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Experimental Oral Immunization with L-Forms of *Vibrio cholerae*

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It has been found that oral administration of lysates of L-forms of *Vibrio cholerae* are antigenic and capable of eliciting an early and high coproantibody response. The L-forms of *V. cholerae* did not produce any booster effect. Coproantibody rise persists to significant levels even six weeks after oral immunization. L-form lysates of *V. cholerae* also produce a considerable rise of circulating antibodies and these persist in significantly high titers up to six weeks.

In an earlier communication (1) we studied the antigenicity of mainly intracellular contents released from L-forms of *Vibrio cholerae* and reported that disrupted L-forms of *V. cholerae* are antigenic after parenteral injection in rabbits. A marked early rise of serum agglutinin and vibriocidin titers was observed. This was followed by an abrupt fall in agglutinin level and a stepwise fall in vibriocidal antibody level. The latter was maintained at a higher threshold for several weeks. We have now investigated local antibody production in gastrointestinal tract (coproantibody) and circulating antibody in blood after oral feeding with L-forms of *V. cholerae* and their lysates. The schedule adopted simulates the heavy immunization program advocated by Freter and Gangrosa (9) for killed oral cholera vaccine.

MATERIALS AND METHODS

Animals. Six healthy male rabbits bred in our animal house and weighing 1.5 kg were employed for immunization.

Production of L-forms of V. cholerae and their lysates. L-forms of *V. cholerae* and their lysates were prepared as described earlier (1). Briefly, *V. cholerae* Ogawa strains S/162/58 and *V. cholera* Inaba B/53/28 were grown in L-form medium containing 2,000 units of penicillin per ml. Sucrose (10%) was also added. The L-forms of these strains had been maintained and passaged weekly in our laboratory for the last 2 yr. Three days growth was scraped and suspended in 0.85% NaCL. Initially, a standard curve correlating dry weight of L-forms of *V. cholerae* and direct turbidity was obtained using a Bausch & Lomb "Spectronic 20" spectrophotometer. L-form suspensions of requisite turbidity or dry weight were disrupted to form a lysate by addition of a few drops of acetone. Lysis of L-forms was confirmed under a Reichert's phase-contrast microscope.

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Immunization procedures. Rabbits were kept fasting for 24 hr prior to immunization. Atropine sulfate, 0.5 ml (0.01 g/ml of solution), was injected subcutaneously in the thigh and the animal was anesthetized with ether. A sterile Ryle's tube was passed into the stomach. Its exact position was ascertained by knowing the distance the Ryle's tube was introduced. A little gastric fluid was withdrawn in a glass syringe and about 10 ml of 5% $NaHCO_3$ solution was injected into the stomach to neutralize gastric acidity. This was followed by 10 ml of L-form lysate through the same tube. The rabbits were kept without food for 2 hr. Initially lysed L-form suspensions [2.6 mg (dry weight)/ml] were fed on five occasions biweekly and later weekly. This immunization program was adopted in view of the extremely variable and intense immunizatcn program followed by some earlier workers for oral immunization.

Collection and processing of stool and blood samples. Metabolic cages were cleaned thoroughly an evening before, and the next morning stool samples were collected and processed further. Precaution was taken to avoid admixture with urine by keeping a piece of fine wire net on a inclined plane inside and the rabbit was made to sit on this inclined plane. Approximately 1 g of stool was mixed with five times (w/v) phosphate-buffered saline, *p*H 8.6 (PBS-8.6). PBS also contained 1 in 10,000 Thiomersal. The stool suspension was thoroughly mixed and homogenized. It was centrifuged at 4 C in a refrigerated centrifuge at 12,000 rev/min for 15 min and the supernatant fluid was collected. Turbid and colored supernatant fluids were clarified by adding activated charcoal, recentrifuging, and collecting the clear supernatant fluid. It was kept at 56 C for 30 min to inactivate any proteolytic enzymes present. Blood samples, 5 ml, were collected in a sterile test tube a day or two after oral feeds. Sera were separated and kept at −20 C.

Determination of coproantibodies in feces and circulating antibodies in blood. Indirect hemagglutination test using tanned red blood cells from sheep, sensitized with ultrasonically disintegrated *V. cholerae* Ogawa or

Inaba strains were employed. A modification of the technique of Kessel et al. (11) was used as follows.

Materials for indirect hemagglutination test included (i) four diluents: physiological saline; phosphate buffer, *p*H 6.4 (PB-6.4); phosphate-buffered saline, *p*H 7.2 (PBS-7.2); and PBS-7.2 containing 1% rabbit serum. The rabbit serum was inactivated for 30 min at 56 C and stored at −20 C. This was labeled as PBS-NRS. (ii) Sheep blood was obtained from external jugular vein. It was mixed with Alsever's solution in a ratio of 1:2:1 (v/v). (iii) Stock solution of tannic acid was made fresh before use in a concentration of 1:1,000 by dissolving 100 mg of tannic acid in 100 ml of distilled water.

For preparation of stool and blood sample dilutions, doubling dilutions of stool supernatant fluids were made in PBS-7.2. The dilutions ranged from 1:4 to 1:1024. Similarly, doubling dilutions of serum, ranging from 1:4 to 1:4,096 were made.

For preparation of sensitized tanned sheep red blood cells, sheep erythrocytes were washed three times with PBS-7.2 and packed by centrifuging at 3,000 rev/min for 30 min. A 2.5% suspension of these red cells was made in PBS-7.2. An equal volume of 1:20,000 tannic acid solution was added (final concentration of tannic acid 1:40,000). The mixture was kept at 0 to 4 C for 15 min. It was shaken intermittently to keep cells in suspension. Tanned red cell suspension (3.75 ml) was centrifuged, washed with PBS-6.4, and suspended in the same volume of buffer. Ultrasonicated antigen (0.25 ml) obtained from *V. cholerae* Ogawa or Inaba was added. Ultrasonically treated antigen was prepared by disintegrating 10 ml of whole *V. cholerae* Ogawa or Inaba strain, 18-hr growth in 3% peptone agar, suspended in PBS-7.2. Opacity was adjusted to 0.4 in a Bausch & Lomb spectrophotometer at 520-nm wavelength. This was disrupted in an MSE ultrasonic disintegrator at 60 kc per sec for 20 min. This was incubated at 37 C for 30 min. It was mixed five to six times during the period of incubation. The sensitized red cells were centrifuged, washed with PBS-NRS, suspended in 4 ml of PBS-NRS, and employed in hemagglutination tests. Unsensitized red cells were used as controls.

With the indirect hemagglutination test technique (before putting up test), the serum and stool supernatant samples were absorbed with packed sheep red cell suspension by mixing equal volumes of serum or stool supernatant samples and packed red cells and incubated for 1 hr at 37 C. This procedure was adopted to remove any nonspecific hemagglutinins. Doubling dilutions of inactivated and red cell adsorbed sera or stool supernatant fluids were made in PBS-7.2 as described before. Sensitized tanned red cell suspensions (0.5 ml) were added to 0.5 ml of different dilutions of stool supernatant fluids or sera. It was incubated at 37 C for 2 hr and kept overnight at 0 to 4 C. In every instance, a separate row of dilutions was put up as control using unsensitized tanned red cell suspensions. The results were read as positive when the cells covered the entire bottom of the tube and negative when they formed a small thick-walled ring. The highest dilution producing a positive pattern was considered as the end point of antibody titration.

RESULTS

Results shown in Tables 1 and 2 indicate that coproantibodies increase within 2 days after the first oral feed of *V. cholerae* L-form lysates. In the majority of rabbits, a second oral feed is followed by an 8- to 16-fold rise in coproantibody titer. A peak titer of 512 was obtained after the third or fourth oral feeds. Further feeds with complete L-forms did not boost the coproantibody response, and there was a gradual fall in coproantibody titer during this period. The coproantibody level fell down to $\frac{1}{32}$ of peak titer 16 in six weeks after immunization. Nearly similar antibody pattern was obtained irrespective of *V. cholerae* subtype administered. Coproantibody titers with hetero-

TABLE 1. *Coproantibody titers after oral feeding of six rabbits with L-form lysates and complete L-forms of V. cholerae Ogawa and Inaba*

Rabbit no.	Oral feed subtype	Subtype used in sensitizing red blood cells	Biweekly oral feeds with L-form lysates					Biweekly oral feeds with L-forms					Weekly oral feeds with L-forms					HA[a] titer after 6 weeks
			1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
R1	Ogawa	Ogawa	16	256	512	512	—[b]	512	256	256	—	—	—	—	—	—	16	16
		Inaba	16	128	512	512	—	512	256	256	—	—	—	—	—	—	16	16
R2	Ogawa	Ogawa	16	128	512	512	—	512	512	128	—	—	—	—	—	—	16	16
		Inaba	16	128	512	512	—	256	256	128	—	—	—	—	—	—	16	16
R3	Ogawa	Ogawa	16	128	256	256	—	512	256	128	—	—	—	—	—	—	32	16
		Inaba	16	64	256	256	—	512	256	128	—	—	—	—	—	—	4	4
R4	Inaba	Ogawa	8	16	128	128	—	256	128	128	—	—	—	—	—	—	16	16
		Inaba	8	16	16	64	—	256	128	128	—	—	—	—	—	—	4	4
R5	Inaba	Ogawa	16	512	512	512	—	512	256	128	—	—	—	—	—	—	16	16
		Inaba	16	256	256	256	—	512	256	128	—	—	—	—	—	—	16	16
R6	Inaba	Ogawa	64	512	512	512	—	512	512	256	—	—	—	—	—	—	32	32
		Inaba	64	512	256	512	—	512	512	256	—	—	—	—	—	—	64	16

[a] Hemagglutination.
[b] —, Not done.

TABLE 2. *Serum antibodies (indirect hemagglutination test) after oral feeding of rabbits with L-form lysates and complete L-forms of V. cholerae Ogawa and Inaba*

Rabbit no.	Oral feed subtype	Subtype used in sensitizing red blood cells	Biweekly oral feeds with L-form lysates					Biweekly oral feeds with L-forms					Weekly oral feeds with L-forms					HA[a] titer after 6 weeks
			1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
R1	Ogawa	Ogawa	16	128	256	512	—[b]	2,048	1,024	1,024	—	1,024	1,024	—	—	—	1,024	256
		Inaba	16	128	256	512	—	1,024	1,024	1,024	—	1,024	512	—	—	—	512	256
R2	Ogawa	Ogawa	16	128	128	512	—	2,048	1,024	1,024	—	1,024	512	—	—	—	256	256
		Inaba	16	32	128	512	—	1,024	1,024	1,024	—	1,024	512	—	—	—	256	256
R3	Ogawa	Ogawa	16	256	128	256	—	1,024	1,024	1,024	—	1,024	512	—	—	—	256	256
		Inaba	16	256	128	256	—	1,024	1,024	1,024	—	1,024	512	—	—	—	256	256
R4	Inaba	Ogawa	16	32	32	128	—	512	512	512	—	512	256	—	—	—	256	128
		Inaba	16	32	32	64	—	256	256	512	—	512	256	—	—	—	256	128
R5	Inaba	Ogawa	16	512	128	512	—	1,024	1,024	1,024	—	1,024	512	—	—	—	512	128
		Inaba	16	512	128	512	—	512	1,024	1,024	—	1,024	512	—	—	—	256	256
R6	Inaba	Ogawa	64	512	512	512	—	1,024	2,048	1,024	—	1,024	1,024	—	—	—	1,024	256
		Inaba	64	512	512	512	—	1,024	2,048	1,024	—	1,024	1,024	—	—	—	1,024	256

[a] Hemagglutination.
[b] —, Not done.

logous antigens were mostly similar to those obtained with homologous antigens. The results of circulating antibodies as determined by indirect hemagglutination test showed that antibodies started appearing after the first oral feed of lysate and from an initial level of 16 there was an 8-, 16-, 32-, 64-, and 128-fold rise after second, third, and fourth feeds. This continued till after the first feed with complete L-forms. Oral administration of complete L-forms did not elicit a booster response. The circulating antibody titer fell to half the peak titer after second or third feed of complete L-forms. This high level of coproantibody was maintained for a week and was later followed by a gradual decline to one-fourth of peak titer in spite of weekly oral feeds of complete L-forms. Six weeks after oral immunization, circulating antibodies were still present in a titer of 256 or 128.

DISCUSSION

The earliest demonstration of coproantibodies in acute experimental cholera was made by Burrows et al. (4) in guinea pigs. Later Burrows and Havens (5) and Koshland and Burrows (12) studied the origin and protective role of coproantibodies in immunity to cholera. Freter (7) produced evidence that coproantibodies, and not circulating antibodies, were protective in experimental acute cholera in guinea pigs. Jenkins and Rowley (10) obtained similar protection in suckling rabbits. It is now well established that coproantibodies may confer a significant degree of protection in enteric disease under certain experimental conditions. There has been, however, some inconsistency in reports regarding presence of coproantibodies. Melnick and Kaplan (13) could not demonstrate them in immunized monkeys. Thind (15) found that coproantibodies (agglutinins) were excreted in rabbits after intragastric feeding with *V. cholerae*. Freter (8) questioned the validity of these findings. Freter and Gangrosa (9) observed that oral immunization in human volunteers who had no previous contact with *V. cholerae* antigen produced coproantibodies to the same extent as in previously vaccinated volunteers injected parenterally. Felsenfeld and Greer (6) found that oral administration of an antigen containing type 2 cholera toxin stimulated a more intensive agglutinating, antitoxic, and vibriocidal activity in mesenteric lymph nodes and intestinal lymphatics than parenteral injection of the same antigen. Bhattacharya and Mukerjee (2) and Bhattacharya et al. (3) observed an increase in antitoxic and antibacterial immunity after oral administration of live cholera vaccine of an avirulent El Tor strain. Subsequently, Sanyal and Mukerjee (14) conducted a small trial on 25 human volunteers with oral cholera vaccine. They found significant increase of vibriocidal titers in serum and demonstrated coproantibodies in stool samples within a week of vaccination. It may be noted that these workers found only a maximum level of 32 for coproantibody in 8 to 9 days and 8 only in 3 months. They have considered these levels as protective. Our experimental results show that within 2 days of oral administration of L-form lysate of *V. cholerae*

subtype Ogawa and Inaba, coproantibodies appeared in stools and a maximum titer of 512 was reached after the third or fourth feed. This is certainly a fairly high titer of coproantibody. Not only the coproantibodies have reached such high titers but the circulating antibodies also reach and maintain a high titer.

If the complete immune coproantibody response is examined, it is seen that in most rabbits a coproantibody level of 16 is found six weeks after immunization. How far this level is protective is difficult to say, although some workers have considered even lower titers as protective. Furthermore, high titers of circulating antibody (1,024 and 2,048) are obtained, and even six weeks after immunization a titer of 256 is present in most rabbits. It is not possible for us to interpret this in terms of protection afforded because most workers have determined serum agglutinin titers. In the end, we feel if a similar coproantibody response can be elicited in human subjects, oral administration of lysates of L-forms can be an effective mode of immunization against cholera.

LITERATURE CITED

1. Agarwal, S. C., and N. K. Ganguly. 1971. Agglutinin and vibriocidal responses of L-forms of *Vibrio cholerae* and El tor. Indian J. Med. Res. 59:866–872.
2. Bhattacharya, P., and S. Mukerjee. 1968. Further studies on the development of a live oral cholera vaccine. J. Hyg. 66:307–318.
3. Bhattacharya, P., A. Narayanaswami, and S. Mukerjee. 1968. Production of antitoxic immunity by live oral cholera vaccine. J. Bacteriol. 95:255–256.
4. Burrows, W., M. E. Elliot, and J. Havens. 1947. The excretion of coproantibody in experimental enteric cholera in guinea pigs. J. Infect. Dis. 82:261–281.
5. Burrows, W., and J. Havens. 1948. Studies on immunity to Asiatic *V. cholera*. The absorption of immune globulin from the bowel and its excretion in the urine and faeces of experimental animals and human volunteers. J. Infect. Dis. 82:231–250.
6. Felsenfeld, O., and W. E. Greer. 1968. Vibriocidal activity, immunoglobulin producing cells and immunoglobulin levels in *Theropithecus gelada* after administration of a vibrio cholera antigen. Immunology 14:319–324.
7. Freter, R. 1956. Coproantibody and bacterial antagonism as protective factors in experimental enteric cholera. J. Exp. Med. 104:419–426.
8. Freter, R. 1962. Detection of coproantibody and its formation after parenteral and oral immunization of human volunteers. J. Infect. Dis. 111:37–48.
9. Freter, R., and E. J. Gangrosa. 1963. Oral immunization and production of coproantibody in human volunteers. J. Immunol. 91:724–729.
10. Jenkins, C. R., and D. Rowley. 1960. The importance of antibody in the prevention of experimental cholera in rabbits. Brit. J. Exp. Pathol. 41:24.
11. Kessel, J. F., W. P. Lewis, C. M. Pasquel, and J. A. Turner. 1965. Indirect hemagglutination and complement fixation tests in amoebiasis. Amer. J. Trop. Med. Hyg. 14:540–549.
12. Koshland, M. E., and W. Burrows. 1950. The origin of faecel antibody and its relationship to immunization with adjuvants. J. Exp. Med. 65:93–103.
13. Melnick, J. L., and A. S. Kaplan. 1953. Quantitative studies of the virus-host relationship in chimpanzees after inapparent infection with coxsackie viruses. J. Exp. Med. 97:367–400.
14. Sanyal, S. C., and S. Mukerjee. 1969. Live oral cholera vaccine. Report of a trial on human volunteer subjects. Bull. W. H. O. 40:503–511.
15. Thind, K. S. 1961. Effect of cortisone acetote on coproantibody formation Ind. J. Med. Res. 49:214–221.