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INFLUENCE OF STAPHYLOCOCCAL ENTEROTOXIN ON INFLUENZA VIRUS INFECTIVITY IN THE EMBRYONATED EGG

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FOREWORD

This report was initiated by the Physiology Branch, Aero Medical Laboratory, Wright Air Development Center. The study upon which this report is based was undertaken by the Biochemistry Section under Project No. 7165, "Health Hazards of Materials and Radiation," Task No. 71837, "Defense against BW/CW Agents." The senior author, William F. Hill, Captain, USAF (MSC), of the Biochemistry Section, was research project officer in charge of the overall study.

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ABSTRACT

The influence of treating embryonated eggs with "cat positive" staphylococcal enterotoxin filtrate on the infectivity of PR8 influenza virus is described. (The production of the enterotoxin is also described.) This study was undertaken in an attempt to elucidate additional properties of staphylococcal enterotoxin whereby new avenues of approach might lead to developing simple and precise methodology for detecting this toxin in suspect food. Available assay procedures lack definitive reliability in that they are either indirect or one-sided. The results of this study indicate that pre- and post-treatment of embryonated eggs with "cat positive" enterotoxin filtrate exerts an inhibitory influence upon virus infectivity. A statistically significant difference at the 0.05 probability level was found between the mean EID_{50} of enterotoxin-treated and control groups of embryonated eggs. The particular mechanism whereby staphylococcal enterotoxin antagonizes virus infectivity remains obscure at present, but probably involves the alteration of host-cell metabolism.

PUBLICATION REVIEW

This report is published for the exchange of information and stimulation of ideas.

FOR THE COMMANDER:

ANDRES I. KARSTENS
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SECTION I

INTRODUCTION

Adequate methodology for detecting staphylococcal enterotoxin either qualitatively or quantitatively in suspect food is seriously lacking in present-day public health technology. The greatest handicap to developing simple and precise methodology lies essentially in the fact that staphylococcal enterotoxin has been rather poorly characterized in terms of its toxicity and pharmacodynamic activity upon susceptible tissues. In view of the paucity of information on tissue susceptibility to staphylococcal enterotoxin, this study was initiated and designed to investigate, on an exploratory basis, the influence of staphylococcal enterotoxin on influenza virus infectivity in the embryonated egg. The basis underlying this approach to the problem of developing a suitable assay procedure rests primarily in an effort directed toward better characterization of the enterotoxin in terms of its activity upon a host-parasite relationship. This type of information could conceivably elucidate a mechanism of action of enterotoxin whereby new avenues of study would ultimately lead to resolution of the problem with resultant development of a simple and precise methodology.

SECTION II

MATERIALS AND METHODS

A. STAPHYLOCOCCAL ENTEROTOXIN

1. Production of Enterotoxin

The stock culture of Staphylococcus aureus strain 196E was obtained from the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio. A fresh culture transfer was made from the stock culture on an agar slant and allowed to incubate for 48 hours at 37° C. The agar slant culture was then transferred to a flask containing 200 ml. of tryptose broth media and incubated for 24 hours after which the entire 200 ml. of culture was added to 1000 ml. of basal media and incubated for 5 days under a 20% atmosphere of carbon dioxide.

Our basal media consisted of the following:

Solution No. 1

Hycase (Sheffield farms) or pancreatic digest of Casein (Difco) -	15.0 gm.
Yeast extract (Difco) -	5.0 gm.
Distilled water to make -	500 ml.

Solution No. 2

Dextrose (Merck) -	2.25 gm.
Distilled water to make -	500 ml.

2. Recovery Schedule for Staphylococcal Enterotoxin

Upon removal from the incubator, the flask containing the 5-day culture was placed in a boiling water bath for 30 minutes. After chilling in the refrigerator, the batch was filtered through a mat of Dicalite-speed flow (a filter aid) in a Buchner funnel. Ammonium sulfate was added to bring the crude filtrate to 50% saturation. After precipitation was complete, the solution was filtered and the precipitate discarded. Next, the filtrate was 100% saturated with ammonium sulfate, thoroughly mixed, and allowed to stand overnight. Again it was filtered, and this time the filtrate was discarded. The precipitate was dissolved in a small amount of water, poured into a sausage casing, and dialyzed against running tap water (at as low a temperature as possible) until free of sulfate. To the solution which had been dialyzed two volumes of ethyl alcohol (95%) were added with thorough mixing. After several hours this solution was filtered and the filtrate discarded. The precipitate was dissolved in as small a quantity of water as feasible, filtered through a Seitz filter, and transferred aseptically to a sterile vaccine bottle. Four hundred units of penicillin and 1000 micrograms of terramycin per milliliter of stock were then added to the vaccine bottle. This stock was the partially purified enterotoxin.

3. Cat Test for Staphylococcal Enterotoxin

This test is of some value in indicating the presence of enterotoxin. The animals were given a moderate-sized meal about half an hour prior to the test. For the test, healthy cats were injected intravenously (saphenous vein) with 2 to 5 ml. of the partially purified enterotoxin. The cats were observed for 2 hours. The presence of enterotoxin is indicated by vomiting and coarse tremors. Mild diarrhea may occur and persist for several hours. Vomiting was the criterion used in this study to give "cat positive" material. Retching action or tremors without vomiting were not considered sufficient evidence of "cat positive" filtrate.

B. VIRUS

The original sample of PR8 influenza virus used in this investigation was obtained from the Department of Bacteriology of the Ohio State University. Subsequently, the virus was maintained by egg passage with pooled infected allantoic fluids from embryonated eggs. Four hundred units of penicillin and 1000 micrograms of terramycin were added per ml. of stock virus suspension to preclude bacterial contamination.

C. EGGS

Fertile white eggs were incubated at 37° C. for 10 days prior to viral titration. The eggs were turned daily.

Prior to inoculation, the eggs were candled to determine not only the viability of the embryo, but also the site in the egg where the chorioallantois was best developed. This was facilitated by marking the egg shell in the area where the allantoic vein is apparent. In addition, the natural air sac was delineated.

D. DRILLING

Immediately before drilling a small window in the egg shell, the area was cleansed with an aqueous solution of 1:1000 merthiolate. The window was made by rotating the disc of the drill back and forth so as to remove the shell without damaging the underlying shell membrane. For best results, the window should be approximately 1.0 cm. inside the edge of the air sac. Following drilling, the window was cleansed with ethyl alcohol (70%).

E. TITRATION

Ten-day embryonated eggs were inoculated with "cat positive" staphylococcal enterotoxin filtrate (0.1 ml. volumes) via the allantoic cavity route before and after introduction of the virus inoculum. Serial 10-fold dilutions of stock virus suspensions were prepared in chilled nutrient broth. For purposes of titration, each of 4 eggs in a group was inoculated with 0.1 ml. of a given viral dilution and then incubated at 37° C. for 48 hours. Following incubation, eggs were chilled and then examined for evidence of infection. The method of calculating 50% infectivity endpoints was that proposed by Reed and Muench (see references 2 and 3). The presence and/or absence of viral haemagglutination of human type 0 erythrocytes was used to determine the infectivity score.

SECTION III

RESULTS AND DISCUSSION

The results of this investigation have been consolidated and recorded in tables I and II. The results of the statistical analysis are presented in table III. The raw data and statistical calculations are located in the Appendices.

Results indicate that pre- and post-treatment of embryonated eggs with "cat positive" staphylococcal enterotoxin filtrate exert an inhibitory influence upon PR8 influenza virus infectivity. A statistically significant difference at the 0.05 probability level was found between the mean EID_{50} of enterotoxin and control groups of embryonated eggs. This indicates that the observed difference in mean EID_{50} is a real difference since a difference of this magnitude would occur due to chance alone only 5% of the time. Although the mechanism whereby staphylococcal enterotoxin antagonizes virus infectivity remains obscure at present (it is not known whether the enterotoxin affects the virus, per se, or only affects host-cell susceptibility), it is quite apparent that the enterotoxin does exert an influence capable of altering the conditions optimal for virus parasitism in the embryonated egg.

TABLE I

INFECTIVITY OF PR8 INFLUENZA VIRUS FOR EGGS TREATED WITH "CAT POSITIVE" STAPHYLOCOCCAL ENTEROTOXIN FILTRATE

Time Interval* (All Eggs Treated with 0.1 ml. Volume)	Egg Infectivity Dose 50 (EID_{50})
60-minute pre-treatment	$10^{-7.50}$
30-minute pre-treatment	$10^{-8.35}$
0-minute treatment**	$10^{-9.00}$
30-minute post-treatment	$10^{-7.75}$
60-minute post-treatment	$10^{-7.75}$

* Time interval of treatment refers to the time the enterotoxin filtrate was introduced into the eggs compared to the time the virus suspension was inoculated.

** Virus and enterotoxin introduced into the eggs at the same time.

TABLE II

INFECTIVITY OF PR8 INFLUENZA VIRUS FOR UNTREATED EGGS AND EGGS TREATED WITH .85% SALINE (CONTROLS)

Treatment of Egg	Egg Infectivity Dose 50 (EID_{50})
0.1 ml. of .85% saline (30-minute pre-treatment)	$10^{-9.23}$
0.1 ml. of .85% saline (0-minute treatment)	$10^{-8.75}$
No treatment	$10^{-9.37}$
No treatment	$10^{-8.76}$

One limiting aspect encountered in the experimental design of this study was the necessity of working with partially purified enterotoxin filtrate. However, at the present time, methods for obtaining completely purified preparations have not been reported. Another limiting aspect was the selection of proper "control" inocula. "Cat negative" filtrates could have been chosen. However, unfortunately "cat negative" filtrate would not indicate the absence of enterotoxin because, in the use of the "cat test" for determining the presence of staphylococcal enterotoxin, only "cat positive" samples give definitive information. In other words, the "cat test," as such, is a one-sided type assay. Consequently, our control groups consisted simply of duplicate EID_{50} titrations with and without inocula. When an inoculum was used, it was 0.85% saline (see table II).

TABLE III

STATISTICAL ANALYSIS OF EID_{50} BETWEEN ENTEROTOXIN-TREATED AND CONTROL GROUPS

(t Test)

	N	Composite σ^2	Mean EID_{50}	95% Confidence Limits	$t_{(7)}$
Treatment Groups	5	0.2545	8.0700	7.5367-8.6033	2.829*
Control Groups	4	0.2545	9.0275	8.4310-9.6239	($P_{.05} < .05$)

* Significant where $P_{.05} = 2.365$ at 7 degrees of freedom.

In keeping with experimental objectivity, it should be borne in mind that a possibility exists that some impurity present in the partially purified "cat positive" filtrate was responsible for the inhibition of the influenza virus infectivity. However, until a completely purified enterotoxin becomes available for studies of this kind, doubt will always exist as to whether the observed effects were due to enterotoxin, per se, or to some other ingredient present in the partially purified preparations.

Finally, it should be understood that the results of this investigation are only applicable to the particular host-parasite system under study. Consequently, extension of the results described above to other virus parasitisms would be highly speculative, for different parasitic relationships are characterized by tissue affinities and responses unique to the system being studied. Therefore, analogies, unless tested, are unpredictable.

SECTION IV

CONCLUSIONS

1. A statistically significant difference at the 0.05 probability level was found between the mean PR8 influenza virus infectivity titer (EID_{50}) of enterotoxin-treated and control groups of embryonated eggs.

2. Pre- and post-treatment of embryonated eggs with "cat positive" staphylococcal enterotoxin filtrate exerts an inhibitory influence upon PR8 influenza virus infectivity.

3. The mechanism by which "cat positive" staphylococcal enterotoxin filtrate antagonizes influenza virus infectivity is not clearly understood but probably involves the alteration of host-cell metabolism.

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APPENDIX I

RAW DATA

INFECTIVITY OF PR8 INFLUENZA VIRUS FOR EGGS TREATED WITH STAPHYLOCOCCAL ENTEROTOXIN FILTRATE

Time Interval	Virus Dilution	Infectivity Score I*	Infectivity Score N**	Subtotals I*	Subtotals N**	Infectivity %	EID_{50}
60-minute pre-treatment	10^{-6}	3	0	7	0	100	$10^{-7.50}$
	10^{-7}	4	0	4	0	100	
	10^{-8}	0	4	0	4	0	
	10^{-9}	0	4	0	8	0	
30-minute pre-treatment	10^{-6}	4	0	11	0	100	$10^{-8.35}$
	10^{-7}	3	1	7	1	87.5	
	10^{-8}	3	1	4	2	66.6	
	10^{-9}	1	2	1	4	20	
	10^{-10}	0	4	0	8	0	
0-minute treatment#	10^{-7}	4	0	8	0	100	$10^{-9.00}$
	10^{-8}	3	0	4	0	100	
	10^{-9}	1	1	1	1	50	
	10^{-10}	0	3	0	4	0	
30-minute post-treatment	10^{-7}	3	0	4	0	100	$10^{-7.75}$
	10^{-8}	1	2	1	2	33.3	
	10^{-9}	0	3	0	5	0	
	10^{-10}	0	4	0	9	0	
60-minute post-treatment	10^{-6}	4	0	8	0	100	$10^{-7.75}$
	10^{-7}	3	0	4	0	100	
	10^{-8}	1	2	1	2	33.3	
	10^{-9}	0	3	0	5	0	

\# Virus and enterotoxin introduced into the eggs at the same time.
* Infection
** Noninfection

INFECTIVITY OF PR8 INFLUENZA VIRUS FOR UNTREATED EGGS AND EGGS TREATED WITH 0.85% NaCl (CONTROLS)

Time Interval	Virus Dilution	Infectivity Score		Subtotals		Infectivity %	EID_{50}
		I*	N**	I*	N**		
No treatment	10^{-7}	4	0	11	0	100	
	10^{-8}	3	1	7	1	87.5	$10^{-9.37}$
	10^{-9}	4	0	4	1	80	
	10^{-10}	0	4	0	5	0	
No treatment	10^{-7}	4	0	9	0	100	
	10^{-8}	3	1	5	1	83.3	$10^{-8.76}$
	10^{-9}	2	2	2	3	40	
	10^{-10}	0	4	0	7	0	
30-minute pre-treatment	10^{-8}	4	0	7	0	100	
	10^{-9}	2	2	3	2	60	$10^{-9.23}$
	10^{-10}	1	3	1	5	16.6	
	10^{-11}	0	3	0	8	0	
0-minute treatment	10^{-7}	3	0	8	0	100	
	10^{-8}	4	0	5	0	100	$10^{-8.75}$
	10^{-9}	1	2	1	2	33.3	
	10^{-10}	0	4	0	6	0	

* Infection
** Noninfection

APPENDIX II

STATISTICAL ANALYSIS OF EID_{50} BETWEEN ENTEROTOXIN-TREATED AND CONTROL GROUPS OF EMBRYONATED EGGS

Enterotoxin-Treated Group T	T^2	Control Group C	C^2
7.50	56.2500	9.37	87.7969
8.35	69.7225	8.76	76.7376
9.00	81.0000	9.23	85.1929
7.75	60.0625	8.75	76.5625
7.75	60.0625	---	---

$\sum T = 40.35$ $\sum T^2 = 327.0975$ $\sum C = 36.11$ $\sum C^2 = 326.2899$

$n_1 = 5$ $n_2 = 4$

$\overline{T} = 8.0700$ $\overline{C} = 9.0275$

$\sum dev^2 = 1.4750$ $\sum dev^2 = 0.30688$

$s^2_T = 0.36875$ $s^2_C = 0.102293$

Composite Variance $\sigma^2 = \frac{(1.475)+(0.30688)}{7} = \frac{1.7818}{7} = 0.2545$

SE of Difference $= \sqrt{\frac{0.2545}{5} + \frac{0.2545}{4}} = \sqrt{0.05090 + 0.06362} = \sqrt{0.11452} = 0.3384$

$t_{(7)} = \frac{0.9575}{0.3384} = 2.829$; $P < .05$

95% Confidence Limits for Each Mean Separately

A. Enterotoxin-Treated Group

Mean EID_{50} = 8.0700

Composite σ^2 = 0.2545

n_1 = 5; DF = 7 $P_{.05}$ = 2.365

SE of Mean = $\sqrt{\frac{0.2545}{5}}$ = $\sqrt{0.05090}$ = 0.2255

95% Confidence = $8.070 \pm (2.365)(0.2255)$

= 8.070 ± 0.5333 = 7.5367 to 8.6033

B. Control Group

Mean EID_{50} = 9.0275

Composite σ^2 = 0.2545

n_2 = 4; DF = 7 $P_{.05}$ = 2.365

SE of Mean = $\sqrt{\frac{0.2545}{4}}$ = $\sqrt{0.06362}$ = 0.2522

95% Confidence = $9.0275 \pm (2.365)(0.2522)$

= 9.0275 ± 0.59645 = 8.4310 to 9.6239