Effect of Tetanolysin on Platelets and Lysosomes

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Tetanolysin, partially purified, caused the lysis of human and rabbit platelets, as determined by a decrease in the optical density of platelet suspensions and the release of serotonin, enzymes, and protein. This lytic activity was neutralized by antitoxin. In addition, a suspension of the lysosome-containing large granule fraction of rabbit liver released hydrolytic enzymes when exposed to tetanolysin. Thus, tetanolysin can be added to the list of bacterial toxins that are lytic for a variety of cellular or subcellular membranes. These findings provide additional data that suggest that tetanolysin may contribute to the pathogenesis of some of the unusual manifestations observed in clinical tetanus.

Several bacterial toxins have been shown to destroy platelets (5) and disrupt lysosomes (4, 17). Some of these toxins (i.e., streptolysin O and the hemolysin of Listeria monocytogenes) belong to the group of bacterial products known as oxygen-labile hemolysins (2). Tetanolysin, the hemolysin produced by Clostridium tetani, shares many of the properties of other oxygen-labile hemolysins (2, 12), but its effect on platelets and lysosomes has not been previously reported. Data are presented in this report to show that a partially purified preparation of tetanolysin can be added to the list of toxins that disrupt a variety of membranes in vitro.

MATERIALS AND METHODS

Preparation of toxins. Crude culture filtrates of C. tetani, Massachusetts C9 strain, were concentrated and applied to Sephadex G-100 columns (2.5 by 85 to 90 cm) as previously described (11). Elution was made with phosphate buffer (pH 7.0), 0.025 M, by upward flow. The eluate was collected in 5-ml fractions, and chloroform (0.025 ml) was added to each tube as a preservative. Fractions were assayed for neurotoxic activity in mice (minimum lethal doses per milliliter) and hemolytic activity using 2% sheep red blood cells (hemolytic units [HU] per milliliter) (11). Fractions in the area of peak neurotoxic activity were called neurotoxin, and those in the area of peak hemolytic activity were called tetanolysin. Control materials were column fractions that were heated in a water bath at 75 C for 30 min, or elution buffer.

Preparation of platelets. New Zealand white rabbits weighing approximately 2,500 g, obtained from the Rodent and Rabbit Production Section, Division of Research Services (DRS), National Institutes of Health (NIH), were bled by cardiac puncture by using plastic syringes. The blood was delivered into one-ninth of its volume of 4% sodium citrate solution contained in siliconized glass tubes. Platelet suspensions were then prepared by using methods similar to those described by Des Prez et al. (7) and Bernheimer and Schwartz (5). The blood was centrifuged for 30 min at 225 × g. The supernatant fluid containing the platelets was transferred to a polycarbonate tube and centrifuged for 10 min at 15,000 × g at 4 C. The platelets were resuspended in one-half their original volume of phosphate-buffered saline, pH 7.4 (PBS). The platelet suspension was recentrifuged at 15,000 × g, and then the platelets were resuspended in PBS by gentle agitation. The optical density at 520 nm (OD520) of the plasma-free platelet suspension was determined in a Beckman DU-2 spectrophotometer by using PBS as a blank. The OD520 of these suspensions ranged from 1.0 to 1.3. Platelet preparations prepared by these procedures contained few other types of cells when examined microscopically. Platelet counts were not performed. They were used the day of preparation. Human platelets in plasma, contained in Fenwall Transpacks, were obtained from the Blood Bank, Clinical Center, NIH. These were treated in the same manner as the rabbit platelets, with the exception that the initial centrifugation at 225 × g was omitted. The OD520 of the suspension was adjusted with PBS to give a reading of 1.3 to 1.9. For maximum disrupted platelet controls (F-T), portions of both the rabbit and the human platelet preparations were subjected to 10 freeze-thaw cycles.

Preparation of lysosome-containing LGF. The method of Weissmann and Thomas (22) was used for the preparation of a liver fraction containing lysosomes. The livers of New Zealand white male rabbits weighing 800 to 1,000 g, obtained from DRS, NIH, were removed and placed in an ice-cold 0.25 M sucrose solution, minced, and washed in the cold sucrose solution. Subsequent procedures were performed at 4 C. A 1:10 (wt/vol) homogenate of wet tissues was prepared in 0.25 M sucrose by grinding in a glass homogenizer equipped with a motor-driven Teflon pestle at 900 rpm. The homogenate was centrifuged at 15,000 × g for 20 min at 4 C. The supernatant was discarded, and the pellet was washed.
twice in the sucrose solution. Then the pellet was resuspended in the sucrose solution, placed in a glass homogenizer and ground by hand to obtain a uniform suspension. The OD520 of the large granule fractions (LGF) was read and adjusted with 0.25 M sucrose to give a reading of less than 1.9. Sucrose solution was used as the blank. The suspension was maintained at 4 C at all times. For maximum disrupted lysosome controls, a portion of the suspension was subjected to 15 freeze-thaw cycles.

**Platelet- or lysosome-column fraction interactions.** To study the effects of various column fractions on platelets or lysosomes, methods similar to those of Bernheimer and Schwartz (4, 5) and Kingdon and Sword (17) were used. In preliminary experiments, varying volumes of platelet suspensions (0.75 to 4.0 ml) were treated with varying volumes of column fractions, heated fractions, or buffer (0.02 to 0.25 ml). The OD520 was read in a Beckman DU-2 spectrophotometer immediately after the addition of test sample and at various intervals for 60 min. These mixtures were centrifuged at 15,000 x g for 25 min, and samples were obtained for assays of enzymes, protein, OD determination from 200 to 300 nm, and serotonin. In addition, platelets were examined by using phase optics before and after treatment with test samples. Definitive experiments were performed by using the following procedure. Two milliliters of the platelet or lysosomal suspension was measured by using plastic pipettes into 3.5-ml matched quartz cells. One milliliter of eluate, heated eluate, or buffer was added to the cell, and the OD was read at once. Changes in the OD520 were observed for 60 min, and the percent decrease in OD was calculated by using the OD value of the suspension prior to the addition of the eluate, etc. PBS was used as a blank for the platelet assays, and 0.25 M sucrose was used as a blank for the lysosome assays. Samples were transferred to polycarbonate tubes and maintained at room temperature between readings. After the 60-min reading, the preparations were centrifuged at 15,000 x g for 25 min. The supernatant fluids were removed and stored at 5 C in glass screw-cap tubes until assayed for enzymes, serotonin, etc.

**Enzyme assays.** Acid phosphatase determinations were made by the colorimetric method described in Sigma technical bulletin no. 104 (Sigma Chemical Co., St. Louis, Mo.). The substrate was p-nitrophenyl phosphate. The number of Sigma units per milliliter of enzyme was based on a curve prepared by using a p-nitrophenol standard. Beta-glucuronidase determinations were made by the colorimetric method described in Sigma technical bulletin no. 105 (Sigma Chemical Co., St. Louis, Mo.). The substrate was phenolphthalein glucuronide. The number of units per milliliter of enzyme was based on a curve prepared by using a phenolphthalein standard.

**Serotonin.** The spectrofluorometric assay of Snyder et al. (20) was used for the determination of serotonin in the supernatant fluid from the platelet-column fraction mixture. The fluorescence of the mixture was then measured in an Amino-Bowman spectrofluorometer at 490 nm after excitation at 385 nm. The amount of serotonin was determined from a curve prepared by using known concentrations of serotonin as the standard.

**Protein determinations.** Protein determinations were performed by using a modification of the Kunkel and Tiselius adaptation of the Folin-Ciocalteau phenol reaction (18).

**Effect of tetanus antitoxin on the platelet-tetanolysin interaction.** The capacity of tetanus antitoxin to alter the effect of tetanolysin was examined by incubating 0.675 ml of tetanolysin (6,000 HU/ml) for 1 h at 25 C with 0.075 ml of U.S. standard tetanus antitoxin containing 5, 2.5, or 0.5 antitoxin units (AU/ml); tetanus antitoxin (equine) containing 4,000, 2,000 or 400 AU/ml; tetanus immune globulin containing 275, 137.5, or 27.5 AU/ml; or PBS, pH 7.4. All dilutions were made in PBS. The tetanolysin-antitoxin mixture (0.5 ml) was added to 1.5 ml of platelet suspension and the OD520 observed for 60 min, as previously described. The acid phosphatase content of the supernatant of this mixture was then assayed.

**RESULTS**

**Effect of tetanolysin on platelets.** Microscopy examination of platelets using phase optics showed that the platelets treated with tetanolysin reacted first by swelling, sending out pseudopodia and blebs, and then becoming small and shriveled. Neither heated tetanolysin nor buffer altered platelet morphology. Table 1 shows the quantities of various platelet components in the supernatant from rabbit platelets treated with tetanolysin, heated tetanolysin, or buffer, and from the supernatants of the frozen and thawed platelets. In addition, the percent decrease in OD520 after 1 h of incubation of the platelet mixtures is shown. More acid phosphatase, serotonin, and protein were present in the supernatants from the platelet-tetanolysin suspensions than in those from the suspensions containing heated tetanolysin or buffer. No beta-glucuronidase was found in any of the supernatants from rabbit platelets. The percent decrease in turbidity of the platelet suspensions, as determined by OD, was almost as great in the suspensions treated with tetanolysin as in the suspensions undergoing freezing and thawing.

When the OD of a 1:15 dilution in PBS of the supernatants from these platelet suspensions was determined over the range of 200 to 300 nm by using the Beckman DU-2, each curve had a peak at 205 nm. The readings at 205 nm of the supernatant dilutions of the F-T platelets, the tetanolysin-treated platelets, heated tetanolysin, or buffer-treated platelets were 0.45, 0.45, 0.15, and 0.20, respectively. Although no attempt was made to specifically identify the substances being detected, amino acids and peptides are known to absorb at this wavelength. The procedure appeared capable of
detecting some platelet component released by freezing and thawing and by treatment with tetanolysin.

To determine that the platelet lysis was caused only by fractions containing tetanolysin, unheated and heated fractions eluted from the column were added to the rabbit platelet suspensions. Figure 1A shows the elution pattern of neurotoxin and tetanolysin in “column L.” (Other substances are present in these fractions, i.e., a gelatinase, a peptidase, and esterases [unpublished data].) Figure 1B shows the amounts of acid phosphatase or serotonin detected in the supernatants from rabbit platelet suspensions treated with unheated fractions L minus the amounts detected in the heated fractions. The supernatants from platelets treated with buffer had values similar to those of the heated fractions. As stated previously, no β-glucuronidase was detected in any of the experiments using rabbit platelets. Figure 1C shows a second elution pattern for tetanolysin (“column P”), whereas Fig. 1D shows the amounts of acid phosphatase, serotonin, and β-glucuronidase detected in the supernatants from human platelet suspensions treated with unheated fractions from this filtration experiment minus the amount detected in the heated fractions.

### Table 1. Effect of tetanolysin (600 HU/ml) on the OD of rabbit platelet suspensions and the release of platelet constituents into the supernatants

<table>
<thead>
<tr>
<th>Plasma-free platelets plus</th>
<th>Decrease (%) in OD₂₅₀ after 60 min</th>
<th>Acid phosphatase</th>
<th>Serotonin</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sigma U/ml % F-T⁺</td>
<td>µg/ml % F-T⁺</td>
<td>µg/ml % F-T⁺</td>
</tr>
<tr>
<td>Tetanolysin</td>
<td>69</td>
<td>0.31</td>
<td>221</td>
<td>1.75</td>
</tr>
<tr>
<td>Tetanolysin, heated</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>0.35</td>
</tr>
<tr>
<td>Buffer</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0.50</td>
</tr>
<tr>
<td>Frozen-thawed platelets</td>
<td>78*</td>
<td>0.14</td>
<td></td>
<td>2.70</td>
</tr>
<tr>
<td>plus buffer</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Percentage of value detected in supernatants from suspensions of frozen-thawed platelets.
* Value before freeze-thaw used for calculating difference.

![Fig. 1](http://iai.asm.org/)

**Fig. 1.** (A) The neurotoxin and tetanolysin content of fractions of culture filtrate of C. tetani separated by G-100 chromatography (column L). (B) The difference in the amounts of enzymes or serotonin detected in the supernatants from suspensions of rabbit platelets or lysosome-containing LGF of rabbit liver treated with unheated and heated fractions of column L. (C) The tetanolysin content of fractions of culture filtrate of C. tetani separated by G-100 chromatography (column P). (D) The difference in the amounts of enzymes or serotonin detected in the supernatants from human platelets treated with unheated and heated fractions of column P.
fractions. Treatment of this lot of human platelets with active fractions resulted in the release of β-glucuronidase. With both types of platelets, an increase in acid phosphatase, serotonin, and/or β-glucuronidase was observed only in the areas of tetanolysin activity.

Figure 2A shows the effect of selected fractions from “column L” (heated and unheated) on the OD of rabbit platelets after 1 h of incubation. The percent decrease in OD was calculated by using the OD of the suspension prior to the addition of the fraction and the value at 60 min. The maximum change occurred in those suspensions treated with the fractions containing maximum hemolytic activity. Most of the change in OD occurred in the first 15 min. The freezing and thawing of the platelet-buffer suspension resulted in an 88% decrease in OD. The OD could not be changed by shaking, suggesting that the decrease in OD was due to lysis of the platelets and not aggregation. Human platelet suspensions treated with column fractions demonstrated the same pattern of change in OD.

Preincubation of the tetanolysin with certain antitoxin preparations altered the capacity of the tetanolysin to lyse human platelets, as determined by a decrease in the OD and increased levels of acid phosphatase in the supernatants (Table 2). A lot of tetanus antitoxin (equine) containing approximately 4,000 AU/ml contained enough antihemolysin activity to prevent the lysis, whereas the U.S. standard antitoxin (equine) containing 5 AU/ml did not prevent the lytic effect. A lot of tetanus immune globulin (TIG) containing 275 AU/ml appeared to have an intermediate effect on the OD. This was most evident at the 30-min reading. However, the lower dose of TIG appeared to increase the enzymatic activity in the supernatant.

**Effect of tetanolysin on lysosome-containing LGF from rabbit liver.** Table 3 shows the quantities of acid phosphatase, β-glucuronidase, and protein present in the supernatants from LGF treated with tetanolysin, heated tetanolysin, or buffer and the quantities in the supernatants of LGF undergoing freezing and thawing followed by treatment with buffers. As with the platelets, more enzyme was present in the supernatants from the tetanolysin-treated suspensions than in those treated with heated tetanolysin or buffer. The quantities of acid phosphatase and β-glucuronidase detected in supernatants from LGF treated with unheated fractions minus the quantities detected in the heated fractions of “column L” are shown in Fig. 1B. As with the platelets, the maximum quantities of enzymes were released by the fractions containing tetanolysin. The secondary peak of β-glucuronidase was not seen in either of two other experiments using fractions from

![Graph](http://iai.asm.org/)

**Fig. 2.** (A) The effect of selected fractions of column L on the OD of rabbit platelets. (B) The effect of selected fractions of column L on the OD of lysosome-containing LGF of rabbit liver.

<p>| Table 2. Effect of tetanus antitoxin and tetanus immune globulin on lysis of human platelets by tetanolysin |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Tetanolysin (HU)</th>
<th>Anti-toxin (AU)</th>
<th>Decrease (%) in OD</th>
<th>Acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,700</td>
<td>0.0625*</td>
<td>61</td>
<td>64</td>
</tr>
<tr>
<td>2,700</td>
<td>0.1250</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>2,700</td>
<td>0.2500</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>2,700</td>
<td>3.4*</td>
<td>54</td>
<td>59</td>
</tr>
<tr>
<td>2,700</td>
<td>6.8</td>
<td>44</td>
<td>54</td>
</tr>
<tr>
<td>2,700</td>
<td>13.8</td>
<td>17</td>
<td>48</td>
</tr>
<tr>
<td>2,700</td>
<td>20c</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>2,700</td>
<td>None</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>2,700</td>
<td>None</td>
<td>60</td>
<td>62</td>
</tr>
</tbody>
</table>

*U.S. standard antitoxin.
* Tetanus immune globulin.
* Tetanus antitoxin.
other "columns." Although there was a slight decrease in the OD of the lysosomal preparations 1 h after treatment with the fractions containing tetanolysin, this change was not comparable to that observed in the platelet preparations (Fig. 2B).

**DISCUSSION**

Data have been presented that indicate that a partially purified preparation of tetanolysin causes the release of serotonin, hydrolytic enzymes, and proteins from both rabbit and human platelets in vitro and the release of lysosome-associated hydrolytic enzymes and protein from the large granule fraction of rabbit liver. (The term "release" is not used here according to the definition of the "platelet release reaction" as discussed by Holmsen and Day [14].) Previous workers have shown that other oxygen-labile hemolysins are disruptive for rabbit platelets (5), rabbit leukocyte lysosomes (4), and rabbit liver lysosomes (4, 17). Bernheimer classified these bacterial products as cytolytic toxins (2) and stated that all toxins that he studied that are hemolytic lyse platelets in vitro, whereas nonhemolytic toxins did not. Our observations on the lytic activities of tetanolysin are therefore consistent with these previous observations.

In addition to tetanolysin and neurotoxin, a gelatinase, a peptidase, and two esterases have been detected in fractions of *C. tetani* culture filtrate obtained by gel filtration (unpublished data). The peak activities of these enzymes have been separated from the peak activities of the hemolysin by this procedure. The esterase activity has been defined by the ability of the fractions to release fatty acid from the emulsifier, Arlacel A (labeled mannide monooleate). Fractions containing hemolytic activity also contain esterase activity, but our unpublished studies suggest the tetanolysin and esterase can be separated. Fractions eluting before the neurotoxin also contain esterase activity, but these fractions did not alter the platelets or lysosomes. In view of the lytic activities reported for the other oxygen-labile hemolysins, it seems likely that the effect on the platelets and lysosomes was due to the tetanolysin. However, none of the preparations used represented pure tetanolysin; therefore, contaminating enzymes or other materials may have contributed to the lytic effects observed. A pure preparation of tetanolysin is needed to further define this problem.

The mechanisms by which cytolytic toxins damage the cellular or subcellular membranes are unknown. Bernheimer and Davidson (3) stated that all cells that are sensitive to streptolysin O, another of these oxygen-labile hemolysins, contain cholesterol as a constituent of their cell membrane. It has been suggested that cholesterol is involved in the lytic action (2, 3). Such a sterol could serve as the receptor site (or substrate) for the toxin (1). Although these toxins may be similar in many ways, the same mechanism of action may not necessarily be found for each member of this group of toxins. In fact, differences in the activities of pneumolysin and streptolysin O were recently suggested by Johnson (15).

None of the oxygen-labile hemolysins have been assigned a role in human disease, although much effort has gone into implicating streptolysin O in the pathogenesis of streptococcal illness (8, 10). A previous report from our laboratory showed that partially purified tetanolysin was cardiotoxic for mice and monkeys, lethal for mice, and induced hemolysis in vivo after intravenous injection, and suggested that some of the complications of clinical tetanus, such as cardiovascular alterations, hemolysis, and anemia might be due, in part, to the tetanolysin (12).

Tetanus in man has been complicated by hemorrhagic and clotting disorders (9, 13, 16, 19). Although the most likely etiology of these complications is the multiple therapeutic measures necessary for the treatment of this disease, it may be reasonable to examine the possibility that some of these problems are the result of the effect of tetanolysin on platelets and other cells in vivo. Okulski reported that in a group of patients with tetanus the mean blood platelet

**Table 3. Effect of tetanolysin (600 HU/ml) on the release of enzymes and protein from the lysosome-containing LGF of rabbit liver**

<table>
<thead>
<tr>
<th>Lysosomes plus</th>
<th>Acid phosphatase</th>
<th>β-glucuronidase</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sigma U/ml</td>
<td>% F.T.</td>
<td>U/ml</td>
</tr>
<tr>
<td>Tetanolysin</td>
<td>0.80</td>
<td>96</td>
<td>14.5</td>
</tr>
<tr>
<td>Tetanolysin, heated</td>
<td>0.3</td>
<td>36</td>
<td>4.5</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.29</td>
<td>47</td>
<td>4.0</td>
</tr>
<tr>
<td>Frozen-thawed lysozymes plus buffer</td>
<td>0.83</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>
counts were lower than normal upon admission (19). This difference, however, was not statistically significant. Harrison et al. (13) stated that thrombocytopenia was a common complication of the treatment of tetanus. The release of vasoactive substances from platelets by tetanolysin could also contribute to the labile blood pressure observed in some patients. This liability has been attributed to overactivity of the sympathetic nervous system (6).

If tetanolysin plays any role in the pathogenesis of any of the complications of tetanus in man, then antibody to tetanolysin may be one of the necessary components of tetanus immune globulin and tetanus antitoxin, both of which are used for therapy or prophylaxis, or both. The studies reported here show that the lytic activity of the tetanolysin for human platelets could be neutralized by antibody if an adequate quantity was used. The lot of commercial tetanus antitoxin (equine) used in this study appeared to contain more anti-tetanolysin activity per unit of antitoxin than did the lot of tetanus immune globulin (human). The antigen administered to the horse for hyperimmunization may have contained more tetanolysin than the antigen used in man. The presence of this antigen in toxoids to be used for active immunization of individuals and in hyperimmunization programs for TIG production may be indicated.

It has been suggested that tetanolysin contributes little or nothing to the toxicity of *C. tetani* (21) in vivo. In view of our previously reported findings (12) and the present data showing that tetanolysin disrupts cellular and subcellular membrane structures with the release of biologically active amines and hydrolytic enzymes, further examination of the role of this bacterial toxin in some of the unusual manifestations of tetanus and the prophylactic and therapeutic need for antibody should be considered.

**LITERATURE CITED**