The Effect of Glutamate on Toxin Production by *Clostridium tetani*

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SUMMARY

The addition of sodium-L-glutamate (1 %, w/v) to the Mueller medium in which *Clostridium tetani* was grown initially stimulated growth and toxin production, then caused early lysis, and resulted finally in a marked suppression in the titre of tetanus toxin in the culture measured after 7 days. The suppression of production of tetanus toxin was not associated with any general suppression of protein production or with a decrease in the production of any major antigen.

INTRODUCTION

Yoneda's group (Tsunashima, *et al.* 1964) showed that the addition of glutamate (1 %, w/v) to the medium in which *Clostridium tetani* was grown greatly decreased the final titre of tetanus toxin in the culture supernatant fluid, while leaving the amounts of the other antigens unaffected. In the present work, this effect of glutamate has been repeated with another (more toxinogenic) strain of *C. tetani* and has been found to be associated with a marked effect on the life-span of the organism. A suppression specifically of toxin production could be of importance in elucidating a physiological function for tetanus toxin in the parent organism.

METHODS

L-Glutamate was British Drug Houses Ltd. laboratory reagent grade, 95 % pure by enzymic assay; NAD and glutamic dehydrogenase were from Boehringer Corporation (London) Ltd; tetanus antitoxin was provided by Dr R. O. Thomson of the Wellcome Research Laboratories and contained 2400 units/ml.

A highly toxinogenic strain of *Clostridium tetani* (variant of the HARVARD strain) was provided by Dr R. O. Thomson (Wellcome Research Laboratories). It was stored in a sloppy agar Mueller medium (Mueller & Miller, 1954) at 4°. The organisms were grown overnight in another quantity of sloppy agar Mueller medium inoculated from one of the store cultures. This inoculum was used to inoculate experimental tubes of Mueller medium (30 ml. in 50 ml. capacity tubes) which were incubated at 35.5°. At the times when toxicity and growth were to be assessed, 5 ml. samples were removed from the cultures. They were centrifuged down at 3000 g at 4°, and the supernatant fluid was kept for the assays. The pellet was now suspended in 5 ml. of 0.15 M-NaCl solution and the suspension was again centrifuged; the supernatant fluid (i.e. the washings) being also kept for assays. The pellet was now suspended in 5 ml. of 1.0 M-NaCl solution and left at 4° for 2 days to extract the intracellular toxin

(Raynaud, 1949). The suspension was then centrifuged and the supernatant fluid used for assays.

The dry weight of organisms of the culture was assessed by reading the extinction at 600 m μ of a suitable dilution of the culture in 0.15 M-NaCl solution. There was found to be a linear relationship between dry weight of organisms and E_{600} for readings below 0.7. Dry weights were determined after heating at 100° for 12 hr. An extinction reading of 0.65 corresponded to a dry weight of organisms of 1.0 mg./ml. The LD 50 dose was determined by intramuscular injection into pairs of albino mice of serial twofold dilutions of the preparations in 0.1 M-phosphate buffer (pH 7.0), containing 0.2 % gelatin. Antibody-combining power (L+) of the toxin was determined by measuring the amount of each preparation which, in the presence of 0.05 unit anti-toxin, killed half the mice injected with toxin.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), on dialysed samples.

Glutamate was determined in samples which had been deproteinized by the addition of perchloric acid which was subsequently neutralized with potassium hydroxide and the insoluble potassium perchlorate centrifuged down (Williamson, Mellanby & Krebs, 1962). Ammonia was removed from the deproteinized samples by boiling them for 10 min. at pH 9. Glutamate was estimated by measuring the change in extinction at 340 m μ when it was reduced to α -oxoglutarate in the presence of glutamic dehydrogenase, NAD and hydrazine, (Bernt & Bergmeyer, 1963).

RESULTS

Growth and toxin production were investigated in cultures of *Clostridium tetani* grown in Mueller medium, either unmodified or supplemented with Na-L-glutamate (final concentration 1 %, w/v). The glutamate-containing cultures grew more rapidly for about the first 24 hr after inoculation, but then started to autolyse; the cultures in the ordinary Mueller medium continued to grow. An experiment to illustrate this is shown in Fig. 1. It can be seen that the culture without added glutamate grew for about 30 hr longer than the glutamate supplemented culture, and achieved about 30 % higher dry weight of organisms. In this experiment, samples of the culture were removed 36 and 89 hr after inoculation and the toxin and the protein in the culture fluid and the toxin within the bacilli (extractable with M-NaCl solution at 4° for 2 days) was measured. The results of these determinations are shown in Table 1. At 36 hr, when in this particular experiment the two cultures contained the same dry weight of organisms (see Fig. 1), the glutamate supplement approximately doubled the yield of toxin. A much higher proportion of this toxin was extracellular in the glutamate culture, which presumably reflected the fact that the bacilli had begun to lyse. However, by 89 hr the glutamate supplement had suppressed toxin production to about one fifth of that in the ordinary Mueller medium. At this time (89 hr), the glutamate culture had been autolysed for some time, while the ordinary culture was in the process of autolysing. Although the glutamate culture lysed at about 40 hr, in the period between 36 and 89 hr its total toxin titre increased threefold.

Although at 36 hr there was twice as much protein in the supernatant fluid of the glutamate culture as in that of the ordinary culture, its protein content did not increase after this time. In contrast, the protein content of the supernatant fluid of the culture

grown in ordinary Mueller medium doubled between 36 and 89 hr. Thus in this experiment, at 89 hr the protein content of the culture supernatant fluids was about the same, whether or not the culture medium was originally supplemented with 1% glutamate. When the immunoelectrophoretic patterns, against mixed antitetanus serum, of ammonium sulphate precipitates (fully saturated, dialysed and freeze-dried) of supernatant fluids from cultures grown for 5 days, in the presence or absence of

Fig. 1. Growth of *Clostridium tetani* in ordinary Mueller medium (full line) or in Mueller medium supplemented with Na-L-glutamate (final concentration 1%, w/v; broken line).

Table 1. Effect of 1% glutamate in the culture medium on production of toxin by *Clostridium tetani*

	After 36 hr growth		After 89 hr growth	
	No glutamate	1% glutamate	No glutamate	1% glutamate
Toxin in supernatant LD ₅₀ /ml.	5,500*	90,000	1,920,000	480,000
Toxin in washings LD ₅₀ /ml.	1,800	36,000	125,000	38,000
Toxin in cells LD ₅₀ /ml.	70,000	50,000	600,000	3,600
Total toxin LD ₅₀ /ml.	77,000	176,000	2,645,000	521,600
Protein in supernatant (mg./ml.)	0.85	1.65	1.80	1.77
Protein in washings (mg./ml.)	0.20	0.32	0.34	0.39

* The results are expressed per ml. of original culture. For experimental details see Methods.

added glutamate showed no differences. The patterns were complex and contained 5 apparently similar major antigens and several minor ones.

The suppression of final toxicity by glutamate was observed consistently. Since it was possible that the decreased yield of lethal toxin might result from toxoiding in the glutamate cultures, the antibody-combining power was also measured in some experiments, and it was found that the values for L+ and LD 50 paralleled each other.

It was apparent in Table 1 that with a culture grown in ordinary Mueller medium most of the toxin formation occurred after about the first 36 hr of incubation. This was confirmed in an experiment (Table 2) where toxin was estimated in the bacilli and supernatant fluid of a culture in ordinary Mueller medium at 13, 37, 62, 86 and 157 hr after inoculation. Here, between 37 and 62 hr the bacilli accumulated about 5,000,000 LD 50 doses of toxin/ml. culture. Between 62 and 86 hr the bacilli autolysed and intracellular toxin was released quantitatively into the medium. Between 86 and 157 hr there was a decrease in toxicity. It is probable that this was the result of the 'toxoiding' which often occurs when lysed cultures are left to incubate. Table 3 shows an experiment in which the toxin content in terms of LD 50 fell by 20 to 40 % between 3 and 5 days, and by more than 99 % between 5 and 9 days, during which times the toxin content, in terms of L+, remained constant.

Table 2. *Growth and tetanus toxin production by Clostridium tetani*

Time grown (hr)	Dry weight of organisms (mg./ml.)	Toxin in culture supernatant fluid LD ₅₀ /ml.	Toxin in bacilli LD ₅₀ /ml.	Total toxin LD ₅₀ /ml.
13	1.45*	450	6,100	6,550
37	3.8	3,200	380,000	383,000
62	6.9	240,000	5,000,000	5,240,000
86	2.2	5,000,000	56,000	5,056,000
157	1.45	1,000,000	2,800	1,003,000

* The results are expressed per ml. of original culture. For experimental details see Methods.

Table 3. *The effect of age of a culture on the yield of tetanus toxin, expressed either as LD 50 or L+ in the culture supernatant fluid*

Time grown (days)	LD ₅₀ /ml.		L+/ml.		LD ₅₀ /L+	
	1	2	1	2	1	2
3	5,000,000*	3,800,000	24	24	208,000	158,000
5	2,000,000	2,000,000	24	24	83,000	83,000
9	50,000	50,000	24	48	3,400	1,700

* The results are expressed per ml. original culture and are from duplicate cultures (1 and 2). For experimental details see Methods.

Table 4. *Removal of glutamate by Clostridium tetani from the culture medium containing 104 µmoles glutamate/ml. at the time of inoculation*

Time grown (hr)	Dry weight of organisms (mg./ml.)	Glutamate removed (%)
8	0.3	22
11	0.8	49
15	2.3	90
35	2.3	100

Since glutamate had such a pronounced effect on growth and toxin production of *Clostridium tetani* it was obviously important to see whether it was removed from the medium by the organisms. Table 4 shows that 90 % of the added glutamate had been removed by 15 hr. The Mueller medium itself contained about 0.05 % glutamate, all of which disappeared from the medium during the first 8 hr of incubation.

DISCUSSION

When tetanus toxin is measured (either as toxicity, or as antibody-combining power L+) in the supernatant fluid of a culture of *Clostridium tetani* after autolysis, the presence of 1 % glutamate in the original medium will have brought about a marked suppression of toxin production, without having any obvious effect on other antigens. Thus for cultures grown for about 4 days or longer, the present results are in agreement with those of Yoneda's group (Tsunashima *et al.* 1964). Yoneda's results did not concern the effect of glutamate in stimulating early growth and bringing about early autolysis, since their results involved only the later stages of growth. The observation that the glutamate-supplemented cultures never formed major amounts of tetanus toxin, is in agreement with the view that a stationary phase is necessary for the production of high titres of tetanus toxin.

The glutamate-supplemented cultures lysed at least 24 hr after they had removed 90 % of the added glutamate. It is therefore unlikely that the reason these cultures lysed early was because they had developed a dependence on glutamate.

In view of the work of Lettl, Nekvasilova, Moravec & Stejskal (1966) which suggested that *Clostridium tetani* contains an insoluble protoxin which is activated during autolysis, it is interesting that with the present strain, grown in Mueller medium, intracellular toxin was released quantitatively on autolysis of the bacilli. However, where the medium was supplemented with 1 % glutamate there was a threefold increase in toxicity in the culture during autolysis. One of the pieces of evidence produced by Lettl, Nekvasilova, Moravec & Stejskal, (1966) in favour of activation of a protoxin during autolysis was that, even after a culture had been made to lyse immediately after the logarithmic phase of growth by addition of penicillin, the titre of toxin continued to increase, though only, it appeared, to about one-tenth of the titre that would have been obtained without penicillin. Glutamate also produces early lysis, decreases the final titre of toxin and yet allows some increase in toxin titre after lysis.

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