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EVENTS IN THE PATHOGENESIS OF EXPERIMENTAL CHOLERA: ROLE OF BACTERIAL ADHERENCE AND MULTIPLICATION

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VIBRIO CHOLERAE, a causative organism of cholera, reaches the intestine by the oral route, where a series of events occurs which leads to induction of disease (Finkelstein, 1975).

Because vibrios do not invade the body and toxin is ultimately responsible for disease, toxin must be released in the intestine. Not much is known about the factors that determine the release of toxin *in vivo*. Studies during the past few years have shown that motility and chemotaxis of vibrios and their ability to adhere to intestine are associated with virulence of *V. cholerae* (LaBrec *et al.*, 1965; Freter, 1969; Guentzel and Berry, 1975; Freter and Jones, 1976; Schrank and Verwey, 1976; Allweiss *et al.*, 1977; Bhattacharjee and Srivastava, 1979). By the use of high-resolution transmission and scanning electron microscopy, adherence of vibrios to the intestine has been demonstrated (Patnaik and Ghosh, 1966; Nelson, Clements and Finkelstein 1976). It is not known whether vibrios adhere to the intestinal mucosa to resist removal from the intestine (Dixon, 1960) or whether adherence plays a more specific role in pathogenesis. Is it essential for vibrios to undergo a few cell divisions before elaborating toxin?

The present work was undertaken to study adherence and multiplication of some wild-type and laboratory-derived mutants of *V. cholerae* in the ligated ileal loops of adult rabbits to assess their role in the pathogenesis of cholera.

MATERIALS AND METHODS

Vibrio strains

These are listed in table I. The overnight nutrient-agar-slope cultures of bacteria were suspended in 8 ml of phosphate-buffered saline (PBS, pH 7.2). The suspension was diluted $\times 50$ in nutrient broth and incubated at 37°C until the viable count was *c.* 10^9 /ml. Such cultures were diluted according to the design of the experiment. The streptomycin-dependent strain (KB605)

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TABLE I
List of vibrio strains

Strain no.	Relevant properties	Motility	Source/Reference
KB207	<i>str</i> ^r , virulent el Tor	Motile	K. Bhaskaran
KB619	<i>str</i> ^r , virulent classical	Motile	This work
C14-S5	A dwarf-colony mutant, <i>str</i> ^r	Feebly motile	Bhaskaran and Sinha (1967)
CD2	Attenuated strain, normal adherence, <i>str</i> ^r	Motile	B. S. Srivastava (unpublished)
CD11	Non-adherent strain derived from KB207, <i>str</i> ^r	Motile	R. Srivastava and B. S. Srivastava (unpublished)
KB365	<i>str</i> ^r variant derived from 569B	Feebly motile	K. Bhaskaran
KB378	<i>str</i> ^r	Non-motile	K. Bhaskaran
KB605	Streptomycin-dependent, derived from KB207	Motile	K. Bhaskaran

str^r = Streptomycin-resistant mutant.

was grown in broth containing streptomycin 1 mg/ml. Before this strain was inoculated into rabbits the culture was washed and incubated in streptomycin-free broth for 30–60 min. at 37°C.

Culture media

Nutrient Broth (Difco, Detroit, Mich.) 0.8% (w/v) was used. Viable counts were made on nutrient-agar plates containing streptomycin 500 µg/ml or 1 mg/ml for strain KB605. Bacterial dilution, washing and homogenisation of the intestine were done in PBS.

Rabbit ileal-loop model

Swiss Albino rabbits weighing 1–1.5 kg were anaesthetised and not more than six ileal loops were made in each rabbit (De and Chatterje, 1953). After the desired period of time, rabbits were anaesthetised and the loops were taken out for examination.

Motility in vitro

Bacterial motility was observed microscopically and measured with the use of "ten-lambda" micropipettes (Kimble Disposable, Illinois) according to Adler (1969, 1973).

Assay of vibrios in the lumen and those adherent to the intestine

Vibrios present in the lumen were collected by washing the loop in PBS and were enumerated after appropriate dilution. In loops containing choleraic fluid, the volume was measured and suitable dilutions were made. The washed loops were homogenised and diluted to obtain viable counts of vibrios adherent to the intestine. Homogenisation did not affect the viable count of vibrios (Freter and Jones, 1976; Bhattacharjee and Srivastava, 1979). The total viable count was the sum of bacteria in the lumen and those adherent to intestine; the percentage adherence was:

$$\frac{\text{number of adherent vibrios}}{\text{total number of vibrios}} \times 100$$

Preparation of toxin in vitro

Crude toxin was prepared by growing *V. cholerae* in "Syncase"-sucrose minimal medium (Finkelstein and LoSpalluto, 1969) for 16 h in a shaker. The culture was centrifuged and the supernatant, which contained the crude toxin, was filtered through a Millipore membrane (Bedford, Mass.; pore size 0.2 μ m) and stored at 4°C.

Assay of toxin in culture filtrates and in intestinal loops

The cholera fluid accumulating in the loops was centrifuged and filtered as described above. The loops without fluid were washed with 10 ml of Brain Heart Infusion broth (BHI, Difco) which was purified by filtration. Serial dilutions were made in BHI, and 0.1-ml samples were injected intradermally in adult albino guinea-pigs (Craig, 1965). The diameter of the indurated lesion was recorded after 10 h and compared with that produced by a standard preparation of cholera toxin. Purified standard toxin was obtained from the Swiss Serum and Vaccine Institute, Berne, through the kind courtesy of Dr R. Germanier.

Immunisation of rabbits

Rabbits weighing 1 kg were immunised with bivalent cholera vaccine prepared on the day of immunisation. Strains KB92 (Ogawa) and KB93 (Inaba) were grown up to 2×10^9 cells/ml, mixed in equal volumes, and incubated in a boiling-water bath for 10 min. One ml of vaccine was given twice by subcutaneous injection, 21 days apart. Rabbits were challenged 1 week after the second dose of vaccine.

RESULTS

Pattern of adherence and multiplication

Approximately 10^5 cells of *V. cholerae* in 1 ml were injected into ileal loops of adult rabbits. Loops were examined after 18 h for vibrios in the lumen and those adherent to the intestine. The results are given in table II. Wild-type and mutant strains multiplied to the same extent in the intestine. The strains, however, differed in their ability to adhere to the intestine and to elicit choleraic fluid. Loops inoculated with the wild-type pathogenic strains KB207 and KB619 were full of fluid and a large number of vibrios adhered to the intestine.

TABLE II

Adherence and multiplication of vibrios, and volume of fluid, in ileal loops of adult rabbits after the injection of virulent and mutant strains of Vibrio cholerae

Strain no.	Number of vibrios			Total number of vibrios in the loop	Percentage of vibrios adherent	Fluid in the loop (ml/cm)
	injected	adherent to the intestine	in the lumen			
KB207	1.7×10^5	1.8×10^9	1.1×10^9	2.9×10^9	62.0	2.10
KB619	1.6×10^5	1.0×10^9	9.5×10^8	1.95×10^9	51.3	2.25
C14-S5	7.6×10^4	9.0×10^7	2.2×10^9	2.29×10^9	3.9	0.00
CD2	2.0×10^5	5.0×10^9	1.1×10^8	5.11×10^9	97.8	0.00
CD11	9.1×10^4	4.0×10^7	1.9×10^9	1.94×10^9	2.1	0.00
KB365	1.0×10^5	1.1×10^8	1.4×10^9	1.51×10^9	7.3	0.60
KB378	2.1×10^5	5.5×10^7	5.1×10^9	5.2×10^9	1.1	0.00

Two attenuated strains were also examined. While strain C14-S5 was poorly adherent, strain CD2 showed good adherence. A percentage adherence of 1–2 was found for the poorly adhering (CD11) and non-motile (KB378) strains, and there was no fluid in the corresponding loops. The adherence of strain KB365 was low, and it proved to be a comparatively poor pathogen. Therefore, irrespective of the type of mutant, all the strains multiplied in the intestine similarly, but little or no pathogenicity was retained by non-motile mutants and those that had lost the capacity to adhere. Strain CD2 was an exception.

To follow the pattern of multiplication and adherence during the early phase of infection, all the strains were injected into ileal loops and every 2 h the loops were examined. The pattern with strains KB207 and KB619 is shown in the figure. Both strains multiplied and reached the stationary phase in 12 h. Adherence is expressed in two ways: (1) absolute count of adherent bacteria and (2) percentage adherent. When the former is considered, it is evident that there was a slow but progressive increase in the number of vibrios adhering to the intestine, but the increase was well marked only after 4 h in the intestine. At 12 h, there was a significant decline. A high percentage of incoming vibrios adhered rapidly to the intestine followed by a significant decline by 6 h. The subsequent increase reached its maximum at 10–11 h, and at 12 h only 1–10% remained adherent; the rest were eluted in the lumen.

Similar patterns of growth for the mutant strains are shown in table III,

FIGURE.—Increase with time in the total number of vibrios and of those adherent to intestine of rabbit: ● = growth and ○ = adherence of strain KB619; ▲ = growth and △ = adherence of KB207; × = adherence of KB619, expressed as the percentage of the total number of vibrios.

TABLE III
Pattern of adherence and multiplication of mutant strains of V. cholerae in the ileal loop of the adult rabbit at time-intervals after injection

Time (h)	Number of vibrios (and percentage adherent) for strain											
	C14-S5		CD2		CD11		KB365		KB378			
	Adherent	Total	Adherent	Total	Adherent	Total	Adherent	Total	Adherent	Total	Adherent	Total
2	1.8×10^4 (6.7)	2.7×10^5	5.5×10^5 (19.6)	2.8×10^6	8.5×10^3 (2.1)	4.0×10^5	5.5×10^3 (0.7)	7.6×10^5	2.6×10^4 (1.7)	1.5×10^6		
4	2.0×10^5	1.9×10^6	2.8×10^6	1.3×10^7	1.5×10^5	1.0×10^6	1.2×10^5	3.7×10^5	1.1×10^6	1.6×10^7		
6	4.0×10^5 (10.5)	2.0×10^7	8.5×10^7 (21.5)	3.7×10^8	4.0×10^5 (15.0)	2.0×10^7	2.2×10^5 (32.4)	1.5×10^7	3.4×10^6 (6.9)	2.0×10^8		
8	2.2×10^6 (2.0)	1.1×10^8	2.5×10^8 (22.9)	5.1×10^8	2.2×10^6 (2.0)	1.1×10^8	2.5×10^6 (1.5)	6.2×10^7	3.5×10^7 (1.7)	1.5×10^9		
10	1.5×10^7 (2.7)	5.6×10^8	1.4×10^9 (49.0)	2.1×10^9	8.0×10^7 (2.0)	9.8×10^8	3.8×10^6 (4.0)	7.9×10^7	2.2×10^8 (2.3)	2.4×10^9		
12	2.5×10^7 (4.4)	5.7×10^8	5.5×10^8 (66.7)	2.3×10^9	1.0×10^8 (8.2)	1.0×10^9	1.0×10^6 (4.8)	1.0×10^8	2.6×10^8 (9.2)	2.7×10^9		
			(23.9)		(10.0)		(1.0)		(9.6)			

TABLE IV.

Adherence and multiplication of vibrios, and volume of fluid, in ileal loops of adult immunised rabbits challenged with virulent strains ($1-2 \times 10^5$ cells)

Rabbit no.	Challenge-strain no.	Number of vibrios		Total number of vibrios in the loop	Percentage of vibrios adherent	Fluid in the loop (ml/cm)
		adherent to the intestine	in the lumen			
1	KB619	1.5×10^6	1.8×10^8	1.81×10^8	0.8	0.00
	KB207	1.5×10^7	5.0×10^9	5.0×10^9	0.3	0.00
2	KB619	9.0×10^6	1.5×10^7	2.4×10^7	37.5	2.23
	KB207	2.0×10^6	1.0×10^7	1.2×10^7	16.7	2.00
3	KB619	7.5×10^5	1.4×10^8	1.4×10^8	0.5	0.00
	KB207	4.0×10^7	8.0×10^7	1.2×10^8	33.3	2.50
4	KB619	2.5×10^5	1.1×10^7	1.2×10^7	2.1	0.00
	KB207	6.0×10^6	4.0×10^8	4.1×10^8	1.5	0.00
5	KB619	1.1×10^8	1.3×10^7	1.2×10^8	91.7	1.71
	KB207	1.2×10^6	1.5×10^8	1.6×10^8	0.8	0.00

resembling the increase obtained with strains KB207 and KB619; but, with the exception of CD2, adherence to the intestine was very low. There was no detectable fluid in the corresponding ileal loops.

Adherence to the intestine in immunised rabbits

Strains KB207 and KB619 were injected into intestinal loops of immunised rabbits. Antibacterial mechanisms seem to have affected the bacterial capacity to adhere (table IV). In comparison with a 50–60% adherence in normal rabbits (table II), adherence was significantly reduced. In loops with choleraic fluid, bacterial adherence to the intestine was high.

*Multiplication and pathogenesis of *V. cholerae**

To examine the role of bacterial multiplication in the pathogenesis of

TABLE V

*Fluid outpouring into the ileal loops of adult rabbits in the absence of multiplication of *V. cholerae* in the loop*

Strain no.	Number of vibrios			Total number of vibrios	Fluid in the loop (ml/cm)
	injected	adherent to the intestine	in the lumen		
KB605	5.0×10^9	3.0×10^9	2.0×10^9	5.0×10^9	2.1
	5.0×10^8	ND	ND	5.0×10^8	0.0
	5.0×10^7	ND	ND	3.8×10^7	0.0
KB207	1.0×10^{10}	4.5×10^9	8.5×10^9	1.3×10^{10}	1.8
KB619	2.2×10^{10}	2.0×10^9	2.0×10^{10}	2.2×10^{10}	2.3

ND = not done.

cholera, high doses of strains KB605, KB207 and KB619 were injected into ileal loops, which were examined after 18 h. As shown in table V, none of the strains multiplied in the lumen, yet loops full of fluid were recorded. The streptomycin-dependent strain KB605 was washed and cultured in streptomycin-free medium before injection into the loop. Clearly, vibrios did not multiply in the loops, yet their pathogenicity is indisputable. Tenfold or greater dilutions of strain KB605 did not cause fluid to accumulate in the loops (table V). This suggested that multiplication *per se* is not essential, and that disease would occur if approximately 10^9 vibrios were present in a loop.

Toxin production in vitro and in vivo

Except for strain KB365, none of the strains released detectable amounts of toxin *in vitro*. Loops with the motile but poorly adhesive strain CD11, and with the non-motile and hence poorly adhesive strain KB378, were without fluid, suggesting that no toxin was released. Indeed, in the lumen there was no trace of toxin. The loops infected with strains KB207 and KB619 were full of fluid; we found minimal quantities of toxin in the fluid, but this caused neither defined indurated lesions in guinea-pigs nor fluid accumulation in ileal loops. Similarly, little toxin is found in the stools of cholera patients and even less in their jejunal aspirates (Aziz and Mosley, 1972).

Because the outpouring of fluid began between the 10th and 11th hour with strains KB207 and KB619, assays for toxin were made between hours 6 and 10; it was never present in detectable amounts. Toxin was detected only when substantial amounts of fluid accumulated in loops.

Toxin in doses of $<0.1 \mu\text{g}$ from different sources (i.e., prepared *in vitro* by us or obtained from the Swiss Serum and Vaccine Institute) neither caused fluid accumulation nor produced well defined induration in guinea-pigs.

DISCUSSION

We have studied adherence and multiplication of *V. cholerae* in the ileal loops of adult rabbits because in the same rabbit several loops can be made and various strains can be compared. The time-course of adherence of vibrios to rabbit intestine was studied earlier under the scanning electron microscope (Nelson *et al.*, 1976) and our quantitative data are in good agreement with the results. As the bacteria multiplied in the lumen, there was a progressive increase in the number of adherent vibrios followed by sudden elution at 12 h.

Strain CD11, motile but poorly adhesive, was non-pathogenic. This study revealed, as others have found (Guentzel and Berry, 1975; Freter and Jones, 1976), that non-motile strains adhered poorly and were not pathogenic. On the other hand, strains capable of adhering with high efficiency were pathogenic. Therefore, there was a good correlation between bacterial adherence and pathogenicity. The only exception was strain CD2 which adhered well but behaved as an attenuated strain.

The above conclusion is further supported by the results of experiments on immunised rabbits. Immunisation with bacterial vaccine did not offer complete protection to challenge. However, a correlation between low adherence and resistance to challenge was observed. Whenever vibrios managed to escape the immunity barrier and became adherent to the intestinal mucosa, fluid outpouring was observed.

The observed correlation between adherence and pathogenicity suggests that adherence of vibrios to the intestine is concerned in the release of toxin *in vivo*. Our results provide support for this hypothesis, as follows: (1) because poorly adhesive strains (CD11 and KB378) were poor pathogens and toxin was not detected in ileal loops infected with these strains, it is likely that non-adherent vibrios do not release toxin; (2) in loops infected with strains KB207 and KB619, toxin was never detected in the lumen during hours 6–10 although fluid outpouring commenced at 10 h. Because the injection of toxin alone into ileal loops causes fluid outpouring in 3 h (data not shown), toxin released in the lumen ought to have been detected during hours 6–10; and (3) vibriocidal antibodies interfere with bacterial adherence (Freter, 1969) and do not interact with vibrios in the lumen or with toxin. If toxin were to be released by non-adherent vibrios into the lumen, antibacterial immunity should provide no protection.

We observed that multiplication of vibrios in the lumen was not affected by the inability of vibrios to adhere to intestinal mucosa, and bacterial multiplication by itself had no role in release of toxin. If the intestine is exposed to an optimum number of vibrios, multiplication will of itself have no role in the pathogenesis of cholera. However, multiplication of vibrios may have a very important role in natural infection in man, in whom the infecting dose is likely to be small in relation to the number of vibrios needed to cause disease. The data shown in the figure illustrate the possible course of natural infection in man.

SUMMARY

Pathogenic, and laboratory-derived non-adherent, non-motile, streptomycin-dependent and attenuated strains of *Vibrio cholerae*, were injected into the ileal loops of adult rabbits. The pattern of bacterial adherence and multiplication was studied. It was shown that all the strains multiplied to the same extent in the intestine; multiplication *per se* had no role in pathogenesis except when the infecting dose was low.

Vibrio strains differed in their capacity to adhere to the intestine. A good correlation was found between adherence and pathogenesis. While adhesive strains were pathogenic, the poorly adhesive strains proved to be poor pathogens. There was no trace of toxin in the ileal loops inoculated with poorly adhesive strains and very little in the diarrhoeal fluid produced by pathogenic strains. Adhesive strains adhered poorly to the intestine of immunised animals. It is suggested that adherence is concerned in the release of toxin and thus plays an important role in the pathogenesis of cholera.

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