INFECTION AND IMMUNITY, May 1970, p. 464-467
Vol. 1, No. 5

Printed in U.S.A.

Antitoxic Immunity in Experimental Cholera: Observations with Purified Antigens and the Ligated Ileal Loop Model

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Received for publication 4 February 1970

Rabbits immunized with purified antigens, choleragen and choleragenoid (cholera exo-enterotoxin and natural toxoid), were found to resist intra-intestinal challenge with a variety of strains of cholera vibrios. The resistance occurred in the absence of a significant antibacterial antibody response. In fact, the antigens were incapable of protecting mice against the septicemic infection of the standard mouse protection test used for bioassay of conventional cholera vaccines. The immunized rabbits did, however, develop a significant antitoxin titer demonstrable by passive hemagglutination, although the presence of antitoxic coproantibody could not be established conclusively. The results suggest the potential value of antitoxic immunity in cholera and are consistent with the existence of only one serological type of cholera exo-enterotoxin.

It is now well established that the pathogenesis of the diarrhea of cholera is dependent on an exo-enterotoxin elaborated by cholera vibrios. Cell-free materials have reproduced the symptomatology in a variety of experimental models (1, 2, 4, 6, 7, 14, 17, 19) including man (3). Laboratory studies have also demonstrated that it is possible to produce immunity to enteric challenge with either living vibrios or toxin by prior parenteral administration of materials containing the toxic protein antigen (5, 11, 18). However, in those studies, the toxin antigen preparations employed were impure. Thus, although protection was obtained against toxin, on the one hand, and against vibrios, on the other, it was impossible to attribute the immunity against vibrio challenge solely to the antitoxic component, especially since it was shown (11) that even the partially purified preparation used also induced the formation of high levels of antivibrio antibodies. In addition, only a single, highly toxigenic, strain was used as a challenge and no evidence was presented that antitoxic immunization would protect against other strains.

We have since been able to purify the exo-enterotoxin, designated choleragen, as well as a natural toxoid, designated choleragenoid (12; R. A. Finkelstein and J. J. LoSpalluto, J. Infec. Dis., *in press*). The purpose of the present communication is to present evidence that immunization with these isolated substances renders rabbits resistant to enteric challenge with a variety of strains of living vibrios, some of which have failed to produce detectable exo-enterotoxin in vitro in our experiments. This protection was obtained in the absence of a significant antibacterial antibody response.

MATERIAL AND METHODS

Purified choleragen and choleragenoid were prepared as described elsewhere (R. A. Finkelstein and J. J. LoSpalluto, J. Infec. Dis., *in press*). Their concentrations were determined and adjusted according to their optical density readings at 280 nm in a Beckman DU spectrophotometer employing an extinction coefficient (1% solution) of 10.0. Rabbits were immunized as described previously (11) with the purified antigens, with or without Freund's complete adjuvant (Difco). Immunized rabbits and controls were challenged, intra-intestinally in ligated ileal loops, with either 10^6 living agar-grown cholera vibrios or choleragen, by a previously described technique (11). An ileal loop, dose-response curve of a less pure and less active preparation of choleragen was published recently (10). The bacterial strains used were selected to represent the diverse kinds of cholera vibrios. Strains SP35 and 26-3 are Inaba and Ogawa serotype El Tor vibrios, respectively, isolated by the author in Thailand and the Philippines and lyophilized shortly after isolation. Strain 26-3 is hemolytic and SP35 is not, but both are hemagglutinative (13). Strains NIH41 and NIH35 are classical cholera vibrios of

Ogawa and Inaba serotypes, respectively, and are the widely used U.S. vaccine strains. Strain 569 B, Inaba, is the highly toxigenic strain we, and others, have used for the preparation of toxin. The other strains have failed to elaborate detectable amounts of choleragen in vitro (16) or in vivo (10). Vibriocidal antibody titrations were performed as described previously (9). The serum antitoxin titers of some of the experimental rabbits were determined by a recently described passive hemagglutination microtest employing sensitized tanned chicken erythrocytes (15). Choleragen and choleragenoid were also tested for their ability to elicit immunity against the septicemic infection of the standard cholera vaccine mouse potency assay (8). The latter tests were performed in the laboratory of W. F. Verwey, The University of Texas Medical Branch, Galveston, to whom we are indebted. Some attempts were made to demonstrate antibody in the intestinal lumen of immunized rabbits. For this purpose, an additional ileal loop, in each animal to be tested, was inoculated with 10 ml of 12% mannitol solution. The fluid, removed from the osmotically dilated loops at the time of sacrifice, was immediately centrifuged under refrigeration and the supernatant fluid was frozen. The fluids were titrated in the hemagglutination test (15).

RESULTS

The results of challenges with living vibrios and choleragen in control and immunized rabbits are summarized in the Table 1. In control animals, only one of the loops failed to respond with outpouring of fluid. In contrast, in the immunized animals, 16 of the 35 vibrio-challenged loops failed to respond with fluid outpouring and the remainder generally responded to a lesser extent than the controls. Culture of the intestinal mucosa by streaking on meat extract agar in each instance yielded virtually pure and confluent growth of the inoculated strain, indicating multiplication of the challenge dose. The contrast between control and immunized loops challenged with choleragen was not as marked as that observed with the vibrio

TABLE 1. *Effect of immunization with choleragenoid or choleragen on response to intestinal challenge with cholera vibrios or choleragen*

Experimental group	Rabbit no.	Challenge[a]					Choleragen
		SP35	569B	NIH41	NIH35	26-3	
Control	69	0.00[b]	1.86	1.70	1.21	1.20	1.50
	70	0.70	0.10	0.42	1.00	0.75	1.74
	71	1.36	2.08	1.36	1.29	1.57	2.50
	72	1.21	2.00	1.10	1.41	1.45	2.59
	73	0.86	1.50	1.53	1.76	1.78	2.78
	74	0.27	0.63	1.22	1.47	1.06	1.80
Mean		0.73	1.36	1.22	1.36	1.30	2.15
Range		0.00–1.36	0.10–2.08	0.42–1.70	1.00–1.76	0.75–1.78	1.50–2.78
Mean of means				1.19			
Range of means				0.73–1.36			
Immunized[c]	33	0.00	0.00	0.00	0.00	0.00	2.13
	36	0.20	0.22	0.10	0.77	0.17	0.09
	60	0.27	1.50	0.64	0.54	1.33	2.13
	61	1.75	2.09	0.20	0.10	0.79	1.18
	62	0.00	0.00	0.00	0.00	0.00	2.27
	63	0.00	0.00	0.00	0.00	0.15	2.15
	68	0.06	0.00	0.00	0.00	0.07	0.09
Mean		0.33	0.54	0.13	0.20	0.36	1.43
Range		0.00–1.75	0.00–2.09	0.00–0.64	0.00–0.77	0.00–1.33	0.09–2.27
Mean of means				0.31			
Range of means				0.13–0.54			

[a] Challenge: approximately 10^6 viable cells of indicated strains or 2 μg of choleragen (5 μg in rabbits 69, 70, and 33).

[b] Fluid volume (ml)/loop length (cm).

[c] Rabbits 33 and 36 received 2 mg of choleragenoid in Freund's complete adjuvant 9 months before challenge. Rabbit 36 received a "booster dose" of 2 mg, 6 weeks before challenge. Rabbits 60 to 63 received 2 mg of choleragenoid without adjuvant (subcutaneously and intramuscularly) 6 weeks before challenge. Rabbit 68 received 1.3 mg of choleragen without adjuvant (subcutaneously and intramuscularly) 6 weeks before challenge.

challenges, although it can be seen that two immunized animals, 36 and 68, responded only minimally. However, the mean fluid volumes did differ substantially between the groups. It should be mentioned that this dose of choleragen represented a rather massive challenge. Culture of the choleragen loops yielded only minimal normal flora.

Vibriocidal assays of the serum from immunized animals revealed only transient fluctuations in titer, of one or two tubes, during the course of immunization. In no instance did the titer reach 10^{-3}, a level which could hardly be considered significant in the highly sensitive test used (9). Similarly, the preparations were found to be completely ineffective in protecting mice against intraperitoneal challenge with living vibrios in mucin (W. F. Verwey, *personal communication*), a test in which immunity is dependent on the lipopolysaccharide somatic antigen. The preparations likewise did not elicit an agglutinating antibody response in rabbits (J. C. Feeley, *personal communication*).

The immunized rabbits of the present study did, however, develop high titers of circulating hemagglutinating antibody against chicken erythrocytes sensitized with choleragen and choleragenoid as summarized elsewhere (15). HA titers ranged from 1:640 to 1:10,240 at the time of sacrifice of the immunized animals. Sera from control rabbits were unreactive at 1:20. The results of HA titrations of loop fluids were somewhat equivocal. Although none of three loop fluids from control rabbits gave any evidence of reactivity at 1:10 dilution, three of five fluids from immunized rabbits gave altered patterns of the sensitized erythrocytes at the same initial dilution but not at higher dilutions.

DISCUSSION

In the present study, small numbers of rabbits were immunized with purified choleragen or choleragenoid, our designations for cholera exo-enterotoxin and natural toxid, respectively. Rigid criteria were employed to insure the purity of these materials (12; R. A. Finkelstein and J. J. LoSpalluto, J. Infec. Dis., *in press*). The immunized rabbits were found to resist intraintestinal challenge with diverse strains of cholera vibrios and, to a lesser extent, rather large doses of purified enterotoxin. The vibrio strains selected for challenge included some which had previously failed to elaborate serologically detectable amounts of choleragen in vitro or in vivo (10, 16). The immunized animals failed to demonstrate a significant vibriocidal antibody response (9). They did, however, develop high levels of circulating antitoxin demonstrable in a passive hemagglutination test employing chicken erythrocytes sensitized with the purified antigens (15). The same purified antigens were found to be completely ineffective in protecting mice against the septicemic infection induced by intraperitoneal inoculation of vibrios in mucin, a system in which minute amounts of (lipopolysaccharide) somatic antigen induce a solid immunity.

These observations are compatible with the following conclusions. (i) The preparations used were, for practical purposes, free from somatic antigens of cholera vibrios. (ii) Since there was no demonstrable antibacterial response and the inoculated strains multiplied in the intestinal loops, the protection observed most likely should be attributed to antitoxic immunity. (iii) Since protection was obtained, under these circumstances, against strains which had previously failed to elaborate detectable amounts of choleragen, it is likely that the techniques employed previously were not sufficiently sensitive to detect the small amounts of toxin, elaborated by the vibrios growing in the loops, which were, however, sufficient to elicit fluid outpouring in control animals. (iv) By the same token, since the immunity elicited by inoculation of these purified antigens protected against challenge with vibrio strains selected to represent all of the known categories of cholera vibrios, it is likely that only a single antigenic type of toxin is involved in the production of the diarrhea of cholera. The results are compatible with the conclusion that choleragen is that toxin. (v) The natural toxid, choleragenoid, antigenically identical with choleragen but smaller, less highly charged, and nontoxic (12; R. A. Finkelstein and J. J. LoSpalluto, J. Infec. Dis., *in press*), also produces an effective antitoxic immunity in the rabbit. (vi) The results suggest the feasibility of producing an antitoxic immunity effective against cholera in man. (vii) The question of whether antibody must be present in the lumen of the gut or whether circulating antitoxic antibody is protective remains unresolved. It is doubtful whether the altered hemagglutination pattern observed with low dilutions of ileal fluids from immunized rabbits truly represents coproantibody. It is possible that it represents trace amounts of serum antibody introduced in obtaining the specimen; it could be completely nonspecific and dependent on the nature of the specimen, or it is also possible that coproantibody which was present was inactivated during collection, processing, and storage. Additional studies are needed to resolve this question, al-

though recent evidence (G. T. Curlin and C. C. J. Carpenter, J. Infec. Dis., *in press*) strongly suggests that circulating antitoxic antibody is indeed protective.

ACKNOWLEDGMENTS

I appreciate the efficient technical assistance of Russell C. Hollingsworth, Johnny W. Peterson, and Beverly Brock during portions of this work.

This investigation was supported by a Public Health Service research grant (AI-08877) under the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases.

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