Toxicity of Papain-digested Tetanus Toxin

PATHOLOGICAL EFFECT OF FRAGMENT B IN THE ABSENCE OF SPASTIC PARALYSIS*

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Fragment B of tetanus toxin (Helting, T. B., and Zwisler, O. (1977) J. Biol. Chem. 252, 187-193), obtained after digestion with papain, gel chromatography, immunoabsorption, and repeated gel chromatography, was atoxic in experimental animals at concentration levels sufficient to induce protective antibody synthesis. However, mice injected with 200 μg of highly purified Fragment B appear normal for approximately 48 h. Between 48 and 72 h after inoculation, the animals develop a characteristic syndrome involving respiratory difficulty, loss of weight, failure to take food, retraction of their abdominal muscles, and finally, death. Convulsions or spastic paralysis of the injected limb, typical features associated with classical tetanus, never developed after administration of Fragment B. In contrast, the muscular activity decreased somewhat in agreement with a more flaccid appearance of the hind legs.

The effect of Fragment B could be abolished by homologous antisera administered together with the antigen. Antisera given at later stages progressively lost their capacity to interfere with the action of Fragment B. Whereas this time dependence of the efficacy of the antibody was similar to that observed for parent tetanus toxin, the action of the latter substance was also inhibited by antisera directed against Fragment C, the second split product of tetanus toxin released upon papain digestion of the molecule. Anti-Fragment C serum did not react with Fragment B and could not interfere with its action.

It is suggested that Fragment B has lost the capacity of the parent toxin molecule to induce the syndrome of spastic paralysis but has retained some characteristic functions of tetanus toxin which may play an important role in interfering with the autonomous nervous system.

A large body of evidence has accumulated that tetanus toxin may supress inhibition in the central nervous system, thereby causing the classical clinical syndrome of spastic paralysis (for a review see Ref. 1). Recent data have indicated that the toxin interferes with the release process of inhibitory transmitters (2, 3) and that it may exert its effect after retrograde axonal transport (4-7) and trans-synaptic migration to a presynaptic target site (8, 9). In addition to α-motor neurons, engagement of the γ-system has been suggested (10). Furthermore, the autonomic nervous system is clearly involved in tetanus pathogenesis and it is increasingly becoming apparent that the disturbance of this system may be a major cause of death due to tetanus intoxication (11, 12).

Recently, evidence was presented that the heavy chain of tetanus toxin contains the receptor site for ganglioside, which may constitute the natural receptor for the toxin in the nervous tissue (19, 20). Furthermore, the degradation of tetanus toxin to yield two polypeptide fragments, B and C, was reported (17, 21). Whereas Fragment C was derived from one portion of the heavy chain polypeptide, Fragment B was shown to contain the remainder of the heavy chain as well as the light chain of tetanus toxin.1

The two fragments were thus derived from nonoverlapping polypeptide sequences of the toxin molecule and were devoid of lethal effects within the ranges tested. Each polypeptide was immunogenic in experimental animals and elicited the formation of antibodies neutralizing the lethal action of tetanus toxin (22).

As more material became available, it was discovered that injection into mice of Fragment B in larger amounts (200 μg or more) does produce a severe pathological effect or even death to the animals. Spastic paralysis of the injected limb, characteristic for tetanus intoxication, never develops after

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1 According to the terminology used in Ref. 17, "Fragment A," which eluted prior to Fragment B and C on Sephadex gel chromatography, was found to be indistinguishable from native tetanus toxin.
intramuscular application of this fragment and death has been attributed to several factors, including general exhaustion and failure to take food or fluids.

The present report describes these hitherto unknown properties of Fragment B. It is proposed that the effects noted are due, at least in part, to the disturbance by this protein fragment of the vegetative nervous system.

**EXPERIMENTAL PROCEDURES**

Fragment B and Fragment C were isolated as described (17, 21), from papain digests of highly purified tetanus toxin. In addition, Fragment B was rechromatographed on a column (2.5 x 250 cm) of Ultrogel AcA 44 gel, eluted with 0.1 M Tris/HCl buffer, pH 8.0, containing 1 M NaCl. The fraction corresponding to Fragment B (Fig. 1A) was treated with rabbit anti-Fragment C serum (immunoglobulin fraction, see below) in order to absorb any trace amount of intact tetanus toxin still present in this fraction. Fragment B was subsequently separated from the immunoglobulins by repeated chromatography on the Ultrogel column.

Antisera directed against Fragment B or C were obtained in rabbits by a sequence of three subcutaneous injections of the antigen (total amount of protein, 0.3 mg) in Freund's complete adjuvant. The immunoglobulin fraction of the sera was prepared after dialysis to its use for absorption purposes. The immunoglobulin fraction, see below, was removed by the immunoabsorption procedure with anti-Fragment C serum, which had no effect on the action of Fragment B, antisera produced against highly purified Fragment B (or against conventional tetanus toxoid) abolished the activity of the antigen. Incomplete protection was noted if these antibodies were administered prior to Fragment B. Whereas anti-Fragment C serum, cannot be ruled out. It appears reasonable, however, to conclude that the spastic paralysis induced in experimental animals by the injection of Fragment B prior to immunosorption is caused by contaminating intact tetanus toxin. The impurity induces a syndrome perfectly in accord with that seen after administration of native tetanus toxin; furthermore, after

**RESULTS**

**Isolation of Fragment B — Purification of Fragment B after digestion of tetanus toxin with papain was conveniently performed by chromatography on large gel columns (Fig. 1). Although homogenous on conventional or sodium dodecyl sulfate-gel electrophoresis, such preparations induced the typical spastic rigidity in the injected limb at concentrations ranging from 1 to 10 μg/mouse. Removal of traces of tetanus toxin by absorption with anti-Fragment C serum, which reacts with tetanus toxin but not with Fragment B, and reisolation of Fragment B by gel chromatography, yielded material which failed to produce any toxic symptoms at such concentration levels.**

**Pathological Effect of Fragment B — Mice injected with 200 μg of Fragment B previously purified by immunosorption appeared normal during 48 h. At 60 h, however, the animals were obviously ill, showing roughing of the fur, weight loss, and an awkward slow movement of the hind legs. At 72 to 96 h, their condition deteriorated further; respiratory difficulty and a peculiar retraction of the abdominal muscles was noted (Fig. 2B). The amount of urine was drastically reduced at 72 h following intoxication, and its protein concentration was increased 3-fold. The animals were unable to take food or water although several attempts at nutritional intake were observed. At these late stages, myograms revealed a diminished electrical activity (Fig. 2C) which corresponded well with the flaccid appearance of the hind legs. There was no evidence for tetanic convulsions nor any sign, during the entire observation period, of spastic paralysis of the injected leg. Death usually ensued at about 96 h and appeared to be due to a combination of several factors, including asphyxia, cardiac arrest, and general exhaustion.

**Effect of Antisera — Table I summarizes the effect of various antisera injected concomitantly with or subsequent to administration of Fragment B. Whereas anti-Fragment C serum had no effect on the action of Fragment B, antisera produced against highly purified Fragment B (or against conventional tetanus toxoid) abolished the activity of the antigen. Incomplete protection was noted if these antibodies were administered 24 h after injection of Fragment B. At 48 h, passive therapy merely prolonged the survival time, and at 72 h, the antisera showed no effect at all. The neutralization by antisera of tetanus toxin exhibited a similar time dependence rechromatography of Fragment B, the contaminating agent partially elutes prior to Fragment B, at a position expected for tetanus toxin. Due to the trailing of the protein, a complete separation from Fragment B by repeated gel chromatography is, however, impractical.
Toxicity of Papain-digested Tetanus Toxin

In a second experiment, tetanus toxin (1.5 mg, approximately 10 nmol) was mixed with anti-Fragment C serum (protective titer, 2000 international units (17)). After incubating for 2 h, aliquots corresponding to 500 µg of the original toxin were injected into three mice and the animals were observed for 14 days. During the entire observation period, no pathological symptoms were recorded. Treatment of Fragment B (10 nmol) with a similar amount of serum followed by injection of 200 µg of this fragment produced the characteristic symptoms on Day 3 followed by death of the animals on Day 4.

**TABLE I**

<table>
<thead>
<tr>
<th>Antigen and antiserum specificity</th>
<th>Time administered</th>
<th>Symptoms</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Anti-Fragment B</td>
<td>0</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>1</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>0</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Anti-Fragment B</td>
<td>0</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>0</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>+ Anti-Fragment C</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

a Day following administration of antigen. Antiserum injected on Day 0 were given simultaneously with the antigen.

b Day following administration of antigen where characteristic symptoms were first observed.

c Symptoms disappeared on Day 10 following injection of antigen.

**Inactivation of Fragment B with Formaldehyde**—Fig. 3 shows the inactivation curve of Fragment B and of tetanus toxin after exposure to formaldehyde for various periods of time. The toxic activity of the two proteins is abolished in a similar fashion, with less than 1% of the original toxicity left after treatment with formaldehyde for 72 h.

**Relative Susceptibility of Various Animals to Fragment B**—Table II shows that based on body weight, guinea pigs are more sensitive to Fragment B than mice. In contrast, rats were not susceptible to Fragment B within the range tested. When given a dose of 2.5 mg of this fragment subcutaneously, rabbits exhibited respiratory difficulties, failure to take food on Day 3 following intoxication and lost 20% of their weight within the 1st week. There were, however, no deaths during the observation period and the animals appeared to recover slowly.

**Postmortem Examination**—Mice intoxicated with 200 µg of Fragment B were killed by cervical dislocation after 80 to 96 h and subjected to pathological examination. Table III summarizes the most important observations. Most conspicuously, the bladders of all intoxicated animals were completely filled with urine. Apart from a slight glomerular atrophy, most organs showed no apparent lesions in the histological examination.

**FIG. 3.** Inactivation of tetanus toxin (○), or Fragment B (×), by treatment with formaldehyde. The proteins were kept at room temperature with 0.05% formaldehyde at pH 6.5 for the periods of time indicated, dialyzed against 0.15 M NaCl, and assayed for toxicity by injection of several dilutions of each sample into groups of mice.

**TABLE II**

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Weight</th>
<th>Amount of Fragment B injected</th>
<th>Onset of symptoms</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>300-350</td>
<td>200</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Mouse</td>
<td>14-16</td>
<td>300</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2500</td>
<td>200</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>Rat</td>
<td>200</td>
<td>2500</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

a Day following administration of Fragment B where symptoms (or death) were first observed.
Toxicity of Papain-digested Tetanus Toxin

<table>
<thead>
<tr>
<th>Organ</th>
<th>Macroscopic finding</th>
<th>Histological finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td>Enlarged</td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>Filled</td>
<td></td>
</tr>
<tr>
<td>Gallbladder</td>
<td>Filled</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>Empty</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>Empty</td>
<td>Normal</td>
</tr>
<tr>
<td>Kidney</td>
<td>Normal</td>
<td>Slight glomerular atrophy</td>
</tr>
<tr>
<td>Brain, heart, lungs, liver, spleen</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Compared to native tetanus toxin, Fragment B, comprising the light chain and a portion of the heavy chain of the intact molecule (17), exhibits only a limited toxicity. The lethal dose required to kill a mouse (about 200 µg of protein) exceeds that of tetanus toxin by several orders of magnitude, and it is important to exclude the possibility that impurities in the preparation of Fragment B may be responsible for the action ascribed to this fragment. On polyacrylamide gel electrophoresis, only one component was observed (Fig. 1). Immuno-diffusion analysis against multivalent horse antitoxin or against antisera raised in rabbits by immunization with Fragment B invariably revealed a single line of precipitation. Furthermore, it may be expected that anti-Fragment C serum, which does not react with Fragment B, would also fail to neutralize impurities (unrelated to tetanus toxin) present in the preparation. However, it is reasonable to assume that such impurities would then remain active after treating samples of tetanus toxin itself, used as starting material for the preparation of Fragment B with anti-Fragment C serum. The finding that anti-Fragment C serum is unable to neutralize purified Fragment B, whereas the toxin preparation used to prepare this fragment may be completely inactivated by the antiserum, strongly suggests that Fragment B itself is responsible for the pathological effects observed.

Fragment B was inactivated by treatment with formaldehyde in much the same fashion as tetanus toxin itself. Further, the degree of protection afforded by administration of homologous antibodies was highly time-dependent. As is the case with tetanus toxin, Fragment B is no longer accessible to neutralizing antibodies given 48 h after injection. A possible explanation of this finding would be the uptake of Fragment B by the nervous tissue. Similarly to the parent toxin molecule, Fragment B would then interfere with the function of the nervous system. The clinical symptoms observed are in accord with this hypothesis. The fact that the morphology of several tissues appeared normal at stages where the animals were severely ill would seem to indicate a functional derangement which has not yet been manifested in organic lesions.

The inability of the animals to take food, although they obviously attempted to eat, may indicate a paralytic syndrome of involuntary muscles. Further, all mice receiving Fragment B were unable to empty their bladders at 72 h following intoxication. This result is consistent with vegetative dysfunction involving increased activity of the sympathetic nervous system.

The relatively low toxicity of Fragment B compared to that of tetanus toxin may conceivably be explained by an impaired capacity to associate with appropriate receptors in the nervous tissue. Whereas the heavy chain of tetanus toxin was shown to bind to ganglioside, no such interaction for Fragment B was observed (19). Therefore, the possibility should be considered that processing of Fragment B within the nervous tissue may be less efficient and that only a small amount of the material injected may reach its site of action. Clearly, tracer studies with Fragment B should be initiated to illuminate this point. In addition, the failure of Fragment B to interact with the putative toxin receptor in the central nervous system might offer a possible explanation for the inability of this fragment to elicit the typical muscular rigidity induced by intact tetanus toxin. One might speculate that binding to the ganglioside, or some similar structure, would constitute a preliminary requisite for eliciting spastic paralysis, whereas nonbound toxin (or Fragment B) occurring in the spinal cord would still cause imbalance to the vegetative centers.

Therapeutic measures aimed at preventing major spasms observed in classical tetanus have only been partially successful in reducing the death rate due to the disease. Increasing attention is being focused on the action of tetanus toxin on the autonomous nervous system (11, 12). In fact, a vegetative dysfunction caused by the toxin may contribute significantly to the high mortality rate of patients under intensive treatment for tetanus. Recent evidence has also been presented that the toxin may exert a pathological activity on the thyroid (24).

While lacking some salient characteristics of the parent molecule, such as the ability to induce muscular rigidity, Fragment B appears to have retained the capacity to interfere with other processes of the nervous system. Therefore, this fragment should constitute a suitable vehicle for studying some aspects of the pathology of tetanus under conditions where the phenomenon of disinhibition of the motor nerve is excluded.

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