# FORMATION OF A SPECIFIC ANTIGEN IN SPINAL CORD SYNAPSES OF RATS WITH LOCAL TETANUS

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Rabbit antisera against various subcellular fractions of spinal cord tissue of rats with local tetanus and reactive with the appropriate antigens in the complement fixation test were obtained. Antibodies reacting specifically with synaptosomes and synaptic membranes isolated from the "tetanus" spinal cord were found in antisera against synaptosomes of the "tetanus" spinal cord after exhaustion with a crude fraction of normal spinal cord mitochondria.

KEY WORDS: tetanus toxin; spinal cord synaptosomes; specific antigens.

Tetanus toxin (TT) is known to disturb mediator secretion in central [2, 7, 9-12] and neuromuscular synapses [5, 6, 11, 12]. However, the primary mechanisms of biochemical disturbances in synapses produced by tetanus toxin have not yet been explained. Previous investigations showed that TT in experiments in vitro and in vivo produces marked changes in the protein metabolism of synaptic structures [3] and inhibits contractile and ATPase activity of the actomyosin-like protein isolated from spinal cord tissue [4].

This paper describes an investigation to study the possibility of formation of proteins with new antigenic properties in the spinal cord synaptosomes of rats with local tetanus.

## EXPERIMENTAL METHOD

Local tetanus was produced in noninbred rats weighing 200-250 g by injection of TT in a dose of 0.1 MLD by multiple injections into the leg and thigh muscles. On the third day after injection of TT the gray matter was removed from the lumbosacral enlargement of the spinal cord on a freezing stage. Fractions of synaptic structures were obtained [8] from the crude mitochondrial fraction (CMF) by centrifugation in a sucrose density gradient. The mixture of cytoplasmic fraction and microsomes (FCM) was isolated by centrifugation at 10,000 g. To obtain immune sera the following fractions were used as antigen: synaptosomes, mitochondria, and FCM. Material was taken from 20 to 30 rats for each immunization. Rabbits were injected with the fraction in a dose of 0.6 mg protein together with Freund's adjuvant (Difco) three times with an interval of one week between injections, given subcutaneously into the plantar pads of the hind limbs and intramuscularly. The rabbits were reimmunized two months after the end of the first course of immunization. On the eighth day after the last injection of the appropriate fractions, blood was taken from the auricular vein and the serum from it was lyophilized. Comparative analysis of the spinal cord antigens from the healthy rats and rats with tetanus was carried out by CFT in the cold. To remove antibodies against heterologous antigens and against the antigens of "healthy" spinal cord tissue, the antisera obtained from rabbits immunized with the "tetanus" material were exhausted by the following fractions respectively: mitochondria (3.8 mg), a mixture of heavy and light synaptosomes (3.8-7 mg), FCM (4-6 mg), and CMF (16-23 mg protein) to 1 ml serum, diluted in the ratio of 1 : 5. The above fractions were sedimented by centri-

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TABLE 1. CFT of Exhausted Rabbit Antisera with Various Tissue Fractions from Healthy and "Tetanus" Rats

| Antigens | Types of antisera and their dilutions | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | against synaptosomes | | | | | | against mitochondria | | | | | | against FCM | | | | | |
| | 16 | 32 | 64 | 128 | 256 | 512 | 16 | 32 | 64 | 128 | 256 | 512 | 16 | 32 | 64 | 128 | 256 | 512 |
| **Normal rate** | | | | | | | | | | | | | | | | | | |
| Spinal cord: | | | | | | | | | | | | | | | | | | |
| MC | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| CMF | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| FCM | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| LS + HS | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Synaptic membranes | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Brain CMF | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| MC: | | | | | | | | | | | | | | | | | | |
| liver | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| kidney | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| muscle | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| heart | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **"Tetanus" rats** | | | | | | | | | | | | | | | | | | |
| Spinal cord: | | | | | | | | | | | | | | | | | | |
| MC | — | — | — | — | — | — | ± | ± | — | — | — | — | — | — | — | — | — | — |
| CMF | 2+ | 1+ | 1+ | 1+ | + | + | — | — | — | — | — | — | — | — | — | — | — | — |
| FCM | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| LS + HS | 3+ | 2+ | 2+ | 2+ | ± | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Brain CMF | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Spinal cord synaptic membranes | 1+ | 1+ | 1+ | 1+ | ± | — | — | — | — | — | — | — | — | — | — | — | — | — |

Legend: CMF – crude mitochondrial fraction; FCM – fraction of cytoplasm + microsomes; LS + HS – light synaptosomes + heavy synaptosomes; MC – mitochondria.

fugation for 15-20 min at 15,000 g, the residue was treated with antiserum (1 ml) in a dilution of 1 : 5 and carefully suspended, and the suspension was allowed to stand for 18 h at 4° C. It was then centrifuged at 15,000 g for 15 min. The supernatant was used in the CFT at 4° C with subcellular fractions of spinal cord tissue of the rats with tetanus. The protein content was determined by Lowry's method. Altogether 10 experiments were performed.

## EXPERIMENTAL RESULTS AND DISCUSSION

The first stage of the work was to detect antibodies against spinal cord in the immune sera and to determine their titer. Antiserum against mitochondria of the "tetanus" spinal cord reacted with the fraction of spinal cord mitochondria, with brain and spinal cord CMF, and with spinal cord FCM of normal rats. The antiserum titer in the reaction with these antigens was 1/128-1/1024. Antiserum against FCM fixed complement on the addition of spinal cord mitochondria and brain and spinal cord CMF, and it reacted correspondingly with spinal cord FCM of normal rats (titer 1 : 64, 1 : 256, 1 : 128). These antisera did not react with synaptosomal and synaptic membrane fractions of normal rat spinal cord. Antiserum against synaptosomes of the "tetanus" spinal cord reacted with a mixture of light and heavy synaptosomes (titer 1 : 256), with spinal cord synaptic membranes (titer 1 : 128), with spinal cord and brain CMF (titer 1 : 1024), with the mitochondrial fraction (titer 1 : 32), and also with normal rat spinal cord FCM (titer 1 : 256). All the sera against brain and spinal cord that were tested contained antibodies to the heterologous antigen, i.e., they gave a positive reaction with antigens from other rat tissues, for example, with fractions of mitochondria from liver (titer 1 : 128), heart (1 : 128), muscle (1 : 64), and kidney (1 : 128).

The second stage of the work was detection of the immunospecific antigen in the synaptic structures of the "tetanus" spinal cord with the aid of exhausted monospecific immune sera. Complete exhaustion of antiserum against mitochondria and FCM of the "tetanus" spinal cord was achieved by the use of a large quantity of material (3.8-6 mg). To exhaust antiserum against synaptosomes of the "tetanus" spinal cord an even greater quantity of material was required. The serum was not exhausted even by 7 mg protein of the synaptosomal fraction. Because of difficulty in obtaining the synaptosomal fraction, normal rat spinal cord was used subsequently for exhaustion. Complete exhaustion of antiserum against synaptosomal antigens of healthy rats was achieved by the addition of CMF in a dose of 16-23 mg protein. Exhausted antisera against mitochondria, synaptosomes, and FCM of the "tetanus" spinal cord did not react with CMF from spinal cord and brain, with mitochondria, synaptosomes, synaptic membranes, with FCM of spinal cord tissue, and also

with mitochondria of the liver, heart, kidney, and muscle of healthy rats (Table 1). Exhausted antiserum against mitochondria of the "tetanus" spinal cord gave partial delay of hemolysis in a dilution of 1:32 with mitochondria and did not react with synaptosomes and other fractions of the "tetanus" spinal cord. Antiserum against FCM of the "tetanus" spinal cord, exhausted with "healthy" FCM, did not react with FCM of the "tetanus" rats. Consequently, the new antigen was found only in the spinal cord and not in the brain of the "tetanus" rats. The grade ± in the CFT is usually regarded as doubtful [1], and the reaction between antiserum against "tetanus" mitochondria and the "tetanus" mitochondria themselves might be regarded as nonspecific due to contamination of the antigens with synaptosomes. However, exhausted serum against "tetanus" synaptosomes did not react with "tetanus" mitochondria and vice versa. The problem of the formation of specific antigens in the mitochondria of the damaged spinal cord requires further investigation.

Since TT reaches the anterior horns of the spinal cord during the development of tetanus [2], it was important to study the following problems: 1) does the serum of rabbits immunized with the damaged spinal cord contain antitoxin, and 2) is the specific antigen that was found identical with the toxin. Titration of the sera was carried out in the usual way by biological tests on mice. The investigations showed that no antitoxic activity was present in the sera and that antiserum against "tetanus" synaptosomes did not react in the CFT with tetanus toxin (tetanospasmin).

It is noteworthy that immunization of rabbits with spinal cord fractions free from myelin fragments did not induce allergic encephalomyelitis in the animals, as is usually observed in the case of immunization with whole brain or spinal cord extract.

The results of these investigations show that in tetanus poisoning (local tetanus) a specific antigen is formed in the affected spinal cord tissue, in which it is localized in synaptic structures (synaptosomes and, evidently, synaptic membranes).

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