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Synergistic Protective Effect in Rabbits of Immunization with *Vibrio cholerae* Lipopolysaccharide and Toxin/Toxoid

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Subcutaneous immunization of rabbits with a combination of *Vibrio cholerae* lipopolysaccharide (LPS) and enterotoxin induced a more than 100-fold-higher degree of protection against intestinal challenge with live cholera vibrios than did vaccination with either of the two antigens alone. Such a synergistic effect was also obtained by immunization with a combination of LPS and choleragenoid. The immunization with LPS and toxin (or toxoid) in combination did not enhance the resistance to toxin challenge above that induced by the toxin component alone. This, together with data from titrations of anti-LPS and antitoxin antibodies in serum and in intestinal washings, contradicts enhanced immune responses due to adjuvant action of the two antigens as the explanation for the synergistic effect of the combined vaccines. A more likely explanation would be that the antibacterial and antitoxic immune responses, without being increased in themselves, function synergistically by interfering with two separate events in cholera pathogenesis.

A protective effect of immunization with *Vibrio cholerae* somatic antigen or purified lipopolysaccharide (LPS) has been well documented in experimental cholera systems as well as in the field (1, 20, 26). However, it is generally recognized that the immunity induced by such vaccines is of a limited magnitude and has a short duration (16, 20). Recent animal experiments have indicated that protection against cholera can be induced by immunization with *V. cholerae* exoenterotoxin or -toxoids (6, 8, 15). This has led to the assumption that a vaccine inducing antibacterial as well as antitoxic immunity would be more effective than the presently available bacterial vaccines (e.g., 4, 14).

Preliminary experiments suggested that immunization with a mixture of purified LPS and purified cholera enterotoxin can give rise to a higher degree of protection against experimental cholera infection in rabbits than the sum protective immunity induced by either antigen alone (A.-M. Svennerholm, Ph.D. thesis, Univ. of Göteborg, Göteborg, Sweden, 1975). In the present study a synergistic protective effect of antibacterial and antitoxic cholera immunity in rabbits is further documented, and the relation between protection and antibody levels in serum and intestinal washings is elucidated.

MATERIALS AND METHODS

Animals. New Zealand rabbits 8 to 10 weeks old at the onset of immunization were used.

Immunogens. Purified cholera toxin, choleragen, prepared by R. A. Finkelstein, Dallas, Tex. (5), was supplied by C. Miller, National Institutes of Health, Bethesda, Md. Purified "natural" cholera toxoid, choleragenoid (5), was a gift from R. A. Finkelstein. Purified LPS of *V. cholerae* 569B Inaba was prepared by the hot phenol-water extraction procedure followed by repeated ultracentrifugation (23). The LPS preparation contained 10% protein as estimated with the method of Lowry et al. (18).

Crude cholera toxin was prepared by filtration of *V. cholerae* culture filtrate (strain 569B, lot no. 4493G, supplied by the National Institutes of Health) on a pellicon membrane (11). The preparation contained about 10% LPS and 0.1% choleragen (wt/wt) after lyophilization.

Immunizations. Nine groups of rabbits, each comprising three to seven animals, were immunized with choleragen, LPS, or combinations of these two antigens in different proportions. Three other groups of rabbits were given, respectively, crude cholera toxin, choleragenoid, and a combination of choleragenoid and LPS. Dissolved in phosphate-buffered saline (PBS), the antigens were injected subcutaneously (s.c.) in two 0.5-ml portions above the right and left posterior legs. Two identical immunizations were given 2 weeks apart in the doses indicated in Fig. 1 and in Tables 1 and 2. An equal number of rabbits injected with PBS and analyzed concurrently with the immunized animals were used as controls.

Sampling of sera and intestinal washings. The animals were bled before immunization and then at the day of challenge. Sera, dispensed into 0.5-ml aliquots, were kept at −20 C and heat inactivated at 56 C for 30 min before use. Intestinal washings were prepared immediately before challenge as follows:

two to three small bowel segments, 15 to 30 cm long, were tied in each animal at different positions along the gut starting 40 cm from pylorus. Five milliliters of PBS was injected into each loop and incubated for at least 10 min. The aspirates from the different segments were pooled and a soybean trypsin inhibitor (Sigma, St. Louis, Mo.) was added to a final concentraton of 0.1 mg/ml. The washings were then centrifuged at 2,000 × *g* for 5 min to eliminate fecal contents and frozen at −70 C until use.

Protection studies. The protection studies were essentially performed as earlier described (8), using a modification of the ileal loop technique (24). Twenty-one 5-cm-long challenge loops were tied in each animal in addition to the described "wash" loops. The latter segments were usually positioned proximally, in the middle, and at the distal ends of the test loops. Each animal was challenged with graded numbers of live 569B vibrios (Inaba) and with various concentrations of *V. cholerae* culture filtrate (NIH lot no. 4493G). Five doses of bacteria, 10^5 to 10^9, as well as of culture filtrate, 0.1 to 10 mg, were tested in duplicates randomly positioned in each animal. A loop injected with 1 ml of PBS was included as a negative control.

Group 50% effective dose (ED_{50}) values for bacteria and culture filtrate were determined as described by Burrows and Musteikis (2). In addition, the ED_{50} values for bacteria and toxin in each individual animal were calculated (Individual ED_{50}).

The protective effect of immunization, referred to as the protection factor, was determined as the ratio between Group ED_{50} values of immunized and concurrently tested control (PBS-injected) animals.

Neutralization test. Toxin-neutralizing antibodies were determined by means of the intradermal assay of Craig (3).

Vibriocidal technique. Titration of vibriocidal antibodies against strain 35A3 (Inaba) was done by means of the spot agar plaque technique (13).

ELISA. Titration of primary binding anti-LPS and anti-exotoxin antibodies of immunoglobulin (Ig)G, IgM, and IgA classes was done by means of the enzyme-linked immunosorbent assay (ELISA), using LPS from strain 35A3 or choleragen as solid-phase antigens (12).

RESULTS

Protective effect of choleragen and LPS alone or in combination. The protective immunogenicity of two s.c. injections of 1.25 mg of LPS, 15 μg of choleragen, or a combination of these antigens was studied in rabbits using toxin (culture filtrate) as well as live vibrios for challenge. The immunization doses used had earlier been found to give rise to maximal serum antibody titers (14).

The immunization with LPS induced, as tested 5 days after a booster antigen injection, a moderately increased resistance to challenge with live vibrios. The Group ED_{50} value for the immunized animals was 3.2-fold that for PBS-injected control animals (Fig. 1A). No significant protection against challenge with toxin was observed after the immunization with LPS (Fig. 1B).

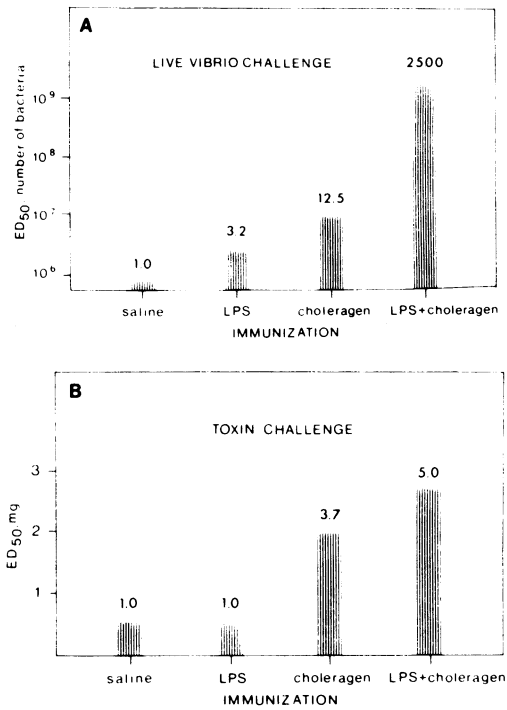

FIG. 1. *Protection by immunization against intestinal challenge with live vibrios (A) or toxin (B). Two s.c. antigen injections were given 2 weeks apart, and the protection was evaluated 5 days after the second immunization. The dose of LPS was 1.25 mg and of choleragen, 15 μg, in each injection. Group ED_{50} values are shown and, in addition, the protection factors, i.e., the ratio between the ED_{50} values of the immunized and the control (saline-injected) groups, are indicated on top of bars.*

Vaccination with choleragen, on the other hand, gave rise to effective immunity against live vibrios as well as against toxin challenge. The protection factor was 12.5 against live vibrios (Fig. 1A) and 3.7 against toxin (Fig. 1B).

Immunization with the combination of choleragen and LPS gave rise, as expected, to increased resistance against both types of challenge. The magnitude of protection against live vibrios in this group was considerably higher than the sum protection induced by each antigen alone, i.e., a synergistic effect (Fig. 1A). The protection factor against toxin challenge was slightly higher than after vaccination with choleragen alone, but comparison of the Individual ED_{50} values by Student's *t* test showed no significant difference between the groups.

Dose dependence of the choleragen compo-

nent. Various amounts of choleragen were tested in combination with a constant dose, 1.25 mg, of LPS. Both against toxin and live vibrio challenge a dose of 4 μg of choleragen in combination with LPS was similarly effective as a fourfold higher dose (Table 1). Lower amounts of choleragen did not appreciably enhance the resistance against bacterial challenge induced by immunization with LPS alone. It is notable that the bacterial but not toxin Individual ED_{50} values varied more in the groups receiving the two highest doses of choleragen than in the other groups (Table 1).

Protection by choleragenoid alone and with LPS. The protective immunogenicity of choleragenoid (15 μg), alone and in combination with LPS, was compared. A superior effect of the combined vaccine against live vibrio challenge was noted, whereas against toxin challenge there was no significant difference in the efficiency of the two types of vaccines (Table 2). Against bacterial challenge the combined vaccine induced a protection factor of 50 compared to a factor of 4 by the toxoid alone; corresponding factors against toxin challenge were 1.8 and 2.1.

Protective effect of crude toxin. The protective immunogenicity of a crude toxin preparation containing a natural mixture of LPS (approximately 10%) and choleragen (approximately 0.1%) was also investigated. This immunogen, when given in two doses of 12.5 mg, gave rise to a protection factor of 200 against live vibrios and 6.2 against challenge with toxin.

Course of protective immunity. The time course of the protective immunity was analyzed for animals given 4 μg of choleragen in combination with LPS. It was found that the protective effect against toxin as well as against live vibrio challenge was similar on days 14 and 5 after reimmunization. After 1 month, however, the protection factor had decreased more than 10-fold in comparison with the maximal protection against toxin challenge. Using 15 μg of choleragen in combination with LPS, the protective effect against live vibrio as well as against toxin challenge was slightly higher on day 14 than on day 5 after reimmunization.

Antibodies in sera and in intestinal washings. The antibody levels in sera and intestinal washings after the various immunization

TABLE 1. *Influence of the amount of choleragen on the protective immunogenicity of combined LPS toxin vaccine*

Immunization[a]	Intestinal challenge[b]			
	Live vibrios		Crude toxin	
	Group ED_{50} ($\times 10^{-6}$)	Individual ED_{50} ($\times 10^{-6}$)	Group ED_{50} (mg)	Individual ED_{50} (mg)
PBS (controls)	0.8	0.99 ± 0.26[c]	0.63	0.65 ± 0.12[c]
LPS	2.5	2.5 ± 0	0.80	0.86 ± 0.13
LPS + 0.25 μg of choleragen	20.0	18.3 ± 6.7	0.63	0.82 ± 0.16
LPS + 1 μg of choleragen	8.0	8.1 ± 1.1	1.6	1.8 ± 0.27
LPS + 4 μg of choleragen	2,500	>1,000 ± >500	5.0	6.6 ± 1.9
LPS + 15 μg of choleragen	2,000	3,270 ± 2,350	2.7	2.6 ± 0.44

[a] Two identical s.c. injections given 2 weeks apart; the dose of LPS was 1.25 mg in each injection.
[b] Small bowel loop technique, 5 days after the second immunization.
[c] Mean value and standard error of the mean.

TABLE 2. *Comparison of protective immunogenicity of choleragenoid alone and in combination with LPS*

Immunization[a]	Intestinal challenge[b]			
	Live vibrios		Crude toxin	
	Group ED_{50} ($\times 10^{-6}$)	Individual ED_{50} ($\times 10^{-6}$)	Group ED_{50} (mg)	Individual ED_{50} (mg)
PBS	0.5	1.2 ± 0.7[c]	1.1	1.2 ± 0.15[c]
Choleragenoid	2.0	2.8 ± 1.3	2.3	2.2 ± 1.1
Choleragenoid + LPS	25.0	170 ± 130	2.0	3.0 ± 1.2

[a] Two identical s.c. injections given 2 weeks apart; the dose of choleragenoid was 15 μg and of LPS, 1.25 mg, in each injection.
[b] Small bowel loop technique, 5 days after the second immunization.
[c] Mean value and standard error of mean.

schedules were analyzed. Titration of antibodies to *V. cholerae* endotoxin was done by the vibriocidal and the ELISA techniques, and antibodies to the exoenterotoxin were determined by means of the intradermal neutralization test and the ELISA.

No or very low antibody titers to these antigens were found in sera (Table 3) or in intestinal washings (Table 4) of the PBS-injected control rabbits.

In animals immunized with LPS only, specific antibodies to LPS were found in high titers in sera and in low amounts in intestinal washings. In serum as well as in intestinal washings, low or absent antibody levels were seen against cholera toxin after such immunization.

Vaccination with choleragen or choleragenoid induced high antitoxin antibody titers in sera and low levels of such antibodies in intestinal washings, whereas no significant immune response could be registered against endotoxin.

Immunization with 15 μg of choleragen or choleragenoid in combination with 1.25 mg of LPS induced serum antitoxin titers of magnitude similar to that measured in animals given the toxin antigens alone, but gave lower serum anti-LPS titers than those induced by LPS only (Table 3). Compared to the individual antigens, the LPS-choleragen combination gave rise to elevated intestinal antibody titers against LPS as well as against toxin. The combination of LPS and choleragenoid, on the other hand, did not induce such increased intestinal antibody levels (Table 4).

By varying the dose of choleragen in the combined vaccine between 0.25 and 15 μg, it was found that a dose of 1 μg in two injections was needed for a measurable antitoxin response. The antitoxin titers in serum increased with the amount of choleragen given, although the differences were not significant between doses of 4 and 15 μg. The depression of the anti-

TABLE 3. *Antibody titers to V. cholerae LPS and choleragen in serum of rabbits immunized with LPS and toxin antigens alone or in combination*

Immunization[a]	Antibody titer[b] to:					
	LPS			Choleragen		
	ELISA		Vibriocidal	ELISA		Neutralizing
	IgG	IgM		IgG	IgM	
PBS (controls)	<10	<10	<4	<10	<10	<2
LPS	770,000	3,800	3,700	<10	50	<2
Choleragen	<10	<10	<4	66,000	80	6,300
Choleragenoid	<10	<10	<4	25,000	ND[c]	ND
LPS + choleragen	4,700	2,600	800	160,000	250	6,800
LPS + choleragenoid	16,000	25,000	ND	40,000	ND	ND

[a] Two identical s.c. antigen injections 14 days apart; the injection dose of LPS was 1.25 mg and of choleragen and choleragenoid, 15 μg.
[b] Arithmetic mean titer.
[c] ND, Not done.

TABLE 4. *Antibody titers to V. cholerae LPS and choleragen in intestinal washings of rabbits immunized with LPS and toxin antigens alone or in combination*

Immunization[a]	Antibody titer[b] to:							
	LPS				Choleragen			
	ELISA			Vibriocidal	ELISA			Neutralizing
	IgG	IgA	IgM		IgG	IgA	IgM	
PBS (controls)	<1	<1	10	<4	3	6	5	<2
LPS	40	3	10	<4	<1	<1	<1	<2
Choleragen	<1	1	3	<4	180	<1	20	<2
Choleragenoid	<1	<1	<1	<4	340	4	14	<2
LPS + choleragen	430	3	80	<4	1,020	30	80	<2
LPS + choleragenoid	20	5	10	<4	160	20	8	<2

[a] Two identical s.c. antigen injections 14 days apart; the injection dose of LPS was 1.25 mg and of choleragen and choleragenoid, 15 μg.
[b] Arithmetic mean titer.

LPS serum antibody response by cholera toxin (Table 3) was restricted to the group receiving 15 μg of choleragen in combination with LPS. It was notable that even though the protection against live vibrio challenge was of the "synergistic" type in the group of rabbits immunized with a combination of LPS and 4 μg of choleragen, neither the serum nor intestinal antibody titers to LPS and choleragen were enhanced above the levels induced by the individual antigens.

DISCUSSION

Immunization with a combination of *V. cholerae* LPS and enterotoxin obviously induces a much higher degree of protection against intestinal challenge with cholera vibrios than does vaccination with either of the two antigens alone. The effect of the combined vaccine is synergistic rather than additive, the protection being more than 100-fold higher than that obtained by the single antigens.

The most likely explanation for the potentiated resistance induced by the combination of LPS and choleragen is that the antibacterial and the antitoxic immune responses, without being increased in themselves, function synergistically by interfering with two separate events in cholera pathogenesis. However, since both LPS and cholera toxin have been shown to possess immunomodulating properties (9, 17, 22), the possibility of enhanced immune responses due to mutual or unidirectional adjuvant actions of the two antigens also has to be considered. An adjuvant effect of the LPS component on the antitoxic immunity is, however, contradicted by the similar resistance to toxin challenge obtained by immunization with choleragen alone and in combination with LPS.

The effects of cholera toxin on immune responses to unrelated antigens depend on the biological activity residing in the H subunit (9). Choleragenoid, lacking this subunit, has been found to have no such immunomodulating influence (9, 17). Therefore, our observation that not only active toxin but also choleragenoid acts synergistically with LPS in inducing protective cholera immunity indicates that an immunomodulating action of the toxin component is not the major cause of the enhanced resistance. Further, the "synergistic protection" obtained by the LPS-choleragenoid as well as by the LPS-4 μg choleragen combinations was not associated with higher antibody titers, neither in serum nor in intestinal washings, than those induced by the individual antigens. However, in the group where the LPS was combined with 15 μg of choleragen, some contribution of an adjuvant effect of the toxin component cannot be entirely excluded, since in this group the intestinal antibody titers were elevated.

Provided that the described pronounced synergism between the antibacterial and the antitoxic cholera immunity in the rabbit is valid for humans, it is clear that an optimally effective cholera vaccine should contain somatic as well as enterotoxin antigens. However, much further work on the appropriate form of both of these antigens is required before an ideal combined vaccine can be prepared. With regard to the somatic antigen component it has been found that most, if not all, of the protective immunogenicity of whole-cell bacterial vaccines resides in the cell wall endotoxin (21, 25, 27). Purified bacterial LPS might therefore be sufficient to induce optimal antibacterial immunity. The development of a suitable cholera toxoid is currently under intense investigation. The limited success experienced with formalin and glutaraldehyde treatment has initiated studies on other, more specific detoxification procedures. Elimination of the H subunit (also called A subunit), which carries the biologically active site but only little of the antigenicity of the toxin (7, 10), or a selective chemical modification of the toxic site in situ by amino acid-specific reagents (19) might be a useful approach to prepare suitable toxoids.

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