INFECTION AND IMMUNITY, July 1972, p. 17-20
Vol. 6, No. 1

Printed in U.S.A.

Oral Immunization with L-Forms of *Vibrio cholerae* in Human Volunteers

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Received for publication 29 December 1971

Oral administration of heat-inactivated lysates of *Vibrio cholerae* Ogawa L-forms elicited significantly high coproantibody, serum indirect hemagglutinating antibody, and vibriocidal antibody responses in human volunteers, against both homologous and heterologous subtypes of *V. cholerae* and *V. El Tor*. Five oral biweekly doses produced an adequate antibody response which persisted for at least 6 weeks after immunization. No untoward side effects were seen.

It was reported earlier that disrupted L-forms of *Vibrio cholerae* are antigenic (1). We also found that lysed *V. cholerae* L-form suspension produced a significant increase in coproantibodies and serum hemagglutinating antibodies after oral administration in rabbits (2). Some investigators have reported that immunological responses to oral administration of *V. cholerae* antigens vary in different animal species, as indicated by coproantibody levels (3). We have, therefore, undertaken the present investigation to determine the immunological responses to oral administration of lysates of *V. cholerae* L-forms in human volunteers. The sequence of immunological responses against homologous and heterologous strains of *V. cholerae* and *V. El Tor* has been measured by estimation of coproantibody in fecal samples and of vibriocidal and indirect hemagglutinating antibody in serum on different days during and after immunization. This has been done mainly to explore the possibility of using L-forms of *V. cholerae* as a monovalent vaccine for human immunization.

MATERIALS AND METHODS

Human volunteers. Six human volunteers were selected from the medical and laboratory staff working in the department. None of the volunteers had received vaccination against cholera during the past 3 to 4 years.

Bacterial strains. *V. cholerae* subtype Ogawa S 162/58 was used for production of L-forms. Bacterial growth suspensions from this strain and other strains, i.e., *V. cholerae* Inaba B/53/28, *V. El Tor* Ogawa Phil. No. 1418, and *V. El Tor* Inaba Phil. No. 6973, were employed for hemagglutination and vibriocidal tests. The bacterial cultures were obtained in a lyophilized form from Central Research Institute, Kasauli, India, and were maintained on brain heart infusion agar slopes.

Production of L-forms of V. cholerae Ogawa and their lysates. L-forms of *V. cholerae* Ogawa strain S 162/58 have been maintained for the past 2.5 years in our laboratory. These were propagated and maintained in L-form agar and were disrupted with acetone to obtain the lysates as described earlier (1). The lysate was filtered in a sintered-glass filter and was inactivated at 60 C for 1 hr. The turbidity was adjusted to an opacity of 0.4 in a Bausch & Lomb spectrophotometer at 520 nm. This was termed as lysate vaccine.

Immunization procedures. At 2 hr after a light breakfast, each volunteer was asked to take two tablets of Alludrox (aluminum hydroxide gel), and 10 min later an oral dose of 10 ml of *V. cholerae* Ogawa L-form lysate (vaccine) was administered. Each dose was given twice a week, and a total of five 10-ml doses were administered.

Collection of samples. Fecal samples for coproantibody estimation were collected 1 day after every oral feed during the period of immunization and later weekly for 6 weeks. On the morning of sample collection, magnesium sulfate, 10 to 15 g dissolved in 2 oz (ca. 60 ml) of water, was administered orally, and 1.5 to 2 hr later the fecal sample was collected in a sterile container. On the same day, 8 ml of blood was collected from the median cubital vein of the arm. Serum was separated for determination of antibodies.

Processing of stool or blood samples and determination of coproantibodies in feces and indirect hemagglutinating antibodies in blood. Techniques similar to those described before (2) were employed.

Determination of vibriocidal antibody titer. The vibriocidal antibody titer was determined by the Kasauli method of Singh and Ahuja (6). The bacterial strains employed for determination of vibriocidal antibody were the same as described above.

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TABLE 1. *Coproantibody levels in fecal samples from human volunteers after oral immunization with Vibrio cholerae Ogawa L-form lysate vaccine*

Volunteer	Antigen used in sensitizing red blood cells[a]	Preimmunization titer	Biweekly oral feeding with L-form lysates					Postimmunization hemagglutination titer					
			1F[b]	2F	3F	4F	5F	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
1	A	<10	40	40	160	320	320	640	640	640	320	—[c]	—
	B	<10	40	40	160	320	320	640	640	320	320	—	—
	C	<10	40	40	160	320	320	640	640	320	320	—	—
	D	<10	40	40	160	320	320	640	640	160	160	—	—
2	A	<10	10	160	320	320	640	640	640	640	320	160	160
	B	20	20	40	160	640	640	640	640	320	320	160	160
	C	40	40	80	320	640	640	640	640	640	320	160	160
	D	10	10	40	160	640	640	640	640	320	320	160	160
3	A	20	20	40	160	640	640	640	640	320	320	160	160
	B	20	20	80	160	640	640	640	640	320	320	320	160
	C	10	10	80	160	640	640	640	640	320	320	160	160
	D	20	20	80	160	640	640	640	640	320	320	160	80
4	A	<10	20	160	320	640	640	640	640	320	320	160	80
	B	<10	10	80	320	640	640	640	640	320	320	320	160
	C	<10	20	160	320	640	640	640	640	640	640	160	80
	D	<10	20	80	160	320	640	640	640	320	320	160	160
5	A	<10	20	160	640	640	640	640	640	640	640	320	160
	B	<10	10	80	320	320	640	640	640	320	320	160	80
	C	<10	10	80	160	640	640	640	640	320	320	160	80
	D	<10	20	80	160	640	640	640	640	320	320	160	160
6	A	<10	40	80	320	640	640	640	640	320	320	160	80
	B	<10	10	80	320	640	640	640	640	320	320	160	160
	C	<10	10	160	640	640	640	640	640	320	320	160	80
	D	<10	20	80	320	640	640	640	640	320	320	160	80

[a] A and B = *V. cholerae* Ogawa and Inaba antigens, respectively; C and D = *V. El Tor* Ogawa and Inaba antigens, respectively.
[b] Oral feed number.
[c] Not done.

The test sera were inactivated at 56 C for 30 min to destroy complement. Ten-fold dilutions of inactivated serum were made in 1% peptone water containing a concentration of guinea-pig complement which did not inhibit the growth of *V. cholerae* or *V. El Tor* as determined earlier. Ogawa and Inaba subtypes of *V. cholerae* and *V. El Tor* were grown for 16 hr at 37 C in 3% peptone water. The turbidity of 16-hr growth of different bacterial strains was adjusted initially to an optical density of 0.4 at 520 nm in a Bausch & Lomb spectrophotometer. This was further diluted so that a 4-mm platinum loop inoculum contained 200 to 500 bacteria, as determined by a viable count. A loopful of diluted 16-hr growth was inoculated into 1 ml of each serum dilution. This was incubated at 37 C for 40 min. One loopful of serum plus bacteria mixture was plated in 3% peptone-agar plates. The colonies in different plates were counted after incubation at 37 C for 24 hr. The highest dilution which killed 50% of the vibrios was taken as the vibriocidal titer. Vibriocidal activity of each serum was tested against *V. cholerae* Ogawa and Inaba subtypes and *V. El Tor* subtypes. A complement and saline control was included with each test.

RESULTS

The results presented in Tables 1–3 show that there was a gradual rise of coproantibodies from a preimmunization level of 10 to a level of 640 during the period of immunization. The coproantibody titer persisted at this level for 3 to 4 weeks after immunization. This was followed by a gradual fall, and the final level at 6 weeks was about 160. Similarly, after oral immunization with L-form lysates of *V. cholerae*, there was a gradual rise in indirect hemagglutinating antibodies in blood. The initial rise of antibody in the blood appeared later than the

TABLE 2. *Indirect hemagglutinating antibody in serum from human volunteers after oral immunization with Vibrio cholerae Ogawa L-form lysate vaccine*

Volunteer[a]	Antigen used for sensitizing red blood cells	Biweekly oral feed with L-form lysate					Postimmunization hemagglutination titer					
		1F[b]	2F	3F	4F	5F	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
1	*V. cholerae* Ogawa	80	80	80	160	640	1,280	2,560	2,560	2,560	—[c]	—
	V. cholerae Inaba	40	80	80	160	320	1,280	2,560	1,280	1,280	—	—
	V. El Tor Ogawa	80	80	80	160	640	1,280	2,560	1,280	1,280	—	—
	V. El Tor Inaba	40	40	80	80	640	1,280	2,560	1,280	1,280	—	—
2	*V. cholerae* Ogawa	10	20	80	320	640	1,280	2,560	2,560	2,560	1,280	1,280
	V. cholerae Inaba	10	20	80	320	640	1,280	2,560	1,280	1,280	1,280	640
	V. El Tor Ogawa	10	20	80	320	640	1,280	2,560	2,560	1,280	640	640
	V. El Tor Inaba	10	20	80	320	640	1,280	2,560	1,280	1,280	640	640
3	*V. cholerae* Ogawa	10	20	80	640	640	1,280	2,560	1,280	1,280	1,280	1,280
	V. cholerae Inaba	10	20	80	320	640	1,280	2,560	2,560	1,280	1,280	640
	V. El Tor Ogawa	10	20	160	320	640	1,280	2,560	2,560	1,280	1,280	1,280
	V. El Tor Inaba	10	20	80	320	640	1,280	2,560	1,280	1,280	1,280	640
4	*V. cholerae* Ogawa	10	40	160	320	640	1,280	2,560	2,560	1,280	1,280	1,280
	V. cholerae Inaba	10	40	160	320	640	1,280	2,560	2,560	1,280	640	640
	V. El Tor Ogawa	10	20	80	160	320	640	2,560	2,560	1,280	1,280	640
	V. El Tor Inaba	10	40	160	320	640	1,280	2,560	2,560	1,280	1,280	640
5	*V. cholerae* Ogawa	10	40	160	320	640	1,280	2,560	2,560	2,560	1,280	1,280
	V. cholerae Inaba	10	40	80	320	640	1,280	2,560	1,280	1,280	640	640
	V. El Tor Ogawa	10	20	160	320	640	1,280	2,560	2,560	1,280	640	320
	V. El Tor Inaba	10	20	160	320	640	1,280	2,560	2,560	1,280	1,280	640
6	*V. cholerae* Ogawa	20	40	160	320	640	1,280	2,560	2,560	1,280	1,280	640
	V. cholerae Inaba	10	20	80	320	640	1,280	2,560	1,280	1,280	640	640
	V. El Tor Ogawa	10	40	160	320	640	1,280	2,560	2,560	1,280	1,280	640
	V. El Tor Inaba	10	20	160	160	640	1,280	2,560	1,280	1,280	1,280	640

[a] In all volunteers, the preimmunization titer was <10.
[b] Oral feed number.
[c] Not done.

coproantibody rise and reached a higher level of 2,560 during the period of immunization. This peak titer of 2,560 persisted for 2 to 3 weeks and then began to fall gradually. Six weeks after immunization, the titer was 640. There was also a considerable increase in vibriocidal antibody during immunization. This increase was maintained during the postimmunization period for at least 6 weeks. Oral immunization with L-form lysates of *V. cholerae* produced a markedly similar heterologous response to *V. cholerae* Inaba and to *V. El Tor* Ogawa and Inaba.

DISCUSSION

Several attempts have been made to develop a cholera vaccine which could be administered orally and produce a good antibody response (3–5). Inconsistent results have been obtained, and the attempts have not been very successful.

Sanyal and Mukerjee (5) reported their results after oral administration of a live cholera vaccine made from an avirulent strain of *V. El Tor*. Their results do not appear very encouraging, since the coproantibody levels were low. Furthermore, they obtained a high preimmunization level of vibriocidal antibodies. The reason for this might be that subclinical infection with *V. cholerae* was high in the West-Bengal population or that the natural bacteriocidal activity of complement was not fully diluted out initially during vibriocidal antibody titration. We have failed to observe such a high preimmunization level of vibriocidal antibodies.

Our results show that *V. cholerae* L-form lysate (vaccine), filtered through sintered glass and heat-inactivated (60 C for 1 hr), has fairly good immunogenicity on oral administration. It produced a gradually increasing coproantibody titer in all six human volunteers. The maximal level reached was also quite high. Sanyal and

TABLE 3. *Vibriocidal antibody titer in blood samples from human volunteers after oral immunization with Vibrio cholerae Ogawa L-form lysate vaccine*

Volunteer[a]	Subtype used in sensitizing red blood cells[b]	Biweekly oral feeds for L-form lysate					Postimmunization hemagglutination titer					
		1F[c]	2F	3F	4F	5F	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
1	A	10^{-1}	10^{-1}	10^{-3}	10^{-3}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-6}	—[d]	—
	B	10^{-1}	10^{-1}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	—	—
	C	10^{-1}	10^{-1}	10^{-3}	10^{-3}	10^{-5}	10^{-5}	10^{-5}	10^{-6}	10^{-5}	—	—
	D	10^{-1}	10^{-1}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-4}	—	—
2	A	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-4}	10^{-4}
	B	10^{-2}	10^{-3}	10^{-2}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-4}
	C	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-5}	10^{-5}	10^{-5}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
	D	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
3	A	10^{-2}	10^{-4}	10^{-4}	10^{-5}	10^{-6}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
	B	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	C	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-3}
	D	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
4	A	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-3}
	B	10^{-1}	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-3}	10^{-2}
	C	10^{-2}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-4}	10^{-3}	10^{-2}
	D	10^{-1}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-3}	10^{-2}
5	A	10^{-2}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	B	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-3}	10^{-2}
	C	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-6}	10^{-5}	10^{-4}	10^{-4}	10^{-4}	10^{-3}
	D	10^{-1}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-4}	10^{-3}	10^{-3}	10^{-2}
6	A	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-6}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	B	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-6}	10^{-6}	10^{-5}	10^{-4}	10^{-4}	10^{-3}
	D	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-4}	10^{-3}	10^{-2}

[a] In all volunteers, the preimmunization titer was $<10^{-1}$.
[b] A and B = *V. cholerae* Ogawa and Inaba antigens, respectively; C and D = *V. El Tor* Ogawa and Inaba antigens, respectively.
[c] Number of oral feed.
[d] Not done.

Mukerjee obtained a maximal titer of 32 in their subjects, as against a maximal titer of 640 in our series. It has been observed that five oral doses are adequate to give a sufficient coproantibody titer after 6 weeks. The serum indirect hemagglutinating antibody and vibriocidal antibody titers were also consistently high, and the latter was in the range of the protective level found by other investigators.

Furthermore, immunogenic responses with *V. cholerae* Ogawa to heterologous subtypes, namely, *V. cholerae* Inaba and *V. El Tor* Ogawa and Inaba, were nearly the same. Therefore, it appears that oral administration of L-form lysates of *V. cholerae* Ogawa is not only effective immunologically but is also cross-reactive against other subtypes of *V. cholerae* or *V. El Tor*. After oral administration of this lysate, none of the human volunteers suffered from any untoward symptoms. Also, if this lysate is used for oral immunization, it will not suffer from the drawback of live oral vaccines with which the attenuated strain may occasionally give rise to virulent variants.

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