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Active and Passive Immunization in the Adult Rabbit Ileal Loop Model as an Assay for Production of Antitoxin Immunity by Cholera Vaccines

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Three field trial cholera vaccines did not induce antitoxic immunity. A choleragenic toxin induced high-serum neutralizing antibody, but protection to loop challenge with toxin was minimal.

The three cholera vaccine field trials carried out in East Pakistan since 1963 have tested two different whole cell vaccines containing both Ogawa and Inaba organisms, and a purified Ogawa lipopolysaccharide (1, 8, 9). These vaccines were protective for several months and the efficacy could be related to the mouse potency and the vibriocidal antibody titers induced in the recipients (10). Although the method of vaccine manufacture was not favorable for toxin production, it was of interest to examine these field trial vaccines to see if they induced antitoxic antibodies or protected animals from toxin challenge since recent studies suggest antitoxic immunity may be significant in cholera (4, 6).

A culture filtrate containing choleragenic activity (designated toxin A4) was prepared from *Vibrio cholera* strain 569B, grown for 18 hr in a shaking water bath at 30 C. The medium was 2% peptone broth (Difco) with 1% sodium chloride initially adjusted to *p*H 7.5. This filtrate was titered for ileal loop toxin activity by the procedure of Burrows and Musteikis (3). Based on these titrations, a challenge dose of 0.005 ml was used in this study. In 109 animals, this dose gave a mean loop fluid output of 1.7 ml per cm.

Four groups of four rabbits each were immunized with weekly intravenous injections for 4 weeks. Group 1 received vaccine B, the 1963 cholera vaccine (10), also designated vaccine R or vaccine CRL in the 1964 trial (2); group 2 received vaccine X, the 1966 trial vaccine (8); group 3 received vaccine T, the 1964 trial purified Ogawa lipopolysaccharide (2); and group 4 received Toxin A4. A 0.5-ml amount of each preparation (125g μ of vaccine T) was administered in each injection. One rabbit in group 3 and two in group 4 died during immunization. One week after the last injection, animals were bled, and 7 to 10 days later ileal loops were challenged with graded doses of Toxin A4 by the method of Finkelstein (6). The sera were titered for ileal loop toxin neutralizing antibodies by the method of Kasai and Burrows (7). The procedure was modified in that the endpoint was defined as the highest dilution of serum giving complete neutralization of the 0.005-ml toxin challenge. Vibriocidal titers were determined according to the microtechnique (1).

The results in Table 1 indicate that the three cholera vaccines did not induce detectable toxin-neutralizing antibodies; however, a good response was seen in the animals receiving Toxin A4. All vaccines induced vibriocidal antibody titers. The animals immunized with the cholera vaccines did not show significant protection against a toxin challenge into the ileal loops (Table 2). The animals immunized with Toxin A4 were protected against the minimal toxin dose (0.005 ml), but when this dose was increased four times the loops were positive. Based on the serum antitoxin titers of the animals receiving Toxin A4, less than 0.03 ml of sera would be required to neutralize 0.02 ml of Toxin A4. The failure of the immunized animal to be protected against this challenge dose suggests that, for the most part, the intravascular antibody was not available for toxin neutralization.

The in vivo neutralization of toxin was further studied in ileal loops of unimmunized rabbits by injecting the loops sequentially with 0.01 ml of Toxin A4 and a 30-fold excess of antitoxin. When antitoxin was introduced into the loop first and toxin was added 3 to 5 min later, the toxin was completely neutralized. By contrast, when toxin was introduced into the loop first and an excess of antitoxin was added after 5 min,

TABLE 1. *Serological responses of rabbits 7 days following immunization with 4 weekly intravenous injections of cholera vaccines or toxin A4*

Rabbit no.	Immunization	Toxin neutralizing titer[a] (Reciprocal)		Vibriocidal Titers[b]			
				Ogawa		Inaba	
		Preimmunization	Postimmunization	Preimmunization	Postimmunization	Preimmunization	Postimmunization
1	Vaccine B	5	5	<10	5,120	<10	5,120
2		<5	<5	<10	5,120	<10	5,120
3		5	<5	<10	1,280	<10	2,560
4		<5	<5	<10	5,120	<10	>20,480
5	Vaccine X	<5	<5	<10	640	<10	1,280
6		<5	<5	<10	1,280	<10	1,280
7		<5	<5	<10	1,280	<10	1,280
8		<5	<5	<10	10,240	<10	5,120
9	Vaccine T	<5	<5	40	1,280	160	160
10		<5	<5	<10	1,280	<10	640
11		<5	<5	<10	1,280	<10	<10
12	Toxin A4	5	316	320	160	320	320
13		<5	316	<10	20,480	<10	>20,480

[a] Highest dilution of sera (in a 2-ml volume) that can completely neutralize 0.005 ml of Toxin A4. Reciprocal titer.
[b] Reciprocal titers.

TABLE 2. *Fluid response to ileal loop challenge of immunized rabbits with graded doses of toxin A4*

Rabbit no.	Immunization	Challenge[a]			
		Buffer[b]	Toxin A4		
			0.005 ml[b]	0.02 ml[b]	0.10 ml[b]
1	Vaccine B	0	1.3	1.6	1.3
2	Vaccine B	0	1.2	1.6	1.8
3	Vaccine B	0	0.7	1.9	N.T.[c]
4	Vaccine B	—[d]	—[c]	—[c]	—[c]
5	Vaccine X	0	1.8	2.2	—[e]
6	Vaccine X	0	0.6	1.3	1.6
7	Vaccine X	0	1.8	1.7	N.T.
8	Vaccine X	0	1.6	2.1	N.T.
9	Vaccine T	0	1.7	3.2	3.1
10	Vaccine T	0	1.1	1.4	1.6
11	Vaccine T	0	1.7	2.2	N.T.
12	Toxin A4	0	0	2.5	2.3
13	Toxin A4	0	0	2.0	2.9

[a] Challenge dose given in a total volume of 2 ml.
[b] Ratio of volume of fluid in loop to length of loop (ml/cm).
[c] N.T., not tested.
[d] Fluid in all loops; animal died many hours before autopsy.
[e] Distended loop burst.

there was no neutralization. These results indicate that the choleragenic toxin is rapidly bound or absorbed to the active site, probably the intestinal epithelial cell, and that after binding or absorption, a high level of antitoxin in the intestinal lumen, or intravascularly in the case of actively immunized animals, will have no effect on the subsequent fluid production. The observations in the adult rabbit ileal loop model, suggesting that effective neutralization of toxin in vivo must occur in the intestinal lumen before the toxin reaches its active site, parallel the findings of Finkelstein in the infant rabbit model (5).

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LITERATURE CITED

1. Benenson, A. S., W. H. Mosley, M. Fahimuddin, and R. O. Oseasohn. 1968. Cholera vaccine field trials in East Pakistan 2. Effectiveness in the field. Bull. World Health Organ. 38:359–372.
2. Benenson, A. S., A. Saad, and W. H. Mosley. 1968. Serological studies in cholera 2. The vibriocidal antibody response of cholera patients determined by a microtechnique. Bull. World Health Organ. 38:277–285.
3. Burrows, W., and G. M. Musteikis. 1966. Cholera infection and toxin in the rabbit ileal loop. J. Infec. Dis. 116:183–190.
4. Curlin, G. T., A. Subong, J. P. Craig, and C. C. J. Carpenter. 1968. Antitoxic Immunity in Experimental Canine Cholera. Trans. Ass. Amer. Physicians Philadelphia 81:314–320.

5. Finkelstein, R. A. 1965. Immunological aspects of experimental cholera. Proc. Cholera Res. Symp., Hawaii. U.S. Govt. Print. Off., p. 58–63.
6. Finkelstein, R. A., and P. Atthasampunna. 1967. Immunity against experimental cholera. Proc. Soc. Exp. Med. Biol. 125:465–468.
7. Kasai, G. J., and W. Burrows. 1966. The titration of cholera toxin and antitoxin in the rabbit ileal loop. J. Infec. Dis. 116:606–614.
8. Mosley, W. H., W. M. McCormack, M. Fahimuddin, K. M. A. Aziz, A. S. M. M. Chowdhury, A. R. Martin, J. C. Feeley, and R. A. Phillips. 1969. Report of the 1966–67 Cholera Vaccine Field Trial in Rural East Pakistan 1. Study design and results of the first year of observation. Bull. Wld Hlth Org. 40:177–186.
9. Oseasohn, R. O., A. S. Benenson, and M. Fahimuddin. 1965. Field trial of cholera vaccine in rural East Pakistan: first year of observation. Lancet 1:450–452.
10. Mosley, W. H. 1969. The role of immunity in cholera. A review of epidemiological and serological studies. Tex. Rep. Biol. Med. 27 (Suppl. 1):227–241.