The Effect of Trypsin on Measles Virus Antigenicity. (26980)

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Recent studies in this laboratory have been directed toward the concentration and purification of measles virus. In the purification studies, enzymatic digestion, which has been used in a number of attempts to purify other viruses(1-5), was investigated. Trypsin, in particular, has been used effectively(2-4). Gresser and Enders(6) have noted the destruction of infectivity and the hemagglutinins of some myxoviruses by trypsin. The experiments reported here demonstrated the deleterious effects of trypsin on the capacity of measles preparations to evoke neutralizing and complement fixing antibodies in guinea pigs.

Materials and methods. Virus. The Philadelphia 26 strain of measles virus, isolated in this laboratory(7), was used for preparation of measles virus-infected fluids in grivet monkey kidney cells. This strain was also passed in stable human amnion cells (8) and used as challenge virus in the serum neutralization test.

Tissue cultures. Trypsinized primary monkey kidney epithelial cultures were grown in medium 199 with 2% calf serum. Bottles were re-fed with the same medium on the fifth or sixth day and infected with measles virus on the seventh day. Following infection, cell cultures were maintained in Eagle's basal medium prepared with Hanks' salt solution (HBME) without serum.

TCF. Fluid was drawn off from settled cell debris on seventh day post inoculation.

Whole TCF. Fluid plus cell debris, seventh day post inoculation.

Serum neutralization test. Stable human amnion cell cultures(8) were used for serum neutralization tests. The cells were dispensed in 1 ml amounts, 35,000 cells per ml, in a medium consisting of Eagle's basal medium prepared with Earle's salt solution (EBME) with 5% calf serum and tubes incubated in stationary racks. The following day the medium was removed and replaced with 2 ml of fresh medium. Serial dilutions of inactivated serum (56°C—30 minutes) in medium 199 at pH 6.8 were incubated for one hour at room temperature with an equal volume of measles virus suspension containing 100 $TCID_{50}$ per 0.1 ml. Following incubation, 0.2 ml of each mixture was added to each of 2 cell culture tubes. The cultures were incubated for 7 days at 36-37°C at which time they were observed for measles cytopathic changes. The titer was calculated as the highest initial dilution of serum which caused complete or near complete suppression of cytopathic change.

Complement fixation (CF) test. The complement fixation (CF) tests were performed according to Osler *et al.*(9), employing five 50% hemolytic doses of guinea pig complement and overnight fixation in the cold. For titration of antigen, four 50% (H_{50}) hemolytic units of guinea pig immune serum were employed. A suspension of partially purified and concentrated measles virus antigen was incubated in each test as a control. CF units, as used in this paper, are defined as the reciprocal of the dilution of antigen required to yield 50% fixation in this test procedure.

Sedimentable CF antigen. CF antigen which is pelleted by one hour centrifugation at 105,000 × g (40,000 rpm, no. 40 rotor, Model L centrifuge (Spinco)).

Non-sedimentable CF antigen. CF antigen remaining in the supernatant fluid after one hour centrifugation at 105,000 × g.

Nitrogen determination. Nitrogen was determined as described by Johnson(10).

Immunogenic potency test. Guinea pigs weighing 400-500 g were used for immunogenic potency tests. Immunization schedule consisted of three 1 ml intramuscular injections of each preparation given at weekly in-

TABLE I. Release of Measles CF Antigen* from Infected Cell Debris.

Description of sample	CF units of clarified fluid	CF units of 105,000 x g supernate	CF units 105,000 x g pellet at equal vol.	% Additional CF antigen (sedimentable)
Clarified TCF	9	<1.7	5.6	—
Suspended cells incubated 1 hr at 37°	<.5	<.2	<.4	<7
Frozen and thawed cells incubated at room temp. 1 hr	<.5	<.2	<.4	<7
Frozen and thawed cells incubated 1 hr at 37°	<.5	<.2	<.4	<7
Frozen and thawed cells 200 μg/ml trypsin,incubated 1 hr at 37°	**	**	4.4	79

* CF values calculated back to original TCF vol.
** Anticomplementary.

tervals. Bleedings were taken 7 days after the third injection.

Results. Release of additional CF antigen. An attempt was made to obtain additional CF antigen from the cell debris at the usual harvest time (seventh day post-seeding). Five hundred ml of whole TCF was centrifuged at low speed and the supernatant TCF decanted. The packed cell debris was washed with 0.15 M NaCl at 5°, and the wash removed by low speed centrifugation. The washed cell debris was suspended in 160 ml of 0.15 M NaCl. A one-fourth volume aliquot of this suspension was set aside without further treatment. The remainder was frozen at —20° and thawed at 37° for a total of 3 cycles. This frozen-thawed suspension was divided into 3 equal parts. One was kept at room temperature for one hour, the second was held at 37° for one hour (as was the original cell suspension), and the third was incubated at 37° for one hour with 200 μg/ml of crystalline trypsin (Worthington). At the end of the one hour period, all 4 aliquots were clarified by low speed centrifugation. A portion of each clarified aliquot was then subjected to one hour centrifugation at 105,000 × g (40,000 rpm, no. 40 rotor, Model L centrifuge (Spinco)). The supernatant fluids were decanted and the pellets suspended in 0.063 M potassium phosphate buffer, pH 7.0, containing 2% NaCl. The results of a representative experiment of this type are shown in Table I.

The data in Table I indicated that an additional 79% (calculated on the basis of the CF antigen sedimented from the clarified TCF) of sedimentable CF antigen was obtained from the cell debris by digestion with trypsin. This CF antigen had not been released into the TCF during the virus growth cycle and was not released from the cell debris by 3 cycles of freezing and thawing. A second digestion with trypsin did not release additional CF antigen. Later work indicated that as little as 5 μg of trypsin per ml of whole TCF was sufficient to release CF antigen. Release of additional CF antigen was similar in magnitude whether or not the cell debris had been frozen and thawed prior to digestion with trypsin. For convenience, subsequent digestions were conducted using whole TCF, rather than separated cell debris, without prior freezing and thawing. Tryptic digestion of infected tissue culture fluid free of cell debris resulted in no change in CF activity. A summary of the results of tryptic digestion of whole measles TCF (10 lots) is shown in Table II. An average increase in measles CF antigen of 138% was observed. All the CF antigen was sedimentable (105,000 × g for one hour). No non-sedimentable antigen was ever observed under the conditions employed.

Higher digestion temperature. Digestion of whole TCF with trypsin, in addition to releasing additional CF antigen, also aided purification by converting contaminating protein to dialyzable digestion products. From the knowledge of the properties of trypsin, it was expected that an increase in the temperature and duration of digestion would increase

TABLE II. Results of Tryptic Digestion* of Whole TCF.

Lot no.	Original fluid (CF units)	Digested fluid (CF units)	% Increase
13	2.8	5.7	103
14	1.0	3.4	240
17	5.7	13.5	137
18	3.4	9.5	180
21	3.4	8.0	135
22	2.8	5.7	103
26	2.8	6.8	142
Pool of 29 and 34	2.0	5.7	185
" " 30, 32, 33, 35	4.8	13.5	182
" " 40, 42, 45	5.7	8.0	40

* All digestions were performed at 37°, 1½–2 hrs, 10 μg/ml trypsin.

the proteolytic effect. Experiments were, therefore, conducted in which whole TCF was digested at 45° for various time periods with 10, 30 or 50 μg/ml of trypsin. The results are shown in Table III.

The data in Table III indicated that incubation at 45° for up to 24 hours (this was extended to 48 hours in later experiments) did not destroy the CF antigen or convert it to non-sedimentable CF antigen. The use of trypsin at this elevated temperature produced a considerable increase in CF antigen while eliminating extraneous protein as shown by the increase in CF per μg N values. Since little, if any, difference existed in the range of 10 to 50 μg/ml trypsin, 30 μg per ml was arbitrarily selected for use.

Potency tests. To determine whether the the proteolytic digestion procedure had affected the antigenic potency of the measles virus, samples were prepared for assay in guinea pigs as indicated below:

Sample A—whole TCF clarified by low speed centrifugation

Sample B—whole TCF kept at 45° for 24 hours, then clarified

Sample C—whole TCF incubated at 45° for 24 hours with 30 μg/ml trypsin, then clarified

Sample D—suspension of ultracentrifugal pellet from A

Sample E—suspension of ultracentrifugal pellet from B

Sample F—suspension of ultracentrifugal pellet from C

The ultracentrifugal pellets were obtained by one hour centrifugation at 105,000 × g and resuspended in 0.15 M NaCl to yield essentially the same CF titer as the fluid from which they were derived. The potency test was conducted as described under *"Materials and Methods"*. The results are shown in Table IV. These data indicate the following: 1. The measles immunogen is stable to heat at 45° for 24 hours. 2. The immunogen is sedimentable from heated (45°) or unheated fluids at 105,000 × g for one hour. 3. The immunizing capacity is reduced by digestion with trypsin at 45° for 24 hours, as measured in guinea pigs. 4. CF antigen (sedimentable) is not necessarily antigenic in guinea pigs and, therefore, sedimentable CF antigen is not necessarily a measure of measles immunogen.

Since the loss of antigenic potency result-

TABLE III. Results of Tryptic Digestion of Whole TCF at 45°.

Trypsin (μg/ml)	Time at* 45° (hr)	Fluid clarified post-incubation (CF units)	Ultracentrifugal supernate (CF units)	Ultracentrifugal pellet (CF units)	Specific activity of pellet (CF units/μg N)
0	0	4.0	<.7	2	.44
0	2	4.8	<.7	4	.93
0	5	5.7	<.7	4	.92
10	2	11.5	<.7	14	4.1
10	5	13.5	<.7	16	8.2
10	24	16.0	<.7	14	12.2
30	2	16.0	<.7	14	5.9
30	5	16.0	<.7	14	5.7
50	2	13.5	<.7	12	4.8
50	5	16	<.7	12	5.5
50	24	19	<.7	16	15.2

* Control sample at 24 hr with no trypsin and 24 hr sample at 30 μg were lost through breakage. All values calculated to original TCF vol.

TABLE IV. Effect of Tryptic Digestion at 45° on Immunogenicity of Whole TCF.

Description of sample	Dilutions injected	Serological conversions obtained	
		Neutralization*	Complement fixation**
A. Whole TCF, clarified	1:5	3/3	3/3
CF units = <3.5	1:25	4/4	3/4
B. Whole TCF kept at 45° for 24 hr, then clarified	1:5	2/2	2/2
CF units = <3.5	1:25	3/4	3/4
C. Whole TCF kept at 45° for 24 hr with 30 μg/ml trypsin, then clarified	1:5	0/3	0/3
CF units = 5	1:25	0/3	0/3
D. Resuspended ultracentrifugal pellet from (A)	1:5	4/4	4/4
	1:25	3/3	3/3
CF units = 7	1:125	3/3	3/3
E. Resuspended ultracentrifugal pellet from (B)	1:5	3/3	3/3
	1:25	3/3	3/3
CF units = 7	1:125	3/3	2/3***
F. Resuspended ultracentrifugal pellet from (C)	1:5	0/4	1/4
	1:25	0/4	0/4
CF units = 14	1:125	0/2	0/2

* No. of guinea pigs with neutralizing titer of 1:2 or greater/no. on test. Range of titers was from 1:2 to 1:64.

** No. of guinea pigs with CF titer of 1:5 or greater/no. on test. Range of titers was from 1:5 to 1:160.

*** One serum anticomplementary.

ing from the use of 30 μg/ml of trypsin at 45° for 24 hours was so marked, a similar potency test was conducted to evaluate the effect of the less rigorous conditions (10 μg/ml trypsin, 37°, 1-3 hours) which had been used in earlier parts of this study (Table II). The results of this experiment are shown in Table V. The adverse effect of trypsin on the measles antigen(s) is still apparent, even when digestion was conducted for only one hour.

Discussion. The data reported here have shown that digestion of whole measles TCF or the infected cell debris therefrom with trypsin at 37° or 45° results in a 2-3 fold increase in the amount of sedimentable (105,000 × g) CF antigen. Although it might have been expected that such an in-

TABLE V. Effect of Tryptic Digestion at 37° on Immunogenicity of Whole TCF.

Description of sample	Dilutions injected	Serological conversions obtained			
		Neutralization*	Geometric mean titer***	Complement fixation**	Geometric mean titer***
Whole TCF kept at 37° for 3 hr, then clarified	1:5	8/8	50	7/8	49
CF units = 14	1:25	7/7	21	7/7	30
Whole TCF kept at 37° for 1 hr with 10 μg/ml trypsin, then clarified	1:5	7/7	22	7/7	20
CF units = 38	1:25	3/6	4	3/6	5
Whole TCF kept at 37° for 3 hr with 10 μg/ml trypsin, then clarified	1:5	4/8	2	2/8	2
CF units = 38	1:25	0/8	—	0/8	—

* No. of guinea pigs with neutralizing titer of 1:2 or greater/no. on test. Range of titers was from 1:4 to 1:128.

** No. of guinea pigs with CF titer of 1:5 or greater/no. on test. Range of titers was from 1:5 to 1:320.

*** In calculating geometric mean titers for those groups with at least one conversion, undetectable neutralization titers and CF titers of <5 were arbitrarily assigned a value of 1.

crease would be paralleled by an increase in antigenic potency, tests in guinea pigs showed that the digestion with trypsin at either 37° or 45° resulted in a marked loss of potency. These data also confirmed the stability of the measles immunogen at 45° (without trypsin) reported previously(11).

In relation to the purification and concentration of measles virus, it is clear that the CF property of measles virus preparations should not be used to evaluate such procedures without frequent animal potency tests.

Summary. Digestion of whole measles fluids with crystalline trypsin at either 37° or 45° results in a 2- to 3-fold increase in amount of sedimentable complement fixing antigen. This treatment, however, markedly reduces the ability of these fluids to induce formation of neutralizing or complement fixing antibodies when injected in guinea pigs.

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A Salmonellosis Resistance Factor For the Guinea Pig.* (26981)

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A relationship between nutritional status and resistance to disease has long been recognized but the nature of this relationship remains obscure(1). Schneider(2) observed decreased mortality among mice infected with *Salmonella enteriditis* when a purified basal diet was supplemented with whole wheat, malted barley or dried egg white. Mortality among guinea pigs subjected to whole-body X-radiation was significantly reduced when the animals consumed raw cabbage or broccoli(3), but it is not clear whether or not the increased survival was the result of increased resistance to infection. Preliminary observations made in our guinea pig colony suggested that consumption of raw cabbage decreased mortality due to salmonellosis. To test this effect under controlled conditions guinea pigs fed a purified diet, with and without cabbage supplementation, were artificially infected and the observations are reported here.

Methods. Female guinea pigs were reared from weaning on the purified basal diet described below or on the basal supplemented with cabbage or cabbage fractions. In so far as possible they were continued on the same diets during the experimental period, but in all cases were fed the experimental diets for at least 2 weeks prior to infection.

Two groups of animals, 10-16 weeks of age and weighing 400 to 600 g, were used in each of 5 trials. They were kept in cages with raised wire floors and housed in a room maintained at 75 ± 5°F. All received a basal purified diet and one-half received in addition a daily supplement of cabbage, *ad libitum*. The basal diet contained, in percent, washed casein 30.0, sucrose 43.3, wood pulp 15.0, salts(4) 4.0, soybean oil 4.0, po-

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