Effect of Formaldehyde on the Immunochemical and Biological Activity of Staphylococcal Endotoxin B

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Staphylococcal enterotoxin B was treated with 0.15 or 0.3% formaldehyde at 37 C and pH 7.3. As early as 8 hr after addition of HCHO, the toxin was unable to precipitate as much antibody from antiserum as untreated toxin; greater amounts of treated toxin were required to reach equivalence and to inhibit precipitation. With increasing time, its reactivity decreased so that at 32 weeks it could precipitate only 20% as much antibody as untreated toxin. When other factors were kept constant, the degree of inactivation increased as the HCHO concentration increased from 0.6 to 1.4%. Treated toxin gave a "reaction of partial identity" by the Ouchterlony double-immunodiffusion technique. The emetic effect of the toxin for the monkey remained essentially unchanged for about 48 hr of treatment, but the amount required to cause death by the intravenous route increased from 25 to over 1,000 μg/kg of body weight. HCHO destroyed the pyrogenic effect for rabbits. The treated toxin remained immunogenic for rabbits even after 4 to 5 weeks of exposure to 0.3% HCHO at 37 C.

Formaldehyde has long been used for the inactivation of toxins without destruction of their antigenic properties. Scattered reports indicate that changes in properties other than inactivation of toxic activity may occur, depending on the reaction or temperature. In crude preparations, components respond differently to HCHO. Dolman and Wilson (5), Minett (11), and Thatcher and Matheson (25) inactivated the α- and β-lysins in crude filtrates obtained from cultures of enterotoxigenic *Staphylococcus aureus*. In all cases these authors observed that enterotoxin activity persisted unless exposure to HCHO was prolonged. Minett found that enterotoxin was resistant to 0.3% Formalin at 37 C for 36 to 40 hr, but was inactivated after 10 days. Dolman and Wilson noted no inactivation by the same concentration of HCHO after 5 to 14 months in a refrigerator. Fisher (7) found that α-hemolysin was more susceptible to inactivation by HCHO than was the protective factor found in crude culture filtrates of a *Staphylococcus* strain. Bergdoll (2) studied the immunological properties of partially purified enterotoxin after inactivation with 0.7% Formalin at pH 8.0. The mixture was incubated either at 50 C for 24 hr or at 37 C for 3 weeks. Inactivation was assessed in rabbits by failure of 0.5 mg of enterotoxin to cause weight loss in excess of 50 g. The alum-adsorbed toxoid induced antibody formation in rabbits and increased the resistance of monkeys to enterotoxin about 100-fold.

Raynaud et al. (18) and Aleksandrov et al. (1), in studies with tetanus and diphtheria toxins, respectively, observed that purified toxin was not as readily inactivated nor was the toxoid as stable as the native toxin. Both groups noted that Formalin-treated purified toxin regained some of its toxicity upon standing.

Enterotoxoid prepared from purified staphylococcal enterotoxin was not studied to determine the effect of HCHO on its immunogenic or other properties. We studied enterotoxoid to determine its immunogenic and antigenic behavior as well as its biological activity in rabbits and monkeys, and to compare these activities with those of untreated enterotoxin. A subsequent report compares immunogenic activity of enterotoxin and its toxoid in monkeys.

MATERIALS AND METHODS

Staphylococcal enterotoxin B, prepared as described by Schantz et al. (20) and free of hemolysins, apyrase, and other metabolic products, was dissolved in 0.02 M phosphate-buffered saline (PBS), pH 7.3. A solution containing 1.0 mg/ml was exposed to HCHO concentrations varying from 0.1 to 1.4% for different time periods. After addition of HCHO, the pH was checked and adjusted to 7.3, if necessary. Subsequent to treatment, excess HCHO was removed by

either dialysis against PBS or by treatment with NaHSO₃.

Changes in the immunochemical activity were followed by the quantitative precipitin technique of Heidelberger and Kendall (9) as described by Mc-Duffie and Kabat (10). The toxin-antitoxin mixtures were incubated at 37 C for 4 hr and allowed to stand at 4 C for 2 to 4 days. The washed precipitates were dissolved in 0.25 N acetic acid, and the optical density of the solutions was determined with a Beckman DU spectrophotometer at 277 nm. The readings were converted to protein nitrogen by the use of a standard curve.

Biological activity was determined by observing emesis or death in *Macaca mulatta* monkeys after injection by the intravenous route. The animals were observed 5 hr for emesis and held for 10 days to determine lethality. Immunogenic properties of toxoid and toxin were compared by immunizing rabbits with identical dosages and schedules. Antibody titers were followed by hemagglutination and by assay of antibody nitrogen. In addition, protective or neutralizing activity of the rabbit antisera was estimated in mice and monkeys. A 10-mg amount of enterotoxin B was dissolved in 2.5 ml of 0.15 M phosphate buffer (pH 8.0) containing 0.3% HCHO (v/v); the mixture was incubated at 37 C for 3 weeks. The toxoid was emulsified with an equal volume of Freund's complete adjuvant so that final concentration of toxoid was 2 mg/ml. A 10-mg amount of untreated enterotoxin B was dissolved in 2.5 ml of buffer and emulsified with 2.5 ml of Freund's complete adjuvant. Groups of four rabbits each were immunized with either the toxin or toxoid. The immunization schedules were identical with both antigens. Four injections were given subcutaneously at 2-week intervals. The rabbits were rested for 7 weeks and again injected at the 14th week. The successive doses were 100, 500, 500, 500, and 750 μg. Each animal received a total of 2.35 mg of toxin or toxoid. Test bleedings were done before each injection.

The antitoxin and antitoxoid were tested in mice challenged with 40 μg of enterotoxin followed by 150 μg of lipopolysaccharide (*Salmonella enteritidis*, Difco) 4 hr later; the antiserum was administered 4 hr before the enterotoxin. All injections were into the peritoneal cavity. The procedure was based on that of Sugiyama et al. (24). The protective value of the sera was determined also in rhesus monkeys (*M. mulatta*). The desired amount of antiserum, as determined by its antibody nitrogen content, was injected intravenously. Three hours later the animals were challenged with 75 μg of enterotoxin or approximately 1 LD₅₀ (25 μg/kg, body weight) per monkey. The animals were observed for 5 hr for emesis and for 7 days to determine the protection obtained against the lethal effect.

RESULTS

Figure 1 shows the effect of HCHO concentration on the precipitin reaction after exposure of enterotoxin to 0.6, 0.9, 1.1, and 1.4% HCHO for 6 days at 37 C. With increasing HCHO concentrations, the toxin was less capable of completely

precipitating antitoxin. The equivalence zone for untreated enterotoxin was between 6.0 and 11.0 μg of toxin nitrogen; that for HCHO-treated toxin was greater than 24 μg of N. Evidence of partial inactivation of antibody by free HCHO is also indicated in Fig. 1. The curve for the reaction with undialyzed toxoid shows less antibody precipitated than was observed after removal of excess HCHO. In single agar immunodiffusion in tubes, little or no change occurred in the rate of diffusion after HCHO exposure; however, the precipitate band was very light and not easily measured. Double-immunodiffusion in agar plates resulted in a "reaction of partial identity" with untreated toxin and HCHO-treated toxin (Fig. 2). The latter formed either a single line or occasionally a
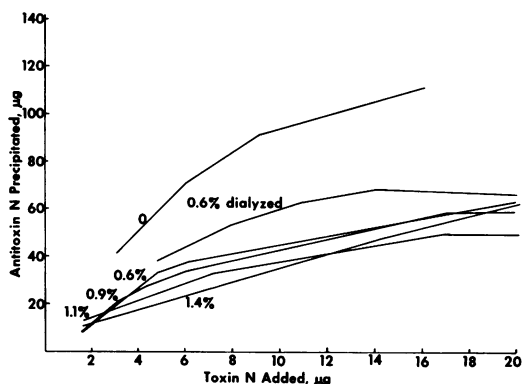

FIG. 1. *Effect of formaldehyde on the immunochemical activity of staphylococcal enteroxtoxin B. Precipitin curves of antitoxin B and toxin treated with the indicated concentration of HCHO at 37 C for 6 days. The effect of free HCHO on precipitation is shown by comparing curve "0.6%" with "0.6% dialyzed."*
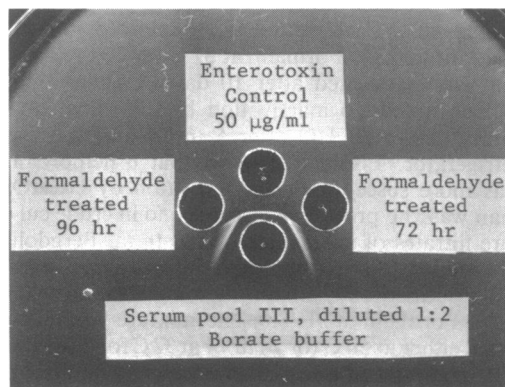

FIG. 2. *Double-immunodiffusion showing spur formation when equal quantities of enterotoxin or of HCHO-treated enterotoxin were reacted with antienterotoxin B antiserum.*

double band of precipitate that formed a spur at the junction with the toxin band. Toxin incubated with 0.6 to 1.4% HCHO for 6 days at 37 C was not emetic for monkeys when 10 µg/kg of body weight was injected intravenously into monkeys. The ED_{50} of the control toxin was 0.1 to 0.3 µg/kg.

Some loss of precipitating activity was observed as soon as 8 hr after exposure to either 0.15 or 0.3% HCHO (Fig. 3). Inactivation increased with time so that HCHO-treated enterotoxin precipitated about one-fifth the amount of precipitable antibody at 32 weeks. As exposure to HCHO increased, more toxin was required to reach equivalence and to inhibit precipitation. Untreated enterotoxin required 16.0 µg of toxin nitrogen to inhibit precipitation. At 48 and 96 hr, 22.0 µg and more than 31.0 µg, respectively, were necessary.

Toxin treated with 0.1 or 0.3% HCHO at 37 C for 8 through 48 hr showed no loss in its emetic effect. There was, however, a decrease in the lethal activity as was indicated by an increase in the intravenous LD_{50} from 24 to over 1,000 µg/kg of body weight (Table 1). The emetic effect was almost completely destroyed at 9 days. Within 3 to 5 days of treatment with 0.3% HCHO at 37 C, enterotoxin lost its pyrogenic activity for rabbits. Although 25.0 µg of native enterotoxin B induced a temperature rise of 3 F or greater, 1.9 mg of toxoid produced no response.

Hemagglutination or immunodiffusion titers, as well as antibody nitrogen content, were higher for antisera obtained from rabbits immunized with toxoid than for the antitoxin (Fig. 4, Table 2). Antibodies appeared sooner after injection of the toxoid, and although the curves were similar when antibody titers were plotted against time, the antitoxoid titers were consistently higher. The mean antibody nitrogen concentrations were 123
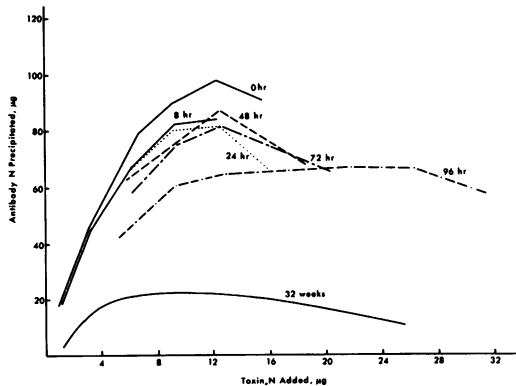
TABLE 1. *Effect of time of exposure of enterotoxin B to 0.3% HCHO on the intravenous emetic and lethal effects for rhesus monkeys*[a]

Toxin (µg)	Duration of HCHO treatment							
	0 hr		8 hr		24 hr		48 hr	
	Emesis	Death	Emesis	Death	Emesis	Death	Emesis	Death
0.1			1/3[b]	1/3	2/3	0/3	3/3	0/3
0.3	3/3	0/3					2/7	2/7
0.5			1/3	0/3	2/3	0/3	2/3	0/3
0.9	3/3	0/3						
2.5			2/3	0/3	3/3	0/3	2/3	0/3
2.7	3/3	0/3					1/3	0/3
30.0			3/3	0/3	3/3	0/3	4/6	0/6
150.0			3/3	0/3	3/3	0/3	3/3	0/3
750.0			3/3	0/3	3/3	0/3	3/3	0/3

[a] Intravenous ED_{50} for untreated toxin is 0.3 µg; intravenous LD_{50} is 24.0 µg (20).

[b] Number responding/number tested.

FIG. 3. *Effect of time on alteration of precipitin curves of enterotoxin B treated with 0.15% HCHO at 37 C.*

and 99 µg/ml for the antitoxoid and antitoxin, respectively. Figure 4 shows the typical anamnestic response obtained with both antitoxin and antitoxoid after the rabbits received a single injection of antigen 7 weeks after the first series of five.

Inhibition of the synergistic effect of enterotoxin for lipopolysaccharide was obtained with less antitoxoid than antitoxin (Table 3). However, the difference is not significant as determined by the *t* test. The protective effect of antitoxin and antitoxoid determined by administration to monkeys 3 hr before challenge is shown in Table 4. No real difference is apparent, although fewer animals receiving antitoxoid vomited than did those receiving an identical dose of antitoxin based on antibody nitrogen. The two deaths that occurred were among the three monkeys that had received the smallest dose (19.1 µg of antibody nitrogen) of antitoxin.

DISCUSSION

The observations that HCHO alters various properties of purified enterotoxin B at different rates are similar to those reported in the literature for other biologically active proteins. Christensen (3) observed that the rates of destruction of toxicity and antigenicity of cobra (*Naja flava*) venom were different. Moroz-Perlmutter et al. (13) observed that, at pH 5.5 to 9.2, the hemorrhagic property of *Vipera palestina* toxin was destroyed but its antigenic property was maintained only at acid pH; both neurotoxic activity and antigenicity were destroyed in the alkaline range. Toxicity
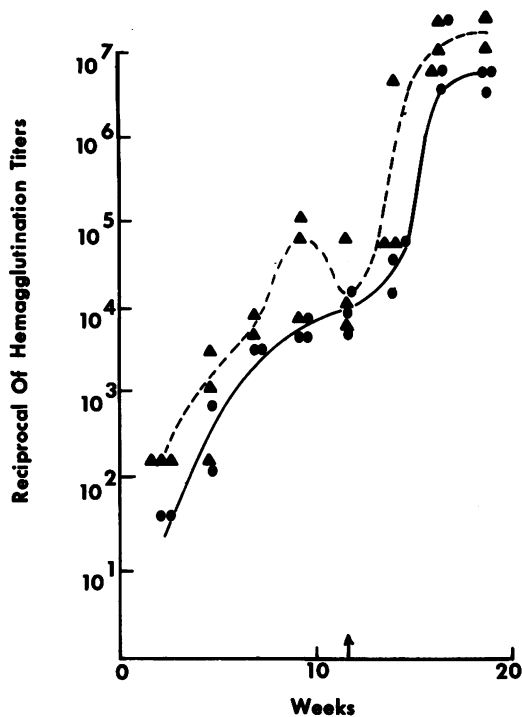
FIG. 4. *Antibody development in rabbits immunized with enterotoxin B or enterotoxoid B. Symbols:* O, *reciprocal of antienterotoxin titer;* ▲, *reciprocal of antitoxoid titer. Arrow indicates time of booster injections.*

of *Echis colorata* venom was incompletely destroyed over a wide pH range, although the hemorrhagic properties were completely destroyed.

Ley et al. (Federation Proc., p. 612, 1966) obtained two fractions by Sephadex G-100 filtration of the flocculating component of tetanus toxoid. Both fractions were antigenic in vivo but the higher-molecular-weight component had only one-half the flocculating activity of the low-molecular-weight component. Miura (12) observed that, at pH 9.5 and 11.0, HCHO inactivated skin toxicity of diphtheria toxin for guinea pigs. The immunogenicity for this animal was greater than that of toxoid prepared at pH 7.4. Munoz and Hestekin (14) found the protective activity of *Bordetella pertussis* extracts to be less susceptible to HCHO than its histamine-sensitizing factor. Neuzil and Pantrizel (15), studying the effect of HCHO on reactions between horse serum as antigen and rabbit antihorse antiserum, found that treatment of either retarded flocculation. The effect on antibody was greater than that on antigen. Like Eagle (6), they observed that the precipitating stage of the reaction was inhibited.

Perez (16) also reported changes in the antigenicity of bovine plasma and crystalline serum albumin. The latter was slightly less active in precipitating antibody after exposure to HCHO. Bovine plasma precipitated more antibody after than before formalinization and showed more

TABLE 2. *Antibody titers of antienterotoxin B and antienterotoxoid B antisera*

Serum	Time[a]	Hemagglutination[b]	Antibody N	Immunodiffusion[c]
	weeks		*μg/ml*	
Antitoxin[d]				
66	2	1,280		
	10	4,000		
	21	6.4×10^6	85.0	10
69	2	>640		
	10	8,000		
	21	3.2×10^6	99.7	20
70	2	>640		
	10	4,000		
	21	6.4×10^6	112.8	40
Antitoxoid[d]				
61	2	3,200		
	10	160,000		
	21	12.8×10^6	148.8	80
63	2	<160		
	10	8,000		
	21		86.4	20
64	2	1,280		
	10	64,000		
	21	25.6×10^6	122.9	80

[a] After initial injection.
[b] Reciprocal of highest dilution giving a "plus" reaction.
[c] Reciprocal of highest dilution causing a precipitation band by Ouchterlony technique.
[d] Subcutaneous injections with either enterotoxin or enterotoxoid emulsified in Freund's complete antigen: 1st week, 100 μg; 3rd week, 500 μg; 5th week, 500 μg; 7th week, 500 μg; 14th week, 750 μg.

TABLE 3. *Protective activity of antienterotoxin B and antienterotoxoid B for mice challenged with enterotoxin B and S. enteriditis lipopolysaccharide*

Antitoxin	PD$_{50}$[a] (μg of antibody N)	Antitoxoid	PD$_{50}$ (μg of antibody N)
69	0.43	61	0.21
70	1.15	64	0.77
66	0.36	63	0.28
Mean	0.65		0.42

[a] PD$_{50}$ = dose to protect 50% of the animals, calculated by procedure of Reed and Muench (19).

TABLE 4. *Protective effect of antitoxin B and antitoxoid B for monkeys challenged with 1 LD$_{50}$ of enterotoxin B*

Serum	Dose (μg of antibody N)	Response (3 animals per group)	
		Emesis	Death
Antitoxoid 61	178.6	0	0
	59.4	2	0
	19.8	2	0
Antitoxin 70	169.0	0	0
	56.3	3	0
	19.1	3	2
Antitoxoid 64	123.0	1	0
	37.0	3	0
	12.3	3	0
Antitoxin 69	120.0	3	0
	40.0	3	0
	14.0	1	0

inhibition in the antigen excess zone. Formaldehyde (0.25%, pH 7.0) alone did not alter the immunogenic properties of ovalbumin after 16 hr of exposure at room temperature nor did it modify the precipitin reaction (17).

The reaction between HCHO and proteins is too complex to enable one to determine which chemical modification has altered the properties of the protein. There is general agreement that the initial reaction is the attachment of a methyl group to the free amino groups, particularly the ϵ-amino group of lysine. Subsequently, there are a variety of inter- and intramolecular linkages formed that result in some polymerization. The importance of the amino groups is shown by the unpublished observation of Schantz that exposure of enterotoxin B to HNO$_2$ produces almost immediate inactivation. He obtained similar conclusions for botulinal toxin, type A (21, 22). Previously, Goldie (8), as well as others, inactivated pepsin and insulin by acetylation of free amino groups with ketene. Goldie was also able to detoxify diphtheria toxin by short exposures to ketene, whereas longer exposure was necessary to destroy its antigenicity.

Enterotoxin is not a protein with unusual chemical structure. It is a single polypeptide chain with one disulfide bridge (23), and whether it is the arrangement of the amino acids, the configuration of the molecule, or specific groupings that render it toxic is unknown. The disulfide bridge is not essential to its biological activity (4). Although the reaction with HCHO is too complex to be useful in determining active groups, it is

likely that the changes in the various activities of enterotoxin involve the same chemical reaction but that the differences in degree of change are quantitative rather than qualitative. The neutralization of enterotoxin by the same concentration of antitoxin and antitoxoid (based on the amount of antibody nitrogen required) suggests that the alteration of the toxin molecule cannot be too radical. It may be that inability of toxoid to precipitate as much antibody N as does toxin is due to masking of antigenic groups by polymerization of the enterotoxin molecule.

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