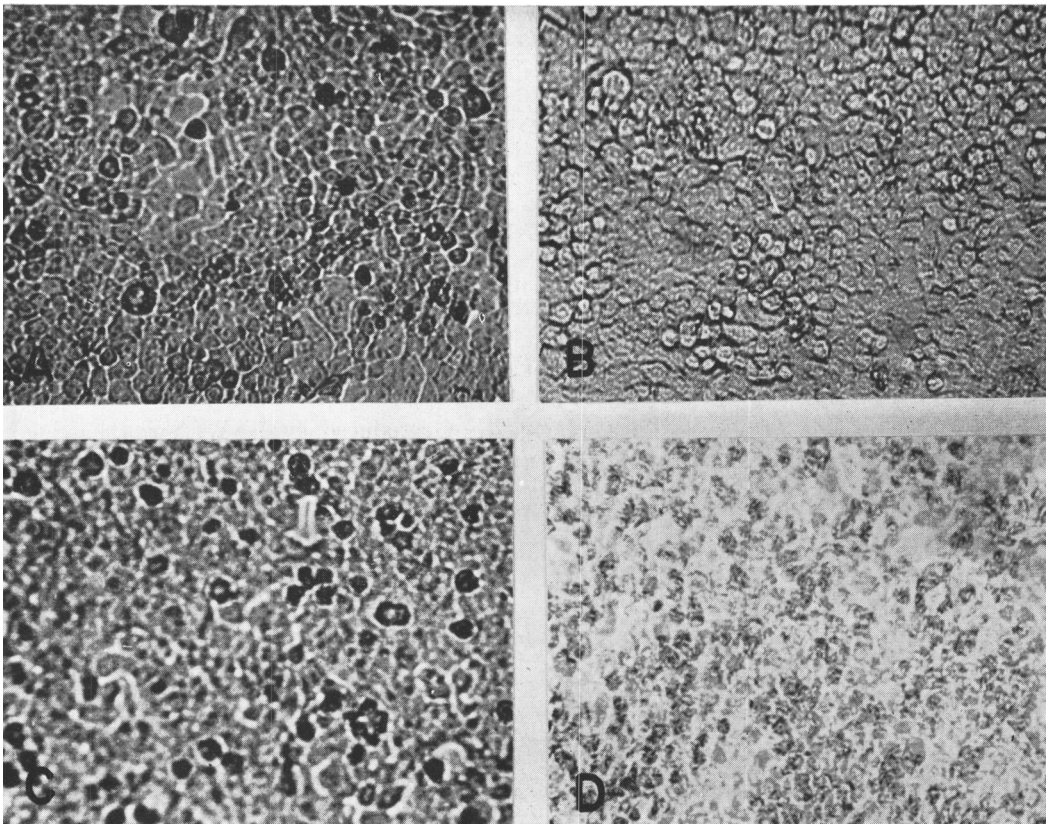

FIG. 1. Cytotoxicity of A2 strain of influenza to HeLa cells after 48 hr incubation. (A) Normal HeLa cells. (B) Cells and antisialidase serum AS10. (C) Cells and A2 virus. (D) Cells, A2 virus and AS10. Tube cultures ×100.

antiserum absorbed with "purified" sialidase continued to enhance cytotoxicity. Thus, the toxin enhancing factor in sialidase antiserum remains to be characterized.

The lack of inhibition of the cytotoxicity of cell cultures and the inhibition of neurotoxicity for mice by sialidase antiserum may be interpreted as due to differences in viral enzyme "receptors" of brain tissue and in the "receptors" of cell cultures or that the mechanism of cytotoxicity and neurotoxicity are completely dissimilar. On the other hand, if the enzyme was solely responsible for inducing neurotoxicity, purified enzyme should be neurotoxic, which has not been demonstrated. The lack of inhibition of cytotoxicity by sialidase serum provides further evidence against participation of sialidase in the toxicity of influenza virus(1). This evidence of enhanced cytotoxicity of HeLa cells by A2 influenza virus and its homologous sialidase antiserum provides a simple *in vitro* system for further investigation of mechanisms of cell injury in microbial and immunologic disease. This system or an analogous cell or tissue culture system may also be applicable for studying the factors causing side reactions following influenza vaccines.

Summary. Sialidase antiserum inhibited neurotoxicity but enhanced cytotoxicity of influenza virus *in vivo* and *in vitro* respectively.

The technical assistance of K. Okuda and discussion of the results with Dr. A. F. Rasmussen is gratefully acknowledged.

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Received December 22, 1964. P.S.E.B.M., 1965, v118.

Effects of Trypsin on Ultraviolet Irradiated-Myosin A.*† (30041)

GEORGE KALDOR AND SANDRA WEINBACH (Introduced by B. W. Volk)
Department of Physical Chemistry, the Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn, N. Y.

It had been reported previously that U.V. irradiation of myosin A led to the formation of dimers and that it also caused alterations in enzymatic activity of the protein(1). To obtain further information about the changes induced by U.V., irradiated protein was subjected to trypsin digestion and the products of this treatment were studied by ultracentrifugation, diffusion, electrophoresis and ATPase measurements.

Materials and methods. Myosin A was prepared as described by Mommaerts(2). The experimental techniques of ultracentrifugation, diffusion and electrophoresis, as well as ATPase determination have been described(1). U.V. irradiation was performed in a cold room (2-4°C) on a 3.2 mm thick (10 mg/ml) myosin A layer under constant slow mechanical stirring. Myosin A was dissolved in a solution containing 0.5 M KCl and 0.1 M tris buffer at pH 7.5. The output of the G15R8 General Electric germicidal lamp was measured by a dose-rate meter(3) and was found to be 8.54×10^{14} quanta/cm^2 second. All samples were spun after irradiation in a Spinco model L ultracentrifuge at 20,000 RPM for 45 minutes. Trypsin digestion was performed at room temperature (23 ± 1°C) for 60 minutes under constant mechanical stirring. The concentration of the myosin A was 7.5 mg/ml and that of trypsin, 0.02 mg/ml. The digestion was stopped by adding 0.03 mg/ml soybean trypsin inhibitor to the mixture. The solubility of the proteins at various ammonium sulphate concentrations was measured as published by Szent-Gyorgyi(4) and the amount of the 5% TCA soluble fraction as described by Mihalyi(5).

Results. After 60 minutes of trypsin digestion of native myosin, the well-known peaks of the heavy and light meromyosins were demonstrated in the analytical ultracentrifuge(5,6,7,8). If, however, the 60-minute irradiated-myosin A was digested by trypsin under identical conditions, a different ultracentrifugal pattern was obtained. Instead of 2 peaks, only one broad peak was observed which migrated with approximately the same velocity as did the slower-moving component (light meromysin) of the digested native myosin A. If actin was also added to the digested myosin (actin:myosin, 1:2, w/w) the faster moving peak disappeared. No change was observed, however, if actin was mixed in a similar proportion with the irradiated-digested myosin A. These experiments showed that while the heavy meromyosin fraction of myosin A retained, even after 60 minutes of trypsin digestion, its actin binding capacity(4,8), the split products of the irradiated-digested myosin have lost this property completely. The preservation of the actin

* This work was supported by N.I.H. grant NB-03600-09.

† The following abbreviations have been used: U.V.-ultraviolet; TCA - trichloracetic acid; ATPase - adenosinetriphosphatase.