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Separation of Neurotoxin and Hemolysin of *Clostridium tetani*. (30195)

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The most prominent manifestations of tetanus are due to a neurotoxin produced by *Clostridium tetani*. Little attention has been given to the possible role of other toxic substances of this bacterium in the pathogenesis of the clinical disease or the need for their presence in prophylactic antigens or their antibodies in therapeutic serum. One other substance to be considered is the hemolysin, tetanolysin.

Shortly after Faber(1) demonstrated the existence of a neurotoxin in filtrates of cultures of *C. tetani*, Ehrlich(2) observed the presence of a hemolysin in cultures of this organism. Characteristics of these substances have been reviewed by Fleming(3). The lysin is set free during the period of active growth of the culture, while most of the toxin liberation occurs after the bacterial multiplication has ceased; the lysin may be absorbed to red blood cells at 0°C but the neurotoxin lacks this property; the hemolysin is more heat and oxygen labile than the neurotoxin; both the lysin and the neurotoxin induce specific antitoxins. Imbriano(4) stated that the hemolysis is the result of esterase or protease activity. The tetanolysin is necrotizing(5), cardiotoxic(6), and is closely related serologically to other oxygen-labile hemolysins, *e.g.*, pneumolysin, streptolysin O, and the theta toxin of *Clostridium perfringens*(6).

Because of the *in vitro* inhibitory activity of normal sera(7) and sterols(4,6) on tetanolysin, *in vivo* effects have been discounted. However, the possibility remains that the hemolysin is contributory to the anemias(8) and the cardiac complications(9) in patients with tetanus.

Previous laboratory studies(3,4,7) of the hemolysin have been made using crude culture filtrates or ammonium sulfate precipitates from filtrates, each of which, no doubt, contains neurotoxin. The activities and properties of the hemolysin could be defined more accurately if it could be separated from the neurotoxin. This paper reports a high degree of separation of the two toxins by use of gel filtration through Sephadex G-100.

Materials and methods. Crude culture filtrates. Five ml of a 48-hour-old culture of the Massachusetts C_2 strain of *C. tetani* in thioglycollate broth were seeded into a dialysis bag (Visking) containing 250 ml of saline immersed in 2500 ml of a protein-free medium(10). The culture was incubated at 37°C for 7 days. The contents of the bag were centrifuged in the cold at 2000 RCF for one hour and the supernatant filtered through a Millipore® filter (HA, 0.45 u). The filtrate was stored at 5°C and then brought to dryness by freeze-drying. Restoration to one-tenth the original volume was made with phosphate buffer, pH 7.0, 0.025 M. The concentrate used to obtain the results given here contained approximately 10^6 mouse MLD and 6×10^5 hemolytic units per ml.

Gel filtration. The chromatographic procedure of gel filtration through Sephadex G-100 (Pharmacia)(11) was used for separation of the hemolysin and the neurotoxin. The Sephadex G-100 was allowed to swell in phosphate buffer, pH 7.0, 0.025 M for 48 hours. After repeated washings and decan-

tation the slurry was poured into a glass column 4 cm in diameter and allowed to pack by gravity to a height of 45 cm. The column was washed with the buffer; then 4 ml of the concentrated filtrate was applied. Elution was made with the phosphate buffer held in a reservoir 20 cm above the top surface of the column. The eluate was collected in 5 ml fractions.

Assay of neurotoxic activity. Two NIH general purpose female mice, weighting 15-18 g, were injected with 0.1 ml of every third fraction in the subcutaneous tissue of the upper portion of the right hind leg. If both mice died within 96 hours, the MLD of the eluate was titrated by the same procedure using 10-fold dilutions in phosphate buffered saline, pH 7.4, containing 0.2% gelatin. The endpoint was designated as the highest dilution that caused death of both mice within 4 days. Since the inoculum was 0.1 ml the number of MLD/ml was 10 times the dilution factor; therefore, activity less than 10 MLD/ml was not measured.

Assay of hemolytic activity. To a series of 10 × 75 mm tubes containing 0.8 ml of phosphate buffer, pH 6.6, with 0.2% (w/v) sodium hydrosulfite, was added 0.2 ml of a 2% suspension of defibrinated sheep RBC. This was followed by 0.2 ml of undiluted or 10-fold dilutions of the test sample. The diluent was the same as used in the MLD assays. The tubes were incubated in a water bath at 37°C for 2 hours. Hemolytic activity, arbitrarily expressed in units (HU), was the reciprocal of the highest final dilution that caused complete visual hemolysis within 2 hours.

Ultraviolet absorption. The optical density of every fourth fraction from the column was determined in a Beckman D-U Spectrophotometer at 280 mu.

Results. The titers of the neurotoxic and hemolytic activities and the optical density (280 mu) of the fractions of a concentrated crude culture filtrate of *C. tetani* eluted from the Sephadex G-100 column are illustrated in Fig. 1. Similar elution patterns were observed in 6 other experiments in which 3 concentrated and 3 unconcentrated filtrates were used.

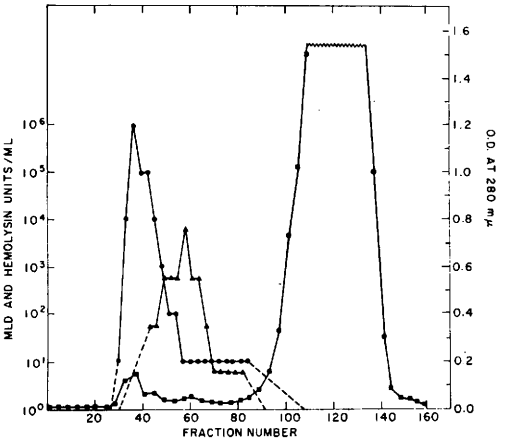

FIG. 1. Lethal neurotoxic activity (●—●), complete hemolytic activity (▲—▲) and ultraviolet absorption (■—■) of fractions of a culture filtrate of *C. tetani* separated by Sephadex G-100 chromatography. The presence of neurotoxin or hemolysin in quantity less than 10 MLD or 6 HU is indicated by dotted lines (- -). All other fractions had no detectable neurotoxic or hemolytic activity.

Animals injected with fractions containing neurotoxin demonstrated the typical signs of experimental ascending tetanus (scoliosis, paralysis of the lower extremities, generalized seizures and death). Neurotoxin was present, in fractions 30 (145-150 ml portion of effluent) through fraction 108 (535-540 ml portion). However, death of the mice was caused only by fractions 30 through 84. Maximum neurotoxic activity, 10^6 MLD/ml, was observed in fraction 36.

Hemolysin was demonstrable in fractions 31 through 91 but compete visual hemolysis was observed only with fractions 43 through 82. As shown in Fig. 1 maximum hemolytic activity, 6×10^3 HU/ml, appeared in tube 58. This fraction contained approximately 1% of the total hemolysin applied to the column. In several experiments it was estimated that 20-50% of the hemolysin applied to the column was recovered.

The portions containing the maximum amount of neurotoxin and hemolysin were the 36th and 58th 5-ml fractions, respectively, or about 100 ml of effluent apart. Tube 36 contained less than 6 HU while tube 58 contained only 10^1 MLD, or 10^5 less than in the fraction of maximum toxicity. In spite of recovery of only 1% of the hemolysin in

this fraction the ratio of hemolysin to neurotoxin was increased a thousand-fold, *i.e.*, from 0.6 HU/1 MLD to 600 HU/1 MLD.

An increase in optical density (280 mu) was observed in the area of the neurotoxin and a slight increase was noted in the area of the hemolysin. Neurotoxic and hemolytic activity were both absent in eluates demonstrating maximum ultraviolet absorption.

Discussion. The results reported illustrate that Sephadex G-100 provided a method for a high degree of separation of the neurotoxin and the hemolysin of *C. tetani* culture filtrates. Although not completely isolated, the major portion of the neurotoxic activity was removed from the fractions containing high hemolytic activity. Neurotoxin, however, was obtained essentially free of hemolytic activity.

The major portion of the hemolytic activity was lost during the process of gel filtration. The efficiency of the procedure therefore is low for the hemolysin recovery. A change in environmental conditions or the character of the eluting buffer, might prevent a loss of this magnitude since the hemolysin is known to be very labile. Addition of the reducing agent, sodium hydrosulfite, to the eluting buffer did not improve recovery. No experiments were conducted to determine the effect of the Sephadex on the hemolysin.

The calculated recovery of neurotoxin exceeded the estimated amount applied to the column. This could be due to the probability of error in the assay procedure. There was no evidence to suggest that an inhibitor of the neurotoxin was removed during the filtration.

Since the hemolysin is eluted after the neurotoxin it may be assumed from the theory of gel filtration(12) that the hemolysin molecule is smaller than the neurotoxin molecule.

The hemolysin and the neurotoxin compose only a minor portion of the material causing increased ultraviolet absorption. This area of increased optical density is due to other low molecular weight nondialyzable nitrogenous materials.

Many methods have been used for separation(13) and purification(14) of the neurotoxin but no method for isolation and recovery of the hemolysin has been found in the literature. Fleming(3) demonstrated that the lysin could be removed from a culture filtrate by the absorption with red blood cells but the lysin was not recovered from the cells by washing. Although an effective separation of the two bacterial products was accomplished, the hemolysin was not recoverable for use in subsequent experiments.

While this work was in progress Latham *et al*(15) reported the use of Sephadex G-100 for preparation of a tetanus antigen for immunization. These workers observed an ultraviolet absorption curve with 4 major components for both tetanus toxin and tetanus toxoid. Hemolytic activity of these components was not mentioned.

Further definition of the properties and specific activities of the hemolysin of *C. tetani* prepared by this method are now in progress. Although the potent neurotoxin has been separated from the hemolysin other significant bacterial products may be present in these fractions.

Summary. Gel filtration through Sephadex G-100 was used to achieve a high degree of separation of the neurotoxin from the hemolysin of a crude concentrated culture filtrate of *C. tetani.* Recovery of the neurotoxin was complete but only 20% of the hemolysin was recovered. The fraction with maximum neurotoxic activity was essentially free of hemolysin while a thousand-fold increase in ratio of hemolytic units to neurotoxin was detected in the fraction with maximum hemolytic activity.

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Some Properties of the Encephalomyocarditis, Columbia SK and Mengo Viruses.* (30196)

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Five strains of virus variously known as encephalomyocarditis (EMC), Columbia SK (COL SK) and Mengo have been studied in this laboratory to compare their characteristics *in vivo* and *in vitro*. These viruses were isolated in widely separated parts of the world from a variety of animal species and have been passaged extensively in rodents and cultured cells. The immunologic relationship of the 5 strains as well as the morphology of plaques which they form in L cell monolayers, sensitivity to inhibitors in agar (presumably sulfated polysaccharides)(1), and hemaggluination properties are considered here. In addition, the results of studies on the pathogenicity of these viruses for laboratory animals are briefly summarized.

Materials and methods. Virus strains. M. Eaton supplied the EMC-M strain which was originally isolated in Florida(2). It had received approximately 80 passages in mice. EMC-L was derived from the Florida isolate and had been transmitted repeatedly in L cells in the laboratories of K. Habel and I. Lowe. A mouse adapted strain of Mengo virus which was isolated in Uganda was made available by L. Kilham(3). The T-61 variant of COL SK virus(4) was obtained from E. Furusawa who had grown it in Ehrlich ascites cells. A strain designated 1138 was isolated in Panama by Murnane *et al* from a naturally infected pig(5). It has been transmitted in mice by the intracerebral (i.c.) and intraperitoneal (i.p.) routes. The L cell adapted r and r^+ variants of EMC virus were supplied by K. Takemoto. The 2 differ in sensitivity to sulfated polysaccharides found in agar(1). Each of the virus strains was passaged in this laboratory using L-939† or primary and secondary mouse embryo (ME) monolayer cell cultures or mice or all 3 systems.

Animal studies. Groups of 12-week-old male Swiss mice were inoculated i.p. with decimal dilutions of virus. The infectivity endpoint was included in all titrations. Signs of illness were recorded as they appeared. The hearts of all animals were removed for histological examination as soon as possible after death or when the experiment was terminated on day 14.

Antiserum. Young rats or guinea pigs were inoculated i.p. on a single occasion with cell culture fluids containing high titers of infectious virus and were bled 2 to 3 weeks later. Sera were routinely adsorbed with a 20% suspension of kaolin.

Hemagglutination (HA) and hemagglutination-inhibition (HI). Tests were carried out by previously described methods(6) using antigens prepared in cultures of ME or L cells and a 0.05 M H_2BO_3 - 0.12 M KCl diluent adjusted to pH 8 with 1 N KOH. Sedimentation conditions are given in Tables I and IV.

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