Advances in Research on Cholera and Related Diarrheas

edited by
S. Kuwahara and N.F. Pierce

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ADVANCES IN RESEARCH ON CHOLERA AND RELATED DIARRHEAS

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PREFACE

The United States–Japan Cooperative Medical Science Program was initiated in 1965 by joint agreement between the President of the United States and the Prime Minister of Japan. The purpose of the Program was to promote cooperative biomedical research between the two countries, especially on health problems of recognized importance in Asia. Cholera was designated as one topic of mutual interest. Panels of scientists from each country were formed, and these met to select priority areas for research. The Cholera Panels initially defined two major goals: 1) improved and simplified therapy for cholera, and 2) better methods for immunization. Progress in the pursuit of these goals led to the recognition that bacteria other than *Vibrio cholerae* are also important causes of acute dehydrating diarrhea which resembles cholera in its manifestations and pathogenesis; most notable among these are enterotoxinogenic strains of *Escherichia coli.* Accordingly, panel guidelines were expanded to include all diarrheal diseases that involve fluid loss caused by an enterotoxin. More recently, studies have shown that vibrios, including *V. cholerae*, have a distinct environmental life cycle that is probably an important factor in the epidemiology of vibrio infections. For this reason, the panel guidelines were again expanded to include studies on the environmental ecology of vibrios.

A major project of the Joint Cholera Panels has been the organization and sponsorship of an annual conference on cholera and related diarrheal diseases. These meetings, held alternatively in Japan and the United States, have provided an unique forum for the presentation of new research on topics falling within the guidelines of the Panels. The very rapid increase in both basic and practical knowledge concerning these diseases, and the fact that this is the only regularly held international meeting on this subject, have added considerably to the value and importance of these conferences.

This volume, the first in a planned series entitled "Advances in Research on Cholera and Related Diarrheas", contains papers presented at the Seventeenth Joint Conference on Cholera, held in Baltimore, Maryland, October 26-28, 1981. The proceedings of the previous 16 conferences, beginning with the first, held in Hawaii in 1965, have also been published by either the United States or Japanese Cholera Panels. It is sincerely hoped that publication in this new format will make these proceedings available to a larger number of researchers, public health officials, and clinicians who are concerned with cholera and related acute diarrheal diseases.

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Chairman, Japanese Cholera Panel

Nathaniel F. Pierce, M. D.
Chairman, United States Cholera Panel

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IMMUNOLOGY AND VACCINE DEVELOPMENT

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ROLE OF COLONIZATION FACTOR ANTIGEN IN IMMUNOPROTECTION AGAINST ENTEROTOXIGENIC *ESCHERICHIA COLI* DIARRHEA

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Colonization factor antigens CFA/I and CFA/II of human-associated enterotoxigenic *E. coli* (ETEC) are good candidates as immunoprotective antigens because of their primary role as ETEC virulence factors which involves specific interaction with the intestinal epithelium. We used the temporary intestinal ligation technique with adult rabbits to investigate the local and systemic immune responses to purified CFA/I and to CFA/I$^+$ ETEC (strain H-10407; 078:Hll:CFA/I; ST$^+$ LT$^+$). Local immune responses were determined by quantitating the number of IgG–, IgM–, –IgA and anti-CFA/I–producing cells in the lamina propria of the test animals; immunoprotection was determined by challenging the antigen-primed animals or rechallenging rabbits which were allowed to survive an initial challenge with the same dose of strain H-10407 (1×10^8 CFU per animal) given 5 to 6 weeks previously (1).

Rabbits (seven; Group II) re-challenged with strain H-10407 showed some protection (4 of 7, or 57%) from diarrhea with significantly ($P<0.0001$) less fluid response than a control group of ten rabbits (Group I) but no protection against colonization of the intestine (1); Table 1. However, rabbits previously exposed to two oral doses of purified CFA/I (1.0 mg antigen per dose for one group of nine animals (Group III) and 5.0 mg antigen per dose for another group of seven animals, Group IV) showed protection against both diarrhea (77% and 100%, respectively) and colonization of the intestine. None of the animals immunized with 5.0 mg doses of CFA/I showed a diarrhea response (Group IV) although a group of five animals similarly immunized with CFA/I (Group V) showed no protection against a challenge dose of a CAF/II-positive ETEC strain (06:H1-6:CFA/II; ST$^+$ LT$^+$), as seen in Table 1.

Comparison of the local and systemic immune response to the purified antigen and to the CFA/I-positive bacteria clearly indicate that the cell-free antigen favored the local response whereas the bacteria favored a systemic response; for example, only the bacteria stimulated significant anti-CFA/I serum titers (data not shown). Protection did not correlate with serum titers (either anti-LT, anti-078 or anti-CFA/I) but did correlate with the local immune response. The oral doses of CFA/I produced a relatively poor response in terms of IgG and IgM-producing cells but did produce a vigorous response in terms of IgA- and anti-CFA/I-producing cells in the lamina propria of the animals (Tables 2 and 3).

Table 1. Diarrhea response of rabbits in five different experimental groups.

Rabbit Group*	Number tested	Fluid accumulation in ml (mean ± standard error)
I	10	65.10 ± 12.05
II	7	16.42 ± 7.22
III	9	13.66 ± 5.24
IV	7	1.85 ± 1.85
V	5	51.60 ± 16.79

*Rabbit groups described in text.

Table 2. Mucosal antibody responses of rabbits immunized perorally with CFA/I or by exposure to CFA/I-positive bacteria.

Rabbit group	Number tested	Number of antibody-producing cells per $mm^3 \times 10^3$		
		IgA	IgM	IgG
I	5	2.472 ± 0.248	1.232 ± 0.160	1.679 ± 0.122
II	7	3.832 ± 0.160	2.146 ± 0.601	4.126 ± 1.348
III	9	7.251 ± 1.240	3.808 ± 0.541	4.098 ± 0.761
IV	7	10.844 ± 2.025	3.924 ± 0.925	4.309 ± 0.828
V	5	11.661 ± 2.523	3.886 ± 0.257	4.987 ± 1.193

Table 3. Mucosal anti-CFA/I response of rabbits immunized perorally with CFA/I or by exposure to CFA/I-positive bacteria.

Rabbit group	Number tested	Number of anti-CFA/I-producing cells per $mm^3 \times 10^3$ (Mean ± standard deviation)
I	10	0.0
II	7	4.685 ± 4.669
III	9	8.403 ± 3.724
IV	5	8.563 ± 2.332
V	5	12.783 ± 5.652

There was a highly significant inverse relationship between the numbers of IgA- and anti-CFA/I-producing cells in the lamina propria of the animals and the diarrhea response to strain H-10407; with correlation coefficients of -0.616 and -0.678, respectively. These results support the hypothesis that oral immunization with purified CFA/I should be immunoprotective in humans.

This work was supported in part by Public Health Service grants AI-13385 (DGE) and AI-15379 (DJEJr) from the National Institute of Allergy and Infectious Diseases, NIH.

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THE ILEAL LOOP TEST ON MICE WHICH WERE ORALLY IMMUNIZED WITH IF30

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We have reported that the oral administration of fraction IF30 of *Vibrio cholerae eltor* Inaba V86 strain protects mice from the intraperitoneal challenge with V86 strain (1, 2, 3). This time we will report the result of the loop test on orally immunized mice and rabbits.

MATERIALS AND METHODS

1. Materials
   a. Animals: Six-week-old closed colony, ddY and ICR strains, of female mice and 2-month-old New Zealand white male rabbits were used.
   b. Antigen: IF30 was used as the antigen. IF30 was made as follows (4, 5, 6): *Vibrio cholerae eltor* Inaba V86 strain was grown on heart infusion agar (Difco), pH 8.0, at 37 C for 18 to 20 hours, harvested in sterile PBS of 1/75 M, pH 7.2, and the same volume of 5 M urea solution was added. The material was sonicated at 20 KC for 20 minutes, and centrifuged to obtain the supernatant. The supernatant was dialyzed against distilled water and centrifuged. Ammonium sulfate was added to the supernatant and the precipitate formed at 30% saturation was obtained. The precipitate was resuspended in PBS of 1/75 M, dialyzed against PB of 0.005 M, pH 7.6, and lyophilized.
   c. Challenging organisms: *V. cholerae eltor* V86 strain was grown on heart infusion agar (pH 8.0) at 37 C for 18 to 20 hours, harvested in sterile saline and suspended in heart infusion broth at desired concentrations.

2. Methods
   a. Oral administration: In case of mice, 1 mg to 10 mg of IF30 was dissolved in 1 ml of sterile distilled water and administered by gastric tubes once a day for three successive days. In case of rabbits, 100 mg or 300 mg of IF30 was dissolved in 50 ml of sterile distilled water and administered by silicon tubes once a day for three successive days.
   b. Immunization by means of drinking water: IF30 was dissolved in sterile distilled water at concentrations of 1 mg, 5 mg and 10 mg/100 ml and was given freely through drinking bottles for one or three weeks.

c. Loop test: In case of mice, 7 to 10 days after the last administration of the antigen, one loop per mouse was made and 0.2 ml of a suspension of V86 which contained 10 to 10^4 organisms was inoculated. Seventeen to 20 hours later mice were sacrificed and swelling was macroscopically observed and then the loops were cut out to measure the length and volume and to calculate the ratio of the volume to length. In case of rabbits, five loops per rabbit were made after 15 days of the last administration of the antigen and 0.5 ml each of V86 suspension which contained 10^2, 10^4 and 10^6 organisms was inoculated in 3 loops, one loop was inoculated with heart infusion broth, and the last one was left as the uninoculated control. Twenty hours later the rabbits were sacrificed and swelling of the loops were observed, for measurement of the volume of fluid in the loops and the length of the loops as well as the ratios of the volume to length.

RESULTS

1. Loop tests in orally immunized ddY strain of mice: As is shown in Table 1, in groups immunized with 10 mg of antigen for 3 successive days and challenged with 1.2 x 10^2 to 4 x 10^2 organisms 44.4% became positive while in those similarly immunized and challenged with 8 to 56 organisms 30% became positive, and these rates were much lower than in the control groups whose rates were 63.6% and 65%, respectively.

2. Loop tests in orally immunized ICR strain of mice: As is shown in Table 2, when challenged with 10^3 organisms no difference was observed between any of the immunized groups and the control group, but the positive rates were much lower than control in groups immunized with 5 mg and 10 mg and challenged with 25 to 100 organisms. The difference was significant with the X^2 test at the 5% level in the 10 mg immunization group.

In the above two experiments, it was shown that when mice were immunized orally with 10 mg of antigen for 3 successive days and challenged with a small number of the organisms the loops were protected from swelling regardless of the mouse strain.

Table 1. Loop tests in orally immunized ddY strain of mice.

Dose per day (Total dose) mg. D.W.	Challenge dose (1.2 ~ 4.2) x 10^2			Challenge dose 8 ~ 56		
	No. of loops examined	(Positive positive	rate,%)	No. of loops examined	(Positive positive	rate,%)
10 (30)	9	4	(44.4)	10	3	(30.0)
5 (15)	6	6	(100)	10	7	(70.0)
2 (6)				10	5	(50.0)
1 (3)	8	6	(75.0)	10	3	(30.0)
Control	11	7	(63.6)	20	13	(65.0)

Table 2. Loop tests in orally immunized ICR strain of mice.

Dose per day (Total dose) mg. D.W.	Challenge dose (1.5 ~ 1.7) x 10^3 No. of loops examined	positive	(Positive rate,%)	Challenge dose 25 ~ 100 No. of loops examined	positive	(Positive rate,%)
10 (30)	7	6	(85.7)	10	3	(30.0)※
5 (15)	6	3	(50.0)	5	2	(40.0)
2 (6)				5	5	(100)
1 (3)	6	5	(83.3)	5	3	(60.0)
Control	9	7	(77.8)	23	16	(69.6) ※

※ : The difference was significant with the X^2 test at the 5 % level.

3. Loop tests in orally immunized rabbits: As is seen in Table 3, when rabbits were orally immunized with one dose of 300 mg, the rates of positive loops were 83.3%, 66.7% and 33.3% after challenge with 10^6, 10^4 and 10^2 organisms, respectively. This result indicates that the rate of positive loops decreased with the decrease of the challenging dose. The v/l rate was also decreased even at the positive loop in the immunized groups. When the immunizing dose was lowered the rate of protection was decreased.

4. Loop tests in ddY strain of mice which were orally immunized by means of drinking water: As is seen in Table 4, in the group immunized with 5 mg/100 ml of the antigen for 3 weeks and challenged with 10^3 to 10^4 organisms or 6 to 20 organisms the rates of positive loops were 25% and 44.4%, respectively, which were lower than in the control groups, but the number of mice used was small, therefore, it will be necessary to add more data before drawing conclusion.

Table 3. Loop tests in orally immunized rabbits.

Dose per day (Total dose) mg. D.W.	Challenge dose (0.8 ~ 1) x 10^6 No.of loops ※1	※2	(Positive rate,%)	Challenge dose (0.8 ~ 1) x 10^4 No.of loops ※1	※2	(Positive rate,%)	Challenge dose (0.9 ~ 1) x 10^2 No.of loops ※1	※2	(Positive rate,%)
300 (900)	6	5	(83.3) [1.52] ※3	6	4	(66.7) [0.90]	6	2	(33.3) [0.74]
100 (300)	6	6	(100) [1.74]	6	6	(100) [1.54]	6	6	(100) [1.15]
Control	7	7	(100) [1.73]	7	7	(100) [1.25]	7	6	(85.7) [1.13]

※1 : No. of loops examined ; ※2 : No. of positive loops.

※3 : Numbers in [ ] are average ratio of volume per length of the loop examined.