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Comparative Protective Action of Unsaturated Fatty Acids for Mice Against Exotoxin, Endotoxin and Snake Venom.* (28078)

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Peripheral vascular collapse in animals can be produced by bacterial exotoxins and endotoxins, and snake venoms. There is evidence that initiating mechanisms responsible for the altered hemodynamic activity differ for each of the foregoing groups of substances. The present study offers further evidence for this conclusion in that injection of unsaturated fatty acids into mice protected against staphylococcal, tetanus and gas-gangrene exotoxins, and also snake venoms, but not against bacterial endotoxins.

Doery and North(1) demonstrated that mice were protected against staphylococcal toxin following an injection of a sublethal amount of venom. We have confirmed this observation, but protection against bacterial endotoxin was not accomplished by this procedure(2). They postulated that this defense was related to the venom enzyme, phosphatidase A, which interacted at the surface of the cells of the host releasing fatty acids and lysophosphatides. The unsaturated fatty acids in turn protected against the bacterial exotoxin.

Materials and methods. Mice. Swiss-Webster white female mice weighing 15-25 g were housed in stainless steel cages in an air-conditioned room. All injections were made into the tail vein in quantities not exceeding 0.5 ml.

Exotoxins.† These included *Staphylococcus* crude toxin (lot #4925-197) with 1:10,000 merthiolate as a preservative; *Clostridium perfringens* crude toxin (lot #W347) and *Clostridium tetani* toxin (lot #T1971M), each with 1:20,000 phenylmercuric acetate as a preservative.

Endotoxins. Standardized amounts of *Escherichia coli* and *Brucella melitensis* endotoxin were used, prepared by methods described elsewhere(3).

Snake venoms.‡ Venoms from *Bothrops jararaca* and *Crotalus terrificus* were employed.

Fatty acids. Solutions of the unsaturated fatty acids, linoleic and oleic acid, were prepared by Dr. Naip Tuna of the Department of Medicine in a concentration of 0.005% in distilled water and 0.01 N NaOH. This basic solution was used in all of the experiments as a diluent for all of the substances injected into mice. No ill effects occurred when mice were injected with 0.5 ml of fatty acid solutions.

Results. Bacterial exotoxins. The results of the protection against staphylococcal, *Cl. perfringens* and *Cl. tetani* toxins, with oleic or linoleic acid are shown in Table I.

The LD_{50} of the staphylococcal toxin was 0.5 ml of a 1:20 dilution. When 0.25 ml of a 1:10 dilution of toxin was added to 0.25 ml of 0.1% of either oleic or linoleic acid, the mixture permitted to stand at room temperature for a few minutes, and then injected into groups of 20 mice, significant protection against the toxin was observed.

When 0.5 ml of titrated *Cl. perfringens* toxin was injected into 20 control mice only 2 out of 20 survived, whereas a mixture of the same amounts of toxin and oleic acid

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TABLE I. Protection of Mice Against Bacterial Toxins with Unsaturated Fatty Acids.

	Material injected	Deaths in 24 hr
1.	.5 ml 1/20 *Staphylococcus* toxin	10/20
2.	.25 ml 1/10 *Staphylococcus* toxin + .25 ml .01% oleic acid	2/20
3.	.25 ml 1/10 *Staphylococcus* toxin +.25 ml 0.01% linoleic acid	1/20
4.	.5 ml *Cl. perfringens* toxin	18/20
5.	.25 ml *Cl. perfringens* toxin + .25 ml .01% oleic acid	0/20
6.	.5 ml 1/400 *Cl. tetani* toxin	19/20
7.	.25 ml 1/200 *Cl. tetani* toxin + .25 ml .01% oleic acid	0/20

given to 20 mice resulted in no deaths. Similarly, *Cl. tetani* toxin produced 19 deaths in 20 mice, while a mixture of the toxin and oleic acid given to the same number of mice gave complete protection.

These results show quite clearly that mice are protected against lethal amounts of bacterial exotoxins if the toxin is mixed with either of 2 unsaturated fatty acids and then injected.

Bacterial endotoxins. In contrast with the protection of mice against exotoxin with the fatty acids, no protection occurred when bacterial endotoxin was employed. The results with *E. coli* endotoxin and with oleic and linoleic acids are shown in Table II. A dose of 0.05 mg of *E. coli* endotoxin caused the death of 34 of 50 mice, and the results of 3 experiments showed that no protection was obtained with oleic or linoleic acid against the endotoxin.

Snake venoms. The results with 2 snake venoms and oleic and linoleic acid are presented in Table III. In the first experiments 1 MLD of venom (*Bothrops jararaca*) was approximately 100 μg. With the amount of oleic acid used no protection against this type of venom was apparent in mice. A second experiment employed an LD_5 of the same venom, which was 25 μg. Under these circumstances only slight, if any, protection was demonstrated.

In experiments carried out with the venom, *Crotalus terrificus,* 1 MLD of venom approximated 10 μg. Excellent protection against this dose of venom was obtained with both oleic and linoleic acid, as noted in Table III.

TABLE II. Failure of Unsaturated Fatty Acids to Protect Mice Against Bacterial Endotoxin.

	Material injected	Deaths in 24 hr
1.	0.5 mg *E. coli* endotoxin	34/40
2.	0.5 mg *E. coli* endotoxin + 0.25 ml 0.01% oleic acid	10/10
3.	0.5 mg *E. coli* endotoxin + 0.25 ml 0.01% oleic acid	19/20
4.	0.5 mg *E. coli* endotoxin + 0.25 ml 0.01% linoleic acid	15/20
5.	2.5 mg *Brucella* endotoxin	13/50
6.	2.5 mg *Brucella* endotoxin + 0.25 ml 0.01% oleic acid	11/15

TABLE III. Influence of Unsaturated Fatty Acids on Survival Rates of Mice Given Snake Venom.

	Material injected	Deaths in 24 hr
1.	100 μg venom (*Bothrops jararaca*)	8/10
2.	*idem* + .25 ml .01% oleic acid	8/10
3.	25 μg venom (*Bothrops jararaca*)	5/10
4.	*idem* + .25 ml .01% oleic acid	4/10
5.	25 μg venom (*Bothrops jararaca*) + .25 ml .01% linoleic acid	3/10
6.	10 μg venom (*Crotalus terrificus*)	8/10
7.	*idem* + .25 ml .01% oleic acid	0/10
8.	10 μg venom (*Crotalus terrificus*) + .25 ml .01% linoleic acid	1/10

Discussion. One of the basic characteristics shared in common by bacterial exotoxins and endotoxins, and by snake venoms is their constricting effect on vascular smooth muscle. It is this property that contributes to fatal peripheral vascular collapse. These studies have demonstrated in mice that the lethal outcome for bacterial exotoxins can be prevented if the exotoxin is injected with an unsaturated fatty acid. A lesser degree of protection was established for snake venom, but no protection occurred for bacterial endotoxins.

Although exotoxins, endotoxins and venoms produce similar hemodynamic alterations the underlying initiating mechanisms are quite different, which are reflected in the results of the present experiments. Staphylococcal toxin acts directly on the mucosal wall of smooth muscle(5). It has been suggested that the toxin mobilizes enzymes of the host, including phosphatidase A, which in turn releases fatty acids and lysophosphatides from

cell surfaces(1). From observations made with snake venoms and endotoxins it is possible that disruption of the cell walls by the mobilization or activation of enzymes by staphylococcal toxin results in liberation of histamine. It is not clearly understood why unsaturated fatty acids injected simultaneously or shortly before toxin protects mice. In the present experiments, the fatty acid was mixed with exotoxin, endotoxin or snake venom before intravenous injection because of technical reasons. The injection of a fatty acid shortly before exotoxin or snake venom also gave protection. Saturated fatty acid offered no defnse against these substances. In the case of snake venoms, it is known that the hemolytic component of many venoms contains phosphatidases, including phosphatidase A, which through cell injury liberates histamine and possibly other vasoactive substances(5).

While the present experiments offer confirmatory evidence that unsaturated fatty acids protect against tetanus toxin, the precise mode of action is not understood. It is possible that the hemolytic factor in the toxin contains a phosphatidase or similar enzymes, which cause cell injury, causing the release of vasoactive substances, including histamine.

There is ample evidence that endotoxin does not act directly on vessel walls, but in an indirect manner in conjunction with a labile serum factor, possibly complement, and probably antibody, liberation of histamine takes place(6). It has been suggested that the lethal action of endotoxin due to peripheral vascular collapse is an anaphylactic effect(7).

The role of fatty acids in the defense mechanism against infectious diseases has had many ramifications in the past. Larson and Nelson(8) exposed tetanus and diphtheritic toxins to castor oil soap and found that guinea pigs withstood 100 lethal doses of each after this procedure. The soap was less effective with botulinus toxin. A 2% solution of the soap mixed with the endotoxin of *Actinomyces gypsoides* completely protected guinea pigs and rabbits against lethal doses of the endotoxin. These workers concluded that the soap "detoxified" these substances by a reduction of surface tension. Subsequently, Nieman(9) reviewed the literature on the effect of fatty acids on the growth of microorganisms, as well as the antibacterial effects. He ascribed a physicochemical action to the fatty acids in which they were absorbed to the surface of the cell membrane and cell permeability was altered. More recent studies including the present experiments would indicate that unrelated toxic substances alter the integrity of the cells, liberating vasoactive substances, which can be prevented with unsaturated fatty acids. It is not clear why fatty acids protect against exotoxins but not against endotoxins. It is possible that the mixture of the fatty acids in the form of soaps with relatively small amounts of exotoxins results in the denaturation of the toxic proteins, thereby altering their lethal properties. Since the endotoxins may be polysaccharide in nature, activity remains after mixing with the fatty acid soaps.

Summary. Swiss-Webster mice were protected against lethal amounts of exotoxins prepared from *Staphylococcus, Cl. perfringens,* and *Cl. tetani* when the toxins were mixed with oleic or linoleic unsaturated fatty acids prior to intravenous injection. Injection of a toxin and unsaturated fatty acid separately also gave protection. Unsaturated fatty acid also protected against *Crotalus terrificus* snake venom. No protection was obtained with oleic acid against *E. coli* or *Br. melitensis* endotoxin.

It is not known in what manner the fatty acids protect against the exotoxins and snake venom. However, the phenomenon may be related to the fact that the toxic substances act directly on vascular smooth muscles, possibly through enzymatic activity. The action of endotoxin, on the other hand, is mediated through a labile component in serum, possibly complement, with the liberation of histamine, and possibly other vasoactive substances. The unsaturated fatty acids in the form of soaps may denature the proteins of the exotoxins, thus altering their lethal activity, but the lethal property of endotoxins remains because of their polysaccharide nature.

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Evidence for a Disequilibrium pH in the Proximal Tubule of Rat Kidney.* (28079)

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Reabsorption of filtered HCO_3^- by the kidney is thought to be mediated by secretion of cellular H^+(1,2), resulting in the following intratubular reaction:

$$\underset{\text{(secreted)}}{H^+} + \underset{\text{(filtered)}}{HCO_3^-} \rightleftarrows H_2CO_3 \rightarrow CO_2 + H_2O \quad \downarrow\uparrow \quad CO_2\,\text{(plasma)}$$

Since dehydration of H_2CO_3 is not instantaneous, excess H_2CO_3 would accumulate in tubular fluid and would not be in equilibrium with plasma CO_2. Consequently, intratubular pH would be lower than that calculated by the Henderson-Hasselbalch equation using plasma pCO_2 and luminal concentration of HCO_3^-(3).

An opposing view postulates that HCO_3^- ions *per se* are reabsorbed directly(4). Were this the mechanism involved, excess H_2CO_3 would not be generated, H_2CO_3 and CO_2 would be in continuous equilibrium, and the intratubular pH would be equal to that predicted by the Henderson-Hasselbalch equation.

In the present experiments the *in vivo* intratubular pH of single proximal tubules perfused with a solution containing 60 mM $NaHCO_3$ was 6.3, or 1.5 pH units below the calculated equilibrium pH of 7.8. These results afford the first direct evidence that HCO_3^- reabsorption in the proximal tubule is mediated *via* H^+ secretion.

Methods. Sprague-Dawley rats were anesthetized and prepared for micropuncture as previously described(5). The exposed surface of the kidney was covered with Ringer's bicarbonate solution continuously equilibrated with 5% CO_2-95% O_2.

Single proximal tubules were perfused by first injecting a drop of silicone oil into the lumen through a micropipette. The oil drop was then split by injecting with a second micropipette a solution containing 90 mM NaCl and 60 mM $NaHCO_3$. The pH of this solution when equilibrated with 5% CO_2 was 7.8.

The *in vivo* intratubular pH was estimated by adding various acid-base indicators to the perfusion fluid in a concentration of 100 mg %. The color changes in the column of injected fluid were observed by direct microscopic visualization. For each indicator 10 to 15 tubules were injected and observed. In a second group of experiments carbonic anhydrase, as well as the acid-base indicators, was added to the perfusion fluid in a concentration of 100 mg %. To evaluate the ability to detect color changes in fluid columns of tubular dimensions (approximately 30 μ in diameter) samples of test solutions with the various acid base indicators were titrated to several different pH values between pH 5

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