Inactivation of Gram-Negative Bacterial Endotoxins by Papain.* (28852)

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It is well established that one or more toxic activities of Gram-negative bacterial endotoxins are associated with lipopolysaccharide molecules. Whether these activities are present in the lipid, the polysaccharide or a specific linkage between the two has not been determined because no endotoxin with pyrogenic or lethal activity has been prepared free of either polysaccharide or lipid(1,2).

Recently we have postulated that the activity of endotoxin may depend upon the immunological state of the host(3). It was suggested that in some hosts, such as the rabbit, a primary toxicity is enhanced by acquisition of a delayed-type hypersensitivity to a common antigenic determinant present in most endotoxins. This enhancement of toxic activity in the rabbit is manifested by an increase in the 3-hour portion of the fever curve, greater susceptibility to lethality, and enhanced skin reactivity.

Although polysaccharides are often implicated, there is no definitive evidence that they can induce delayed hypersensitivity. Recently Holborow and Loewi(4) treated blood-group substances with papain and completely abolished the delayed hypersensitivity reaction without destroying the hemagglutination inhibition activity. It was concluded that a polypeptide and not the polysaccharide was responsible for the delayed hypersensitivity reactivity observed. Likewise, Tremaine(5) has shown a correlation between the protein content of pneumococcal polysaccharides and their activity in delayed hypersensitivity reactions.

Because of these observations and the suggested role of delayed hypersensitivity in endotoxin activity, the effect of crystalline papain on the pyrogenic and lethal activity of endotoxin was determined.

Materials and methods. Endotoxins. The purified *Escherichia coli* 08 $COO8_{2158}S_5$ (COO8) endotoxin was kindly donated by Dr. Otto Westphal, Max Planck Institute for Immunobiology, Freiburg, West Germany. The purified endotoxin of *Salmonella enteritidis* Se 289/273, original (Se) and Se 289/273 R, purified (SeR) were generously supplied by Dr. Edgar Ribi, Rocky Mountain Laboratory, Hamilton, Montana(6). The purified toxins were dissolved in 1 ml of pyrogen-free distilled water and heated in a boiling water bath for 2 minutes; these were diluted in sodium phosphate-buffered saline, pH 7.0, 0.15 M to give a toxin concentration of 1 mg/ml. Activity was measured in MPD-3 units(3). This unit measures the febrile response 3 hours after injection and is a quantitative measurement of pyrogenicity. The MPD-3 of the toxins in μg/kg were as follows: COO8 0.008, Se 0.002, and SeR 0.0016.

Papain inactivation. Twice crystallized preparations of papain obtained from Worthington Biochemical Corp., Freehold, N. J., and Sigma Chemical Co., St. Louis, Mo., were equally active. Because papain acts only in the reduced state, it was possible to include an additional control involving non-activated papain. Each experiment, therefore, included endotoxin plus activated papain, endotoxin plus nonactivated papain, and endotoxin plus reduced buffer. The experiments were run as follows: a. *Activated papain.* 1 ml of endotoxin (1 mg/ml) was mixed with 9 ml of reduced buffered solution containing 100 μg/ml of 2 × crystallized papain; this buffer contained 0.04 M acetate at pH 4.0 or 0.04 M phosphate at pH 7.0, NaCl 0.15 M, L-cysteine HCl 0.005 M, and ethylenediamine-tetraacetic acid, tetrasodium salt (EDTA) 0.001 M. b. *Non-activated papain.* 1 ml of endotoxin solution (1 mg/ml) was mixed with 9 ml of non-reduced buffered solution containing 100 μg/ml of papain; this buffered solution did not contain the cysteine HCl and EDTA. c. *Reduced buffer.*

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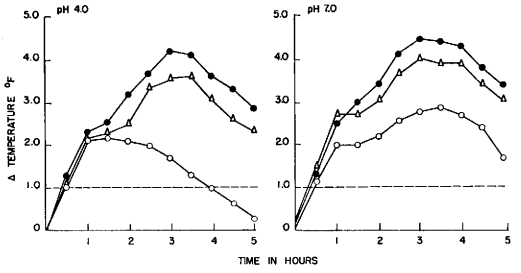

FIG. 1. Inactivation of pyrogenic activity of endotoxin by papain at pH 4.0 and 7.0. Each curve represents the mean febrile response of 5 adult rabbits (1.0 to 1.2 kg) injected intravenously with 1 μg/kg of endotoxin (COO8) treated with: ○, activated papain; ●, non-activated papain; △, reduced buffered saline.

1 ml of endotoxin (1 mg/ml) was added to 9 ml of reduced buffered saline; this was the same as (a) without the addition of papain. Incubation was at 65°C for 16 hours. Any remaining enzyme was destroyed by heating in a boiling water bath for 2 minutes. These original solutions were diluted with pyrogen-free phosphate buffered saline at pH 7.0 before testing for pyrogenic activity.

Activity determinations. Pyrogenicity. For each diluted preparation, 5 adult American Dutch rabbits (1.0 to 1.2 kg) were injected intravenously with 1 ml/kg. If there had been no inactivation, each rabbit received 1 μg/kg of COO8 or 0.2 μg/kg of Se or SeR. Rectal temperatures were recorded at 30-minute intervals for 5 hours as previously described(3). From a standard curve for each toxin, the number of MPD-3 units was determined for each preparation. *Lethality.* Adult American Dutch rabbits $>$ 1.5 kg were injected intravenously with 100 μg/kg of COO8 and 50 μg/kg of Se; this represents the concentration of toxin before treatment. The animals were observed for a period of 2 days although they usually died within 24 hours.

Results. Fig. 1 gives the results of a representative experiment where inactivation, determined by pyrogenicity, was carried out at pH 4.0 and 7.0. The control curves, at both pH values, given by the injection of COO8 endotoxin treated with non-activated papain and reduced buffer, are characteristically biphasic reaching a maximum at 3 hours. As observed in pyrogenic tolerance (3), there is little difference at 1 hour in the response to the 3 preparations. At 3 hours, however, significant inactivation was evident by the suppressed fever response in the preparation treated with activated papain in relation to the controls. Where the reduced buffer curve is used as a control, the relative inactivation at pH 4.0 is 97% and at pH 7.0 the endotoxin is approximately 87% inactivated. As shown in Fig. 1 and in repeated experiments, there was greater inactivation at the lower pH values. The differences in responses observed between the controls suggest a slight inactivation due to the reducing agent cysteine and EDTA. This is insignificant compared with activated papain.

Endotoxins such as COO8 of Westphal, prepared by the phenol method(7) usually contain amino acids indicative of small quantities of peptides. The endotoxins from *S. enteritidis* were prepared by the aqueous ether method(6,8); the Se contained amino acids and peptides, but the SeR was primarily polysaccharide and lipid with no detectable amino acids. All the nitrogen, within the experimental error of the methods, could be accounted for as hexosamine nitrogen(6). The MPD-3 activity of the Se was 0.002 μg/kg and the SeR 0.0016 μg/kg. Therefore, there was no apparent correlation between the nitrogen content and their pyrogenic activity. As given in Table I, both the Se and the SeR toxins were readily inactivated by papain.

In a previous study(3), a good correlation was observed in rabbits between the 3-hour fever response and the lethal effects of endotoxin. In the present experiments, papain reduced the 3-hour febrile response and therefore it was important to determine the effect

TABLE I. Effect of Nitrogen Content of Endotoxins on Inactivation by Papain.

Treatment	Recovery of pyrogenicity (MPD-3/kg)*	
	Se original (4.12% N)	Se purified (.20% N)
Activated papain	5	4
Non-activated papain	100	125

* Before treatment the concentration of the Se original was 100 MPD-3/kg and Se purified 125 MPD-3/kg.

TABLE II. Inactivation of Lethal Effect of Endotoxin by Papain.

Endotoxin	Dose before treatment, μg/kg	Treatment	No. of animals	No. of deaths
E. coli 08 (CO08)	100	Activated papain	5	0
		Non-activated papain	5	4
S. enteritidis (Se)	50	Activated papain	5	1
		Non-activated papain	5	5

of papain on the lethal effects of endotoxin. The results given in Table II show a decrease in lethal activity of endotoxin after treatment with activated papain.

Discussion. The inactivation of the pyrogenic and lethal activities of endotoxin by reduced crystalline papain and the failure of non-reduced enzyme or the reduced buffer containing EDTA and cysteine to inactivate significantly the toxin suggests that the observed inactivation is due to enzymatic activity and not to non-specific complex formation. The greater inactivation at pH 4.0 when compared to pH 7.0 is not inconsistent with the observation that the optimal pH for papain activity varies with the nature of the substrate(9).

Although papain was able to inactivate endotoxins in which amino acids or peptide linkages could not be detected, these results do not preclude the possible role of small amounts of amino acids closely associated with the molecular structure of the polysaccharide or lipid. It has been pointed out to us by Doctor Ribi, that because of the small quantities of the SR endotoxin available for analysis, it is not possible to detect amounts of amino acids represented by 0.025% N which is the difference between total nitrogen and that present in the hexosamines(6). If the secondary toxicity of endotoxin is due to a delayed hypersensitivity reaction to specific groups within the endotoxin, a peptide would be the most likely determinant. We have also suggested that the primary toxicity may be more closely associated with the lipid portion of the macromolecule(3). Lipid A (1) contains long chain fatty acids bound to glucosamine by ester and amide linkages. Papain is capable of splitting linkages of both types(9), and, therefore, if primary toxicity of endotoxin is associated with ester or amide linkages, one would anticipate the observed inactivation by papain.

Even though this crystalline enzyme has the potential to split more than one type of linkage known to be present in the endotoxin molecule, this enzyme may be a useful tool in determining the nature of the active sites responsible for one or more activities of endotoxins.

Summary. Reduced crystalline papain inactivated the pyrogenic and lethal activities of purified Gram-negative bacterial endotoxins. Because of the possible presence of peptide, ester and amide linkages within the macromolecular toxin and the known ability of the enzyme to split linkages of all 3 types, it was not possible from these results to implicate a specific linkage or group in endotoxin activity. It was suggested that this purified enzyme may prove useful for further studies on the chemical nature of the active groups within the endotoxin molecule.

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