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Further studies on the development of a live oral cholera vaccine

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The inadequacy of the injectable cholera vaccine presently in use for prophylaxis against cholera has been brought out in recent field trials. The *W.H.O. Cholera Information* no. 4 (1965) in its editorial on the results of these trials observed that a safe and effective vaccine had still to be developed for obtaining a high degree of protection against cholera in man. While one way of improving the vaccine would be to lower the toxicity without decreasing the protective effect, it was suggested that more effective immunization might be possible by routes other than the parenteral.

Mukerjee (1963) studied the possibility of developing a live cholera vaccine for administration by the oral route and obtained evidence, both by *in vitro* and *in vivo* experiments, indicating the suitability for this purpose of an El Tor vibrio strain isolated from the Middle East or from water sources in Calcutta in the absence of cholera. In a subsequent publication Mukerjee (1965) reported the stability of the avirulent character of the proposed vaccine strains on repeated propagation in animals or artificial lysogenization with phages from virulent cholera cultures. These strains have been further examined with special reference to their ability to multiply in the intestine, which is considered a prerequisite for stimulating the development of local cellular immunity (Suter & Ramseir, 1964). These studies as well as the results of further work on the pathogenicity and immunizing value of the vaccine strains and the nature of immunity produced in laboratory animals by live vaccination by oral or intra-intestinal routes are reported in this communication.

MATERIALS AND METHODS

Vibrio strains

The *Vibrio cholerae* strains used in these experiments had been isolated from patients in India. The two El Tor strains proposed to be used as oral vaccine are ME-7 isolated in a Middle East country and EW-6 from a water source in Calcutta in 1958 (Mukerjee, 1963). These strains were used in the present series of tests also. They will be referred to as 'vaccine strains'. The *V. eltor* (case) strains had been isolated in infected areas from either patients or carriers. All *V. cholerae* strains and the *V. eltor* (case) strain no. HK 130 belonged to the Inaba serotype, the rest of the

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El Tor strains being Ogawa. *V. eltor* strains in this series were haemolytic at the time of test and non-susceptible to group IV cholera phage, while the *V. cholerae* strains were non-haemolytic and lysable by this phage.

Animals

The rabbits used in these experiments were obtained from local suppliers and were heterozygotic. Some of the infant rabbits, however, were bred in our animal house from pure stock obtained from the Haffkine Institute, Bombay, and Central Drug Research Institute, Lucknow.

Pathogenicity test in adult rabbits

The test was carried out according to the method of De & Chatterjee (1953) and has been described in detail by Mukerjee (1963).

Pathogenicity test in infant rabbits

The test was carried out according to the method of Dutta & Habbu (1955). A 3 hr. broth culture (diluted in saline or undiluted) containing approximately $2{\cdot}5 \times 10^4$–10^9 viable vibrios was injected in the proximal part of the small intestine. As a rule, higher doses were used in tests with apathogenic strains.

Protection test in adult rabbit

A 3 hr. broth culture of ME-7 containing approximately 5×10^8 viable cells in 0·5 ml. was injected into the proximal part of the small intestine under ether anaesthesia. After 3–6 days the immunity of the rabbit to challenges with *V. cholerae* and *V. eltor* (case) strains was tested in ligated intestinal loops.

Protection test in infant rabbit

Using a rubber catheter, infant rabbits were given by mouth 100, 1000 and 10,000 viable vibrio cells from a 3 hr. culture of the ME-7 strain on the 3rd, 5th and 7th days respectively after birth. The rabbits were challenged intra-intestinally between the 10th and 12th day with virulent cultures of an El Tor (case) strain.

Tests for stability of the avirulent character of vaccine strains after animal passage

The ME-7 and EW-6 strains were used in two different series of experiments. These strains were serially propagated and re-isolated from the ligated intestinal loops of adult rabbits and intestinal tract of infant rabbits. In each series propagation was repeated ten times and the virulence of the strains was carefully examined for any alteration.

Tests on the multiplication of the vaccine strains in adult rabbits

ME-7, GS 1/65 and GS 9/65 were used as representative strains of apathogenic *V. eltor*, *V. cholerae* and pathogenic *V. eltor* respectively. Three-hour growth of a vibrio strain diluted in nutrient broth was injected into the intestinal loop of an

adult rabbit. Viable counts were made from samples used for the injection. Twenty-four hours after injections the contents of the loops were washed out in saline and a viable vibrio count of each sample was made after plating.

Tests on the multiplication of the vaccine strain in infant rabbits

ME-7 and GS 9/65 were taken as representative apathogenic and case El Tor strains. Half ml. quantities of diluted 3 hr. growth of the strains in nutrient broth were injected into the proximal part of the small intestine under ether anaesthesia. Viable counts of the vibrio cells per ml. of the samples injected were made. After 24 hr. the reactions in the gut were noted. The contents of the small and large intestine were washed out in a fixed volume of normal saline and the viable vibrios in the sample counted after plating on nutrient agar. The total viable cells found in the intestinal contents as compared with the number injected indicated the rate of multiplication.

Tests on multiplication of the vibrios in gut of adult rabbit after intra-intestinal immunization

Adult rabbits were immunized by injection, into the upper part of the small intestine, of 2·5 ml. of a saline suspension of an 18 hr. growth of ME-7 containing about $2{\cdot}0 \times 10^{11}$ viable cells. On the eighth or ninth day of immunization known numbers of viable cells of *V. cholerae* (569B or GS 1/65) and *V. eltor* (pathogenic and apathogenic types) were injected into three isolated loops of small intestine of equal length in each rabbit. After 4, 8 and 24 hr. or in some experiments only after 24 hr. the rabbits were killed, reaction in the loops noted and viable counts of the loop contents were made. With each immunized animal one normal animal was generally inoculated with the same number of vibrios as a control.

RESULTS

Pathogenicity of the vaccine strain

The degree of pathogenicity for laboratory animals of the Middle East and water El Tor strains as compared with that of *V. cholerae* and *V. eltor* strains isolated from cholera cases was retested in an extended series of experiments. The results are given in Tables 1 and 2. It may be seen from Table 1 that all *V. cholerae* and *V. eltor* (case) strains gave rise to inflammatory reactions in all ligated intestinal loops of the thirty rabbits tested. On the other hand, all loop tests of ME-7 and EW-6 strains in 34 rabbits failed to produce any reaction excepting in one instance where injection of the EW-6 strain caused slight swelling. Table 2 shows that all infant rabbits receiving cultures of *V. cholerae* and *V. eltor* (case) strains showed a positive reaction, while no reaction was noted in 4 rabbits injected with EW-6 and only 1 of the 19 rabbits receiving ME-7 showed slight distension of the large gut with fluid.

Mukerjee (1963) reported that the typical reaction of profuse diarrhoea and extreme dehydration described by Dutta & Habbu (1955) could not be found in infant rabbits of the heterozygotic type obtained from suppliers in Calcutta.

Identical observations were made in the present series even when litters of pure-bred stock raised in the laboratory were used. A positive reaction was accordingly judged by the death of the animal and typical distension of the large intestine with fluid.

Table 1. *Tests for gut inflammatory reaction in normal adult rabbits*

Vibrio type	Strain no.	Area of isolation	Total no. of tests	No. of tests showing positive reactions
V. cholerae	H 86/62	Calcutta	5	5
	H 96/64	Calcutta	4	4
V. eltor (case)	Baroda 6/64	India	2	2
	Baroda 7/64	India	1	1
	Bg 79/64	India	6	6
	Bom 1/65	India	1	1
	Bom 2/65	India	1	1
	Bom 3/65	India	1	1
	Gs 9/65	India	1	1
	H 16/64	India	1	1
	HK 130	Hong Kong	2	2
	Phil 32/62	Phillipines	2	2
V. eltor (water strains)	EW-6	Calcutta (water)	14	0*
	ME-7	Middle East	20	0

* One ligated loop showed slight swelling

Table 2. *Pathogenicity to infant rabbits*

Type of vibrio	Strain no.	Area of isolation	Total no. of tests	No. of tests showing positive reactions
V. cholerae	H 86/62	Calcutta	1	1
	H 96/64	Calcutta	2	2
V. eltor	Baroda 6/64	India	1	1
	Baroda 7/64	India	1	1
	Bg 79/64	India	1	1
	GS 9/65	India	5	5
V. eltor (water strains)	ME-7	Middle East	19	0*
	EW-6	Calcutta (water)	4	0

* One rabbit showed slight distension of the large gut with fluid.

Tests for protection by the vaccine in rabbits

It was found that a single dose of live vaccine administered intra-intestinally in adult rabbits could protect the animals against gut-inflammatory reaction on subsequent challenge with *V. cholerae* and *V. eltor* (case) strains in ligated intestinal loops. Out of 16 rabbits thus immunized, all were found to resist gut-inflammatory

reaction when challenged with a *V. eltor* (case) strain. On challenge with *V. cholerae*, 12 out of 16 rabbits showed complete immunity, 3 showed partial immunity and in 1 no immunity was found.

Out of 20 infant rabbits immunized orally with live vaccine and subsequently challenged with an El Tor (case) strain, all but 3 died showing typical distension of the large intestine.

Table 3. *Multiplication of* Vibrio cholerae *and* V. eltor (*case and 'vaccine' strains*) *in the gut of normal adult and infant rabbits*

Experimental model	Rabbit no.	Strain no.	Total no. of vibrios injected	Total no. recovered
Intestinal loop of adult rabbit	1	ME-7	$2{\cdot}6 \times 10^{7}$	$1{\cdot}8 \times 10^{9}$
		GS 1/65	$1{\cdot}3 \times 10^{7}$	$4{\cdot}0 \times 10^{8}$
	2	ME-7	$3{\cdot}6 \times 10^{5}$	$4{\cdot}5 \times 10^{9}$
		GS 1/65	$3{\cdot}9 \times 10^{3}$	$2{\cdot}8 \times 10^{8}$
	3	ME-7	$1{\cdot}3 \times 10^{4}$	$2{\cdot}0 \times 10^{8}$
		GS 1/65	$2{\cdot}2 \times 10^{4}$	$3{\cdot}1 \times 10^{7}$
		GS 9/65	$2{\cdot}0 \times 10^{4}$	$5{\cdot}6 \times 10^{7}$
	4	ME-7	$5{\cdot}2 \times 10^{4}$	$2{\cdot}7 \times 10^{9}$
		GS 9/65	$9{\cdot}8 \times 10^{3}$	$4{\cdot}2 \times 10^{8}$
	5	ME-7	$1{\cdot}5 \times 10^{4}$	$1{\cdot}8 \times 10^{12}$
		GS 1/65	$5{\cdot}0 \times 10^{4}$	$1{\cdot}0 \times 10^{6}$
		GS 9/65	$4{\cdot}0 \times 10^{4}$	$5{\cdot}0 \times 10^{10}$
	6	ME-7	$1{\cdot}5 \times 10^{4}$	$3{\cdot}6 \times 10^{11}$
		GS 1/65	$5{\cdot}0 \times 10^{4}$	$1{\cdot}0 \times 10^{6}$
		GS 9/65	$4{\cdot}0 \times 10^{4}$	$1{\cdot}9 \times 10^{10}$
Infant rabbit	1	GS 9/65	$2{\cdot}5 \times 10^{4}$	$1{\cdot}8 \times 10^{9}$
	2	ME-7	$1{\cdot}3 \times 10^{5}$	$1{\cdot}8 \times 10^{8}$
	3	ME-7	$1{\cdot}3 \times 10^{5}$	$8{\cdot}0 \times 10^{7}$
	4	ME-7	$5{\cdot}0 \times 10^{4}$	$6{\cdot}9 \times 10^{7}$
	5	ME-7	$5{\cdot}0 \times 10^{4}$	$1{\cdot}0 \times 10^{8}$
	6	GS 9/65	$7{\cdot}0 \times 10^{4}$	$3{\cdot}3 \times 10^{9}$
	7	GS 9/65	$7{\cdot}0 \times 10^{4}$	$6{\cdot}1 \times 10^{9}$
	8	GS 9/65	$3{\cdot}6 \times 10^{5}$	$3{\cdot}5 \times 10^{7}$

Stability of the avirulent character of the vaccine strains on animal passage

Neither of the proposed vaccine strains showed enhancement of pathogenicity after ten serial passages in the ligated loops of small intestine of adult rabbits. Identical results were also obtained when the vibrio strains were propagated ten times serially in infant rabbits by the intestinal route.

Multiplication of the vaccine strains in rabbits

From the results given in Table 3 it may be seen that in the ligated loop of intestine of adult rabbits ME-7 multiplied more rapidly than GS 1/65, a *V. cholerae* strain, and to about the same extent as GS 9/65, an El Tor (case) strain. It may also be seen from the same Table that ME-7 definitely multiplied in the infant rabbit's intestine also, although the growth rate was lower than that of GS 9/65.

Tests on multiplication of the vibrios in gut of adult rabbits after intra-intestinal immunization

From the results given in Table 4 it may be seen that the vibrios multiplied in the ligated intestinal loops of the immunized rabbits but the multiplication was much slower than in normal rabbits. It is also of interest that although the virulent strains multiplied in immunized rabbits there was no gut-inflammatory reaction in the loops even after 24 hr., whereas reaction was invariably produced in normal rabbits.

Table 4. *Comparative rates of multiplication of vibrios in intestinal loops of normal and immunized* rabbits*

Experiment	Strain no.	Inoculum (0 hr.)	Viable count at 24 hr.	
			Normal†	Immunized‡
1	2	3	4	5
1	GS 9/65	$4{\cdot}5 \times 10^{2}$	$1{\cdot}1 \times 10^{11}$	$6{\cdot}0 \times 10^{8}$
	ME-7	$5{\cdot}0 \times 10^{2}$	$3{\cdot}1 \times 10^{9}$	$4{\cdot}7 \times 10^{6}$
	569B	$3{\cdot}5 \times 10^{2}$	$9{\cdot}0 \times 10^{7}$	$2{\cdot}7 \times 10^{7}$
2	GS 9/65	$4{\cdot}5 \times 10^{3}$	$6{\cdot}3 \times 10^{9}$	$2{\cdot}4 \times 10^{7}$
	ME-7	$5{\cdot}0 \times 10^{3}$	$3{\cdot}8 \times 10^{9}$	$1{\cdot}3 \times 10^{7}$
	569B	$3{\cdot}5 \times 10^{3}$	$8{\cdot}3 \times 10^{7}$	$9{\cdot}5 \times 10^{5}$
3	GS 9/65	$4{\cdot}5 \times 10^{4}$	$6{\cdot}0 \times 10^{9}$	$4{\cdot}4 \times 10^{7}$
	ME-7	$5{\cdot}0 \times 10^{4}$	$4{\cdot}3 \times 10^{8}$	$3{\cdot}4 \times 10^{7}$
	569B	$3{\cdot}5 \times 10^{4}$	$4{\cdot}5 \times 10^{8}$	$5{\cdot}1 \times 10^{4}$
4	GS 9/65	$3{\cdot}4 \times 10^{4}$	$9{\cdot}1 \times 10^{9}$	$1{\cdot}2 \times 10^{9}$
	ME-7	$5{\cdot}6 \times 10^{4}$	$1{\cdot}5 \times 10^{10}$	$8{\cdot}3 \times 10^{8}$
	569B	$8{\cdot}0 \times 10^{4}$	$1{\cdot}3 \times 10^{10}$	$2{\cdot}8 \times 10^{9}$
5	GS 9/65	$2{\cdot}8 \times 10^{4}$	$3{\cdot}9 \times 10^{9}$	$7{\cdot}0 \times 10^{7}$
	ME-7	$1{\cdot}0 \times 10^{5}$	$7{\cdot}8 \times 10^{9}$	$2{\cdot}0 \times 10^{6}$
	GS 1/65	$1{\cdot}6 \times 10^{5}$	$1{\cdot}3 \times 10^{8}$	$5{\cdot}0 \times 10^{5}$
6	GS 9/65	$1{\cdot}3 \times 10^{6}$	$2{\cdot}3 \times 10^{8}$	$1{\cdot}2 \times 10^{7}$
	ME-7	$2{\cdot}6 \times 10^{6}$	$3{\cdot}3 \times 10^{9}$	$5{\cdot}9 \times 10^{7}$
	569B	$1{\cdot}5 \times 10^{6}$	$6{\cdot}0 \times 10^{9}$	$3{\cdot}6 \times 10^{7}$
7	GS 9/65	$1{\cdot}4 \times 10^{4}$	—	$6{\cdot}0 \times 10^{6}$
	ME-7	$7{\cdot}3 \times 10^{4}$	—	$1{\cdot}2 \times 10^{7}$
	569B	$9{\cdot}0 \times 10^{4}$	—	$1{\cdot}3 \times 10^{6}$

* Immunization was carried out by intra-intestinal injection of viable ME-7 cells 8–9 days before challenge as described under Materials and Methods.

† There was reaction in all the loops injected with GS 9/65, GS 1/65 and 569B.

‡ There was no reaction in any loop.

From the data on the rate of growth of the vibrios in intestinal loops of normal and immunized rabbits presented in Table 5 and plotted in Figs. 1–3 it is evident that in the normal rabbit the *V. eltor* (case) strain multiplied about 1000 times in 8 hr. and thereafter the viable count remained stationary up to 24 hr., whereas in the immunized rabbit loop there was only a threefold multiplication in 8 hr. and about 50-fold multiplication in 24 hr. (Fig. 1). The *V. cholerae* strain multiplied about 100-fold in 8 hr. and 1000-fold in 24 hr. in normal rabbit loops, whereas in

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the loops of immunized rabbits the count showed a fivefold increase in 8 hr. and a 25-fold increase in 24 hr. (Fig. 2). In the normal loop the *V. eltor* vaccine strain multiplied 300-fold in 8 hr. and 1000-fold in 24 hr. whereas in immunized rabbits there was practically no increase in viable count even at 24 hr (Fig. 3).

All the loops of the normal animal injected with *V. cholerae* and El Tor pathogenic strain showed the typical gut-inflammatory reactions from 8 hr. onwards but in no case was there any reaction in the intestinal loops of immunized rabbits.

Table 5. *Comparative rate of multiplication of vibrios in intestinal loops of normal and immunized* rabbits*

Experiment no.	Strain no.	Viable count at: 0 hr.	4 hr. Normal‡	4 hr. Immunized‡	8 hr. Normal†	8 hr. Immunized‡	24 hr. Normal†	24 hr. Immunized‡
A	GS 9/65	$2{\cdot}0 \times 10^6$	$3{\cdot}5 \times 10^8$	$6{\cdot}0 \times 10^6$	$1{\cdot}9 \times 10^9$	$3{\cdot}6 \times 10^7$	$4{\cdot}9 \times 10^9$	$7{\cdot}6 \times 10^8$
	569B	$1{\cdot}3 \times 10^6$	$7{\cdot}0 \times 10^7$	$9{\cdot}5 \times 10^6$	$1{\cdot}8 \times 10^7$	$1{\cdot}5 \times 10^6$	$3{\cdot}2 \times 10^9$	$9{\cdot}5 \times 10^7$
	ME-7	$3{\cdot}0 \times 10^6$	$2{\cdot}3 \times 10^7$	$3{\cdot}5 \times 10^6$	$4{\cdot}3 \times 10^7$	$1{\cdot}6 \times 10^7$	$1{\cdot}1 \times 10^9$	$5{\cdot}5 \times 10^6$
B	GS 9/65	$3{\cdot}5 \times 10^6$	$1{\cdot}0 \times 10^9$	$1{\cdot}2 \times 10^6$	$5{\cdot}5 \times 10^9$	$2{\cdot}2 \times 10^6$	$1{\cdot}2 \times 10^9$	$2{\cdot}7 \times 10^7$
	569B	$3{\cdot}5 \times 10^6$	$3{\cdot}9 \times 10^8$	$1{\cdot}0 \times 10^5$	$1{\cdot}5 \times 10^9$	$5{\cdot}8 \times 10^7$	$3{\cdot}6 \times 10^9$	$2{\cdot}5 \times 10^6$
	ME-7	$1{\cdot}9 \times 10^6$	$1{\cdot}3 \times 10^8$	$2{\cdot}0 \times 10^7$	$1{\cdot}3 \times 10^{10}$	$5{\cdot}5 \times 10^4$	$1{\cdot}1 \times 10^{10}$	$6{\cdot}5 \times 10^5$
C	GS 9/65	$2{\cdot}6 \times 10^6$	$9{\cdot}0 \times 10^5$	$7{\cdot}5 \times 10^4$	$2{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^4$	$2{\cdot}3 \times 10^8$	$5{\cdot}9 \times 10^7$
	GS 1/65	$1{\cdot}5 \times 10^6$	$1{\cdot}6 \times 10^8$	$2{\cdot}1 \times 10^4$	$1{\cdot}1 \times 10^9$	$5{\cdot}3 \times 10^6$	$3{\cdot}6 \times 10^9$	$6{\cdot}0 \times 10^7$
	ME-7	$2{\cdot}5 \times 10^5$	$2{\cdot}1 \times 10^6$	$4{\cdot}5 \times 10^4$	$2{\cdot}2 \times 10^9$	0	$3{\cdot}3 \times 10^9$	0

* Immunization was carried out by intra-intestinal injection of viable ME-7 cells 8–9 days before challenge as described under Materials and Methods.

† There was reaction in all the loops injected with GS 9/65, GS 1/65 and 569B.

‡ There was no reaction in any loop.

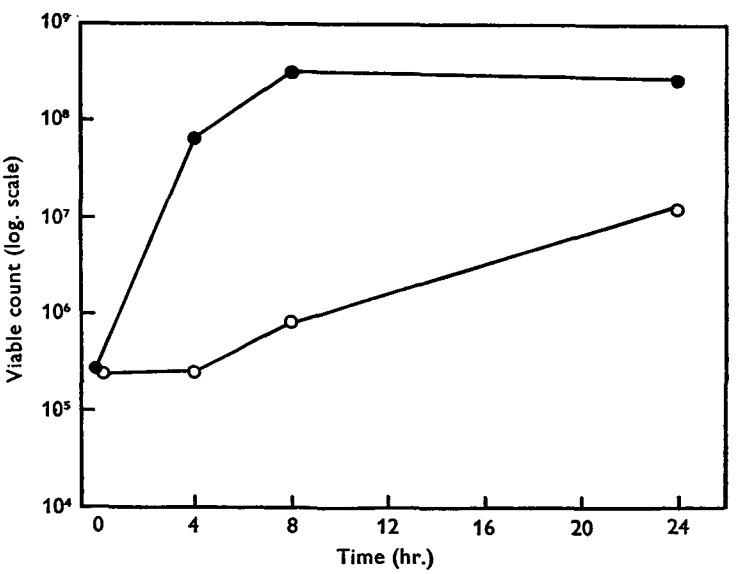

Fig. 1. Growth of pathogenic *Vibrio eltor* (GS 9/65) in the intestinal loop of normal and immunized rabbits. ●, Normal rabbit; ○, immunized rabbit.

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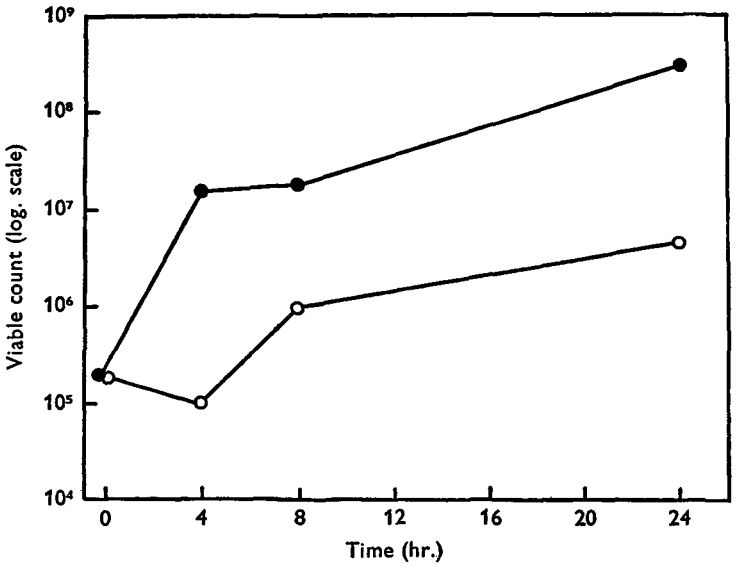

Fig. 2. Growth of *Vibrio cholerae* (569B) in the intestinal loop of normal and immunized rabbits. ●, Normal rabbit; ○, immunized rabbit.

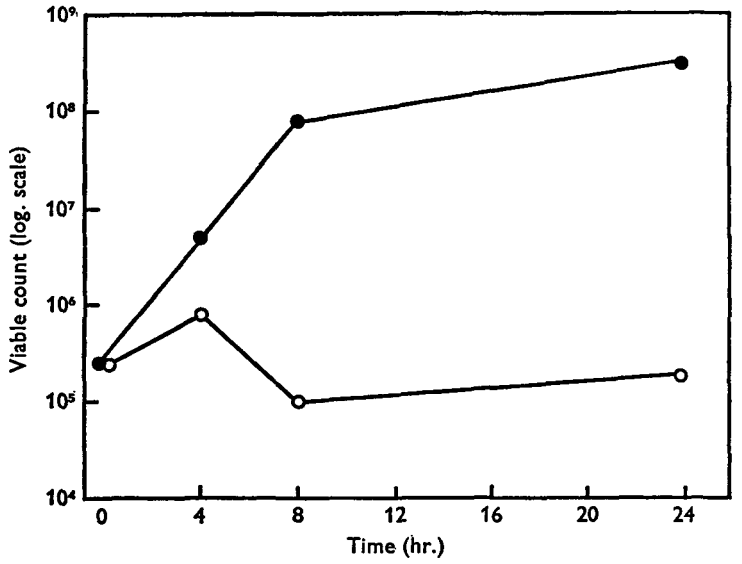

Fig. 3. Growth of the vaccine strain (ME-7) in the intestinal loop of normal and immunized rabbits. ●, Normal rabbit; ○, immunized rabbit.

DISCUSSION

It may be seen from the results in Tables 1 and 2 that the El Tor vaccine strains ME-7 and EW-6 proved apathogenic both in the ligated intestinal loops of adult rabbits and on intra-intestinal challenge in infant rabbits in marked contrast to cultures of *V. cholerae* and *V. eltor* strains isolated from cholera patients. Absence

of pathogenicity of the vaccine strains was confirmed to be a stable characteristic which remained unaffected by serial animal passages. However, the results of pathogenicity tests with the vaccine strains appears to have differed to some extent in different laboratories, which may be due to some difference in susceptibility to experimental cholera of rabbits from different stocks. The results presented here show that injection of a dose as high as $1{\cdot}0 \times 10^9$ viable cells of the vaccine strains failed to produce choleraic reaction in infant rabbits. The relative avirulence of the vaccine strains has been confirmed by Finkelstein, Norris & Dutta (1964). Dr John C. Feeley of the National Institute of Health, Bethesda, U.S.A., Dr Rolf Freter of the University of Michigan Medical School, U.S.A., and Dr H. Ogonuki of Chiba Serum Institute, Japan, to whom cultures of the water strains were sent for pathogenicity tests, also found them avirulent. J. C. Feeley (personal communication), after injecting over $1{\cdot}0 \times 10^9$ viable cells of ME-7 and EW-6 directly into the small intestines of infant rabbits or administering a similar number of vibrios orally, could not elicit diarrhoea, whereas diarrhoea was produced in infant rabbits given $1{\cdot}2 \times 10^2$ and $1{\cdot}5 \times 10^4$ living cells of a known virulent strain (VC 12). But he could obtain positive cultures at autopsy from all animals which had been given the water strains, except in one case of intra-intestinal challenge with ME-7; however, the latter animal was positive by rectal culture at 48 hr. after challenge, as were all other animals.

R. Freter (personal communication) after injecting $1{\cdot}0 \times 10^6$ viable cells directly into the small intestine, found that all animals receiving EW-6 and ME-7 survived the 20 hr. experimental period. There was a small amount of fluid accumulating in all the three animals receiving EW-6 and in one of the three receiving ME-7, but the quantity of fluid found was much less than with the two virulent strains he used.

H. Ogonuki (personal communication) also found ME-7 and EW-6 strains in heavy dose to be apathogenic in the infant rabbit test.

The finding of an occasional mild reaction produced by the vaccine strains is in accord with Mukerjee's observation (Mukerjee, 1963). Oza and Dutta's observation (Oza & Dutta, 1965) also indicates that these strains are not completely devoid of pathogenicity in experimental animals as the virulence of these strains could be enhanced by adding mucin, and EW-6 was found to be choleragenic even without addition of mucin. They used an unusually heavy inoculum (10^9 vibrios/100 g. of body weight of infant rabbit).

The residual virulence, which the water strains appear to possess, is an essential requirement for a live vaccine strain intended to stimulate the immunologically competent cells at the site of multiplication (Emel'janova, 1957). A completely avirulent strain is likely to be eliminated as an inert substance from the intestinal tract without causing any immunogenic reaction in the intestinal mucous membrane.

The vaccine strains administered by the intra-intestinal route have been found to protect adult rabbits against gut inflammatory reaction on subsequent challenge in ligated intestinal loops with both *V. cholerae* and *V. eltor* (case) strains. Similar protective immunity was not produced by oral vaccination of infant rabbits. The

failure of the vaccine to protect the infant rabbits was presumably due to their immaturity; it is known that the immunity-forming mechanism is not well developed in immature animals (Good & Papermaster, 1964).

Freter in recent years has done considerable work on the possibility of developing an oral cholera vaccine, but he used killed vaccines in his studies. Freter (1965) agreed that a live oral cholera vaccine should be better than killed cultures provided that the vaccine strain is capable of multiplication in the human intestinal tract. He felt that the low virulence of the vaccine strains proposed by Mukerjee was an expression of their inability to multiply in the lumen of the intestine. However, from the results reported in this paper it is clear that the vaccine strains are capable of multiplying in the gut of adult and infant rabbits. Dr Feeley observed that the strains survived in the intestine of infant rabbits at least up to 5 days (the period of his observation). The multiplication of the strains without giving rise to pathological reactions indicates that they are incapable of producing choleragenic toxin in the intestinal tract in sufficient concentration to give rise to an observable reaction in the animal model.

It is clear from the results presented here that multiplication of all the vibrio strains was markedly slower in immunized rabbits' gut than in the normal rabbits' gut. The difference was most marked during the first 4–8 hr., when in normal rabbits all the vibrio strains multiplied rapidly while in the immunized rabbits the multiplication was very sluggish. The total viable count of the vibrios in the intestinal loops of immunized rabbits at the end of 24 hr. also was less than in the loops of normal rabbits. The rates of growth of the vaccine strain, pathogenic *V. eltor* and *V. cholerae* in the immunized rabbits' loop increased in that order.

The inhibition of vibrio growth in immunized rabbits' intestinal loops may be due either to the soluble antibodies present in the lumen or to the phagocytic activity of the reticulo-endothelial cells of the intestine, the phagocytic index of which might have been increased by the previous exposure to antigen. However, the interval during which growth is slowed down was found to be constant (4 hr.) irrespective of the vibrio count in the inoculum, whereas if the initial phase of inhibition had been due to either specific antibodies or phagocytosis slowing down growth till such time as the antibacterial or phagocytic mechanism became overwhelmed, the period of growth-inhibition should have varied according to the size of the challenge dose of the vibrios.

The slower rate of vibrio multiplication is not likely to be the principal reason for the absence of gut-inflammatory reaction in immunized rabbits on challenge with virulent strains, since a total viable count of $1{\cdot}0 \times 10^8$ cells of pathogenic vibrios invariably produced reactions in intestinal loops of the normal rabbit, whereas in immunized rabbits' loops the same strain of vibrio multiplied to counts as high as $2{\cdot}8 \times 10^9$ cells in 24 hr. and yet failed to cause any reaction. This result suggests that apart from antibacterial antibodies, some degree of antitoxic immunity was produced as a result of multiplication of the vaccine strain in the intestine of vaccinated animals. Although it has not been possible to demonstrate production of soluble toxin by the vaccine strains in *in vitro* culture (R. A. Finkelstein and J. C. Feeley, personal communication), this does not constitute

unequivocal evidence that the vaccine strains are incapable of producing toxin in subminimal concentrations under suitable conditions. The failure to demonstrate choleragenic toxin in culture filtrate may equally well be explained on the supposition that the toxin concentration was insufficient to cause choleragenic symptoms in rabbit models, and a more sensitive method of assay might have enabled its detection. An alternative explanation of the absence of choleraic reaction in the intestinal loops of immunized rabbits is that the metabolism of the pathogenic vibrios may have been altered in such a way that they do not get the proper nutrient conditions required for the production of choleragenic toxin. An alteration in metabolic capacities is not unlikely in view of the observed effect on the dynamics of growth, and the work of Finkelstein *et al.* (1964) has established that even a prolifically toxinogenic strain forms toxin only when specific nutrient requirements are available in the culture medium.

The vaccine strains thus appear to be capable of getting established in the intestine and of multiplying there without causing disease. As a result of their multiplication, there is production of protective immunity in the intestine, which comprises both antibacterial and antitoxic activities. The naturally avirulent El Tor strains selected for study therefore appear to fulfil all the requirements of a live vaccine strain, as judged from the present laboratory tests.

SUMMARY

The possibility of developing a living oral cholera vaccine with naturally avirulent El Tor cultures isolated in a Middle East country or from water sources in Calcutta in the absence of cholera El Tor in these areas has been further studied.

In an extended series of experiments the markedly low pathogenicity of the proposed vaccine strains in laboratory animals has been confirmed. The vaccine strains have been shown to get established and multiply regularly in the ligated intestinal loops of adult rabbits and in the intestine of infant rabbits without producing pathogenic reactions.

The apathogenic character of the vaccine strains has been found to be stable. When propagated serially in these two laboratory models of experimental cholera the vaccine strains show no enhancement of pathogenicity.

Intra-intestinal administration of the live vaccine has been shown to protect adult rabbits fully in the intestinal loop test. Immunized animals were also protected against challenge with *V. cholerae* strains, though to a somewhat lesser extent. Protective immunity could not, however, be demonstrated in infant rabbits probably because of the immunity-forming mechanism being still rudimentary.

Immunization with live vaccine was found to inhibit the growth of homologous strains in the ligated intestinal loop of the adult rabbit. The growth rate of pathogenic *V. eltor* and *V. cholerae* strains was also seen to be markedly reduced throughout the 24 hr. period of observation, but more markedly during the first 4–8 hr. However, the total count of pathogenic *V. eltor* and *V. cholerae* strains at 24 hr. after inoculation in ligated ileal loops of immunized rabbits reached levels that invariably caused inflammation and accumulation of fluid in normal rabbits'

loops. The absence of gut-inflammatory reaction in immunized rabbits under these conditions has been discussed in relation to the nature of the immunity produced by administration of live vaccine.

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