Effect of Chemical and Heat Inactivation on the Antigenicity and Immunogenicity of *Vibrio cholerae*

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The effects of heat and chemical inactivation on the antigenicity and immunogenicity of *Vibrio cholerae* 1418 in rabbits were studied. *V. cholerae* 1418 was inactivated with heat and chemical inactivants (phenol or Formalin) alone or in combination. Enzyme-linked immunoassay systems employing whole cells of *V. cholerae* 1418, lipopolysaccharide, or flagella as immobilized antigens were used to measure the antibody response (immunoglobulins G and M) after parenteral immunization of rabbits with various inactivated whole-cell preparations. The "classical" whole-cell vaccine, produced by phenol treatment, was found to be a comparatively poor immunogen. When Formalin was used instead of phenol, the antibody response to all three enzyme-linked immunosorbent assay antigens was greatly increased. Immunoglobulin G titers to intact *V. cholerae* cells were as much as 100-fold higher in rabbits immunized with the Formalin-inactivated preparation as compared to the classical phenol-inactivated vaccine. Furthermore, antibody produced against the Formalin-inactivated preparation was capable of recognizing antigenic determinants expressed on the cell surface of several heterologous strains of *V. cholerae*. These results indicate that the antigenicity and immunogenicity of *V. cholerae* are greatly affected by the inactivation conditions employed for vaccine production and that Formalin is much superior to phenol as an inactivant under the conditions employed in the present study.

The action of cholera toxin, produced by *Vibrio cholerae* which have colonized the small bowel, is responsible for the symptomatic fluid outpouring associated with cholera (8, 13). Both antitoxic (7, 11, 22) and antibacterial (15, 17, 30) immunities have been shown to be protective. Although the relative contributory role that antibacterial versus antitoxic immunity may play in prevention of disease in humans is unknown, results from human volunteer studies suggest that antibacterial immunity is of prime importance (15).

The phenol-inactivated, parenterally administered whole-cell vaccine currently in use has been found to afford only short-term protection in controlled field trials (2, 17, 18). The precise reason(s) for poor vaccine efficacy remains unknown. However, several possible explanations exist, including poor vaccine immunogenicity, evidenced by increased protection when employed with an adjuvant (2), and the ability of a parenterally administered vaccine to evoke a protective gut immunity (8, 9). Additionally, the phenol inactivation process employed for vaccine production may result in destruction or alteration of somatic protective antigens. Antibody to lipopolysaccharide (LPS) (13, 25, 27)

and cell surface proteins (21, 24, 31) have been shown to be protective in experimental and human (3) cholera, though their stability to heat and chemical inactivants is unknown. Furthermore, the antigenicity, and perhaps immunogenicity, of bacterial LPS is believed to be dependent upon association with cell-surface proteins (28) which may be disrupted upon inactivation.

The present study was conducted in an attempt to define inactivation conditions which would produce a preparation retaining a high degree of native antigenicity. The antibody response elicited by parenteral immunization of rabbits with different inactivated whole-cell preparations was studied by means of enzyme-linked immunosorbent assay (ELISA) systems, using *V. cholerae* LPS, flagella, and intact cells as immobilized antigens as a means to gauge the effect of different inactivation procedures on the immunogenicity and antigenicity of *V. cholerae*.

MATERIALS AND METHODS

Bacteria and growth conditions. *V. cholerae* CA 401 (Inaba) was provided by C. D. Parker, University of Missouri, Columbia. *V. cholerae* 1418 (Ogawa, El Tor) was the gift of T. P. Pesigan, Department of Public Health, Manila, the Philippines. *V. cholerae* V 86

(Inaba, El Tor) was provided by Y. Watanabe, World Health Organization, Geneva, Switzerland. *V. cholerae* NIH 35 (Inaba) and NIH 41 (Ogawa) were obtained from J. Feeley, National Institutes of Health, Bethesda, Md. All strains were routinely grown in CB medium (pH 7.5) (23 g of Hy Case SF [Humko Sheffield, Memphis, Tenn.], 9.5 g of NaCl, and 3 g of yeast extract per liter). For vaccine production *V. cholerae* 1418 was grown in an 8-liter fermentor under high aeration (1.6 liters of O_2 per minute) and agitation (600 rpm). The fermentor was inoculated with a mid-log-phase culture (1%, vol/vol) and incubated for 18 h at 30°C. The pH was maintained at 7.5 by the addition of acetic acid. The final culture contained approximately 10^{10} colony-forming units per ml. Cells for use in the CSA-ELISA (see below) were grown in CB medium in 125-ml Erlenmeyer flasks at 37°C with vigorous shaking.

Vaccine production. The fermentor-grown culture was harvested in 2-liter flasks (400 ml per flask). Formalin (37% stock) or phenol (86% stock) was added to the appropriate concentration (vol/vol) where indicated. Cultures were placed in a 65°C water bath and gently mixed. Treated cultures were then allowed to cool at room temperature. Samples were tested for sterility by dilution and plating on brain heart infusion agar. The inactivated preparations were aliquoted and stored at −20°C.

Immunization of rabbits. Antiserum against live vibrios was prepared in rabbits by repeated intramuscular injections of phosphate-buffered saline (PBS)-washed viable (late log to early stationary phase) *V. cholerae* 1418 cells grown in CB medium. The first immunization consisted of 10^8 cells, followed by 10^9- and 10^{10}-cell doses at 2-week intervals. Sera from three rabbits were collected 1 week after the final immunization and pooled (ALC-1). For immunization with vaccine, rabbits (in groups of two) were given 10^{10} killed cells intramuscularly at two different sites in 0.5-ml volumes. In 2 weeks, the rabbits received a second dose of 10^{10} cells in the same manner. Sera were collected and pooled 2 weeks after the second immunization.

Purification of LPS. LPS from stationary-phase cultures of *V. cholerae* 1418 grown in CB medium was prepared by phenol-water extraction (29). Extracted LPS was subjected to two rounds of ultracentrifugation at 100,000 × *g* for 1 h. The final LPS pellet was suspended in PBS and lyophilized. Purity of the final product was confirmed by obtaining an absorption spectrum from 220 to 400 nm and by determination of the protein content by the method of Lowry et al. (16). LPS purified in such a manner contained undetectable amounts of protein.

Flagella preparations. Crude flagella were prepared by a slight modification of reported methods (24). A 20-ml portion of an overnight culture of *V. cholerae* CA 401 was used to inoculate 1 liter of brain heart infusion medium in a 10-liter carboy. The cultures (5 liters) were gently shaken at 37°C until the absorbance at 540 nm (brain heart infusion blank) reached 0.3. The cells were collected by centrifugation at 6,000 × *g* for 10 min at 4°C. Flagella were obtained as previously described (24), but Formalin was not added to the final product. The flagella preparation contained less than 0.1% LPS (wt/wt) as determined by a *Limulus* lysate assay.

ELISA. An ELISA, employing intact NaN_3-killed

vibrios as immobilized antigens to study cell surface antigens (CSA; CSA-ELISA), was performed essentially as described elsewhere (6). Briefly, polystyrene tubes (13 by 100 mm) were coated with 10^8 washed, NaN_3-killed, late log-phase cells in 1 ml of PBS–0.02% NaN_3 buffer by incubation at 37°C for 3 h. Polystyrene tubes were coated with 10 μg of flagellar protein in 1 ml of 0.1 M sodium carbonate buffer (pH 9.2) by incubation at 37°C for 3 h. Polystyrene tubes were also coated with 50 μg of *V. cholerae* 1418 LPS (12) in 1 ml of 5 mM sodium phosphate buffer (pH 7.4) by incubation at 37°C for 3 h. All coated tubes were stored at 4°C until used (usually within 2 weeks).

ELISA was performed as follows: coated tubes were washed three times with PBS containing 0.02% Tween 20 (PBS-T). Serum dilutions (1 ml in PBS-T) were added, and the tubes were incubated for 6 h at room temperature. Tubes were again washed with PBS-T. For measurement of immunoglobulin G (IgG), 1 ml of a horseradish peroxidase-*Staphylococcus* protein A complex prepared by the method of Nakane (20) was added. For quantitation of IgM, 1 ml of a peroxidase-labeled goat anti-rabbit IgM (Biogenzia Lemania, Lausanne, Switzerland) diluted 1:500 in PBS-T was used. Both mixtures were incubated overnight at 4°C. After three washes with PBS-T, 1 ml of fresh substrate solution [containing 10 mg of 2,2′-azino-di(3-ethyl-benzol-thiozoline) sulfonic acid-6; Boehringer AG, Mannheim, West Germany], dissolved in 50 ml of 0.1 M sodium phosphate-hydrochloride buffer (pH 4.0) with 125 μl of 10% H_2O_2, was added. The color was developed at room temperature for 1 h, and then 100 μl of 0.04% NaN_3 was added to terminate the reaction. Samples were read at 410 nm. Titers for all ELISA assays were expressed as the reciprocal of the serum dilution giving 50% of the absorption obtained with the standard antiserum ALC-1 run in parallel. As measured in the CSA-ELISA, approximately 75% of the antibody present in ALC-1 was directed against LPS and 25% was directed against non-LPS surface antigens.

RESULTS

The CSA-ELISA was selected as an initial screening method to monitor IgG antibody production in response to parenteral immunization with various inactivated preparations because this assay can detect antibodies against both LPS and non-LPS CSAs. Furthermore, by using

TABLE 1. Effect of various inactivation procedures on the immune response to parenteral immunization as measured by CSA-ELISA[a]

Inactivation procedure	CSA-ELISA titer[b]
1. 0.4% phenol, 65°C, 60 min	1.55
2. 0.4% Formalin, 65°C, 60 min	71.5
3. 1.5% Formalin, 65°C, 60 min	156.0
4. Heat alone: 65°C, 15 min	17.5
5. Heat alone: 65°C, 60 min	3.0
6. Standard antiserum ALC-1	720

[a] Inactivated preparations were used to immunize rabbits as described in the text.

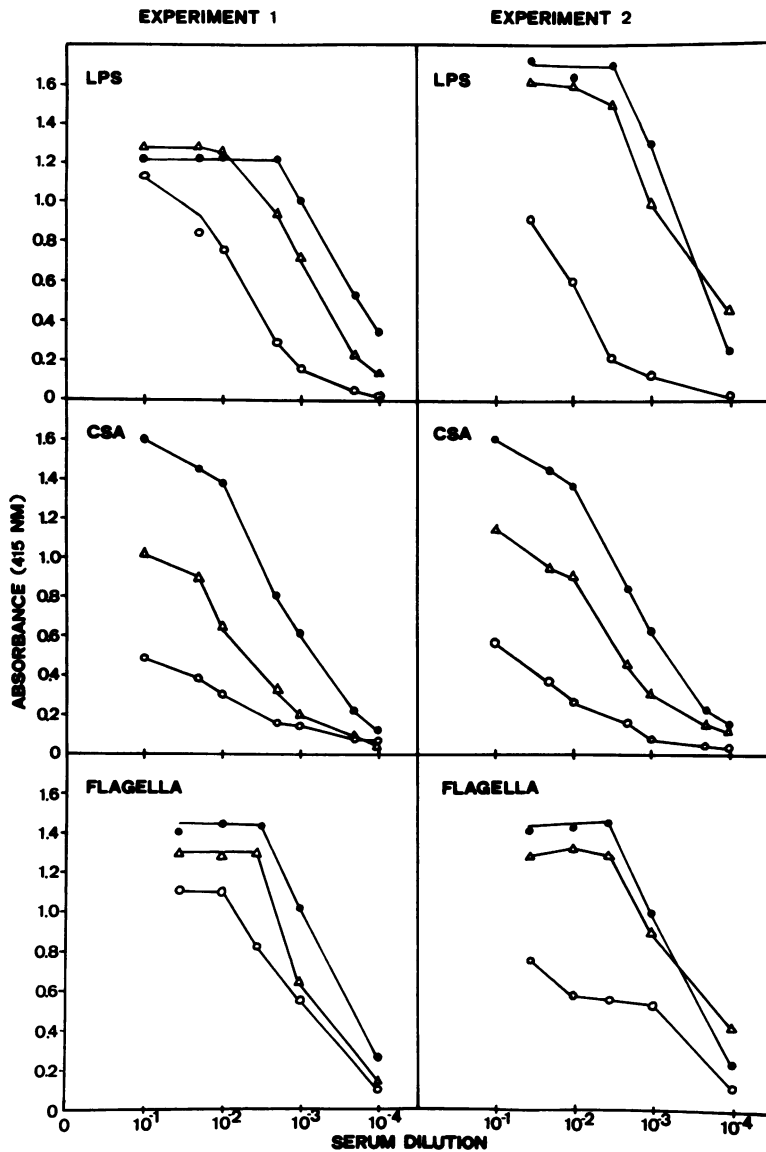[b] Mean of two independent determinations.

FIG. 1. Representative titration curves for ALC-1 (●) and antisera raised against Formalin-inactivated (△) and phenol-inactivated (○) preparations in ELISA systems employing LPS, *V. cholerae* 1418 whole cells (CSA), and flagella as immobilized antigens.

whole cells inactivated by a relatively gentle procedure (NaN$_3$ killing) as immobilized antigens, a greater proportion of native antigenic determinants would be preserved. The IgG titers obtained when various inactivated preparations of *V. cholerae* 1418 were used as immunogens are shown in Table 1. When cells were inactivated by treatment with phenol and heat (0.4% phenol; 65°C for 60 min; preparation 1, Table 1), very low antibody titers were found. By substitution of Formalin for phenol as an inactivating agent, an almost 50-fold increase in titer was

obtained (preparation 2, Table 1). By increasing the concentration of Formalin to 1.5% (preparation 3, Table 1), a further twofold increase in titer was observed. A further increase in the Formalin concentration had no beneficial effects on the antibody titers obtained (data not shown). When cultures were inactivated by heating alone at 65°C for either 15 or 60 min, titers in response to immunization were much lower than when Formalin was included. These results indicate that the use of Formalin in place of phenol, in conjunction with heating, produces an inactivat-

TABLE 2. IgG titers to *V. cholerae* surface antigens in response to immunization with inactivated preparations of *V. cholerae* 1418

Inactivation procedure[a]	IgG titers[b]					
	CSA-ELISA[c]		LPS ELISA[d]		Flagella ELISA[e]	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
0.4% phenol, 65°C, 60 min	0.76 (0.66)	3 (1.7)	83.3 (9.1)	55 (7.4)	346 (18.6)	115 (10.7)
1.5% Formalin, 65°C, 60 min	102 (10)	220 (14.8)	1,233 (35.1)	3,033 (55)	1,550 (39.3)	2,316 (48.1)
ALC-1	758 (27.5)		2,300 (47.9)		2,230 (47.2)	

[a] *V. cholerae* 1418 cells were inactivated as indicated before immunization of rabbits as described in the text.

[b] Titers are expressed as described in the text. Values given represent the geometric mean of three independent determinations, with the standard deviation in parentheses.

[c] Tubes were coated with 10^8 *V. cholerae* 1418.

[d] Tubes were coated with 50 μg of *V. cholerae* 1418 LPS.

[e] Tubes were coated with 5 μg of *V. cholerae* CA 401 flagella.

ed preparation which is effective in eliciting high titers of IgG antibody which recognizes native cell surface determinants.

Antibody to several *V. cholerae* somatic antigens have been shown to be protective in experimental animal models including flagella (24, 31) and LPS (13, 27). It was of interest to compare the antibody response to these antigens when *V. cholerae* cultures were inactivated with phenol (procedure 1, Table 1) or Formalin (procedure 3, Table 1). To verify that the results obtained were reproducible, two independent inactivation experiments were performed, starting with different culture lots through immunization of rabbits (experiments 1 and 2, Fig. 1 and Table 2). Antiserum ALC-1, produced in response to repeated injections of live vibrios, was used as a reference serum. Representative titration curves of serum samples obtained from both experiments in the CSA, LPS, and flagella ELISAs are shown in Fig. 1. The phenol-inactivated preparation elicited antibody titers substantially lower than those of the Formalin-inactivated preparation in all three assay systems in both experiments. Although the configuration of the curves varied depending upon both the assay system and experiment, the trend was constant. The CSA, flagella, and LPS IgG antibody titers for both experiments are shown in Table 2. The greatest difference in titer was seen in the CSA-ELISA, in which the Formalin-inactivated preparation evoked titers approximately 100-fold higher than those of the phenol-inactivated preparation in both experiments. Marked differences in titer were also observed in the LPS and flagella ELISAs. The anti-LPS titer was from 15-fold (experiment 1) to 55-fold (experiment 2) higher when the Formalin-inactivated preparation was used as an immunogen. A similar trend was also seen in anti-flagella titers (Table 2). ALC-1 possessed the highest titer in all three assays, although the difference, as compared to the response elicited against the Formalin-inacti-

vated preparation, was less than twofold in the LPS and flagella ELISAs and approximately two- to sevenfold less in the CSA-ELISA.

The IgM antibodies present in antisera raised against the phenol- and Formalin-inactivated preparations were also determined in the three ELISA systems (data not shown). Although the differences in IgM titers were not of the same magnitude as seen with the IgG titers, the trend was the same.

Next, the antisera raised against phenol- and Formalin-inactivated *V. cholerae* 1418 (inactivation procedures 1 and 3, respectively, from Table 1) were tested for reactivity against several strains of *V. cholerae* representing various combinations of serotype and biotype (Table 3). Again, as when the homologous strain was used as an immobilized antigen, the antisera produced in response to the Formalin-inactivated preparation possessed titers at least 60-fold higher than those evoked by the phenol-inactivated preparation for all strains tested.

DISCUSSION

There can be little doubt that antibacterial immunity contributes significantly to protection against cholera in humans (2, 5, 15, 17, 30). However, relatively little is known about the protective somatic antigens of *V. cholerae*, especially relating to their expression by viable cells (14) and their susceptibility to alteration or destruction by heat or chemical means of inactivation used for vaccine production. Recently, several protective somatic antigens have been identified, including LPS and flagella (21, 24, 27, 31).

Results from controlled field trials employing phenol-inactivated whole-cell vaccines have been generally disappointing. Protection was found to be limited to certain populations and to be of short duration (2, 17). Several explanations have been put forth to explain these results,

TABLE 3. CSA-ELISA IgG titers to heterologous strains of *V. cholerae* after immunization with inactivated preparations of *V. cholerae* 1418[a]

Inactivation procedure	Titer with *V. cholerae* strain as immobilized antigen[a]:		
	V 86 (Inaba, El Tor)	NIH 35 (Inaba, classical)	NIH 41 (Ogawa, classical)
0.4% phenol, 65°C, 60 min	1.83 (1.35)	2.6 (1.61)	2.2 (1.49)
1.5% Formalin, 65°C, 60 min	166 (12.9)	65 (12.8)	170 (13)
ALC-1	390 (19.7)	220 (14.8)	480 (21.9)

[a] Tubes were coated with 10^8 cells of the indicated strain. Titers are expressed as described in the text. Values given represent the geometric mean of three independent determinations, with the standard deviation in parentheses. Sera tested were from experiment 1.

including: (i) poor immunogenicity of the vaccine (2); (ii) selection of vaccine strains with low antigenicity (8); and (iii) suboptimal route of immunization (8, 9, 17).

Although it is widely accepted that the currently employed phenol-inactivated whole-cell vaccine offers only marginal protection, few studies have been performed in an attempt to improve the efficacy of inactivated whole-cell vaccines (19, 26). To date, there have been no studies aimed at comparing the effect of different inactivating procedures on the immunogenicity-antigenicity of whole-cell vaccines. In the present report we have studied the immune response in rabbits to parenteral immunization with various killed whole-cell preparations of *V. cholerae* 1418. Since several *V. cholerae* somatic antigens have been implicated as protective antigens, the antibody response against two such antigens (LPS and flagella) in addition to NaN₃-killed whole cells was evaluated by using sensitive and specific ELISA systems.

V. cholerae inactivated with phenol and heat was found to be a comparatively poor immunogen. The replacement of phenol with Formalin greatly increased the antibody titers in all three assay systems. Both the concentration of Formalin and its use together with heat appeared to be important for the production of an optimal immunogen. The greater antibody response obtained with Formalin- as compared to phenol-inactivated cells is probably due to changes in cellular antigenicity, immunogenicity, or both, induced by the chemical inactivants. Considering the mechanism by which Formalin acts upon proteins (1, 4), its effects on surface proteins would be expected to be much less severe than those of phenol, which readily denatures proteins. The different reactivity of these chemicals towards proteins could account for the differences seen in the flagella and CSA-ELISAs, in which antibody to protein(s) is measured.

It was somewhat unexpected to see that anti-LPS titers were also greatly affected depending upon which chemical inactivant was employed. Since the LPS employed as an immobilized

antigen in the ELISA was purified by phenol-water extraction, phenol-induced antigenic alterations to cell-associated LPS would not be expected to account solely for the differences in titers observed. One possibility is that phenol treatment extracts a portion of the LPS from cells during the inactivation process. Such extracted LPS may possess a lower antigenic potency than cell-associated LPS. Perhaps of more importance are the alterations caused to the cell surface by the removal of LPS alone or together with associated proteins. Formalin treatment would not be expected to extract appreciable amounts of LPS, thereby limiting these types of cell surface alterations.

It is interesting that antiserum raised against the Formalin-inactivated preparation was capable of recognizing cell surface antigenic determinants possessed by heterologous strains of *V. cholerae*. The IgG titers against all three heterologous strains were only about twofold less than those of the reference antiserum ALC-1. Again, antiserum produced against the phenol-inactivated preparation had very low titers to all three heterologous strains. It would therefore appear that the Formalin inactivation procedure conserves antigenic determinants shared by different strains of *V. cholerae*.

Although only one alternative chemical inactivant (Formalin) was compared with phenol for production of a whole-cell vaccine, the results obtained, as concerns the immune response to parenteral immunization, were promising. It remains to be determined whether the higher levels of antibody produced against the Formalin-inactivated preparation are protective and how effective the Formalin-inactivated preparation is as an oral immunogen. However, the results obtained offer at least one plausible explanation for the poor showing of previously tested phenol-inactivated vaccines. Furthermore, the use of a highly immunogenic-antigenic killed-cell preparation, perhaps in conjunction with a cholera toxoid (10, 23), as a vaccine may prove effective for inducing both antibacterial and antitoxic immunity.

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