Cytotoxicity of Cobra (*Naja naja kaouthia*) Venom on Rabbit Red Blood Cells and S-180 Tumor Cells in the Presence of Tetracaine, Lidocaine and Procaine

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The cytocidal action of *Naja naja kaouthia* venom on rabbit red blood cells and S-180 tumor cells treated with local anesthetics (tetracaine, lidocaine and procaine) were studied. The S-180 cells were more sensitive to the venom than the red blood cells which required albumin for efficient hemolysis in the 10 minute assay. All three local anesthetics at lower concentrations protected both cell types against venom hemolysis. At higher concentrations the local anesthetics enhanced the cell lysis to 100%. The effectiveness of the local anesthetics for both the inhibition and enhancement phases of cytotoxicity was tetracaine > lidocaine > procaine. This is the same order as their anesthetic effectiveness, lipid solubility and their protein binding.

Introduction

The cytolytic action of cobra venom on washed red blood cells (RBC) in isotonic medium is due primarily to the synergistic action of phospholipase A_2 and membrane active, basic polypeptide cytotoxins [1–4]. The cytolytic action of venom varies depending on the animal source of red blood cells [1] and the sensitivity of various animal cells to the cytolytic action of venom varies markedly by several orders of magnitude [5]. Certain tumor cells are specially sensitive [6].

The interaction of low concentrations of basic (+), lipophilic local anesthetics (tetracaine, lidocaine, procaine) with cell membranes induces stabilization against osmotic lysis [7, 8], produces membrane swelling [9, 10], displaces membrane bound calcium [11] and inhibits hydrolysis of membrane phospholipids by phospholipase A [12–14]. Therefore, such anesthetics would be expected to inhibit the cytolytic action of venom.

The purpose of this present study is to investigate the relative cytotoxicity of *N. N. kaouthia* venom on rabbit RBC and S-180 tumor cells in the presence of tetracaine, lidocaine and procaine.

Materials and Methods

Lyophilyzed *N. N. kaouthia* (Bangkok, Thailand) venom was collected by means of electric venom extraction [15]. Cytotoxicity was determined as the dose of venom which would hemolyze 50% of RBC or kill 50% of the S-180 cells in 10 minutes at 37 °C, and at otherwise constant conditions. The LD_{50} value of the venom for the S-180 cells, and the 50% hemolytic dose (HD_{50}) for the rabbit RBC under the experimental conditions in this report were controlled with every experimental series. The stock venom concentration was 0.6 mg per ml for rabbit RBC experiments and was 0.3 mg per ml for S-180 cell experiments. The venom cytotoxic activity in these solutions was stable at least for 16 h at room temperature.

Heparinized rabbit blood was drawn from healthy adult New Zealand white rabbits. The RBC were washed three times with ten volumes of Tyrode solution. Two volumes of packed RBC were then mixed with five volumes of Tyrode solution with or without 2% bovine serum albumin (BSA). This stock RBC suspension had a concentration of approximately 3×10^9 RBC/ml and was used within 36 h.

S-180 tumor cells were obtained from Dr. John Hibbs. These tumor cells were maintained in Swiss-Webster *ICR* mice and transplanted once each week intraperitoneally. The tumor cells were washed with Tyrode solution and freed from blood cells by repeated washing and diluted according to the method of Hibbs *et al.* [16]. The final cell concentration in Tyrode solution was 10^6 cells/ml. The cells were used within six hours.

Local anesthetics tetracaine, lidocaine and procaine were dissolved in Tyrode solution. These stock

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solutions were spectrophotometrically stable for at least four days at room temperature.

Determination of the local anesthetic effects on the N. N. kaouthia venom cytotoxicity to rabbit RBC: A 0.7 ml stock RBC suspension was mixed with 0.2 ml anesthetic solution and pre-incubated at 37 °C for five minutes. A 0.1 ml of stock *N. N. kaouthia* venom solution was than added to the RBC-anesthetic mixture. One ml of the final RBC suspension then contained 1% BSA, 60 µg *N. N. kaouthia* venom, 1.8 mM $CaCl_2$ and various concentrations of anesthetics (0–200 mM). After incubation for 10 minutes, 5 ml of 0.1 M EDTA (~ 270 mOSM) was added to stop the hemolysis. Following centrifugation at 1000×*g* for 10 minutes, the absorbance of the supernatant at 540 nm was measured without delay on a Cary 16 spectrophotometer. The supernatant of RBC suspension without snake venom was used as a reference blank. The rabbit RBC hemolysis at higher anesthetic concentration, without *N. N. kaouthia* venom, was measured with every experimental series. The 100 percent hemolysis was measured by the same procedure, except 5 ml distilled water was added instead of EDTA solution.

Determination of the effects of local anesthetics on the N. N. kaouthia venom cytotoxicity to S-180 tumor cells: 0.1 ml S-180 stock cell suspension was mixed with 0.1 ml anesthetics of various concentrations in a test tube. 0.1 ml *N. N. kaouthia* venom without BSA was added to the mixture and incubated at 37 °C for 10 minutes. The concentration of cells in the final volume of 0.3 ml Tyrode solution was about 10^6 cells/ml. The snake venom concentration was 100 µg/ml which is the LD_{50} value of *N. N. kaouthia* venom for S-180 cells for that particular batch. The anesthetic concentration varied from 0 to 10 mM. The anesthetics alone, at this concentration range, cause no apparent damage to the S-180 cells. At the end of the incubation period, 0.1 ml 0.5% trypan blue was added to the cell suspension. The dye-treated cells were counted within 5 minutes determined by direct count on two fields on 100 cells.

Results

Rabbit red blood cells: All hemolysis experiments with rabbit RBC were repeated at least 3 times. The variation of percent hemolysis in various experimen-

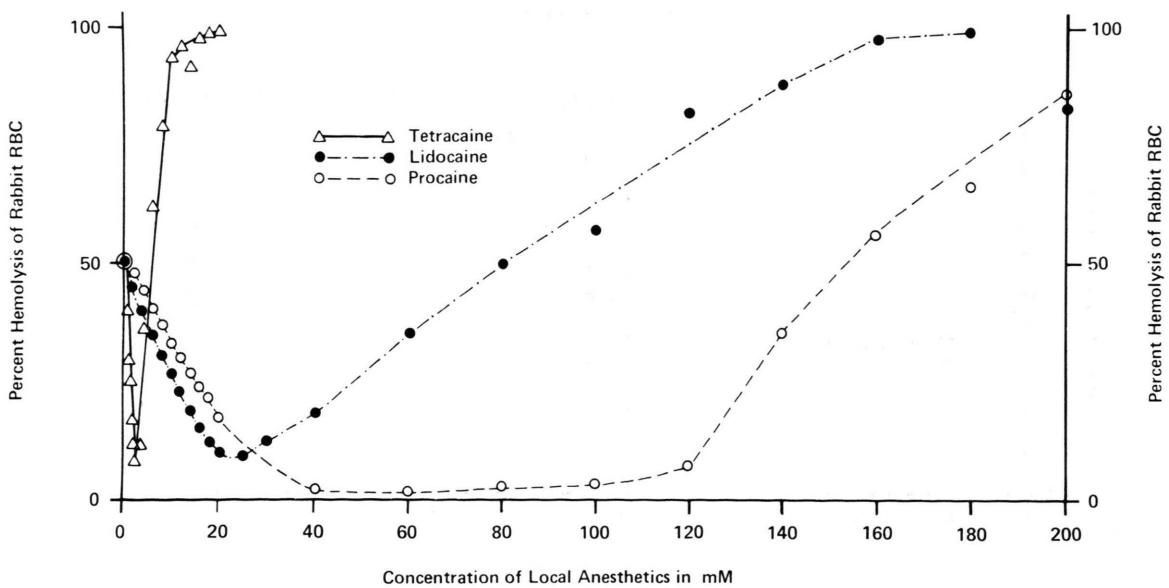

Fig. 1. Effects of tetracaine, lidocaine and procaine on the NNK venom hemolysis of rabbit RBC. The rabbit RBC were in Tyrode solution containing 1% BSA, 60 µg NNK venom/ml and were incubated for 10 minutes at 37 °C. The experiments with each local anesthetic were repeated at least three times. The variation of percent hemolysis in various experimental series reached a maximum of ± 5%. Figure shows a typical experimantal results.

tal series reached a maximum of ± 5%. The results from a typical experiment are shown in Fig. 1.

In the presence of 1% BSA, tetracaine, lidocaine and procaine in each case first prevented hemolysis and subsequently increased hemolysis in the medium of *N. N. kaouthia* venom (Fig. 1). The maximal protection against *N. N. kaouthia* venom hemolysis was reached at the concentration of 2 ± 0.4 mM of tetracaine, 23 ± 2 mM of lidocaine and 40 mM procaine. Tetracaine and lidocaine at the above concentration suppressed the *N. N. kaouthia* venom hemolysis from 50% to 8%. Procaine at the concentration range between 40 and 80 mM prevented the rabbit RBC hemolysis completely.

Tetracaine > 2 mM, lidocaine > 23 mM and procaine > 100 mM hemolyzed the rabbit RBC in the absence of the *N. N. kaouthia* venom. The rapid increase in hemolysis (Fig. 1) which reached 100% was caused by the combined effects of *N. N. kaouthia* venom and local anesthetic. The venom treated rabbit RBC hemolyzed 100% at 20 – 22 mM of tetracaine and at about 160 mM of lidocaine. Procaine needed to be at a concentration higher than 200 mM to reach 100% hemolysis.

Tyrode solution which contained 1.8 mM Ca^{2+} was used in these studies. This was compared to glycylglycine buffer which was used by Gul *et al.* [17] and Lankisch [18] with 1.8 mM and 10 mM Ca^{2+}. The HD_{50} value of *N. N. kaouthia* venom hemolysis of rabbit RBC in Tyrode solution was higher than that in glycylglycine buffer with 1.8 mM Ca^{2+}. The local anesthetics at the similar concentration range as in this present report, but in glycylglycine buffer with 1.8 mM Ca^{2+}, protected the rabbit RBC completely against *N. N. kaouthia* venom hemolysis; the protection of tetracaine and lidocaine in Tyrode solution was incomplete (Fig. 1). Higher Ca^{2+} concentration (10 mM) in glycylglycine buffer required slightly higher concentration of the three local anesthetics for maximal protection. BSA always was required in both buffer system, and at both Ca^{2+} concentrations of 1.8 mM and 10 mM for efficient hemolysis in 10 minutes.

S-180 tumor cells: The S-180 cells killing experiments were afflicted by large variation in the LD_{50} value. The LD_{50} value of NNK venom for this cell type varied ± 30% in different experimental series. The experiments were repeated at least 3 times, up

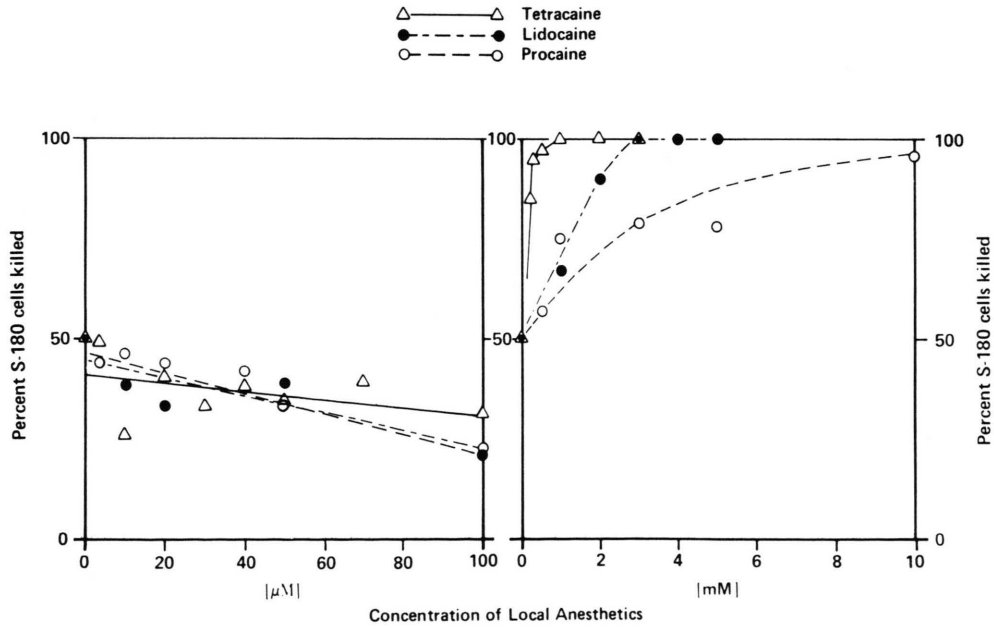

Fig. 2. Effects of tetracaine, lidocaine and procaine on the NNK venom killing of S-180 tumor cells. 100 μg NNK venom/ml was in the final incubation medium. The LD_{50} value, 100 μg/ml, of NNK venom for S-180 cells varied ± 30% in different experimental series. The experiments were repeated at least three times, up to seven times at the lower anesthetic concentration range. The results shown are from a typical experimantal series.

to seven times at the lower concentration ranges. Figs 2 a and 2 b show results of a typical experiment.

The effects of local anesthetics on *N. N. kaouthia* venom cytotoxicity of S-180 tumor cells were shown in Fig. 2. Note that BSA was not required for efficient cytolysis of S-180 cells in contrast to RBC hemolysis. All three local anesthetics protected the S-180 cells from *N. N. kaouthia* venom damage at lower concentrations (< 100 μM) (Fig. 2 a). However, the protection was much weaker in comparison to the inhibition of RBC hemolysis.

The cytotoxicity at higher anesthetic concentration (> 100 μM) increased with a concentration characteristic for each anesthetic (Fig. 2 b). The local anesthetics enhanced the cytotoxicity on S-180 cells in the following order: tetracaine > lidocaine > procaine. This was the same relative order found for enhancing RBC hemolysis, but the effect on S-180 cells occurred at a much lower absolute concentration. The anesthetic concentration ranges for 100% S-180 cells killed by venom in 10 minutes were tetracaine (1 mM), lidocaine (2 – 5 mM), and procaine (> 10 mM). Doses of the local anesthetics alone up to 10 mM did not destroy the S-180 cells under these experimental conditions.

Discussions

The results show that S-180 cells are more sensitive to *N. N. kaouthia* venom than rabbit RBC. The S-180 cells are lysed efficiently by *N. N. kaouthia* venom acting on membrane in the absence of BSA. Rabbit RBC are hemolyzed by *N. N. kaouthia* venom much less efficiently, and BSA is required. The effect of BSA on the hemolysis of rabbit RBC by *N. N. kaouthia* venom may be explained by the finding of Gul *et al.* [17, 19]. The mechanism may involve the binding and removal of phospholipid hydrolysis products (fatty acids) from the membrane by albumin. Since S-180 cells do not require albumin for efficient lysis, perhaps their lipid constituents are much more fluid and loosely held. Lankisch *et al.* [20] demonstrated that cytotoxin and phospholipase A_2 of *Naja naja* venom hemolyzed the human RBC efficiently at a Ca^{2+} concentration of 5 mM without albumin presence if the incubation time was increased to 30 – 40 minutes. This difference might be caused partly by different venom sources. Dimari *et al.* [21] pointed out that some cobra venoms contain more basic cytotoxins which are more effective lytic agents.

The relative efficiency of the local anesthetics to inhibit the hemolysis corresponds to their relative lipid solubility and protein binding as well as their anesthetic potency [22]. Local anesthetics at lower concentrations (10^{-3} M) are known to protect mammalian RBC from osmotic hemolysis through membrane expansion [8] and to inhibit hydrolysis of membrane phospholipids by phospholipase A through fluidization of the lipid layer [12 – 14].

The studies of cobra venom cytotoxins and phospholipase A_2 and their synergistic action on RBC membranes in recent years [2, 4] indicate that cobra venom hemolysis is due to a direct effect of the cytotoxin and a phospholipid splitting facilitating action of the cytotoxin with the hydrolysis action of phospholipase A_2. The mechanism of the cobra venom hemolysis and the osmotic hemolysis are certainly different. The protective action of local anesthetics on both kinds of hemolysis suggests that the membrane expansion and membrane fluidization induced by local anesthetics at lower concentrations also inhibit the cytolytic action of *N. N. kaouthia* venom on rabbit RBC.

In the case of S-180 cells there was a rapidly increasing lytic effect above 0.1 mM anesthetic concentration with *N. N. kaouthia* venom. This is in the concentration range that local anesthetics alone do not destroy the cells. This difference in sensitivity may reflect a fundamental difference in membrane lipid-protein composition and arrangement in S-180 cells compared to rabbit RBC. The data suggest that the lytic action of *N. N. kaouthia* venom and the action of local anesthetics both are on the hydrophobic lipid/protein base of the cell membranes and that further investigation of their combined effect on cell membrane may be beneficial in understanding the mechanism of both *N. N. kaouthia* venom action and anesthetic action.

Acknowledgement

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