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6 Changes in Tissue Cyclic AMP Concentrations following an Intravenous Lethal Dose of Cholera Enterotoxin in Rabbits

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Running head: IV Cholera Enterotoxin and cAMP in Rabbits

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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As a human disease, cholera is widespread in southeast Asia and the Indian subcontinent. It is a severely debilitating disease which is contracted orally, causing dehydration and electrolyte losses. Evidence has also shown that in some patients death was associated with cardiac lesions [1] and acute renal tubular necrosis [2]. Although these pathologic changes were considered as a secondary result of severe hypotension or acidosis plus K depletion [2], the possibility of an intestinal absorption of cholera enterotoxin in man cannot be completely ruled out. In fact, intestinal absorption of cholera enterotoxin has been demonstrated in rabbits [3-5] and guinea pigs [1].

Since cholera enterotoxin causes death when administered to animals either orally or iv, the latter route becomes desirable for the study of toxicity. Using this iv route, increases of hepatic adenylyl cyclase (6 hr postinoculation) and serum alkaline phosphatase were demonstrated, respectively, in rats [6] and dogs [7]. Furthermore, recent studies showed that iv cholera enterotoxin produced diffuse hemorrhage in monkeys [8]. The most striking changes were found in heart. Alterations in tissue water and electrolytes were also seen in rabbits given cholera enterotoxin by the iv route [9].

It has been well-established that intestinal losses of water and electrolytes during cholera are caused by an activation of intestinal mucosal adenylyl cyclase, which increases the conversion rate of intracellular ATP to cAMP. Since cAMP has been proposed as a "second messenger" mediating the effects of a variety of hormones, and iv injection of a pharmacologic dose of cAMP causes vasodilation and hypotension [10], the main purpose of this study was to test the hypothesis that a generalized increase in plasma and tissue cAMP may be associated with cholera enterotoxin-induced death in Dutch rabbits.

Materials and Methods

Source of cholera enterotoxin. Highly purified cholera enterotoxin was obtained from a commercial source (Schwartz-Mann, Orangeburg, N.Y.). Based upon the available analysis report with the toxin powder (Lot No. EZ 3399) in several vials containing 1 mg of toxin (protein) each, the following information was given: (1) When the 1 mg of cholera toxin is reconstituted to 1 ml with distilled water, this toxin (protein) suspension is in 0.05 M Tris, 0.001 M Na₂-EDTA, 0.003 M NaN₃, and 0.2 M NaCl. The final pH of the suspension is 7.5. (2) The concentration of protein (cholera enterotoxin) was determined by extinction at 280 nm and the purity was evidenced by a single major band in disc electrophoresis. (3) The biological activity of cholera toxin was determined as 26.0 LB (limit of blueing)/ μ g Lowry protein. The LB dose of toxin is that amount of Permeability Factor which, when mixed with one unit of antitoxin in a total volume of 0.1 ml and injection into the clipped skin of rabbits, evokes on the average, an area of increased vascular permeability 4 mm in diameter.

Animal experimentation. Fifteen male Dutch rabbits weighing 1.8-2.2 kg were allocated as control (n = 9) and challenge groups (n = 13). The challenged rabbits were injected via the marginal ear vein with a low (100 μ g/kg, n = 7) or a high (200 μ g/kg, n = 6) dose of purified cholera enterotoxin, the control rabbits received only saline injections.

Toxin-inoculated rabbits were maintained in individual cages for frequent observations until death appeared imminent (20-30 hr after inoculation). At this time, a laparotomy was performed under halothane (Halocarbon Lab, Inc., Hackensack, N.J.) or diethyl ether anesthesia. The arterial blood flow was interrupted by taking blood samples from the dorsal aorta. Samples from 13 tissues including heart (left ventricle),

diaphragm, skeletal muscle, skin, liver, lung, stomach, jejunum, renal cortex, renal medulla, thalamus-hypothalamus complex, cerebellum, and spinal cord were excised immediately after death [11]. Plasma was separated from RBC after centrifugation and a urine sample was taken from the bladder. The same procedures were also applied to control rabbits.

Tissue samples were placed first into liquefied nitrogen, weighed, and then transferred to a Tris/EDTA buffer solution (pH = 7.5) for homogenizing (Ultra-Turrax, Tekmar, Cincinnati, Ohio). The tissue homogenates were heated in a boiling water bath for 3 min. After centrifugation, the supernatant was removed and stored in a freezer for cAMP determination.

cAMP determination. Cyclic AMP concentrations in various tissue extracts, urine, and plasma were determined with a cyclic AMP assay kit (Amersham Corp., Arlington Heights, Ill.). The assay is based on the competitive binding of a given protein to either tritiated or unlabeled cAMP. To this mixture charcoal was added to adsorb the unbound cAMP; the protein-bound cAMP (labeled) was counted in a scintillation counter (Searle Analytic, Inc., Mark III, 6880 Liquid Scintillation System, Des Plaines, Ill.). Since the affinity for protein binding is the same, the distribution of protein bound to labeled and unlabeled cAMP is equal. Thus, the concentration of unlabeled cAMP bound to protein is inversely related to the concentration of labeled cAMP bound to protein. The ratio of labeled to unlabeled cAMP was determined and used to find the concentration of cAMP in the sample, using a simultaneously established standard curve. The concentration of cAMP in the tissue sample and urine or plasma was expressed as pmoles/g wet tissue and pmoles/ml, respectively.

Statistical analyses. Statistical comparisons of the data between control and cholera enterotoxin-challenged rabbits were made using independent t test and standard error of means were also calculated. Significant differences were accepted at $P < 0.05$.

Results

Normal values of cAMP concentrations in 13 rabbit tissues are summarized in Table 1. In these tissues, cAMP concentrations arbitrarily were assigned to three levels. Tissues with the highest cAMP concentrations were cerebellum, heart, thalamus-hypothalamus complex and lung. Intermediate cAMP values were measured for the renal medulla, stomach, skin, diaphragm and renal cortex. The lowest concentration of cAMP was observed in the spinal cord, jejunum, muscle and liver.

Effect of cholera enterotoxin. There were no significant differences between the control rabbits and rabbits challenged with 100 $\mu\text{g}/\text{kg}$ of cholera enterotoxin when compared on the basis of cAMP concentrations in plasma, urine and 13 different tissues. A higher dose of cholera enterotoxin (200 $\mu\text{g}/\text{kg}$) caused significant increases in cAMP values of skeletal muscle, liver, lung, renal cortex, renal medulla, and spinal cord (Table 1).

Discussion

It has been well-established that the mechanism for orally or locally cholera enterotoxin-induced intestinal hypersecretion of water and electrolyte is an activation of adenyl cyclase activity of mucosal cells [12-15]. However, the question as to why iv injection of the same enterotoxin causes hypotension, diffuse hemorrhage and death in rhesus monkeys [3] has not been answered. If a small quantity of cholera enterotoxin can be absorbed into the circulation from the infected

intestine in man, it is important to understand how cholera enterotoxin acts systemically in an animal model. For the treatment of cholera in human patients, a continuous iv administration of water, electrolytes and bicarbonate usually reverses the disease processes of severe dehydration [16-18]. However, some cholera patients still die, despite the conventional fluid treatment [1, 2].

Using the Dutch rabbit as an animal model, it was possible to examine whether a universal increase in tissue cAMP concentrations was produced at a given time, 20-30 hr after a lethal iv injection of cholera enterotoxin. In the present study, a single dose of 100 $\mu\text{g}/\text{kg}$ failed to alter significantly tissue, urine or plasma concentrations of cAMP. When the dosage was increased to 200 $\mu\text{g}/\text{kg}$, all measured tissue cAMP concentrations showed little changes except for renal cortex, renal medulla, liver, lung, skeletal muscle, and spinal cord, in which the cAMP concentrations were significantly increased. Surprisingly, the usual target organ, small intestine, did not respond to either dosage of the systemically administered cholera enterotoxin. The cAMP concentration in the small intestine was unaltered and watery diarrhea was not observed in the intoxicated Dutch rabbits.

Commercially available kits are commonly used for the determination of cAMP concentrations in tissue extract or body fluids. Vossenburt et al. [19] compared results from four different kits. Their investigations indicated that the principle for using the Amersham product was based upon a competitive protein-binding and that the procedure for cAMP determination was simple with a maximal intra-assay variability of 4.1%. Since the Amersham kit for cAMP determination was used in the present study, it appears that the determined values of cAMP in various tissues, plasma, and urine in control and toxin-treated Dutch rabbits

are valid. Although determined cAMP concentrations in some tissue samples showed large standard errors, urine values of cAMP revealed the greatest variation. This variation of urine cAMP concentrations was not caused by the applied techniques for cAMP determination, but rather by the renal excretion of large and varying amounts of cAMP in the urine [20, 21] in order to maintain a relatively constant plasma cAMP concentration.

The mechanism for increases in concentrations of cAMP in selective tissues following a lethal iv dose of cholera enterotoxin remains obscure. Since this enterotoxin is a protein, its entrance into the cell is based upon the assumption that only the "subunit A" can be transported across the cell membrane [22, 23]. According to the present findings, the process of cellular internalization of cholera toxin should not be considered uniform in different body cells. The selectivity for cholera toxin penetrating into the cell may be the key factor for an increased activity of adenylyl cyclase, which in turn produces cAMP from ATP within the cell [12-15].

Since the increase in tissue cAMP concentration was selective after an iv injection of a high dose of cholera enterotoxin in Dutch rabbits, the proposed hypothesis of a universal increase in tissue cAMP concentration during cholera toxemia could not be totally supported. Despite the fact that tissue cAMP concentrations were not determined in an earlier stage after toxin inoculation, other theories or hypotheses are also needed to explain the cause of death induced by cholera enterotoxin in order to improve clinical management of cholera in man.

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TABLE 1. Cyclic AMP concentrations in plasma, urine and tissues of control and cholera-intoxicated rabbits.

Sample	pmoles/g or ml					
	Control (n = 9)		100 μ g/kg (n = 7)		200 μ g/kg (n = 6)	
Skin	262.5 \pm	49.8	208.0 \pm	52.2	566.4 \pm	233.1
Muscle (rectus abdominis)	136.3 \pm	26.1	179.5 \pm	48.7	512.9 \pm	65.3 ^{***}
Diaphragm	338.1 \pm	104.4	248.0 \pm	45.6	605.6 \pm	189.3
Heart (left ventricle)	819.2 \pm	239.9	333.9 \pm	92.7	1060.8 \pm	345.2
Liver	117.8 \pm	20.2	218.8 \pm	59.6	506.3 \pm	127.7 ^{**}
Lung	391.8 \pm	80.1	441.7 \pm	68.2	1699.8 \pm	703.4 [*]
Renal cortex	245.0 \pm	44.8	152.6 \pm	28.3	641.4 \pm	146.5 ^{**}
Renal medulla	378.5 \pm	61.9	342.1 \pm	91.6	781.6 \pm	188.6 [*]
Stomach	303.5 \pm	79.1	277.8 \pm	86.8	503.8 \pm	180.6
Jejunum	194.6 \pm	29.7	211.3 \pm	35.7	1155.9 \pm	699.9
Cerebellum	1097.0 \pm	371.9	419.6 \pm	131.1	622.8 \pm	117.9
Thalamus-hypothalamns	647.9 \pm	90.2	617.5 \pm	132.0	1307.4 \pm	511.7
Spinal cord	225.6 \pm	24.8	380.7 \pm	140.4	1201.0 \pm	469.8 [*]
Plasma	35.5 \pm	6.3	55.5 \pm	16.4	50.8 \pm	6.7
Urine	21815	\pm 16505	67551	\pm 60080	77410	\pm 36427

* P < 0.05

** P < 0.01

*** P < 0.001

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The purpose of this study was to test the hypothesis that a generalized increase in tissue cAMP concentrations after an IV injection of cholera enterotoxin may play a role in the development of biochemical and pathological changes leading to death. Dutch rabbits allocated as control (n=9) and challenged (n=13) groups were injected IV with highly purified cholera enterotoxin at a dose of 100 or 200 µg/kg. When death appeared imminent (20-30 hr after inoculation), rabbits were anesthetized; plasma		

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urine and 13 idfferent tissue samples were obtained for cAMP determintions. When results from toxin-injected rabbits (100 $\mu\text{g}/\text{kg}$) were compared with those of controls, no significant changes were observed in any tissue studied. A higher dose of enterotoxin (200 $\mu\text{g}/\text{kg}$) produced significant increases in cAMP concentrations in lung, spinal cord, skeletal muscle, renal cortex, renal medulla and liver. The elevation of cAMP values in selected tissues suggests differences in tissue sensitivity to cholera enterotoxin; the data fail to support in toto the proposed hypothesis.

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