ACTION OF POLYMYXIN ON CHOLERA VIBRIOS

II. EFFECT ON EL TOR VIBRIOS

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GAN and TJIA (1), POWELL and FINKELSTEIN (2) and ROY, MRIDHA and MUKERJEE (3) have found classical cholera vibrios to be universally sensitive and El Tor vibrios generally insensitive to polymyxin B. The last group of workers have further reported that in media containing polymyxin B El Tor strains show an initial phase of inhibition of growth before the normal growth pattern is re-established. Further studies on the action of polymyxin B on El Tor vibrios are reported in this paper.

MATERIALS AND METHODS

Strains. Twenty strains of *Vibrio el tor* collected from different geographical areas over different periods of time were used. Of these, strain nos. Philippine 42/63 and Makassar 757 were most commonly used.

Polymyxin B. "Aerosporin", polymyxin B sulfate, manufactured by Burroughs Wellcome & Co. was used. One μg of it was equivalent to 6 units.

Estimation of the effect of polymyxin. A 24-hr culture from a Roux flask was washed and suspended in nutrient broth and diluted to contain approximately 10^8 cells/ml. This suspension gave a reading of 25 (approximately) with a 540 $\text{m}\mu$ filter in a Klett-Summerson photo-electric colorimeter. To 10 ml of this suspension varying concentrations of polymyxin B solution were added. The suspension was then incubated at 37° for 5 hr in a conical flask with a side-tube. The growth of the culture was estimated from the optical density of the bacterial suspension which was measured photometrically every hour by tipping the culture suspension into the side-tube which was placed in the colorimeter directly.

Bioassay of polymyxin. A bioassay method was devised for the detection of polymyxin. A lawn of a *V. cholerae* strain was prepared by plating 0.05 ml of a 2-hr-broth culture of the vibrio giving an optical density of about 0.2, in 4 ml of soft agar (0.7% agar in nutrient broth). In the lawn a well was dug with a cork-borer and a 0.1 ml portion of the test material

Fig. 1. Effect of polymyxin B ($5 \mu\text{g/ml}$) on El Tor vibrios.

Control {	{	--- Philippine 42/63	5 $\mu\text{g/ml}$ Polymyxin {	--- Philippine 42/63
		--- Hongkong 23		--- Hongkong 23
		--- Makassar 36		--- Makassar 36
		--- Classical El Tor		--- Classical El Tor

was placed in the well. A clearing of growth around the well indicated the presence of polymyxin. The method was found to be sufficiently sensitive to detect polymyxin in dilutions to $1 \mu\text{g/ml}$.

Fig. 2. Varying concentrations of polymyxin B on Mak. 757.

Numbers on the curves represent polymyxin concentrations in $\mu\text{g/ml}$.

RESULTS

Effect of polymyxin B on the growth-curve of El Tor vibrios

The effect of polymyxin B was studied on twenty representative El Tor strains, which differed in their haemolytic and pathogenic characters. These strains were treated with $5\mu\text{g}$ polymyxin B per ml of suspension in broth and their growth pattern followed for about 5 hr. In the control samples, without polymyxin, the normal growth-curve of the vibrios consisted of an initial phase of slow rise followed by an exponential phase which tended to level off slowly. On addition of polymyxin, there was in every case, except with the rough strains, a fall in the optical density from 0 hr to about 2 hr after which the vibrios grew exponentially, the growth-curve of the treated

Fig. 3. Effect of polymyxin B on Phil. 42/63 passaged once in 25 $\mu\text{g/ml}$ polymyxin medium.

Numbers on the curves represent polymyxin concentrations in $\mu\text{g/ml}$.

culture becoming parallel to that of the control sample (Fig. 1). Viable vibrio counts of the polymyxin treated cultures at 0 hr and at the time of the maximum fall showed a drop of 70-90%. The strains which were rough showed continuous fall in the optical density.

Effect of varying concentrations of polymyxin B

A wide range of concentrations of polymyxin B (2-100 $\mu\text{g/ml}$ of bacterial

Fig. 4. Effect of polymyxin B on Mak. 757 passaged five times in 5 $\mu\text{g/ml}$ polymyxin B.

Numbers on the curves represent polymyxin concentration in $\mu\text{g/ml}$.

suspension) was used to see how El Tor vibrios responded to them. The results are shown in Fig. 2. From these it may be seen that with increasing concentrations of polymyxin the inhibition phase became longer. The turbidity of the bacterial suspension was also found to be progressively more decreased indicating lysis of an increasing number of cells. As the concentration of the viable cells in the bacterial suspension became lowered, it took proportionately longer for the normal growth to be restored.

Fig. 5. Effect of repeated addition of polymyxin B on Mak. 757.

a: Polymyxin B was added continually at the rate of 5 $\mu\text{g/ml/hr}$ (up to 25 $\mu\text{g/ml}$).

b and c: Polymyxin B was added initially at the concentrations of 5 and 25 $\mu\text{g/ml}$, respectively.

Behavior of strains passaged in polymyxin

A. Effect of polymyxin on strains passaged in polymyxin. It was thought that the initial fall in growth rate and its restoration after a definite time interval could reflect an inhomogeneity in the bacterial population of the culture arising from the presence of genetic mutants or other variants differing in their degree of susceptibility to polymyxin B. To test this possibility Mak. 757 and Phil. 42/63 were grown once in liquid media containing 5, 10 and 25 $\mu\text{g/ml}$ of polymyxin. When the growth entered the logarithmic phase at the end of the initial period of inhibition, a small portion of the culture was removed and used to seed a Roux flask. After 24 hr growth, the vibrios were tested in the usual manner for the effect of polymyxin at concentrations of 5, 10, 25 and 50 $\mu\text{g/ml}$. There did not appear to be any

Fig. 6. Polymyxin B with varying concentrations of inocula of Phil. 42/63..

Numbers represent concentrations of inocula in ml bacteria/50 ml broth.

perceptible difference in behavior between passaged and unpassaged bacteria (Fig. 3).

B. Effect of polymyxin on strains serially sub-cultured in polymyxin-containing media. Experiments were also carried out using Mak. 36 and Mak. 757 strains to see if any resistant mutants could be selected. These two strains were serially sub-cultured five times in polymyxin-containing media ($5 \mu\text{g/ml}$) with a view to selecting the mutants which were resistant to this concentration of the antibiotic. The passaged strains were then tested for their response to polymyxin B at 5 and $10 \mu\text{g/ml}$. The results plotted in Fig. 4 show that the serially sub-cultured vibrios did not differ in their response to polymyxin B from the original untreated cultures. Therefore, mutants varying in susceptibility to polymyxin did not appear to be present.

Effect of repeated addition of polymyxin B

As stated earlier, the length of time during which growth is inhibited is in direct proportion to the concentration of polymyxin used. This could be due to the fact that the vibrios metabolised the polymyxin to an inactive form and the inhibition period lasted until the polymyxin in the medium was exhausted. On adding 5 $\mu\text{g/ml}$ polymyxin every hour (to a total of 25 $\mu\text{g/ml}$) to the culture of El Tor strains Mak. 757 and Phil. 42/63, no difference in the pattern of growth could be observed from that when 5 $\mu\text{g/ml}$ polymyxin was added only at 0 hr (Fig. 5). However, when 25 $\mu\text{g/ml}$ was added at 0 hr, the growth pattern was quite different. After a greater and longer initial fall than in the above cases, the optical density was increased. It appears that a vigorously growing culture can remain unaffected by the addition of polymyxin in concentrations of the order of 5 $\mu\text{g/ml}$.

Effect of polymyxin B on varying sizes of the inoculum

An experiment was conducted in which the same concentration of polymyxin (5 $\mu\text{g/ml}$) was added to bacterial suspensions made with different amounts of inocula.

In untreated cultures, the size of the inoculum made no difference to the shape of the growth curves. However, in polymyxin-treated cultures (5 μg polymyxin per ml bacterial suspension), the pattern of the growth-curve varied with the number of cells in the inoculum (Fig. 6). With increasing size of the inoculum the extent and duration of growth inhibitions decreased, indicating that the inhibitory effect is determined by a proportion between the number of cells (24 hr) and the concentration of polymyxin present.

Effect of polymyxin on a young culture

A comparatively small number of cells of Phil. 42/63 was inoculated in broth and allowed to grow for 2 hr. This culture was diluted with broth to give a reading of 20 to 30 in the Klett-Summerson colorimeter, using 540 $\text{m}\mu$ filter. Polymyxin B was then added to a concentration of 5 $\mu\text{g/ml}$. On measuring the growth kinetics it was found that the antibiotic had no inhibitory effect at all on this culture (Table 1).

Detection of polymyxin in the medium of a treated culture

A. *Effect of various concentrations of polymyxin.* A Mak. 757 culture was treated with polymyxin in concentrations of 5, 10, 50 and 100 $\mu\text{g/ml}$, in the usual way. After 3-hr incubation, culture medium was separated by centrifugation and bioassayed for polymyxin B against classical cholera strain no. 154. The results obtained are illustrated in Table 2. No polymyxin was detected in the cases where 5 and 10 $\mu\text{g/ml}$ were used. At higher concentrations the antibiotic was found to be present.

Table 1. Effect of polymyxin on a young culture.

Sample	Klett-reading at 540 m μ				
	Time in hours after addition of polymyxin				
	0	1	2	3	4
Control	25	70	80	100	120
Polymyxin-treated	31	65	82	100	136

Table 2. Presence of polymyxin in culture medium of Mak. 757 treated with various concentrations of polymyxin B.

Concentration of bacterial suspension (no. of cells/ml)	Concentration of polymyxin (μ g/ml)	Klett-reading at 540 m μ				Bioassay result 3 hr after incubation
		Time in hours after addition of polymyxin				
		0	1	2	3	
10 ⁸	0	27	54	80	125	—
10 ⁸	5	28	28	32	55	—
10 ⁸	10	23	23	35	40	—
10 ⁸	50	26	23	18	15	‡‡
10 ⁸	100	18	10	9	9	‡‡
0	5	—	—	—	—	‡‡

B. *Effect of time interval after addition of polymyxin.* A Mak. 757 culture was treated with 5 μ g/ml polymyxin in a series of screw capped test tubes. At short time intervals the tubes were removed to cold temperature, centrifuged and aliquots of the supernatants were bioassayed to see when the antibiotic disappeared from the medium. The results are recorded in Table 3. Within 15 min of addition the concentration of polymyxin in the medium was markedly reduced and at 30 min no polymyxin could be detected.

Polymyxin became undetectable long before the normal growth rate was resumed, that is, before its inhibitory action ceased. This suggests that the antibiotic in the medium was taken up by the cells rapidly, and continued to exert its inhibitory action after absorption by the cells.

Polymyxin-inhibiting agent in Phil. 42/63

A 2-hr growth culture of Phil. 42/63 was filtered through a candle. The cell-free filtrate (Filtrate I) was then used in the experiments. A similar culture filtrate (Filtrate II) was obtained from Phil. 42/63 grown in polymyxin containing medium. The classical cholera strain no. 154 was then grown in both these filtrates, with and without polymyxin (5 μ g/ml) added to them.

Table 3. Presence of polymyxin in the culture media of polymyxin-treated Mak. 757 in relation to time.

Sample	Klett-reading at 540 m μ				Bioassay result			
	Time (min)				Time (min)			
	0	15	30	60	0	15	30	60
Polymyxin control	—	—	—	—	##	##	##	##
Mak. 757 control	20	21	22	24	—	—	—	—
Mak. 757 polymyxin	22	22	18	20	##	+	—	—

Table 4. Klett-readings at 540 m μ of the growth of *V. cholerae* in the presence of El Tor culture filtrate.

Growth medium of strain no. 154	Time in hours					
	0	1	2	3	4	5
Broth+polymyxin	25	25	22	19	19	19
Filtrate I	22	46	56	94	115	135
Filtrate I+polymyxin	29	21	18	15	15	15
Filtrate II	23	30	32	56	80	113
Filtrate II+polymyxin	24	28	22	24	19	20

The growth kinetics of strain no. 154 was then followed over a 5-hr period. The Klett-readings marking the growth rate are recorded in Table 4. It can be seen that the action of polymyxin on the classical cholera vibrios was not inhibited by the culture filtrates of the El Tor Phil. 42/63. This result may be interpreted as indicating that there is no extracellular agent which brings about the inactivation of polymyxin in the culture medium. An intracellular mechanism of inactivation of the antibiotic is suggested.

DISCUSSION

The degree of resistance of El Tor vibrios to polymyxin seemed to be dependent partly on the proportion between the polymyxin present in the incubation mixture and the number of El Tor cells (Figs. 2, 6). No evidence was found to indicate the presence of mutants differing genetically in their response to the antibiotic (Figs. 3, 4). However, the physiological

state of the bacteria appeared to play a role in determining the degree of resistance offered. The growth-curve of a 2 hr culture was completely unaffected by the addition of polymyxin (Table 1), while a 24-hr culture showed an initial phase of inhibition before resumption of the logarithmic phase (Fig. 1). A suspension of resting cells in saline containing polymyxin, on the other hand, was highly sensitive to the antibiotic (3).

The difference in sensitivity between rough and smooth strains indicated that the state of the bacterial surface was involved in determining the response of the El Tor vibrios to polymyxin. This is in line with the finding that the mode of action of polymyxin is similar to that of surface active agents (4). Using a fluorescent derivative of polymyxin, NEWTON (5) found that the antibiotic combined specifically with the phospholipid-rich membrane particles in *Pseudomonas aeruginosa* and *Bacillus megaterium*. HALDAR's (6) work with classical cholera vibrio strain was consistent with this finding.

It is generally accepted that classical cholera and El Tor vibrios possess the same lipo-polysaccharide antigens. Further, the surface antigens of the cells in the various growth stages of an El Tor culture are of identical nature. But the action of polymyxin at low concentration appeared to be different not only as between *V. cholerae* and *V. el tor* but also between El Tor vibrios at different phases of growth. Smooth strains of El Tor vibrios in the logarithmic phase of growth showed the maximum resistance to the antibiotic. Since smooth El Tor strains are known to have the maximum rates of growth and metabolism among the vibrios under study, it appears possible to correlate polymyxin resistance at low concentration of the antibiotic to growth and metabolic rates. Such correlation is further evidenced by the results of experiments in which polymyxin at the concentration of 5 μ g/ml was added hourly for 5 hr to the actively growing El Tor cultures without causing any change in the growth rates. Some metabolic process resulting in inactivation of the polymyxin appears also to be involved since the same concentration of polymyxin per cell acted differently on resting and actively multiplying cells. Investigations on these problems are in progress.

SUMMARY

The action of polymyxin on El Tor vibrios was studied by following the growth pattern of the vibrios in polymyxin-containing media under various conditions.

The results suggest that the antibiotic gets adsorbed on the cell surface whereafter its activity is determined by the metabolic state of the El Tor cells. The resting cells proved to be sensitive, a 24-hr old culture showed an initial period of inhibition followed by normal growth and a 2-hr old culture continued to grow normally in presence of the antibiotic.

The degree of sensitivity appeared to be dependent also on the propor-

tion between the number of cells and the concentration of polymyxin.

It is suggested that an intracellular metabolic process which inactivates the polymyxin after its adsorption on the cell surface may be responsible for the resistance to the antibiotic shown by growing El Tor cells.

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