Factors Influencing the Survival and Multiplication of Bacteriophages in Calves and in Their Environment

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(Received 18 September 1986)

Seven phages were fairly susceptible *in vitro* to the lethal effect of acidified whey, more so than the enteropathogenic *Escherichia coli* strains on which they were active. The low acidity that prevailed in the abomasum contents of calves shortly after a milk feed had little harmful effect on orally administered organisms of these phages; they flooded into the small intestine. The high acidity that prevailed later was lethal to orally administered phage organisms; few entered the small intestine. The lethal effect could be counteracted by giving CaCO_3 in the feed. Low concentrations of phage-neutralizing antibodies were found in some serum samples from human beings, cattle and pigs. Antibodies to one of the seven phages were common in the human samples and antibodies to another, phage B44/1, were common in the cattle and pig samples and in bovine colostrum. Phage B44/1 antibodies in a sample of colostrum whey were destroyed at pH 3.25 or less. Giving colostrum containing phage B44/1 antibodies with CaCO_3 to a calf greatly reduced the numbers of orally administered phage B44/1 organisms in its alimentary tract. Antibodies to another phage were induced in the serum of a calf suffering from *E. coli* diarrhoea by treating it with that phage. The phages were as susceptible as the *E. coli* strains to the lethal action of formaldehyde and sodium hypochlorite. In contrast to the *E. coli* strains, they were almost completely resistant to phenol and chloroxylonol. The *in vitro* virulence of 21 phages varied according to the temperature at which tests were performed. The majority were almost avirulent at 20 °C and 43 °C and most virulent at 37 °C. Experiments in calves suggested that their body temperatures were sufficiently high to adversely influence the virulence of some phages.

INTRODUCTION

During recent studies in which phages were successfully used to treat generalized and cerebral *Escherichia coli* infections of mice (Smith & Huggins, 1982) and *E. coli* diarrhoea in calves, lambs and piglets (Smith & Huggins, 1983), it became increasingly evident that factors not directly involved in the phage/bacterium conflict might influence the successful control of bacterial infections by means of phage. These included the presence of phage antibodies in the blood of the infected animal and its body temperature. Others, probably only applicable to the therapy of intestinal bacterial infections, were the acidity of the stomach contents at the time of phage administration and, in the very young mammal, the presence of colostrum antibodies in the alimentary tract. The ability of phages to survive outside the animal body is also important and this might be influenced by disinfectants which are frequently used in high-disease-risk environments.

It was decided to investigate some of these factors in parallel with further studies that were pursued on phage therapy (accompanying paper: Smith *et al.*, 1987). We felt that such investigations might provide not only explanations for the failures or successes of phage therapy but new information that could be exploited to improve the efficacy of phage therapy. Phages active on *E. coli* strains enteropathogenic for calves, lambs, piglets or human beings were

employed in the investigation; whenever it was thought relevant, the enteropathogenic *E. coli* strains themselves were included.

METHODS

Bacterial and phage strains. The phages were selected for study because they were active on enteropathogenic strains of *E. coli* that had been isolated from human beings (H), calves (B), lambs (S) or piglets (P). A group of seven was used in most experiments, each identified by the number of the *E. coli* strain against which it was active followed by a numeral, its isolation number. They were B41/1, B44/1, B44/3, S13/1, P433/1, H19/6 and H130/92; B44/3 was not active on *E. coli* B44 itself but on its K⁻ mutants, as was phage B44/5, a phage used in one experiment. The antigenic formulas of the *E. coli* strains were B41, O101:K30,99; B44, O9:K30,99; S13, O8:K85,99; P433, O20:K101,987P; H19, O26:K60:H11; and H130, O78:H12. Strains B41, B44, S13 and P433 produced heat-stable (ST) enterotoxin and H130 produced ST and heat-labile (LT) enterotoxin. A phage, MW/R (Smith & Huggins, 1982), active on a human bacteraemic *E. coli* strain of antigenic formula O18:K1:H7 was included in some of the experiments.

The methods whereby the phages were originally isolated and how they and the *E. coli* strains were propagated, maintained, counted and used in *in vitro* virulence tests are described in the accompanying paper (Smith *et al.*, 1987). So are the method of producing *E. coli* diarrhoea in calves, their source and subsequent management and the technique of performing microbiological examinations upon them. Unlike those used by Smith *et al.* (1987), most of the calves in the present investigation were aged 5–7 d when they were put under experiment. In experiments in which their fate was studied in the calf alimentary tract, phage organisms were given in milk by means of a bottle and teat to ensure that most of them went directly into the abomasum.

Survival of phages and *E. coli* in acidified whey. Whey was obtained by adding rennin to pasteurized cows' milk according to the manufacturer's instructions. The large clots were allowed to settle, the supernatant fluid was removed, and 5 ml samples were adjusted to the required pH values by the addition of HCl. The phages, 5×10^5 viable organisms ml⁻¹ of each, were added to one set of samples of different pH values and the *E. coli* strains, 5×10^4 viable organisms ml⁻¹ of each, added to another. The samples were held in a static water bath at 37 °C and 0.3 ml amounts removed at intervals and counted, approximately 10% (w/v) CaCO₃ being added to the first dilution tube to counteract acidity.

Survival of phages and *E. coli* in disinfectants. Phages, 5×10^5 viable organisms ml⁻¹ of each, or *E. coli* strains, 5×10^4 viable organisms ml⁻¹ of each, were added to a 10% (w/v) aqueous suspension of cow's faeces that had previously been held at 80 °C for 20 min. Doubly falling concentrations of the disinfectants were made in 5 ml samples of the phage- and *E. coli*-containing faecal suspensions held at 37 °C in a static water bath. The samples were counted 1 h later. The same specimen of cow's faeces was used throughout.

Detection of phage-neutralizing antibody in serum and colostrum. Approximately 10^5 viable organisms of each phage were added to the same 1.0 ml sample of serum or colostrum held at 37 °C in a static water bath. The numbers of organisms of the different phages in the samples were counted 3 h later. Samples in which the numbers of organisms of a phage had been reduced at least tenfold were recorded as antibody-positive for that phage. During the conduct of these tests, negative controls consisting of serum from colostrum-deprived calves or heat-sterilized cows' milk were always kept – a reduction in phage count was never observed in them.

RESULTS

*Susceptibility of phages and *E. coli* to acidity*

No decrease in the numbers of phages B41/1, B44/1, B44/3, S13/1, P433/1, H19/6 or H130/92 was observed when they were held at 37 °C for 1 h in milk whey adjusted to pH 6.8, 4.0 or 3.5 with HCl. Some decrease in the numbers of all the phages was observed at pH 3.0; at pH 2.5 and 2.0 the decrease was great (Table 1*a*). Phages S13/1 and H19/6 were more resistant than the other phages. Small numbers of organisms of these two phages were isolated 5 min, but not 10 min, after they were exposed to pH 2.0; the other five phages did not survive for 0.5 min at this pH. With the exception of *E. coli* H130, the *E. coli* strains upon which the phages were active were more resistant to acidified whey than were the phages (Table 1*b*).

Whey had been chosen as the medium for the above tests because it simulated to some extent the gastric contents of suckled animals. However, in an experiment in which five of the phages were held at 37 °C in samples of nutrient broth of different pH values for 1 h, the results were similar to those obtained with whey.

A calf was given a mixture of the seven phages 5 h after a milk feed and examined 30 min later. Its abomasum contents, pH 3.9, contained as many phage organisms as did the abomasum

Table 1. *Survival time of phages and E. coli in whey of different pH*

Samples (5 ml) of the same specimen of whey were adjusted to the required pH values with HCl and (a) 5×10^5 viable organisms ml^{-1} of each phage, or (b) 5×10^4 viable organisms of each *E. coli* strain, were added to each sample at 37 °C. Volumes of 0.3 ml were withdrawn at 0.5, 5, 10, 20, 30, 45 and 60 min, added to broth containing 10% (w/v) CaCO_3 , and their phage or bacterial content assayed.

pH of whey sample	Time (min) required to reduce, approx. 100-fold, the no. of (a) <i>Viable organisms of phage:</i>						
	B41/1	B44/1	B44/3	S13/1	P433/1	H19/6	H130/92
3.5	> 60	> 60	> 60	> 60	> 60	> 60	> 60
3.0	60	60	> 60	> 60	30	> 60	60
2.5	0.5	0.5	30	> 60	0.5	> 60	5
2.0	0.5	0.5	0.5	5	0.5	5	0.5

	(b) <i>Viable organisms of E. coli strain:</i>						
	B41	B44	S13	P433	H19	H130	
2.5	> 60	> 60	> 60	> 60	> 60	< 60	
2.0	10	20	> 60	> 60	> 60	5	
1.5	5	5	> 60	20	> 60	0.5	
1.0	5	5	5	5	10	< 0.5	

Table 2. *Counts on contents of different parts of the alimentary tract of calves given phages 5 or 10 h after a feed of milk, to which CaCO_3 had or had not been added, and examined 30 min later*

Six calves that had been fasted for 24 h were given 1.5 l of milk containing or not containing 30% (w/v) CaCO_3 . After 5 or 10 h they were given 10^8 viable organisms of each of phages B41/1, B44/1, B44/3, S13/1, P433/1, H19/6 and H130/92 in 20 ml of milk and examined 30 min later. The small intestine was divided into seven equal parts for counting, part 1 being next to the abomasum and part 7 next to the colon; the results for parts 4-7 are not shown because very little phage-containing chyme had progressed beyond part 3 in the 30 min after phage administration. Only the results for phages B41/1 and H19/6 are shown because those for phage S13/1 resembled those for phage H19/6 and those for the other phages resembled those for phage B41/1.

Interval between feeding and phage administration (h)	CaCO_3 in feed	pH of abomasum contents	Phage enumerated	No. of organisms ($\log_{10}[\text{no. (g contents)}^{-1}]$) in			
				Abomasum	Small intestine part:		
					1	2	3
5	No	3.9	B41/1	4.8	3.5	1.8	1.5
			H19/6	5.2	4.2	3.2	2.4
5	Yes	4.9	B41/1	4.8	4.0	3.8	3.8
			H19/6	4.4	3.8	3.8	3.5
10	No	2.3	B41/1	1.5	3.6	< 1.5	< 1.5
			H19/6	3.6	4.4	4.5	3.6
10	No	2.2	B41/1	< 1.5	< 1.5	< 1.5	< 1.5
			H19/6	< 1.5	< 1.5	< 1.5	< 1.5
10	No	1.6	B41/1	< 1.5	< 1.5	< 1.5	< 1.5
			H19/6	< 1.5	< 1.5	< 1.5	< 1.5
10	Yes	5.0	B41/1	5.6	5.2	5.0	2.6
			H19/6	5.5	5.0	5.0	2.3

contents, pH 4.9, of a similarly treated calf whose milk feed had CaCO_3 added to it (Table 2). The phage organisms had flooded into the small intestine. The pH of the abomasum contents of three calves given the phages 10 h, instead of 5 h, after the milk feed was much lower, 2.3, 2.2 and 1.6. No phage organisms were detected in either the abomasum or small intestinal contents of two of them. In the third, the numbers were much lower than in a similarly treated calf whose

Table 3. *Counts on the contents of different parts of the alimentary tract of calves at different times after they were given phages in a feed of milk*

Four calves were fasted for 24 h, given 10^{10} viable organisms of each of the seven phages featured in Table 2 in 1.5 l of milk and examined at 0.3, 2.5, 5.0 or 10.0 h later. For other details see Table 2; for reasons given in that table, only the results for phages B41/1 and H19/6 are shown. In general, the results for parts 2, 4 and 6 of the small intestine were intermediate between those for parts 1 and 3, 3 and 5 and 5 and 7 respectively.

Time after giving phages (h)	pH of abomasum contents	Phage enumerated	No. of organisms ($\log_{10}[\text{no. (g contents)}^{-1}]$) in				
			Abomasum	Small intestine part:			
				1	3	5	7
0.3	4.4	B41/1	6.5	6.3	6.2	5.7	2.8
		H19/6	6.9	6.7	6.7	6.3	<1.5
2.5	3.9	B41/1	6.0	6.2	6.0	6.0	6.6
		H19/6	6.8	6.8	6.5	6.8	6.8
5	1.8	B41/1	<1.5	1.8	2.4	3.3	8.0
		H19/6	3.8	4.2	4.0	4.4	8.0
10	1.2	B41/1	<1.5	1.8	<1.5	3.5	5.3
		H19/6	<1.5	2.2	2.0	3.4	6.2

Table 4. *Incidence of phage-neutralizing antibodies in serum samples of animals of different species*

Animal species	No. of samples examined	No. of samples with antibodies to phage							
		B41/1	B44/1	B44/3	S13/1	P433/1	H19/6	H130/92	MW/R
Humans	20	1	0	1	1	8	0	1	—
Pigs	24	0	13	0	3	0	0	2	0
Cattle	16	0	11	0	3	0	0	3	2

milk feed had contained CaCO_3 . The volume of the abomasal contents of these four calves was much less than the case in the two calves examined 5 h after the milk feed.

The phage-lethal effect of the acid in the abomasum was also demonstrated by examining four calves at different times after they had been given a milk feed containing a mixture of the seven phages (Table 3). Little or no destruction had occurred in the abomasum contents (pH 4.4 and 3.9) of the two examined 0.3 or 2.5 h after feeding and the phage organisms had flooded into the small intestine. Destruction was great in the two killed 5 or 10 h after feeding. Only organisms of phages H19/6 and S13/1, the two phages found to be the most acid resistant *in vitro*, were detected in the abomasal contents (pH 1.8) of the calf examined at 5 h; no phage organisms were detected in the abomasum contents (pH 1.2) of the one examined at 10 h and only low numbers were found in its anterior small intestine.

Incidence of phage-neutralizing antibodies in the serum of animals of different species and in bovine colostrum

The results of examining serum samples of 'unrelated' pigs, cattle and human beings for neutralizing antibodies to the seven phages and to phage MW/R, the phage active on a human bacteraemic *E. coli* strain, are summarized in Table 4. Those for the cattle and pig samples were similar but different from those for the human samples; in particular, antibodies to phage B44/1, common in the cattle and pig samples, were not found in the human samples and antibodies to phage P443/1, common in the human samples, were not found in the pig and cattle samples. No antibodies were detected in 11 of 28 antibody-positive samples diluted 3 times, in another 13 diluted 10 times or in the remaining four samples diluted 30 times.

Colostrum samples from 14 cows kept on three different farms were examined for antibodies to phages B44/1, B44/3, H19/6 and H130/92. The number positive for antibodies to these four

Table 5. Counts on the contents of different parts of the alimentary tract of calves given phages after a feed of colostrum containing phage antibody or of milk

Two calves were fasted for 24 h and then given 1.5 l of milk or colostrum containing 30% (w/v) CaCO_3 . Five minutes later they were given 10^8 viable organisms of each of phages B41/1, B44/1, B44/3, P433/1, S13/1, H19/6 and H130/92 in 20 ml of milk. The colostrum contained antibody against phage B44/1 but not against the other six phages. The calves were examined 4 h later. Only the results for phages B44/1 and B41/1 are given; those for the other five phages closely resembled those for B41/1. The pH of the abomasum contents of the calf given colostrum was 4.5 and that of the calf given milk was 5.1.

Contents examined	No. of organisms ($\log_{10}[\text{no. (g contents)}^{-1}]$) in calf given			
	Colostrum + CaCO_3		Milk + CaCO_3	
	Phage B44/1	Phage B41/1	Phage B44/1	Phage B41/1
Abomasum	<1.5	3.8	3.3	3.5
Small intestine, part:				
1	<1.5	3.5	3.2	3.5
2	<1.5	3.8	3.5	3.4
3	<1.5	4.0	3.5	3.4
4	<1.5	4.7	3.8	3.8
5	<1.5	4.8	4.2	4.6
6	<1.5	6.0	4.5	5.0
7	<1.5	5.5	5.6	5.8

phages was 13, 5, 7 and 1 respectively. Samples of whey, with an antibody titre for phage B44/1 of 1 in 30, prepared from one of the colostrum samples were adjusted to different pH values with HCl and held at 37 °C for 1 h. After the addition of CaCO_3 their antibody content for phage B44/1 was assessed. No antibody was detected in the samples that had been held at pH 3.25 or lower. There was evidence of some antibody destruction in the sample held at pH 3.5 but not in the specimens held at pH 3.75 and above. To determine whether phage antibody might be active in the alimentary tract, a calf was given the seven phages after a feed of colostrum containing antibodies to phage B44/1, but not to the other six phages, and examined 4 h later. To negate the phage-destructive and antibody-destructive effect of the acid in the abomasum contents, CaCO_3 had been added to the colostrum; the experiment was repeated in another calf, the colostrum being replaced by milk (Table 5). No phage B44/1 was detected in the contents of the abomasum or small intestine of the calf that had been given colostrum but it was found in these organs in the calf given milk in concentrations similar to those of the other six phages; the concentrations of each of the six were similar in both calves.

The development of serum antibody to orally administered phage was studied in a calf treated with phage B41/1 when it was suffering from experimental *E. coli* B41 diarrhoea. No antibody to the phage was detected 1 d after administration but it was detected at the subsequent examinations made 15, 23 and 32 d later. No antibody to phages B44/1, B44/3, S13/1, H19/6 and H130/92 was found at any of these examinations; antibodies to phages P433/1 and MW/R were present in the calf's serum at the commencement of the experiment but their concentration did not increase during its course. The serum obtained 32 d after the administration of phage B41/1 gave a positive reaction for antibodies to that phage when diluted 10, but not 100, times. Much higher concentrations of phage antibody were obtained by parenteral inoculation of phage. For example, the serum of a calf 10 d after being given 10^{10} viable organisms of phage B41/1 intravenously on seven occasions during a period of 14 d was positive for antibody to phage B41/1 when diluted 1000, but not 10000, times; it did not contain antibodies against the other six phages.

Activity of disinfectants against phages and *E. coli*

Sodium hypochlorite and formaldehyde were highly lethal for the seven phages and the strains of *E. coli* on which they were active (Table 6). The phages were much more resistant than the *E. coli* to phenol and, particularly, chloroxylonol.

Table 6. *Activity of different disinfectants against phages and E. coli*

Seven phages, 5×10^5 viable organisms ml^{-1} of each (a), or six *E. coli* strains, 5×10^4 viable organisms of each (b), were added to a 10% (w/v) aqueous suspension of cow's faeces that had been held at 80 °C for 20 min. Doubly falling concentrations of the disinfectants were made in 5 ml samples of the faecal suspension held at 37 °C. The phage or bacteria in the samples were counted 1 h later.

Disinfectant	Concn (% w/v) required to reduce, approx. 100-fold, the no. of (a) Viable organisms of phage:						
	B41/1	B44/1	B44/3	S13/1	P433/1	H19/6	H130/92
Formaldehyde	0.03	0.004	0.03	0.008	0.03	0.004	0.03
Sodium hypochlorite	0.25	0.12	0.06	0.12	0.12	0.12	0.03
Chloroxylenol*	> 4.0	> 4.0	2.0	4.0	> 4.0	> 4.0	4.0
Phenol	8.0	8.0	8.0	8.0	8.0	8.0	4.0

	(b) Viable organisms of <i>E. coli</i> strain:					
	B41	B44	S13	P433	H19	H130
Formaldehyde	0.03	0.06	0.06	0.03	0.03	0.03
Sodium hypochlorite	0.06	0.06	0.06	0.06	0.06	0.06
Chloroxylenol*	0.06	0.06	0.06	0.06	0.06	0.06
Phenol	1.0	1.0	1.0	1.0	1.0	1.0

* Dettol (Reckitt and Colman).

Table 7. *In vitro* virulence of phages at different temperatures

Ten ml amounts of nutrient broth were inoculated with 10^8 viable *E. coli* organisms and threefold falling numbers of phage organisms and incubated for 5 h at different temperatures; 0.03% (w/v) CaCl_2 was added to the broth used in the tests with phage S48/2 because this phage needed it for lysis. The smallest number of phage organisms required to lyse the *E. coli* culture was recorded as the MLD.

Phage	MLD at					
	20 °C	24 °C	37 °C	40 °C	41 °C	43 °C
H19/6	10^6	10^6	10^5	—	10^5	10^6
MW/R	$> 10^9$	3×10^4	10	—	10	10
B44/5	$> 10^8$	$> 10^8$	$> 10^8$	—	$> 10^8$	30
B44/3	10^2	10	10	—	10^6	10^8
B117/1	15	5	2	2	2	5×10^8
P433/1	$> 3 \times 10^8$	10^5	3	—	3	3×10^6
S48/1	$> 2 \times 10^8$	—	20	2×10^4	—	$> 2 \times 10^8$
S48/2	—	—	10^2	10^2	—	—
B117/2TS	75	2	5	50	$> 5 \times 10^8$	$> 5 \times 10^8$
B117/2TR	10^5	3×10^2	2	2	2	$> 5 \times 10^8$

In vitro virulence of phages at different temperatures

The minimum number of phage organisms required to lyse cultures of *E. coli* (MLD) at different temperatures was determined for 21 phages; the results for a selection of them, chosen mainly to illustrate the principal behaviour patterns observed, are given in Table 7. The MLD for phage H19/6 was fairly high at all the temperatures tested. Phage MW/R was completely avirulent at 20 °C, of low virulence at 24 °C and very virulent at all the higher temperatures. Phage B44/5 was only virulent at the highest temperature tested, 43 °C. The opposite was the case with phages B44/3 and B117/1 although their virulence at 41 °C differed. Other phages, such as P433/1, were of low virulence at both the higher and the lower temperatures but very virulent at the intermediate temperatures. Phage S48/1 was more virulent than phage S48/2 at 37 °C and less virulent than it at 40 °C. They were, therefore, of interest because the rectal temperatures of the calves used in this and the related investigation (Smith *et al.*, 1987) varied between 37.1 °C and 39.5 °C (median 38.8 °C). Much lower numbers of phage S48/1 than of S48/2 were found in the small intestinal contents of a calf suffering from experimental *E. coli* S48

Table 8. Counts on the contents of different parts of the small intestine of a calf suffering from *E. coli* diarrhoea and treated with a mixture of two phages of differing *in vitro* virulence

Shortly after the onset of diarrhoea caused by the oral administration of *E. coli* S48 (O101:K28,99), a calf was given 10^5 viable organisms of each of phages S48/1 and S48/2; it was examined 24 h later when it was recovering. The MLD of phage S48/1 for *E. coli* S48 at 40 °C and 37 °C was 2×10^4 and 20 viable organisms respectively; the corresponding figures for phage S48/2 were 10^2 and 10^2 viable organisms respectively.

Contents of small intestine, part:	No. of organisms ($\log_{10}[\text{no. (g contents)}^{-1}]$) of phage	
	S48/1	S48/2
1	<2.3	4.2
2	6.2	6.2
3	6.5	7.8
4	6.4	8.5
5	6.4	8.2
6	6.2	8.0
7	6.7	8.7

diarrhoea 24 h after it had been treated with a mixture of equal numbers of the two phages (Table 8). Phage B117/2TS (Table 7) was avirulent at 41 °C. Phage B117/2TR was a mutant obtained from it by passage in broth cultures of *E. coli* B117 at 41 °C – the mutants were highly virulent at 41 °C. Mutants resembling B117/2TR became dominant in the alimentary tract of two calves that had been infected with *E. coli* B117 and treated with phage B117/2TS.

The colonial morphology of six bovine and ovine enteropathogenic *E. coli* strains used to determine the *in vitro* virulence of six phages that were less virulent at 41 °C than at 37 °C and almost avirulent at 43 °C and 20 °C was compared at these four temperatures on nutrient agar and MacConkey's agar. At 37 °C and 20 °C the colonies of the six strains were very mucoid, indicative of the production of much capsular material. At 41 °C the colonies appeared slightly less mucoid. At 43 °C the mucoid appearance was almost completely lost, the colonies tending to resemble those of K⁻ mutants of the strains.

DISCUSSION

The present investigation has elucidated several factors that could adversely influence the successful use of phages to control bacterial diseases. The *in vitro* experiments and, more particularly, the experiments in which the numbers of orally administered phage organisms in the alimentary tract were estimated at different times in relation to feeding revealed that, at the low pH which sometimes prevails in the partially empty abomasum, phage populations could be rapidly and perhaps completely destroyed. The time of their administration, if individual therapy were to be adopted, therefore, could be important should they be used for controlling diseases of the alimentary tract, their most likely application. The results of the *in vivo* experiments indicate that the phages should be given with a meal or with alkali. In the very young animal, though, the problem of gastric destruction would probably not be important because in the first day or so of life the pH of the contents of the stomach or abomasum is usually high (Smith & Jones, 1963; Smith, 1965); most of the animals used by Smith & Huggins (1983) and Smith *et al.* (1987) in their studies on the phage control of *E. coli* diarrhoea were less than 24 h old when they were given phage.

The impact that phage-neutralizing antibodies might have on phage therapy under practical conditions is difficult to assess. Certainly such antibodies in low concentrations were found in some of the serum samples we examined and in the serum of a calf to which they had been given orally to cure experimentally produced *E. coli* B41 diarrhoea. Furthermore, antibodies stimulated by previously inoculated phage MW/R prevented this phage from curing mice

infected with *E. coli* MW (Smith & Huggins, 1982). It seems unlikely that low concentrations of serum antibody would destroy phages in the lumen of the alimentary tract. This would be more likely to occur in very young animals that had received colostrum containing antibodies. We did, in fact, demonstrate such destruction of phage B44/1 in a calf given colostrum containing antibody to it, CaCO₃ being given at the same time to prevent not only destruction of the phage but also of the antibody itself, by the acid abomasum contents. Nearly all the bovine colostrum samples tested contained antibodies to phage B44/1. It is reasonable to assume then that the colostrum consumed by most of the calves in experiments that demonstrated the therapeutic potential of this phage (Smith & Huggins, 1983) and of a related one, B44/4, which was also neutralized by B44/1 antibody (Smith *et al.*, 1987), would have contained these antibodies because it came from the same sources as the colostrum samples used in the tests; there was no suggestion that phage antibodies complicated those experiments. Even so, if antibody to a certain phage were to 'build up' in a community as a consequence of frequent exposure to that phage to the extent that it complicated the therapeutic use of that phage, then the situation might be overcome by use of an antigenically different phage.

The main reason for determining the susceptibility to disinfectants of phages and the *E. coli* strains upon which they were active was the finding that *E. coli* diarrhoea could be controlled simply by keeping the experimentally infected calves in an environment deliberately contaminated with phages active on the causal organisms (Smith *et al.*, 1987). If this finding were ever to be exploited in the field, care would be needed in the choice of disinfectants to be used for any purpose in the phage-contaminated environment. Clearly, formaldehyde and sodium hypochlorite would have to be avoided because they were lethal to the phages in low concentration. The position with regard to phenol and, especially, chloroxylenol was different. The phages, but not the *E. coli* strains, were very resistant to them. The great difference in susceptibility of the phages and the *E. coli* strains to chloroxylenol was such that, on theoretical grounds, it would be possible, with a relatively high degree of safety, to use this agent in a deliberately phage-contaminated environment at concentrations adequate for killing enteropathogenic *E. coli*, and probably many other bacteria, without having any deleterious effect on the phage organisms.

The limited *in vivo* studies that were performed tended to confirm the observations made *in vitro* that at temperatures not a great deal higher than 37 °C, temperatures near the norm for some animal species, the virulence of some phages is reduced. Other phages were not so temperature sensitive, and these may be of greater practical value. Pyrexia is not a feature of *E. coli* diarrhoea, the disease we have mainly studied, but it may well have to be taken into account when considering the phage therapy of other diseases. The fact that the *in vitro* virulence of most of the phages tested declined sharply at 20 °C, and sometimes at 24 °C, suggested that environmental temperature would be a limiting factor in determining the ability of phages to multiply outside the animal body, at least in temperate countries. For this reason alone, it is improbable that their numbers would greatly increase in deliberately phage-contaminated environments. An explanation for the phages active on the bovine and ovine enteropathogenic strains being avirulent at 43 °C may lie in the fact that the strains produced little capsular material at that temperature and the capsule is believed to be the attack point of these phages (Smith & Huggins, 1983; Smith *et al.*, 1987). This cannot provide an explanation for most of them being avirulent at 20 °C because capsular production did not appear to be impaired at that temperature. It is of interest in this respect that phage B44/3 was also avirulent at 43 °C whereas phage B44/5 was avirulent at all temperatures except 43 °C – both phages were active on the bovine and ovine enteropathogenic *E. coli* B44 but only on its non-capsulated mutants. These facts, together with the great variety of behavioural patterns exhibited by the other phages in the relevant tests, suggest that many factors are involved in determining the activity of different phages at different temperatures.

We are grateful to Mrs Teresa Berrill, Professor P. M. Biggs, Mr Geoffery Hart, Miss Margaret Lovell and Mrs Shirley Pilcher for assistance in a variety of ways. The expenses of this work were defrayed, in part, by a grant from the Wellcome Trust; we thank them.

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