The Frog Test for Staphylococcal Enterotoxin. (18999)

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The recognized need for a more readily available laboratory animal which might satisfactorily substitute for the kitten in the testing of materials believed to contain staphylococcal enterotoxin appeared to be met when Robinton(1,2) reported that frogs could be used for this purpose. She observed that the feeding of staphylococcal culture filtrate to intact frogs induced, after 45 minutes to 7 hours, a specific "spasm" reaction consisting of a series of gaping and swallowing movements performed in a distinctive posture over a 5-10-minute period, climaxed by slow wiping of the face and the sides of the body with fore- and hind-feet respectively. In decerebrate animals the introduction of enterotoxin via the oesophagus into the exposed stomach induced reverse peristalsis either immediately or within 30 minutes depending upon the concentration of the material. Non-enterotoxic filtrates and sterile culture medium had no comparable effects upon intact or decerebrate frogs.

It seemed to us worthwhile to investigate the frog test further, not only because of its possible importance in the study of enterotoxin, but also because it might lend itself to an instructive laboratory exercise for students.

Materials and methods. Seven strains of *Micrococcus pyogenes*, var. *aureus* from various human sources were used. Six strains produced alpha hemolysin and were coagulase positive; 3 of these produced dermonecrotoxin. One strain was coagulase negative and produced no demonstrable alpha hemolysin or dermonecrotoxin. The organisms were inoculated into 125 ml Erlenmeyer flasks containing 35 ml of heart infusion broth with 5% neopeptone and 0.3% agar added, initial pH 7.2. After incubation for 3 days at 37°C in an atmosphere of 30% carbon dioxide (by volume) the cultures were spun for 30 minutes in the cold room at 3600 rpm, using a Size 1 International centrifuge fitted with an angle head. Each culture supernate was decanted and stored separately in a sterile stoppered tube in the cold room. Control supernatant fluid was similarly prepared from uninoculated medium. Portions of the unconcentrated culture supernates and control supernate were concentrated to one fifth of their original volume by evaporation through sterile cellophane sacs placed in front of a fan. Each unconcentrated culture supernate was tested for enterotoxic activity in kittens by the intraperitoneal injection(3) of 2 ml after the first experiment with frogs was completed. Difco Nutrient Gelatin containing 120 g per liter of Bacto-gelatin was used. The honey was a Louisiana product purchased in a local grocery; it was later sampled by us with no ill effects. The frogs used in the early experiments were from Wisconsin (*R. pipiens*) while in later tests Louisiana frogs were employed (the majority *R. clamitans*, a few undoubtedly *R. pipiens sphenocephala*). They were established in the laboratory each in a small museum jar containing a little water and cleaned daily. They were forcibly fed, once a week,* a small ball of ground beef (in the last experiment small pieces of liver were given) and experiments were started 4 hours after feeding. The culture supernates and control media were given orally by medicine dropper and the frogs were watched until it was certain they had swallowed the material. After the liquids were administered the animals were observed steadily for 1 or 2 hours, thereafter at frequent intervals up to 7 or 8 hours while other work was carried on in the same room. Frogs

1. Robinton, E. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 265.

2. Robinton, E. D., *Yale J. Biol. and Med.*, 1950. v23, 94.

3. Dolman, C. E., and Wilson, R. J., *Canad. J. Pub. Health*, 1940, v31, 68.

*As recommended in Carworth Farms Quarterly Letter, No. 11, Oct. 1, 1948.

TABLE I. Results of Administration of Staphylococcal Culture- and Control-Supernates to Kittens and Frogs.

Toxin	Unconcentrated culture supernates	Concentrated culture supernates			
		Intact frogs,* 4/19 and 4/22/51		Decerebrate frogs,* 4/26 and 5/4/51	
	Kittens, 4/3/51	Frog No.	Result	Frog No.	Degree of reverse peristalsis
1	Diarrhea, vomiting	9	Spasm, 6 hr†		
		10	" 3 hr	10	Slight
2	" "	11	No reaction		
		12	Spasm, 1 hr		
3	No reaction	13	Spasm, 10 min		
		14	No reaction	14	Violent
4	Died	15	No reaction	15	Moderate
		16	Doubtful	16	Slight
5	No reaction	17	No reaction	17	None
		18	" "		
6	" "	19	Spasm, 6 hr		
		20	" 5 min	21‡	Strong
7	Slight diarrhea	21	No reaction		
		22	" "	22	None
Medium Controls	No reaction	23	Spasm, 5 hr	23	Doubtful
		24	" 5½ hr	24	Slight

* Frogs fed meat 4 hr before supernates administered.
† Time elapsed between introduction of supernate and spasm.
‡ This survivor of experiment with toxin No. 7 on 4/22/51 was used to test toxin No. 6 a week later.

that survived these experiments were kept and at a later date, 4 hours after being fed meat, they were decerebrated and the stomachs exposed. The test liquids, in amounts from 0.2-1.0 ml, were introduced via oesophagus directly into their stomachs which were observed for peristalsis. When the experiment lasted for a period of more than 10-15 minutes, physiologic saline was applied to the entire frog at intervals to prevent dehydration.

Results. In a preliminary trial, no reactions were seen in the short period during which unfed frogs were observed after they had been given the unconcentrated culture supernates or control supernate. Twenty-four hours later when food was offered (but not forcibly fed) to the animals, spasms were exhibited by: (1) a frog given an unconcentrated culture supernate demonstrated to produce diarrhea and vomiting in a kitten; (2) a frog given an unconcentrated culture supernate which was negative by kitten test. Two frogs given unconcentrated culture supernates which were enterotoxic for kittens showed no reaction. Under the conditions of this experiment there was no high degree of correlation between the results of the tests in the two species of animals.

Since Robinton had been able by concentrating the filtrates to shorten the period between administration of toxin and the occurence of a reaction, a second group of frogs was tested with the concentrated culture supernates and concentrated control supernate; each supernate was administered to 2 frogs by dropper. The results are summarized in Table I. Frogs 9, 19 and 20 exhibited only short spasms with a few very rapid wiping motions; frogs 10, 12, 13, 20 and control frogs 23 and 24 performed the slowly repeated motions considered most typical. Three of 6 frogs which died within 24 hours were noted to have vomited the meat, practically undigested. The survivors were tested a week or so later in the decerebrate state. Five of the 7 decerebrate frogs tested with concentrated culture supernates and 1 of the 2 given concentrated control supernate showed reverse peristalsis varying from 1 to 8 small upward waves in the pyloric region to a few large waves, the distance traversed varying from about ⅛ inch to one third or one half the

TABLE II. Results of Administration of Concentrated Control Supernates and Other Materials to Frogs.

Material administered	Frog No.	Intact frogs* Date	Intact frogs* Result	Surviving frogs* Date	Surviving frogs* Prepared by	Surviving frogs* Result
Concentrated control supernate	26	6/1	No reaction	6/5	Decerebration	Slight reverse peristalsis
	27		Doubtful		"	" " "
	28		Spasm		"	Strong " "
	29		No reaction		"	Spontaneous reverse peristalsis upon exposure of stomach after which introduction of material elicited either no response or both upward and downward waves
	30		Spasm	6/6†	"	
	31		Doubtful		"	
Untreated semisolid medium	32	6/19	No reaction	6/26	Curare	Spontaneous reverse peristalsis
	33		Spasm		"	Doubtful " "
	34		"		Decerebration	" " " "
	35		No reaction		"	Spontaneous " "
Honey	36		Spasm			
	37		Spasm			
Gelatin	38		No reaction		Curare	Slight " "
	39		Spasm		Decerebration	Spontaneous " "

* Frogs fed meat 4 hr before material administered.
† Frogs 30 and 31 were decerebrated the following day when their stomachs were empty.

length of the stomach, with the food sometimes being pushed into the upper half of the stomach. Again, no marked correlation with the kitten test for enterotoxin was evident.

Because of the fact that the 2 frogs which had received the concentrated control supernate exhibited spasms, a third group of 6 frogs was tested with the concentrated control supernate only; of these, 2 showed complete spasms and 2 gave doubtful reactions,—gaping and swallowing for a short period or at intervals without the wiping of the face or the abdominal wall. Following decerebration some degree of reverse peristalsis occurred in at least 3 of the frogs. Table II summarizes the observations on this group as well as a fourth group of animals tested with untreated semisolid medium, honey or gelatin. Two of the 4 frogs given untreated semisolid medium, 1 of 2 given gelatin and both of the frogs given honey exhibited spasms. The 2 that received honey vomited within 15 to 30 minutes; both were extremely ill and died. The survivors were tested a week later, with the variation that half of the frogs were injected with 5 units of curare 10 minutes before the stomachs were exposed, instead of being decerebrated, in an attempt to rule out the possibility that reverse peristalsis resulted from the shock of decerebration. However, in frog 32 there was some reversal and contraction of the pylorus before any medium was given; after relaxation of the pylorus, insertion of material was followed only by normal downward waves from the cardia. The other 2 curarized frogs did not show reversal or contraction until after test material was introduced. In decerebrate frogs 35 and 39 strong spontaneous contractions of the pylorus lasting 20 and 40 minutes respectively occurred upon exposure of the stomachs and no reaction was elicited when medium was introduced, possibly because the muscles were still tonically contracted or in the refractory phase.

From Table III which summarizes the findings in frogs tested with materials other than staphylococcal culture supernates, it may be seen that spasms occurred in about half of the intact frogs and that reverse peristalsis resulted in about one third of the decerebrate animals tested with preparations considered to be free of enterotoxin.

Discussion. A satisfactory explanation is not at hand to account for the origin of the spasms and the delay in their onset sometimes observed. Robinton(1), who decapitated frogs at the height of spasm following administration of staphylococcal filtrate, observed reverse peristalsis occurring; in control frogs decapitated at the same time reverse motion of the stomach was not seen. On the other hand, 6 of our decerebrate control frogs

TABLE III. Summary of Experiments with Materials Other Than Staphylococcal Culture Supernates.

Test material	Observation of spasm in: Intact frogs — No. tested	Positive	Doubtful	Negative	Frogs decerebrate or curarized — No. tested	Positive	Doubtful	Negative
Unconcentrated control supernate	1*			1				
Concentrated control supernate	8	4	2	2	8	4	1	3
Untreated semi-solid medium	4	2		2	4		3	1
Honey	2	2						
Gelatin	2	1		1	2	1		1
Totals	17	9	2	6	14	5	4	5

* Not fed before material given.

showed spontaneous reversal; Mellinger(4) also had observed irregular contractions of the stomach in frogs immediately after pithing. The cause of the reverse peristalsis is unknown.

The consideration that the consistency of the material administered might be of some importance led to the trial of other substances of like property. The concentrated culture supernates and concentrated control supernate were of the viscosity and appearance of maple syrup or honey. The untreated semi-solid medium was not as syrupy as the concentrated supernates, the gelatin even less so, but the honey was quite as glutinous and produced an immediate and prolonged response lasting over a period of hours. These results suggest an action on gastric tone and motility, the reasons for which are not apparent.

Robinton(2) noted a decrease in frequency of spasms occurring in frogs fed toxins during November and complete absence of such responses in December and January. Mellinger (4) found the ability of frogs to vomit upon administration of emetics was absent in January and February, diminished in October and November. His experiments were most successful in the months of June and July and this would seem to imply that there was not at any time 100% response to emetics but a high percentage in summer decreasing to zero in the winter. Our first experiment was performed on March 10th. By April 19th, 50% of the frogs including the controls exhibited spasms and that percentage was maintained through the last experiment on June 19th. Some factor associated with season probably contributes greatly to the higher incidence of spasms during the Spring.

Conclusion. The occurrence of a spasm in frogs fed staphylococcal culture products is either a non-specific response to various substances of an especially viscid nature which is elicited more readily at certain seasons, or is a response at a particular season to some stimulus not yet determined.

4. Mellinger, C., *Arch. f. d. ges. Physiol.*, 1881, v24, 232.

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