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Exocytotic and phagocytotic activities of *Tetrahymena pyriformis* are not influenced by *Clostridium botulinum* neurotoxins

N. M. Staender, W. Schroedl and M. Krueger

Institut für Bakteriologie und Mykologie, Veterinärmedizinische Fakultät der Universität Leipzig

Summary:

The ciliate *Tetrahymena pyriformis* GL was tested for its applicability to the detection of botulinum neurotoxins. Botulinum neurotoxins attack different proteins of the SNARE-complex, which is involved in fusion processes of the cellular membrane traffic. The exocytosis of enzymes and the phagocytosis of germs include several presumptive SNARE-dependent pathways within *T. pyriformis*. Acid phosphatase was chosen as indicator enzyme for the quantification of the exocytotic activity. It was determined by photometric measurement after the addition of the substrate *p*-nitrophenylphosphate. The phagocytotic activity was quantified with *Escherichia coli* as prey germ. Botulinum neurotoxins were produced by cultivating reference strains of the seven *Clostridium botulinum* toxovars A to G in TPGY broth with and without the addition of trypsin. The success of the neurotoxin production was tested by the mouse bioassay, which is the standard test for botulinum neurotoxins. The neurotoxins were added to the assays used for the determination of the exocytotic and the phagocytotic activity of *T. pyriformis* in final concentrations of $1.5 \cdot 10^2$ mouse lethal doses$\cdot$ml^{-1}, except E: 1.5 mouse lethal doses $\cdot$ml^{-1}. No significant influence of the toxins could be detected. Hence, *T. pyriformis* cannot be used as a test-organism for the detection of botulinum neurotoxins.

Key words: BoNTs, botulinum neurotoxins, exocytosis, indicator organism, phagocytosis, *Tetrahymena pyriformis* GL

Abbreviations: BoNT(s) = botulinum neurotoxin(s), cfu = colony-forming units, DIN = Deutsche Industrie Norm, MLD = mouse lethal dose, NSF = *N*-Ethylmaleimid sensitive fusion protein, SD = standard deviation, SNARE = Soluble NSF acceptor protein receptor

Introduction

Tetrahymena pyriformis is a very popular test and indicator organism for various substances (Dayeh et al., 2004; Muller et al., 2006; Leitgib et al., 2007), including bacterial toxins (Schlimme et al., 1999). This is due to minor demands on cultivation conditions and a responsiveness comparable to that of higher eukaryotic cells.

The botulinum neurotoxins A to G, according to the nomenclature of the seven toxovars, are produced by the anaerobic, spore-forming bacterium *Clostridium botulinum*. They act as metalloproteinases and are considered to be the most poisonous poisons (Lamanna, 1959). Furthermore, these toxins are shown to be the most selective proteases according to their substrate specificity (Arndt et al., 2006). They interact with different proteins of the SNARE-complex (Singh, 2000). These proteins mediate homo- and hetero-typic vacuolar fusion processes within eukaryotic cells (Nichols et al., 1997) and are highly conserved (Ferro-Novick and Jahn, 1994). The points of attack differ depending on the type of toxin. As a result of the interaction, SNARE-dependent membrane fusion processes, such as the exocytosis of neurotransmitter, are stopped (Rossetto et al., 2001), which is the reason for the clinical symptoms of the widespread disease "botulism" (Bohnel and Gessler, 2005).

Besides their pivotal role in the exocytosis, SNARE-proteins were proven to be essential for the phagocytosis: it was inhibited in murine macrophages by the application of BoNT B (Hackam et al., 1998). Their relevance for the fusion of endosomes and lysosomes was also demonstrated in rabbit alveolar macrophages (Ward et al., 2000).

To date, there is no satisfying assay allowing one to detect BoNTs in biological matrices because of the minimal amount of an effective dose. The mouse bioassay is the only test accredited by law to prove BoNTs in suspicious samples in Germany. However, it involves different problems, namely, ethical aspects (the mice die slowly with preserved sensorium), high limits of detection (cattle is about 13 times more sensitive; Moeller et al., 2003) and the impossibility to standardize this test. Alternative detection tests are urgently needed, also in order to meet new diagnostic challenges, such as the "visceral botulism" (Bohnel and Gessler, 2005).

Some authors (Gräf, 1985; De Waart et al., 1972) have already discussed the applicability of *T. pyriformis* to the detection of BoNTs. Their approaches were unsuccessful, since they decided to choose the growth pattern of a protozoan culture as a presumably sensitive parameter. However, there is hitherto no evidence of an involvement of the SNARE-proteins in growth pattern and cell division.

Encouraged by the detection of SNARE-dependent steps during the membrane traffic in protozoa as different as *Giardia intestinalis* (Dacks and Doolittle, 2002), *Paramecium tetraurelia* (Kissmehl et al., 2002; Froissard et al., 2002) and *Dictyostelium discoideum* (Bogdanovic et al., 2002), we studied again the applicability of *T. pyriformis* to indication of BoNTs, but focused on exocytosis and phagocytosis as presumably SNARE-dependent and sensitive parameters.

The exocytosis of the acid phosphatase is mainly constitutive and quite insensitive to alterations in the medium compared to the other enzymes released by *T. pyriformis* (Banno et al., 1987). The process of release seems to be analogous to the release of neurotransmitter in metazoans (Hutton, 1997).

The phagocytotic activitiy in *T. pyriformis* can be determined in different ways, e.g. with Indian ink (Kovacs et al., 1986), latex particles (Batz and Wunderlich, 1976) or spores of *Glugea* spp. (Weidner and Sibley, 1985). *Escherichia coli* was also used (Watson et al., 1981; Burkharin and Nemtseva, 2001).

Material and Methods

Tetrahymena pyriformis

T. pyriformis strain GL was used. The cells were counted in a Neubauer chamber. They were cultivated in 25 cmI-cell culture-flasks (TPP AG Trasadingen, Switzerland) filled with 10 ml of PPYG (20 g proteose-peptone, 1 g yeast-extract, 10 g glucose ad 1 l aqua dest.).

Escherichia coli

The apathogenic strain K12 of *E. coli* was chosen as prey germ and stored at -80°C. Before using them, the bacteria were incubated overnight on a blood agar plate at 37°C. Prior to the experiment they were washed down from the agar with VOLVIC®-water. They were quantified photometrically as suspension in VOLVIC®-water as described by Schroedl et al., 2003.

Botulinum neurotoxins

The BoNTs were produced according to the DIN 10102. Briefly, two media were used: TPGY (50 g casein-peptone, 5 g mixed peptone, 4 g glucose, 1 g sodium thioglycolate, 20 g yeast extract ad 1 l aqua dest.) without and with (1:15) trypsin solution (porcine trypsin, activity 1:250, 0.15 g ad 10 ml aqua dest.).

Reference strains of the *Cl. botulinum* toxovars A to G and of *Cl. sporogenes* were incubated anaerobically on blood agar plates for three days at 37°C. Afterwards, the cells were washed down and one half of this suspension was inoculated into 100 ml of TPGY with trypsin solution and the other one into 100 ml of TPGY without it. These cultures were

incubated for seven days at 25°C under anaerobic conditions. Afterwards, the suspensions were centrifuged (1500 g for 15 min, 8°C) and the supernatants were sterile-filtrated. The concentrations of neurotoxins in these supernatants were determined in the mouse bioassay. Blank media without inocula were treated in the same manner in order to serve as controls (control 1). The amount of protein was measured photometrically.

Preliminary investigations

The viability of the protozoa during both of our assays was assessed by light optical microscopy at 0 h and after 1h, 2h, 4h and 5h.

The culture supernatants and blank media controls were incubated with an *E. coli* K12-suspension in VOLVIC®-water (7.00 $\log_{10}$cfu·ml^{-1}) at 28°C for 4h. The effective concentrations of neurotoxins were the same as in the assay to determine the phagocytotic activities. Before and after the incubation period, samples were taken to determine the cfu·ml^{-1} via microdilution method (plate count).

The activity of acid phosphatase was measured as follows: a culture of *T. pyriformis* in PPYG (6.00 $\log_{10}$cells·ml^{-1}) was centrifuged (1500 g for 5 min) and sterile-filtrated. The supernatants and blank media controls were added to the sterile filtrate using the same effective concentrations as in the assay to determine the exocytotic activities. Samples of 50 µl were taken after 5 h and diluted 1:4.3 with 100 mM sodium acetate (pH 4.0). To 200 µl of this solution, 5 µl of 300 mM *p*-nitrophenylphosphate were added. After 15 min of shaking (400 U/min at room temperature), the reaction was stopped by 50 µl of 1 N NaOH. The extinction was measured at 405 nm (620 nm as reference). PPYG without protozoa was treated in the same way in order to serve as control for unspecific background signals, which were then subtracted.

Exocytotic activity

Cells in the early static growth phase from cultures with PPYG were centrifuged (1500 g for 5 min). The pellet was resuspended in fresh PPYG and the cells were adjusted to a concentration of 5.35 $\log_{10}$cells·ml^{-1}. Seventy µl of this suspension were incubated at 28°C with 35 µl of the supernatants and the blank media. Samples with pure PPYG instead of PPYG with protozoa served as controls for unspecific background signals again. After an incubation period of 5 h, 100 mM sodium acetate (pH 4.0) were added (1:4.3), which immobilized but did not destroy the cells. After centrifugation (1500 g for 5 min), 5 µl of 300 mM *p*-nitrophenylphosphate were added to 200 µl of the supernatants. The activity of the acid phosphatase released into the medium during the incubation period was measured as described above.

The supernatant of toxovar E was detoxified by heating (100°C, 15 min).

Phagocytotic activity

The possible influence of protozoal secretion products on the bacteria was examined first. An overnight culture of *T. pyriformis* in VOLVIC®-water in the beginning of the exponential growth phase was centrifuged (1500 *g* for 5 min) and the pellet was resuspended in VOLVIC®-water. This culture was adjusted to 4.60 $\log_{10}$cells·ml^{-1} according to the assay-setting for the determination of the phagocytotic activity. The protozoa were removed by sterile filtration. The filtrate was incubated with bacteria in VOLVIC®-water (7.00 $\log_{10}$cfu·ml^{-1}). A sample with pure VOLVIC®-water instead of the sterile filtrate served as control. After an incubation period of 4 h at 28°C, cfu·ml^{-1} of *E. coli* were determined as described above. The results obtained with the sterile filtrate were compared to the results of the control.

The supernatants and blank media controls were tested afterwards. They were incubated with protozoa (prepared as above) for 1 h at 28°C before the bacteria were added. This mixture containing 4.60 $\log_{10}$cells·ml^{-1} of *T. pyriformis* and 7.00 $\log_{10}$cfu·ml^{-1} of *E. coli* was incubated further at 28°C for another 4 h. Afterwards, the remaining cfu·ml^{-1} of the bacteria in the suspensions were determined as described above. Since the concentration of the bacteria during the incubation with the supernatants and blank media controls did not vary and since protozoal secretion products had no effect, either, a decrease of the cfu·ml^{-1} had to be due to the phagocytosis of *T. pyriformis* solely. Thus, the phagocytotic activity could be calculated by a comparison of the cfu·ml^{-1} added at the beginning of the incubation period and the cfu·ml^{-1} in the suspension after incubation with protozoa. The possible effect of the neurotoxins on the phagocytosis was determined by comparing the results of the approaches with neurotoxins to the results of the approaches with the blank media controls (control 1) and the supernatant of *Cl. sporogenes* (control 2).

The supernatants of toxovar E were detoxified as described above.

Statistical analysis

The results of the assays aimed at the determination of an influence of the BoNTs on the exocytotic

and phagocytotic activity were analysed statistically. The Mann-Whitney Rank Sum Test was used to show significant differences between the approaches with culture supernatants and those with blank media controls.

Results

Botulinum neurotoxins

The reference strains of the toxovars A – D, F and G produced BoNTs in concentrations of $5 \cdot 10^2$ MLD·ml^{-1} in Trypticase-peptone-glucose-yeast-extract broth with addition of trypsin solution (TPGYT), as well as in the broth without it (TPGY). Strain E produced a concentration of only 5 MLD·ml^{-1} in the trypsinized variant (TPGYT) and no detectable BoNT in the non-trypsinized one (TPGY).

The culture supernatants of *Cl. sporogenes* (TPGY and TPGYT), which should serve as another non-neurotoxin-containing control (= control 2), did not show any mouse toxicity.

T. pyriformis and BoNTs

Preliminary investigations

The microscopical examination demonstrated that there was no negative influence of the BoNTs either on the viability or on the quantity of the protozoa, when these approaches were compared to the controls (controls 1 and 2).

The growth of the prey germ *E. coli* K12 in suspensions with the culture supernatants of the *Cl. botulinum* toxovars and *Cl. sporogenes* did not differ from the growth in suspensions with blank media (TPGY and TPGYT, control 1 without neurotoxins) after an incubation period of 4h (Table 1). Furthermore, there was no difference in the measurable enzymatic activity of acid phosphatase between the approaches incubated with the blank media controls for 5 h and the ones incubated with culture supernatants of the *Cl. botulinum* toxovars and *Cl. sporogenes* (Table 2).

Exocytotic activity

The results of the photometrical measurements of the amounts of acid phosphatase released into the medium were initially adjusted by subtracting the unspecific background-signals. Afterwards, the results of the assays with the blank media controls were defined as 100% exocytosis-activity and the results of the approaches with the neurotoxin-containing supernatants of the *Cl. botulinum* toxovars and the supernatants of *Cl. sporogenes* were adjusted accordingly.

The results of the approaches with the supernatants of the *Cl. sporogenes* cultures did not differ from the results with the blank media controls.

The number of cells of *T. pyriformis* remained constant during the assay determining the exocytotic activity (Table 3).

In the assays with the different neurotoxins, the trypsin-added culture supernatant of toxovar E was

Table 1. Log_{10}cfu·ml^{-1} of *E. coli* K12 after incubation in a suspension with blank media (control) and with culture supernatants of *Cl. botulinum* and *Cl. sporogenes* for 4h.

Cultivation medium	Control	Mean (n=3), log_{10}cfu·ml^{-1}	SD	Supernatant of	Mean (n=3), log_{10}cfu·ml^{-1}	SD
Without trypsin (TPGY)	Blank medium	6.90	0.14	Toxovar A	7.17	0.27
	Blank medium	7.50	0.27	Toxovar B	7.50	0.27
	Blank medium	7.03	0.11	Toxovar C	7.06	0.11
	Blank medium	7.81	0.16	Toxovar D	7.58	0.31
	Blank medium	8.16	0.12	Toxovar E	7.89	0.24
	Blank medium	6.90	0.14	Toxovar F	7.24	0.21
	Blank medium	7.81	0.16	Toxovar G	7.75	0.28
	Blank medium	7.42	0.09	*Cl. sporogenes*	7.36	0.08
With trypsin (TPGYT)	Blank medium	7.78	0.10	Toxovar A	7.89	0.11
	Blank medium	7.78	0.10	Toxovar B	7.59	0.19
	Blank medium	7.92	0.43	Toxovar C	7.90	0.17
	Blank medium	7.92	0.43	Toxovar D	7.81	0.12
	Blank medium	8.24	0.08	Toxovar E	7.93	0.30
	Blank medium	7.78	0.10	Toxovar F	7.91	0.12
	Blank medium	7.92	0.43	Toxovar G	7.93	0.08
	Blank medium	7.37	0.04	*Cl. sporogenes*	7.23	0.10

Table 2. Milli-optical density of the approaches to determine the enzymatic activity of the acid phosphatase after the addition of the substrate in sterile filtrates of a *T. pyriformis* culture after incubation with blank media (control) and with culture supernatants of *Cl. botulinum* and *Cl. sporogenes* for 5h, unspecific background signals subtracted.

Cultivation medium	Control	Mean (n=3), mOD*	SD	Supernatant of	Mean (n=3), mOD*	SD
Without trypsin (TPGY)	Blank medium	1535	31	Toxovar A	1515	66
	Blank medium	1535	31	Toxovar B	1495	38
	Blank medium	1459	31	Toxovar C	1464	16
	Blank medium	1459	31	Toxovar D	1463	43
	Blank medium	1606	56	Toxovar E	1598	57
	Blank medium	1069	33	Toxovar F	1062	3
	Blank medium	1459	31	Toxovar G	1459	106
	Blank medium	1380	40	*Cl. sporogenes*	1362	29
With trypsin (TPGYT)	Blank medium	1059	23	Toxovar A	1031	58
	Blank medium	1059	23	Toxovar B	1040	18
	Blank medium	1531	47	Toxovar C	1508	37
	Blank medium	1531	47	Toxovar D	1530	69
	Blank medium	1816	105	Toxovar E	1859	102
	Blank medium	2027	100	Toxovar F	2072	154
	Blank medium	1891	85	Toxovar G	2039	225
	Blank medium	1816	105	*Cl. sporogenes*	1805	94

* mOD = milli-optical density

the only one to cause a significantly lower exocytosis-activity (Table 4). After detoxification of the supernatant, a significantly lower exocytosis-activity was still observed. The measurement of the protein amounts in the supernatant and in the blank medium control (TPGYT) revealed differences (145 $mg \cdot ml^{-1}$ and 46 $mg \cdot ml^{-1}$, resp.). They were adjusted by dilution. Afterwards, the exocytotic activities did not differ significantly anymore (Table 5).

Phagocytotic activity

Sterile filtrates of protozoan cultures did not show any detectable influence on the concentration of the bacteria (Table 6), compared with the control (VOLVIC®-water instead of sterile filtrate).

Table 3. Number of cells of *T. pyriformis* at the beginning and the end of the assays to determine the exocytotic and phagocytotic activity.

Group	Time in h	Mean (n=3) $log_{10}cfu \cdot ml^{-1}$	SD
Exocytosis assay	0	5.22	0.02
	5	5.24	0.03
Phagocytosis assay	0	4.53	0.08
	4	4.45	0.13

Therefore, the decrease of the cfu of *E. coli* K12 in a suspension with *T. pyriformis* was only due to the

Table 4. Exocytotic activity of *T. pyriformis* after incubation with culture supernatants of *Cl. botulinum* and *Cl. sporogenes*, resp., for 5 h, unspecific background signals subtracted, propotional (exocytotic activity in the blank media controls = 100%).

Cultivation medium	Supernatant of	Mean (n=9), %	SD
Without trypsin (TPGY)	Toxovar A	84	6
	Toxovar B	88	3
	Toxovar C	109	8
	Toxovar D	116	7
	Toxovar E	88	17
	Toxovar F	91	18
	Toxovar G	103	12
	Cl. sporogenes	94	9
With trypsin (TPGYT)	Toxovar A	89	14
	Toxovar B	92	2
	Toxovar C	100	12
	Toxovar D	102	8
	Toxovar E	66*	2
	Toxovar F	99	14
	Toxovar G	96	22
	Cl. sporogenes	87	8

* $p < 0,01$

Table 5. Exocytotic activity of *T. pyriformis* after incubation with culture supernatants of *Cl. botulinum* for 5 h, unspecific background signals subtracted, propotional (exovytotic activity in the blank media controls = 100%).

Cultivation medium	Supernatant of	Mean (n=9), %	SD
With trypsin (TPGYT)	Toxovar E	66*	1
	Toxovar E, detoxified	65*	10
	Toxovar E, protein amount adjusted	98	24

* $p < 0,01$

phagocytotic activity of the protozoa. The number of cells remained constant during the assay determining the phagocytotic activity (Table 3).

The culture supernatants of *Cl. sporogenes* did not affect the phagocytotic activity of the protozoa compared with the blank media controls. The neurotoxin-containing supernatants of the different *Cl. botulinum* toxovars did not significantly affect the phagocytotic activity of *T. pyriformis*, either, neither in the trypsinized nor in the non-trypsinized variant,

Table 6. $Log_{10}cfu \cdot ml^{-1}$ of E. coli K12 after incubation with a sterile filtrate of a *T. pyriformis* culture and VOLVIC®-water, resp., for 4 h.

Group	Mean (n=3), $log_{10}cfu \cdot ml^{-1}$	SD
Sterile filtrate	6.58	0.22
VOLVIC®-water control	6.79	0.20

except the supernatants of toxovar E (Table 7). The detoxified variants still impaired the phagocytotic activities in the same way. The amount of protein in the non-trypsinized supernatant of toxovar E differed from the blank medium control (TPGY) as well (100 $mg \cdot ml^{-1}$ and 43 $mg \cdot ml^{-1}$, resp.). The protein concentrations in the culture supernatants were adjusted by dilution. No further effect on the phagocytotic activities was observed (Table 8).

Discussion

The addition of trypsin to the culture medium of the *Cl. botulinum* strains is shown to enhance the toxicity of the neurotoxins A (Krysinski and Sugi-

Table 7. $Log_{10}cfu \cdot ml^{-1}$ of *E. coli* K12 after incubation with *T. pyriformis* and blank media (control) and culture supernatants of *Cl. botulinum* and *Cl. sporogenes*, resp., for 4 h.

Cultivation medium	Control	Mean (n=9), $log_{10}cfu \cdot ml^{-1}$	SD	Supernatant of	Mean (n=9), $log_{10}cfu \cdot ml^{-1}$	SD
Without trypsin	Blank medium	4.39	0.22	Toxovar A	4.37	0.18
(TPGY)	Blank medium	4.33	0.19	Toxovar B	4.47	0.14
	Blank medium	4.73	0.27	Toxovar C	4.76	0.24
	Blank medium	4.61	0.40	Toxovar D	4.62	0.32
	Blank medium	5.23	0.36	Toxovar E	7.51*	0.28
	Blank medium	4.46	0.16	Toxovar F	4.56	0.14
	Blank medium	4.61	0.40	Toxovar G	5.05	0.56
	Blank medium	4.71	0.28	*Cl. sporogenes*	5.13	0.25
With trypsin	Blank medium	4.48	0.17	Toxovar A	4.76	0.42
(TPGYT)	Blank medium	4.48	0.17	Toxovar B	4.59	0.19
	Blank medium	4.61	0.22	Toxovar C	4.67	0.25
	Blank medium	4.61	0.22	Toxovar D	4.64	0.40
	Blank medium	4.40	0.47	Toxovar E	7.03*	0.19
	Blank medium	4.48	0.17	Toxovar F	4.55	0.14
	Blank medium	4.61	0.22	Toxovar G	4.50	0.37
	Blank medium	4.99	0.43	*Cl. sporogenes*	4.69	0.29

* $p < 0,001$

Table 8. $Log_{10}cfu \cdot ml^{-1}$ of *E. coli* K12 after incubation with *T. pyriformis* and blank media (control) and culture supernatants of *Cl. botulinum* toxovar E, resp., for 4 h.

Cultivation medium	Control	Mean (n=9), $log_{10}cfu \cdot ml^{-1}$	SD	Supernatant of	Mean (n=9), $log_{10}cfu \cdot ml^{-1}$	SD
Without trypsin	Blank medium	5.23	0.36	Toxovar E	7.51*	0.28
(TPGY)	Blank medium	5.09	0.53	Toxovar E, detoxified	7.46*	0.25
	Blank medium	4.79	0.70	Toxovar E, protein amount adjusted	4.97	0.42
With trypsin	Blank medium	4.45	0.38	Toxovar E	7.03*	0.19
(TPGYT)	Blank medium	4.34	0.14	Toxovar E, detoxified	7.00*	0.50
	Blank medium	4.68	0.48	Toxovar E, protein amount adjusted	4.57	0.33

yama, 1981); B (Hallis et al., 1996); D (Miyazaki et al., 1977); E (Duff et al., 1957); F (Holdeman and Smith, 1965) and G (Giménez and Ciccarelli, 1970). There are controversial discussions about toxovar C enhancement (Baumgart, 1970), no effect (Eklund and Poyski, 1972), nevertheless, it was treated like the others.

Differences in the mouse bioassay due to the addition of trypsin were only seen in toxovar E, but BoNT concentrations were not specified further than the decimal power for ethical reasons, and so it is possible that the differences caused by the addition of trypsin were not revealed. The different toxovars produced their toxins in the same amounts, except toxovar E. Since no other strain of toxovar E was available, we had to work with a lower toxin concentration.

Cl. sporogenes served as additional unspecific control because of its similarity to *Cl. botulinum*. There is a strong DNA-homology and it is not possible to distinguish between those two species just by metabolic or biochemical parameters (Cato et al., 1986). Therefore, we concluded that *Cl. sporogenes* releases into the medium the same or very similar products as *Cl. botulinum*, except the neurotoxins.

Due to the assay design, the effective doses of the neurotoxins were not as high as those detected in the mouse bioassay because they were automatically diluted by the usage in both of our assays. Thus, the effective doses were $1.5 \cdot 10^2$ $MLD \cdot ml^{-1}$ (toxovars A-D, F and G) and 1.5 $MLD \cdot ml^{-1}$ (toxovar E).

All available BoNT-containing supernatants were tested in both of our assays for the exocytotic and phagocytotic activities. Though the non-trypsinized toxovar E did not show any toxicity in the mouse bioassay, it cannot be excluded that our assays could have been more sensitive.

The microscopical examination of protozoan cultures that were exposed to the different neurotoxins revealed no differences compared to the controls without BoNTs in contrast to the results published by Gräf (1985) and De Waart et al. (1972). Although our observation period was shorter than in their assays (4 and 5 h, resp. compared to 1–5 days), there is no hint in the literature that SNARE-proteins play any role in cell division.

The acid phosphatase is transported to the exocytotic sites via lysosomes (Tiedtke et al., 1993). The amount of enzyme released into the medium can be determined by its activity (Rasmussen et al., 1992) and is influenced by the age of a *Tetrahymena* culture. Cells in a static growth phase release more acid phosphatase than those in an exponentially growing phase. Therefore, we used older cultures in the exocytosis assay than in the phagocytosis assay.

Since the detoxified supernatant of toxovar E impaired the release of acid phosphatase in a similar degree as the one with active neurotoxin, the lower performance had to be attributed to other reasons. The considerably higher amount of protein in the supernatant compared to the blank medium control could be proven to cause the differing exocytotic activities.

The usage of a vital prey germ offered several advantages. The phagocytotic activity is more than the simple incorporation of particles. A complex interaction of different membrane fusion processes is required. All of them are presumably mediated by

proteins of the SNARE-complex: the transport of membrane vesicles from the cytoplasm to the cytostome to form the future food or digestive vacuole (Hausmann and Radek, 1996), the lysosomal traffic from the derivation in the endoplasmatic reticulum via processing in the Golgi apparatus to the fusion with the digestive vacuole and the process of egestion (Nilsson, 1987).

Rasmussen et al. (1992) assumed a contribution of secretion products to the extracellular digestion. Therefore, their influence on the bacteria was tested. The amount of enzymes released by the number of cells which we used was not sufficient to degrade vital germs of *E. coli* K12.

It was shown that the phagocytotic activity of *T. pyriformis* was not affected by BoNTs but was affected by the amount of protein introduced into the assay. The additional protein entry seemed to be more critical for the phagocytosis than for the exocytotic activity, since the non-trypsinized supernatant of toxovar E impaired the phagocytosis but not the release of acid phosphatase.

There might be several reasons why no effect of the neurotoxins either on the exocytotic or on the phagocytotic activity of *T. pyriformis* could be measured.

Firstly, the concentration of the BoNTs might have been insufficient. As they could be easily detected in the mouse bioassay, it did not seem necessary to use higher concentrations when looking for an alternative assay.

It is also not clear whether the BoNTs could reach their substrates in an appropriate way. But even if *Tetrahymena* does not possess receptors to take up the BoNTs via endocytosis as other eukaryotic cells, the neurotoxins might have gained access to their substrates at least via phagocytosis of medium or during the engulfment of the bacteria. Since the contents of the digestive vacuoles also undergo acidification, the conditions in them are probably similar to those in the vesicles of the motor nerve cells (Singh, 2000). Nevertheless, this remains speculative, for we did not examine the way of the BoNTs into or through the protozoa.

There is also a probability that SNARE-proteins are not involved in the exocytosis and phagocytosis of *T. pyriformis*. However, since such an involvement is described in many eukaryotic cells (Brumell et al., 1995; Desjardins et al., 1997; Hackam et al., 1998; Bogdanovic et al., 2002; Kissmehl et al., 2002; Froissard et al., 2002), it is more likely that the effect was not visible due to mutations in the binding or cleaving sites of the presumable SNARE-isoforms in *T. pyriformis*.

Thus, *T. pyriformis* cannot provide an alternative for the detection of BoNTs.

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Address for correspondence: Norman Martin Staender. Institut für Bakteriologie und Mykologie, Veterinärmedizinische Fakultät der Universität Leipzig, An den Tierkliniken 29, 04103 Leipzig, e-mail: staender@vetmed.uni-leipzig.de