Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1964, 31, 825-834

Comparison of Immune Mechanisms in Various Experimental Models of Cholera *

ROLF FRETER, Ph.D.[1]

Two of the main features of human cholera—induction of diarrhoea and confinement of the infection to the lumen of the intestinal tract—may be reproduced in three experimental models: (a) the streptomycin-treated, starved guinea-pig; (b) the intestinal loop in the adult rabbit; and (c) the suckling rabbit. In this paper the author compares the two last-mentioned models with his earlier work in guinea-pigs.

Intestinal antibody (coproantibody) was highly protective, while circulating antibody had little or no effect, in all three models. The protective coproantibody was specific for the heat-stable vibrio antigens. It did not affect the growth of vibrios in the intestine, and its function may possibly be regarded as antitoxic rather than antibacterial. Oral vaccination protected adult rabbits against challenge by the loop technique. Heat-killed vaccine was as effective in this respect as live vaccine.

The author feels that the present uncertainty concerning the protective value of cholera vaccination may be due to the fact that conventional vaccine is designed to induce high serum titres only. He considers that since oral vaccine has been shown to induce and maintain the production of coproantibody in human volunteers, a field trial should be carried out to determine whether coproantibody is as protective in man as it has proved to be in the experimental models.

INTRODUCTION

The problem of immunity to enteric infections such as cholera, shigellosis, etc. was first attacked by Pfeiffer in 1895 with his classical demonstration of intraperitoneal bacteriolysins in the guinea-pig. However, little basic progress was made in this field until about 50 years later, when Burrows, Elliot & Havens (1947) advanced the concept that intestinal antibody, which they termed "coproantibody", may be the protective agent in enteric diseases. Early work by the present writer (Freter, 1954, 1956b) on a cholera model in the starved, streptomycin-treated guinea-pig showed for the first time that circulating antibody was ineffective in preventing the fatal outcome of an experimental enteric disease. However, good protection was found in this model when the antibody was located in the lumen of the intestine. In applying these findings to the problem of immunity to human cholera, one must consider that the conventional parenteral cholera vaccine induces the production of coproantibody in man for only two to three weeks (Freter, 1962). Consequently, the doubtful value of parenteral cholera vaccine may be due to its failure to maintain an adequate level of coproantibody.

More recent work from this laboratory has laid the foundation for the actual testing of this concept in man by showing that coproantibody may be induced and maintained in human volunteers by means of a simple heat-killed oral cholera vaccine (Freter, 1962; Freter & Gangarosa, 1963). Consequently, the next logical step in this matter would be an actual field test in a human population of the effect of coproantibody, induced by an oral vaccine. This is obviously the only way of obtaining a decisive answer to the entire problem. However, a number of detailed questions should

* This study was aided by contract No. DA-49-193-MD-2307 with the US Army Research and Development Command, Department of the Army. The able technical assistance of Marianna Wakulowska and Sergey Harkaway is greatly appreciated.

[1] Department of Microbiology, Jefferson Medical College, Philadelphia, Pa., USA.

be settled before one can justify the considerable expense in time and funds which such a field trial would entail.

The first question concerns the nature of the protective antibody. This information is necessary to decide which bacterial antigen(s) should be included in the human vaccine to be field-tested. This problem has become acute in the past ten years with the discovery of certain "toxins" or "active substances" in experimental enteric infections (De & Ghose, 1960; Dutta, Panse & Kularni, 1959; Oza & Dutta, 1963; Finkelstein & Norris, 1964), and with the findings of antigenically distinct fimbriae (Duguid, 1959) and of "adherence factors", possibly related to a slime layer (Bales & Lankford, 1961), in various enteric pathogens. The latter antigens enable micro-organisms to adhere to red blood cells and to suspensions of mucosal cells. *In vivo* adhesion of bacteria to the intestinal wall would almost certainly constitute a major virulence factor for enteric pathogens. Consequently, if such a phenomenon could be demonstrated *in vivo*, attempts should be made to include "adherence factor" antigens in an enteric vaccine.

Our earlier work on guinea-pigs had shown that antibody to heat-stable antigen was protective if it was present in the intestinal tract. Since that time, two other experimental models of enteric infections have been studied extensively by others—namely, the suckling rabbit (Dutta & Habbu, 1955) and the intestinal loop model in the adult rabbit (De & Chatterjee, 1953). These two models differ considerably—among themselves and as compared with the guinea-pig model—in the experimental manipulations that are necessary before cholera-like disease can be induced. The models are thus likely to differ also in the mechanisms of pathogenicity. Consequently, the probability that a given experimental finding applies to human disease seems much greater when this finding is consistently obtained in all three of these models, in contrast to results which are peculiar to only one experimental design. The present paper therefore describes an attempt to compare the effect of serum and intestinal antibody in the suckling rabbit and in the intestinal loop models with our earlier findings in guinea-pigs. A similar comparative study of antibody quality will also be described. As will be seen, the general pattern which is consistent in all three models shows that circulating antibody had very little, if any, effect, while coproantibody was highly protective. Coproantibody to the heat-stable antigens, or an oral vaccine containing heat-killed bacteria, were sufficient in all three systems, while the addition of heat-labile antigens to the vaccine did not enhance protection. As will be discussed later, these findings seem sufficient to justify a field trial in a human population of an oral vaccine containing heat-killed vibrios.

MATERIALS AND METHODS

The suckling rabbit model

The method of Dutta & Habbu (1955) was followed with minor modifications: 0.5 ml of a 10-hour-old *Vibrio cholerae* broth culture, diluted in saline, was injected directly into the small intestine of lightly chloroformed 9- to 12-day-old rabbits. In some experiments the inoculum also contained 0.05 ml of diluted normal or immune rabbit serum, which was mixed with the culture immediately before being injected into each animal. The minimum infective dose of vibrios was not determined accurately, but it was established that a dose of 1000 vibrios infected 100% of the animals. In all the experiments reported here, a dose of about 10 000 to 20 000 vibrios per animal was given, which thus constitutes at least ten and probably considerably more infective doses. The animals were sacrificed 16-18 hours after infection. A positive result ("diarrhoea") was recorded when large amounts of fluid had accumulated in the caecum. Frequent discharge of fluid stools could be observed in most positive animals. On autopsy, such rabbits also showed large amounts of fluid in the small intestine as well as in the caecum. Control experiments with sterile broth inocula showed normal intestines in all animals tested. Veal infusion broth or trypticase soy broth were used throughout the present study.

The isolated intestinal loop model in the adult rabbit

The animals used were female white rabbits weighing about 6 lb (3 kg). Food and water were withheld on the day before infection. Four loops about 10 cm long were prepared in the lower small intestine of each rabbit, using chloroform anaesthesia. The loops were injected with 10-hour-old broth cultures of *V. cholerae* diluted in saline. Two of the loops received an inoculum of about 10×10^6 cells and two an inoculum of about 1×10^6 cells, the total volume injected into each rabbit

being 2 ml. Ten milligrams of streptomycin per loop were added to each inoculum to suppress normal flora. Injection of the inocula was made through a 27-gauge (0.40-mm) needle inserted at one end of the loop. The area containing the puncture wound was then isolated from the main body of the loop by tying it off with a thread. This precaution was suggested by Sasaki, Ghoda & Kobari (personal communication, 1963) to minimize erratic results. It was frequently observed in the present studies that the segment containing the puncture wound was distended with fluid even when the two adjacent loops were negative. The animals were sacrificed 16-18 hours after infection. Positive loops were distended with large amounts of fluids. Sterile inocula never induced positive loops.

The strain used for all infections was a streptomycin-resistant *V. cholerae*, Ogawa type—the same strain as that used in earlier studies (Freter, 1956b). In order to ensure continued high virulence, the strain was frequently re-isolated from strongly positive non-immunized control animals. The effect of possible day-to-day variations in virulence of the inoculum and/or susceptibility of the experimental animals was minimized by including an equal number of immunized animals and of non-immunized controls in each single experiment. A single experiment was always carried out within a time interval of 4 hours or less, and usually comprised a total of 6-8 suckling rabbits or 4 adult rabbits (containing a total of 16 intestinal loops). All the tables presented in this paper therefore show the combined data from several single experiments. The data on the effect of antiserum in the lumen of the intestinal loop were obtained by preparing two loops containing antiserum and two loops with normal serum in the same animal. The position of these loops in individual animals was randomized. Antiserum was added to the inocula immediately before injection into the loops. Bacterial counts to determine *in vivo* growth of vibrios were made by removing the entire intestine from infected suckling rabbits (or the entire loop from adult rabbits), homogenizing it in 30 ml of broth in a Virtis "45" homogenizer, and plating appropriate saline dilutions of the homogenate on veal infusion agar containing 1 mg/ml streptomycin. The walls of the homogenizing vessels were treated with Dow Antifoam A. Each vessel contained a small crystal of *n*-octadecyl alcohol to further reduce foaming.

Oral vaccination

The vaccines were prepared from saline suspensions of agar-grown vibrios of the same strain as that used for the challenge infections. The preparations were lyophilized either directly (live vaccine) or after boiling in flowing steam for 20 minutes (heat-killed vaccine). The dried powder was then dispensed into No. 5 gelatin capsules, which were fed to the animals through a glass tube. Each capsule contained about 35 mg of the live vaccine or 12 mg of the heat-killed material.

The rabbits were fed an average of 15 daily doses. They were challenged on the 4th day after the last dose of vaccine.

Antiserum

Two batches of hyperimmune rabbit serum (No. 69 and No. 70) were used throughout the study. These had been prepared with the same strain of *V. cholerae* as was used for challenge infections. The immunizing antigen for these sera was a washed saline suspension of agar-grown vibrios heated in flowing steam for two hours. The sera were sterilized by Seitz filtration and inactivated by heating at 56°C for 45 minutes.

Haemagglutination

Red blood cell suspensions from various species were prepared from freshly drawn citrated blood by washing three times in Tris[1]-buffered saline, pH 7.5. A concentration of 5% was used in routine titrations. Serial twofold dilutions of the bacteria to be titrated were prepared in Tris-buffered saline in 13 mm × 100 mm tubes, and mixed with equal volumes of red cell suspensions. The mixtures were incubated at 37°C for 1 hour. Haemagglutination was read either immediately or after the tubes had been allowed to stand overnight at 4°C. A positive reaction was indicated by a characteristic "pattern" of red cells covering the entire bottom of the tube.

In order to correlate haemagglutination with adsorption to the intestinal wall, several million bacterial cells were injected directly into the duodenum of chloroformed mice, guinea-pigs or rabbits. The haemagglutinin titres of the suspensions used for animal inoculation were determined before each experiment. Haemagglutinating strains always showed titres of 1:256 to 1:1024 in suspensions containing about 10^9 vibrios per ml. The inoculum also contained a non-absorbable

[1] Tris(hydroxymethyl)aminomethane.

radioactive tracer, ^{131}I *p*-toluidine polyvinylpyrrolidone (^{131}I-PVP). The relative distribution of tracer and bacteria some hours after injection was used as a measure of retention of the micro-organisms. Distribution was determined by dividing the small intestine into three portions of equal length. The caecum constituted the fourth portion. These four specimens were homogenized separately in a Virtis blender and assayed for ^{131}I and bacterial concentration.

RESULTS

Immunity in the suckling rabbit model

The results obtained are shown in Table 1. As may be seen, 0.0005 ml of *V. cholerae* O-antiserum protected 8 out of 9 animals, while all rabbits receiving normal rabbit serum showed diarrhoea.

TABLE 1

PROTECTIVE EFFECT OF *VIBRIO CHOLERAE* O-ANTISERUM No. 69 IN THE SUCKLING RABBIT

Type of serum	Amount of serum (ml)	Route of administration	Animals with diarrhoea	
			No.[a]	%
Anti-O	0.0005	Into lumen of intestine	1/9	11
Normal	,,	,,	8/8	100
Anti-O	0.0005	intraperitoneal	8/8	100
Normal	,,	,,	11/11	100
Anti-O	0.05	intracardial	12/13	92
Normal	,,	,,	9/10	90

[a] Number of animals with diarrhoea/number examined.

When the same amount of antiserum was given intraperitoneally four hours before infection, all animals showed diarrhoea which was as strong as that of the controls. One hundred times this amount of antiserum (0.05 ml) had no protective effect when injected intracardially at the time of infection. The above data indicate that antibody, in order to be protective, had to be located in the intestinal lumen (i.e., had to be coproantibody), while systemic antibody had no demonstrable effect.

The serum used contained antibody only to the heat-stable vibrio antigens (O-antibody). It therefore seemed possible that the protective effect of intestinal antibody might be due to some antibacterial mechanism. Table 2 compares the numbers of vibrios recovered from immunized and non-immunized rabbits at different times after infection.

TABLE 2

EFFECT OF *VIBRIO CHOLERAE* O-ANTISERUM No. 70 ON THE NUMBER OF VIBRIOS RECOVERED FROM THE INTESTINE OF SUCKLING RABBITS AT DIFFERENT TIMES AFTER INFECTION

Number of vibrios ($\times 10^{-6}$) recovered from:	
Animals with antiserum [a]	Animals with normal serum [a]
4 hours after infection	
0.56	1.2
0.96	2.4
6.7	3.6
7.4	4.4
12.4	6.5
12.8	14.2
19.4	15.9
34.3	27.1
52.0	
19 hours after infection	
1.8	2 400
16.6	2 880
24.0	3 200
28.0	3 520
64.0	4 000
65.2	6 400
132	13 200
400	

[a] 0.005 ml given with the inoculum directly into the intestinal lumen.

The data indicate that antibody did not inhibit the initial growth of vibrios in the immunized animals. This confirms earlier results in guinea-pigs and mice (Freter, 1955, 1956a). Only late in the course of infection were much greater numbers of vibrios recovered from the non-immunized animals than from the immunized ones (Table 2), i.e., after the

TABLE 3

EFFECT OF *VIBRIO CHOLERAE* O-ANTISERUM No. 69 ON THE DEVELOPMENT OF POSITIVE INTESTINAL LOOPS IN ADULT RABBITS CHALLENGED WITH *V. CHOLERAE*

	Antiserum			Normal serum (all routes)
	into lumen of intestine		intravenous	
	(0.002 ml/loop = 0.004 ml/animal)	(0.2 ml/loop = 0.4 ml/animal)	(0.4 ml/animal)	
Number of positive loops/number examined	8/14	1/18	15/32	41/60
Percentage of positive loops	57	5.5	47	68
P [a]	>0.05	<0.001	<0.05	—

[a] Significance of difference from control group (normal serum), determined by the χ^2 test.

non-immune host had reacted by the outpouring of fluids which presumably constituted a good growth medium for further multiplication of the vibrios. One must therefore conclude that the antibody protected the animals by a mechanism which was not antibacterial. Most likely, the antibody interfered with the production or action of a substance which was involved in the mechanism of causing diarrhoea in suckling rabbits.

Immunity in the intestinal loop model in adult rabbits

Table 3 indicates that almost complete protection may be achieved with an amount of antiserum somewhere between 0.002 ml and 0.2 ml per loop. This is a much larger amount of antiserum than that required to protect a suckling rabbit (Table 1). Again, as with the suckling rabbit model, antiserum administered parenterally had little effect. In order to assess the effect of intestinal *versus* systemic antibody with more accuracy, these experiments were repeated with a large pool of serum (batch No. 70). The results (Table 4) indicate that about 1000 times more systemic antibody (4 ml/animal) was required to achieve the same degree of protection as that afforded by coproantibody (0.004 ml/animal). However, after an intravenous injection of as much as 4 ml of hyperimmune antiserum, the amount of antibody leaking into the intestinal lumen may become significant (Burrows & Havens, 1948). Consequently, much or perhaps even all of the protective effect of passive intravenous immunization

TABLE 4

EFFECT OF *VIBRIO CHOLERAE* O-ANTISERUM No. 70 ON THE DEVELOPMENT OF POSITIVE INTESTINAL LOOPS IN ADULT RABBITS CHALLENGED WITH *V. CHOLERAE*

	0.002 ml serum into loop = 0.004 ml serum per animal		4 ml serum per animal; intravenous	
	Anti-serum	Normal serum	Anti-serum	Normal serum
Number of positive loops/ number examined	13/32	25/32	11/32	19/28
Percentage of positive loops	40.6	78.1	34.3	67.9
P [a]	<0.01	—	<0.01	—

[a] Significance of difference from control groups (normal serum), determined by the χ^2 test.

(Table 4) may actually have been due to coproantibody derived by diffusion.

The effect of oral vaccine is shown in Table 5. As may be seen, oral vaccination reduced the frequency of positive loops by a factor of 2-3. Heat-killed vaccine was as effective as live vaccine (the difference between the two vaccinated groups is not statistically significant). As mentioned above, much less coproantibody was needed to protect a suckling rabbit than to protect an intestinal loop in the adult rabbit. One may consequently assume that the amount of coproantibody induced by oral vaccination in the adult rabbits (Table 5) would

TABLE 5

OCCURRENCE OF POSITIVE LOOPS, ONE DAY AFTER CHALLENGE WITH *VIBRIO CHOLERAE*, IN RABBITS RECEIVING 15 DAILY DOSES OF ORAL CHOLERA VACCINE

Vaccine	Number of positive loops/number examined	Percentage of positive loops	P [a]
Live vibrios	15/40	37.5	<0.001
Boiled vibrios	5/20	25	<0.001
Non-vaccinated controls	\45/64	70	—

[a] Significance of difference from control group, determined by the χ^2 test.

have been sufficient to effect 100% protection in suckling rabbits.

Table 6 shows that intra-intestinal administration of antiserum, in amounts that were highly protective, did not reduce the *in vivo* growth rate of the vibrios. As in the suckling rabbit model, the protective effect of antibody was therefore not based on an antibacterial mechanism.

TABLE 6

EFFECT OF *VIBRIO CHOLERAE* O-ANTISERUM No. 70 ON THE NUMBER OF VIBRIOS RECOVERED FROM ISOLATED INTESTINAL LOOPS OF ADULT RABBITS FOUR HOURS AFTER INFECTION

Number of vibrios ($\times 10^{-6}$) recovered from:	
Loops with antiserum [a]	Loops with normal rabbit serum [a]
Inoculum: 5×10^6 cells	
0.51	1.33
12.5	17.6
57.8	40.8
153	81.6
173	98.6
228	316.2
Inoculum: 750 000 cells	
0.81	0.37
11.2	1.60
11.6	4.69
17.8	6.90
20.9	8.97
54.4	20.0

[a] 0.2 ml per loop given with the inoculum directly into the lumen

Haemagglutination and adsorption on the intestinal wall

Several strains of vibrios and one strain of *Escherichia coli* were tested for *in vitro* adherence to red blood cells (haemagglutination) of several animal species. All four smooth *V. cholerae* strains tested reacted with rabbit and mouse but not with guinea-pig cells. Three rugose *V. cholerae* strains reacted with cells from all three species and the one *Esch. coli* strain did not agglutinate cells of any of the species tested. In the rugose vibrios the haemagglutinating property was associated with the bacterial cells. Washed suspensions of vibrios retained their original reactivity. Broth cultures lost their haemagglutinating properties after removal of the bacteria by centrifugation. The haemagglutinin in saline suspensions of agar-grown rugose vibrios was partially destroyed by heating at 56°C for 15 minutes. Heating in flowing steam resulted in complete destruction. *In vitro* haemagglutination was not affected by changes in the pH of the system within the range of pH 6.1-7.5.

Three strains were selected for the *in vivo* studies:

one rugose strain of *V. cholerae* (44R);
one smooth strain of *V. cholerae*;
one non-haemagglutinating *Esch. coli* (C 25).

Representative data obtained with guinea-pigs are shown in Table 7. Within the limits of experimental error, there was no difference in the distribution of the two bacterial species. Retention of bacteria in the uppermost specimen (S1) of the small intestine was actually somewhat higher (higher B:I ratio) for the non-haemagglutinating *Esch. coli*. However, this difference was not significant. Similar results were obtained with all three bacterial strains mentioned above, in a total of about 80 animals of the three species studied.

One must therefore conclude that the ability of the bacterial cells to adsorb *in vitro* on certain red blood cells had no obvious counterpart in the intestinal tract of intact animals. Consequently, inclusion of antigenic "adhesion factors" into enteric vaccines does not seem necessary unless the need for such inclusion could be demonstrated in

TABLE 7

RECOVERY OF HAEMAGGLUTINATING OR NON-HAEMAGGLUTINATING BACTERIA AND RADIOACTIVE TRACER (^{131}I-PVP) FROM THE INTESTINE OF GUINEA-PIGS THREE HOURS AFTER INJECTION OF BACTERIA INTO THE DUODENUM

Section of intestine [a]	*Vibrio cholerae* 44R (haemagglutinating)			*Escherichia coli* C25 (non-haemagglutinating)		
	Percentage recovery of:		B: I ratio	Percentage recovery of:		B: I ratio
	bacteria (B)	^{131}I (I)		bacteria (B)	^{131}I (I)	
S 1	1.49	0.62	2.4	20.0	9.9	2.02
S 2	32.7	22.2	1.47	50.0	50.5	1.0
S 3	62.3	48.5	1.29	30.0	35.4	0.85
C	3.71	28.5	0.13	0	0.64	—
S 1	1.68	2.66	0.63	2.43	1.74	1.40
S 2	12.2	7.1	1.72	23.9	22.2	1.08
S 3	85.0	86.0	1.0	72.8	47.2	1.54
C	0.96	4.5	0.21	0.80	27.3	?
S 1	0.11	0.44	0.25	6.8	7.6	0.89
S 2	0.96	0.70	1.37	81.0	84.0	0.96
S 3	50.0	18.3	2.73	11.85	8.0	1.48
C	48.6	80.3	0.61	0.08	0.31	0.26
S 1	1.7	5.0	0.34	0.96	0.76	1.26
S 2	8.45	30.5	0.28	99.1	98.8	1.0
S 3	66.2	63.0	1.05	0	0.11	—
C	23.8	1.52	15.6	0	0.24	—

[a] S 1, S 2, and S 3 = upper, middle, and lower third of small intestine; C = caecum.

future studies. This finding does not exclude the possibility, postulated earlier by various authors (Stoerk, 1916; Lankford, 1960; Freter, Smith & Sweeney, 1961), that cholera vibrios may adhere to the intestinal wall during cholera. Such a phenomenon has now actually been observed by LaBrec & Formal (personal communication, 1964), who found the mucosa of infected guinea-pigs to be covered with vibrios that could be demonstrated by the fluorescent antibody technique. The present data do suggest, however, that the mechanism of such *in vivo* adherence may differ from that causing *in vitro* haemagglutination.

DISCUSSION

A comparison of the above results with previous data obtained in guinea-pigs indicates that the following observations apply to all three experimental cholera models.

(*a*) Coproantibody was protective while circulating antibody had little or no effect.

(*b*) Antibody to the heat-stable vibrio antigens was sufficient to induce protection.

(*c*) The mechanism by which coproantibody against heat-stable antigens protected these animals was not antibacterial, i.e., it did not reduce the *in vivo* growth rate of vibrios.

The importance of antibody to heat-stable antigens was further underlined by the present finding that oral vaccine containing boiled vibrios was at least as effective as live oral vaccine.[1]

[1] These results are analogous to earlier findings by Sasaki, Ghoda & Kobari (personal communication, 1963), who were able to protect rabbits against challenge with *Shigella* by means of acetone-killed oral *Shigella* vaccines.

The present results are entirely compatible with those reported by Jenkin & Rowley (1960) and by Panse, Jhala & Dutta (1964). However, the conclusions reached by the latter authors differ from those outlined above. The reason for this discrepancy must be sought in the fact that the Indian workers used passive immunization administered by parenteral routes only. According to the present data, this type of administration is not very effective. Their conclusion that antibody against the heat-stable vibrio antigens (O-antibody) is not protective is therefore valid only for circulating antibody and does not apply to coproantibody of the same specificity, the high effectiveness of which was demonstrated in the present paper. The protective value of an anti-live-vibrio serum reported by these authors may be attributed to the fact that this particular serum had by far the highest titre of O-antibodies (agglutinins, etc.) of the sera used in their study. Consequently, with the enormous amounts of antiserum injected parenterally (about 10 000 times the protective dose of coproantibody), the amount of O-antibody diffusing into the gut may have been sufficient to be protective. Thus, while it is very probable that vibrio antigens other than the heat-stable ones may also induce protection against experimental cholera, this has not been demonstrated conclusively at the present time.

The present data show that a high degree of protection may be obtained with coproantibody directed against the heat-stable vibrio antigens. Since this is consistent in all three experimental models, the probability is high that a similar effect may be obtained in man also. This probability is further increased by the recent finding that coproantibody appears regularly in cholera convalescents (Freter, 1964). As demonstrated earlier, the only practicable means of maintaining coproantibody in man is by the use of an oral vaccine. A rather interesting, though not necessarily valid, comparison may be made by considering that the median titre of coproantibody in the stools of orally vaccinated human volunteers was about 1:1.5 Freter, 1962; Freter & Gangarosa, 1963). One can then calculate that the amount of antibody necessary to protect a suckling rabbit in the present study was contained in about 100 ml of liquid stool from the human volunteers. Assuming the amount of fluid that passes the human small intestine every day to be about 20 litres, one may calculate that an orally vaccinated volunteer produced every 7 minutes an amount of coproantibody that was sufficient to neutralize an inoculum of 10 000 to 20 000 vibrios in the suckling rabbit. However, since coproantibody was not antibacterial, its protective action in the suckling rabbit probably occurred several hours after infection, i.e., at a time when proteolysis and peristalsis had reduced the amount of serum remaining to a small fraction of the amount originally given. It is therefore probable that the above comparison considerably underestimates the relative effectiveness of human coproantibody production.

As pointed out above, the very small amounts of antiserum introduced into the intestinal lumen in the present experiments cannot be expected to remain there indefinitely. Since the effect of O-antibody was not antibacterial, one must assume that the passive protection demonstrated in the present experiments was temporary, lasting only until the moment when the original amount of antibody had been destroyed by intestinal enzymes, or until it had been neutralized by continued production of bacterial antigen. For this reason the choice of 16 to 18 hours after infection as the duration of the present protection studies may have been important. Obviously, such considerations do not apply to individuals actively immunized with oral vaccines, since there is continuous production of coproantibody under these circumstances.

One must conclude from the above discussion that only a field trial will decide whether the amount of coproantibody that can be induced in man by oral vaccination is sufficient to confer protection against natural cholera. The present results do indicate, however, that there is a high probability of obtaining positive results even when the oral vaccines used in such a trial contain nothing more complex than heat-killed vibrios. A killed vaccine would circumvent the potential risk and the difficulties in production and storage associated with trials of live attenuated strains. There is at present no evidence in experimental or natural cholera for any "cellular" or "local tissue" immunity other than that mediated by coproantibody. Consequently, the only definite advantage to be gained by the use of a live oral vaccine would be a possible wider spacing of successive vaccine doses that might be permissible if the vaccine contained strains which actually multiply in the human intestine. In other words, growth of a vaccine strain in the intestinal lumen might be expected to substitute for one or several

subsequent oral doses of vaccine which would have to be given in the absence of such growth. Unfortunately, however, bacteria introduced into the enteric flora do not usually multiply to any extent (Sears, Brownlee & Uchiyama, 1950 ; Freter, 1956a ; Zubrzyki, 1958). Indeed, the relatively low virulence of certain El Tor strains, recently recommended for use in a live oral vaccine, was a function of their inability to multiply in the lumen of the intestine (Mukerjee, 1963). The development of vibrio strains suitable for use in a live attenuated oral vaccine may therefore present some difficulties.

In summary, then, a field trial comparing oral cholera vaccine containing heat-killed vibrios with the conventional parenteral preparations is, at present, the most promising approach towards elucidation of the possible protective effects of coproantibody and systemic antibody in human cholera. Once this basic question has been decided, further efforts to simplify the technical aspects of oral immunization would be justified. Such later efforts might be directed at minimizing the number of doses of oral vaccine that are necessary to induce protection. This might be accomplished by developing live attenuated vibrio strains that are capable of extensive *in vivo* multiplication, or by the use of parenteral vaccination to *induce* copro-antibody, followed by widely spaced doses of oral vaccine to *maintain* the production of coproantibody in the immunized individual. Other protective antigens, if and when they are discovered, might also be included in the then-existing type of vaccine.

RÉSUMÉ

Dans un travail antérieur, l'auteur avait démontré expérimentalement — sur le cobaye dénutri traité par la streptomycine — l'opinion selon laquelle les anticorps intestinaux, dits copro-anticorps, jouent un rôle essentiel dans la protection contre le choléra, dont les anticorps circulants sont impuissants à empêcher l'issue fatale.

Le vaccin anticholérique administré par voie parentérale ne stimule la production de copro-anticorps que pendant 2-3 semaines. Il est possible que la valeur incertaine de ce vaccin soit due à son incapacité de maintenir assez longtemps un taux de copro-anticorps assez élevé. Des essais effectués sur l'homme ont montré d'autre part qu'un vaccin anticholérique tué par la chaleur, administré par voie buccale, provoque la formation et assure la persistance de copro-anticorps.

L'auteur a poursuivi l'étude de la question sur deux autres modèles expérimentaux utilisés dans ce type de recherches: l'anse intestinale de lapin isolée par ligature, et le lapereau non sevré. Il a comparé ces résultats à ceux qu'il avait obtenus antérieurement sur le cobaye.

Dans les trois modèles, l'immunisation passive par voie parentérale a été sans action, tandis que de petites quantités de copro-anticorps assuraient une bonne protection. Les copro-anticorps agissent contre les antigènes thermostables du vibrion. Le mécanisme de protection n'est pas antibactérien: tout en empêchant la diarrhée, les copro-anticorps ne diminuent pas le taux de multiplication du vibrion dans l'intestin; leur action semble donc plutôt antitoxique. Le vaccin oral contenant des vibrions tués par la chaleur a protégé le lapin contre l'infection d'épreuve — par le test de l'anse intestinale. Un vaccin vivant ne s'est pas montré plus efficace que le vaccin tué.

Du fait que les trois méthodes expérimentales en usage indiquent l'inefficacité des anticorps circulants et le degré élevé de protection conféré par les copro-anticorps, l'auteur conclut que l'étape suivante des recherches sur l'immunologie du choléra consisterait à organiser une épreuve pratique dans une collectivité humaine, en vue de comparer l'action des anticorps circulants à celle des copro-anticorps, c'est-à-dire l'effet d'un vaccin buccal contenant des vibrions tués par la chaleur à celui des préparations administrées par voie parentérale, actuellement en usage.

Certaines questions cependant doivent être élucidées avant que l'on ne s'engage dans cette voie, en particulier celle de la diminution du nombre de doses de vaccin buccal nécessaires — peut-être par l'emploi d'un vaccin vivant atténué — et celle de la combinaison éventuelle d'une vaccination parentérale et d'une vaccination orale, destinée à maintenir la production de copro-anticorps pendant un temps suffisant.

REFERENCES

Bales, G. L. & Lankford, C. E. (1961) *Bact. Proc.*, p. 118

Burrows, W., Elliott, M. E. & Havens, J. (1947) *J. infect. Dis.*, **81**, 261

Burrows, W. & Havens, I. (1948) *J. infect. Dis.*, **82**, 231

De, S. N. & Chatterjee, D. N. (1953) *J. Path. Bact.*, **66**, 559

De, S. N. & Ghose, M. L. (1960) *J. Path. Bact.*, **79**, 373
Duguid, J. P. (1959) *J. gen. Microbiol.*, **21**, 271
Dutta, N. K. & Habbu, M. K. (1955) *Brit. J. Pharmacol.*, **10**, 153
Dutta, N. K., Panse, M. V. & Kularni, D. R. (1959) *J. Bact.*, **78**, 594
Finkelstein, R. A. & Norris, H. T. (1964) *Fed. Proc.*, **23**, 144
Freter, R. (1954) *Bact. Proc.*, p. 56
Freter, R. (1955) *J, infect. Dis.*, **97**, 57
Freter, R. (1956a) *J. exp. Med.*, **104**, 411
Freter, R. (1956b) *J. exp. Med.*, **104**, 419
Freter, R. (1962) *J. infect. Dis.*, **111**, 37
Freter, R. (1964) *J. infect. Dis.* (in press)
Freter, R. & Gangarosa, E. J. (1963) *J. Immunol.*, **91**, 724
Freter, R., Smith, H. L., jr & Sweeney, F. J., jr (1961) *J. infect. Dis.*, **109**, 35
Jenkin, C. R. & Rowley, D. (1960) *Brit. J. exp. Path.*, **41**, 24
Lankford, C. E. (1960) *Ann. N.Y. Acad. Sci.*, **88**, 1203
Mukerjee, S. (1963) *Bull. Wld Hlth Org.*, **29**, 753
Oza, N. B. & Dutta, N. K. (1963) *J. Bact.*, **85**, 497
Panse, M. V., Jhala, H. I. & Dutta, N. K. (1964) *J. infect. Dis.*, **114**, 26
Pfeiffer, R. (1895) *Z. Hyg. Infekt.-Kr.*, **19**, 75
Sears, H. J., Brownlee, I. & Uchiyama, J. K. (1950) *J. Bact.*, **59**, 293
Stoerk, O. (1916) *Beitr. path. Anat.*, **62**, 121
Zubrzyki, L. J. (1958) *Development and application of the continuous flow principle to the study of normal human fecal flora*, Philadelphia (Doctoral Dissertation, Temple University)