The Toxic Role of Alpha-haemolysin in the Pathogenesis of Experimental *Escherichia coli* Infection in Mice

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Filtrates from strains of *Escherichia coli* possessing plasmid-cloned haemolysin (Hly) genes and from strains possessing 'wild' Hly plasmids were lethal for mice on intravenous inoculation; similar doses of preparations from derivatives of these strains in which the Hly genes had been rendered non-functional or which did not possess the 'wild' plasmids were not. Live cultures of both kinds of Hly⁺ strain usually had a lower lethal dose for mice on intraperitoneal inoculation than the corresponding Hly⁻ forms. Mice that had been inoculated with Hly⁺ forms had shorter survival times and lower numbers of organisms in peritoneal washings, lungs and blood at point of death than mice that had been inoculated with the corresponding Hly⁻ forms; this was also so for mice pre-treated with FeSO₄, a procedure which rendered mice equally susceptible to the lethal effects of the Hly⁺ and Hly⁻ forms of a strain. In FeSO₄-treated mice the numbers of organisms in the tissues of those dying from infection with Hly⁺ organisms were no higher than they were at the same time after inoculation in others given the corresponding Hly⁻ forms; before mice of the latter category died the numbers of organisms in their tissues increased greatly. The clinical and pathological signs exhibited by mice inoculated with Hly⁺ organisms, but not with Hly⁻ organisms, resembled those exhibited by mice inoculated with bacteria-free haemolysin preparations. These results suggest that haemolysin played a significant role in the pathogenesis of the disease produced by the Hly⁺ organisms by having a direct toxic action on the host.

INTRODUCTION

Following their discovery of the transferable nature of the haemolysin (Hly) genes in many alpha-haemolytic strains of *Escherichia coli*, Smith & Halls (1967) demonstrated by intravenous (i.v.) inoculation of bacteria-free fluids of cultures of laboratory-constructed *E. coli* strains differing in the presence or absence of the Hly plasmid that haemolysin was toxic for mice. Using this and other approaches, several workers have produced evidence that the haemolysin is toxic to cultured animal and human fibroblasts (Chaturvedi *et al.*, 1969; Cooke & Ewins, 1975; Cavalieri & Snyder, 1982*a*) and rabbit and human leucocytes (Emödy *et al.*, 1980; Cavalieri & Snyder, 1982*b, c*; Gadeberg *et al.*, 1983). It has been shown in several animal models that viable Hly⁺ forms of a strain are more virulent than Hly⁻ forms (Fried *et al.*, 1971; Smith & Linggood, 1971; Kétyi *et al.*, 1978; Emödy *et al.*, 1980; van den Bosch *et al.*, 1981, 1982; Linggood & Ingram, 1982; Waalwijk *et al.*, 1982). Smith & Linggood (1971) mainly studied Hly⁺ and Hly⁻ forms of an O141:K85,88 porcine enteropathogenic *E. coli* strain, called P5. They found that the Hly⁺ forms were more lethal for mice by intraperitoneal (i.p.) inoculation than the corresponding Hly⁻ forms. This observation with P5 was confirmed by Linggood & Ingram (1982). These workers, however, reported that such toxicity as they observed in mice inoculated with bacteria-free preparations of cultures of *E. coli* K12 to which Hly plasmids had been transferred did not correlate with the haemolysin titres of their preparations. In fact, their subsequent experiments

Abbreviations: i.p., intraperitoneal(ly); i.v., intravenous(ly).

with derivatives of P5 led them to the conclusion that haemolysin enhanced virulence by enabling *E. coli* to obtain iron necessary for growth by lysis of erythrocytes in infected mice. Rapid bacterial proliferation then occurred leading to death of the animals. Their main evidence for this role for haemolysin was the finding that Hly⁻ organisms multiplied to the same rapid extent as Hly⁺ organisms of the same strain when both kinds of organisms were inoculated together into mice or when the Hly⁻ organisms were inoculated with haemolysin or iron but not when they were inoculated alone. Additional evidence was that the presence of anti-haemolysin protected mice against death by inhibiting the proliferation of normally virulent haemolytic organisms.

Because techniques such as plasmid manipulation had been employed to obtain the strains used in many of the above-mentioned experiments, they would be expected to yield more reliable information than would a comparison of wild haemolytic and non-haemolytic strains of *E. coli*. Nevertheless, the possibility still exists that mutants of a strain that had acquired or lost Hly genes might concurrently have acquired or lost other genes, genes that might themselves influence virulence. Welch *et al.* (1981) overcame this problem by comparing the virulence of an *E. coli* strain possessing a plasmid in which Hly genes had been cloned with that of an ampicillin resistance insertional plasmid mutant of that strain which no longer conferred the haemolytic phenotype; they found that the Hly⁺ parent strain, inoculated i.p., was more virulent for rats than the mutant. Using somewhat similar approaches but in different animal models, Hacker *et al.* (1983), Waalwijk & de Graaff (1983) and Waalwijk *et al.* (1983) obtained results similar to those of Welch *et al.* (1981). In addition, Waalwijk *et al.* (1983), using a mouse haematogenous pyelonephritis model, produced evidence suggesting, as Linggood & Ingram (1982) had done, that haemolysin enhances virulence mainly by increasing the amount of available iron.

Because Dr Welch kindly made his strains available to us we decided to use them and some of our strains containing 'wild' Hly plasmids to re-investigate the toxicity of haemolysin for mice and the role that haemolysin plays in the pathogenesis of experimentally produced generalized *E. coli* infections in mice.

METHODS

Bacterial strains. *E. coli* strains provided by Dr Welch (Welch *et al.*, 1981), were as follows. WAF107 was a derivative of a strain J198, containing plasmid pSF4000 which had been constructed by insertion of an 11.7 kb chromosomal restriction endonuclease fragment encoding haemolysin into a plasmid vector. WAF108 was WAF107 containing a Tn1 insertional mutant of its plasmid which no longer conferred the haemolysin phenotype. WAF111 was J198 containing plasmid pAN202-312 in which the Hly determinants of a 'wild' plasmid had been cloned by Goebel & Hedgpeth (1982). Other *E. coli* strains employed were either porcine enteropathogens (prefix P), whose haemolysin genes were located in 'wild' plasmids, plasmid-free (Pla⁻) derivatives of them and non-haemolytic strains to which the Hly plasmids had been transferred; P5, the O141:K85,88 strain previously studied by Smith & Linggood (1971) and Linggood & Ingram (1982) was used in its 88⁻,Col⁻ form. The non-haemolytic strains included spontaneous antibiotic-resistant mutants of a prototrophic K12 strain; of strain H209 (O9:K31:H4), that had been isolated from the faeces of a healthy human being; of strain MW (O18:K1:H7), that had caused meningitis in a baby (Smith & Huggins, 1980); and strain B188 (O78), that had caused generalized infection in a calf. Originally, MW and B188 possessed ColV plasmids but ColV⁻ derivatives were used in these studies because Hly plasmids could not be transferred to the ColV⁺ forms. The particular plasmid inserted in MW had been mobilized by transfer factor F from P102, a Tra⁻ O141:K85 strain, transferred to MW and then F eliminated by treatment with sodium lauryl sulphate. The methods of transferring Hly plasmids and of isolating Pla⁻ mutants from Hly⁺ strains were those of Smith & Linggood (1971).

Culture media and cultural conditions. Unless stated, all inoculated culture media were incubated at 37 °C for 24 h, liquid media, except when used for plasmid transfer, being shaken in a water bath (100 r.p.m.). Broth cultures consisting of organisms grown in 10 ml nutrient broth, Oxoid No. 2 (CM67), contained 1.5–3.0 × 10⁹ viable organisms ml⁻¹. Those of Hly⁺ organisms contained very little or no cell-free haemolysin. Alkaline extract broth, used for haemolysin production, was prepared according to the method of Smith (1963). Chloramphenicol (20 µg ml⁻¹) was added to all media used for growing strains WAF107, WAF108 and WAF111 because their plasmids, which contained genes for chloramphenicol resistance, were unstable.

Haemolysin preparations. Alkaline extract broth (70 ml) in 250 ml Erlenmeyer flasks was inoculated with 7 ml of a broth culture of *E. coli* and incubated for 3 h. It was then centrifuged at 6000 g for 30 min and the haemolysin precipitated from the supernate with 20% (w/v) ammonium sulphate at 5 °C for 1 h. The haemolysin was then separated by centrifugation at 6000 g for 30 min and re-dissolved in normal saline to 1/20 its original volume. It

was then centrifuged at 2500 *g* for 30 min and the supernate passed through a Millipore membrane filter (450 nm pore diameter). These filtrates, referred to as haemolysin preparations, usually contained about 10 times more haemolysin than the extracellular fluids of the alkaline broth cultures from which they had been prepared; their titres were expressed as the dilution which haemolysed 50% of a 2% suspension of sheep erythrocytes (Smith, 1963).

Experimental animals. Eight-week-old White Swiss mice of both sexes (approx. 30 g), bred at this institute, were used.

Testing haemolysin preparations for toxicity and living organisms for virulence. The toxicity of the haemolysin preparations was determined by inoculating groups of mice with twofold dilutions in normal saline into a tail vein. The virulence of live Hly⁺ and Hly⁻ organisms was assessed by inoculating groups of mice i.p. with three or tenfold dilutions of broth cultures in normal saline. Some of these groups were inoculated i.p. with 0.1 ml of 0.2% (w/v) FeSO₄ 10 min before they were inoculated with the broth cultures (Linggood & Ingram, 1982). The clinical signs and survival times of the mice were recorded. So were the macroscopic lesions in many that died.

Bacterial counts. The numbers of viable *E. coli* organisms in broth cultures and in heart blood, lungs and peritoneal washings of i.p. inoculated mice, were assessed by the method of Miles & Misra using MacConkey agar (Oxoid, CM7). Lungs were first ground with sterile sand in a pestle and mortar. Peritoneal washings were obtained by rinsing the abdomen with 1 ml of normal saline and retrieving as much of the fluid as possible.

RESULTS

Inoculation of mice with haemolysin preparations

Intravenously inoculated haemolysin preparations of the two *E. coli* J198 derivatives that possessed plasmid-cloned Hly determinants, WAF107 and WAF111, were lethal for mice, those of WAF107, the stronger haemolysin producer (reciprocal titre 1600), being more lethal than those of WAF111 (reciprocal titre 160). For example, 16 of 20 mice inoculated with 0.1 ml WAF107 preparation died, compared with 9 of 20 mice inoculated with 0.5 ml WAF111 preparation. Similar preparations of WAF108, the non-haemolytic insertional mutant of WAF107 and of Pla⁻ mutants of WAF107, WAF108 and WAF111 and J198 itself, even at the highest test dose of 0.5 ml, given to groups of 20 mice, had little obvious harmful effect.

Similar results were obtained when 'wild' Hly plasmids in their natural antigenically different pig enteropathogenic *E. coli* hosts and in *E. coli* K12 were studied. The haemolysin preparations of these pig and K12 strains possessed approximately the same concentration of haemolysin and when inoculated i.v. they were about equally lethal for mice, LD₅₀ ranging from 0.025 to 0.2 ml. Apart from 9 of the 90 mice given the highest test dose (0.5 ml) none of the mice inoculated with any of the preparations of the nine Pla⁻ derivatives of the pig and K12 strains died. A direct correlation between haemolysin titre and lethality was noted when haemolysin preparations of *E. coli* B188 containing Hly plasmids from four antigenically-different pig enteropathogens (strains P5, P102, P233 and P316) were inoculated i.v. into groups of mice. All those given 0.1 ml of the preparations of two of the Pla⁺ derivatives with a haemolysin titre of 1 in 1600 died. In contrast only 2 of the 10 given this dose of a preparation of another Pla⁺ derivative with a titre of 1 in 800 died. The titre of the preparation of the fourth derivative was 1 in 400; none of the mice given 0.1 or 0.2 ml of this preparation died but 4 of the 10 given 0.5 ml died.

Mice given the larger doses of haemolysin in all the previous experiments usually died 0.5–1 h after inoculation without exhibiting any characteristic clinical or pathological signs. Those given lower but still lethal doses survived longer, but rarely for more than 5 h. The majority of them exhibited a progressively severe dyspnoea; intense haemoglobinuria was frequently observed. These signs were common to mice given haemolysin preparations of both the WAF *E. coli* strains containing the plasmid-cloned Hly determinants and *E. coli* strains containing the 'wild' Hly plasmids.

Inoculation of mice with cultures of Hly⁺ and Hly⁻ derivatives of E. coli J198: effect of FeSO₄

When inoculated i.p., WAF107 and, to a lesser extent, WAF111, the weaker producer of haemolysin *in vitro*, were more virulent for mice than the Hly⁻ derivatives of these strains and of J198 itself (Table 1). When FeSO₄ was given i.p. before infection, the virulence of all the strains increased to about the same level. A striking observation was that survival times of mice that died following inoculation with Hly⁺ derivatives were much shorter than those of mice that died

Table 1. *Mortality in mice inoculated i.p. with cultures of derivatives (WAF) of E. coli J198 of different plasmid constitution: effect of FeSO₄*

Pre-treatment with FeSO ₄ *	Derivative inoculated†	Hly status	No. of 10 mice that died after receiving 10 ⁷ × the following no. of viable organisms			
			30	10	3	1
No	WAF107	+	10	5	0	0
	WAF108	—	0	0	—	—
	Pla ⁻ WAF107	—	1	0	—	—
	Pla ⁻ WAF108	—	1	0	—	—
	WAF111	+	7	0	—	—
	Pla ⁻ WAF111	—	0	0	—	—
	J198	—	1	0	—	—
Yes	WAF107	+	10	10	6	2
	WAF108	—	10	9	0	0
	Pla ⁻ WAF107	—	9	9	3	0
	Pla ⁻ WAF108	—	10	9	0	0
	WAF111	+	10	10	2	0
	Pla ⁻ WAF111	—	10	9	5	0
	J198	—	10	10	4	0

—, Not applicable.

* Where applicable, mice were given 200 µg FeSO₄ i.p. 10 min before receiving the *E. coli* inoculum.

† Pla⁻, plasmid-free. The plasmids of WAF107 and WAF111 contained different cloned Hly determinants; the plasmid of WAF108 was an insertional mutant of the plasmid of WAF107.

Table 2. *Survival times of mice inoculated i.p. with 3 × 10⁸ viable organisms of the derivatives of E. coli J198*

For other details see Table 1.

Pre-treatment with FeSO ₄	Derivative inoculated	Hly status	No. of 20 mice that had died by the following times (h) after inoculation														
			3	4	5	7	9	11	13	14	17	19	22	24	30	48	168
No	WAF107	+	0	8	15	17	18	18	18	18	18	18	18	18	18	18	18
	WAF108	—	0	0	0	0	0	0	0	0	1	2	3	4	5	6	6
	Pla ⁻ WAF107	—	0	0	0	0	0	0	0	0	0	0	0	0	2	4	4
	Pla ⁻ WAF108	—	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2
	WAF111	+	0	0	0	0	0	2	2	4	7	11	11	14	14	16	16
	Pla ⁻ WAF111	—	0	0	0	0	0	0	0	0	0	0	0	0	1	5	5
	J198	—	0	0	0	0	0	0	0	0	0	0	0	0	2	6	6
Yes	WAF107	+	0	10	19	19	19	19	19	19	19	19	19	19	19	19	19
	WAF108	—	0	0	0	0	0	1	8	12	13	14	18	18	18	19	19
	Pla ⁻ WAF107	—	0	0	0	0	0	4	6	9	15	20	20	20	20	20	20
	Pla ⁻ WAF108	—	0	0	0	0	0	2	8	10	14	15	19	19	19	20	20
	WAF111	+	0	0	0	3	8	13	14	15	16	16	17	17	18	19	19
	Pla ⁻ WAF111	—	0	0	0	0	0	0	0	4	4	7	14	18	18	18	18
	J198	—	0	0	0	0	0	0	0	6	10	11	16	17	17	17	17

following inoculation with the Hly⁻ derivatives. This was well illustrated in another experiment in which groups of 20 mice were inoculated with 3×10^8 viable organisms of the different derivatives and their survival times recorded at frequent intervals (Table 2). As far as the FeSO₄-treated mice were concerned, most of those given WAF107 had died by 5 h after inoculation. This situation was reached in the mice given WAF111 by 11 h but it was not reached in the mice given the Hly⁻ derivatives until 17 h or more, despite the fact that the lethality of the Hly⁺ and Hly⁻ derivatives for mice pre-treated with FeSO₄ was about the same (Table 1).

In the above studies with live cultures of the J198 derivatives and in subsequent ones with them and with strains possessing 'wild' Hly plasmids, the clinical and pathological signs exhibited by mice inoculated with Hly⁺ organisms resembled those previously noted in mice

Table 3. *Bacterial counts on tissues of mice inoculated i.p. with 3×10^8 viable organisms of the different derivatives of E. coli J198*

Five mice given each treatment were killed and the number of organisms in their peritoneal washings, lungs and blood were counted; peritoneal washings were obtained by rinsing out the abdomen with 1 ml of normal saline. The median for each set of five results is shown. The results for five mice examined 3 min after inoculation with WAF107 were: peritoneal washings, 7.7; lung, 3.3; blood, 3.5.

Pre-treatment with FeSO ₄	Derivative inoculated	Hly status	Time (h) after inoculation*	log ₁₀ [No. of organisms (g material) ⁻¹] in:					
				Peritoneal washings		Lung		Blood	
				3.5	13-16	3.5	13-16	3.5	13-16
No	WAF107	+		9.0	NA	4.8	NA	4.3	NA
	WAF108	-		8.6	6.0	5.5	4.0	6.0	<2.0
	Pla ⁻ WAF107	-		9.0	7.0	6.7	5.6	6.8	3.7
	Pla ⁻ WAF108	-		9.0	6.4	6.2	4.9	6.0	3.5
	WAF111	+		8.3	6.5	4.5	5.5	4.7	3.5
	Pla ⁻ WAF111	-		8.9	6.7	6.2	5.4	6.3	6.7
	J198	-		8.7	8.0	6.2	6.7	5.9	6.0
Yes	WAF107	+		9.0	NA	4.5	NA	5.0	NA
	WAF108	-		9.0	9.9	5.9	7.2	6.2	7.2
	Pla ⁻ WAF107	-		9.2	9.9	6.3	7.8	6.8	6.9
	Pla ⁻ WAF108	-		9.2	10.3	6.2	7.5	5.9	7.2
	WAF111	+		8.9	NA	4.0	NA	4.0	NA
	Pla ⁻ WAF111	-		9.2	10.0	6.2	7.3	6.3	7.3
	J198	-		9.2	10.0	5.9	7.3	6.0	6.8

NA, Not applicable because mice so inoculated did not survive for this period of time.

* 3.5 h was the time when mice were dying having received the WAF107 derivative with or without FeSO₄; 13-16 h was the time when mice were dying having received the Hly⁻ derivatives with FeSO₄.

inoculated with the sterile haemolysin preparations to the extent that they had severe progressive dyspnoea and many that survived for more than 5 h had intense haemoglobinuria. Serous pleural and peritoneal effusions were noted in some mice. These signs were not observed in mice that died following inoculation with Hly⁻ organisms. They exhibited a progressive lethargy and ultimately became moribund and died. Their respiration was slow and shallow and towards the termination of the disease they had a pronounced ocular discharge. No macroscopic pathological lesions were noted in them at autopsy.

Numbers of organisms in tissues of mice after i.p. inoculation with cultures of different derivatives of E. coli J198

Groups of mice, with or without FeSO₄ pre-treatment, were inoculated i.p. with 3×10^8 viable organisms of different derivatives of *E. coli* J198. Mice from each group were examined 3.5 h later when those given the Hly⁺ derivative WAF107 with or without FeSO₄ were dying and 13-16 h later when those given the Hly⁻ derivatives with FeSO₄ or WAF111 without FeSO₄ were dying (Table 3); groups not mentioned at these particular times were not gravely ill at that time. At 3.5 h the numbers of organisms in the peritoneal washings of the mice given WAF107 were no higher than they were in most of the other mice; the numbers in their lungs and blood and in the lungs and blood of the mice given the other Hly⁺ derivative WAF111 were usually 10 or more times lower than they were in the mice given the Hly⁻ derivatives (Table 3). Counts on liver were not meaningful because relatively high numbers of organisms were found on liver surfaces immediately after i.p. inoculation. At 6 h after inoculation (data not shown), the mice given WAF111 with FeSO₄ had only slightly higher numbers of organisms in their peritoneal washings, lungs and blood than did the mice near to death at 3.5 h after inoculation with WAF107. Counts of their peritoneal washings were no higher than those of the mice given the Hly⁻ derivatives and FeSO₄; their lung and blood counts were about 10 times lower. In general, the counts obtained at 6 h from groups of mice not given FeSO₄, including those inoculated with

Table 4. *Survival times of groups of 10 mice pre-treated or not with FeSO₄ and inoculated i.p. with forms of different strains of E. coli that possessed or did not possess 'wild' Hly plasmids*

Where applicable, mice were given 200 µg of FeSO₄ i.p. 10 min before receiving the *E. coli* inoculum. Deaths were recorded at approx. hourly intervals between 1 and 32 h post infection. Survival time was recorded when 6 out of 10 mice from the group had died.

<i>E. coli</i> strain	log ₁₀ (No. of viable organisms inoculated)	Survival time (h) of mice			
		- FeSO ₄		+ FeSO ₄	
		Hly ⁺ form	Hly ⁻ form	Hly ⁺ form	Hly ⁻ form
MW	8.5	3	10	2	9
	7.5	6	18	3	10
	6.5	10	-	4	8
	5.5	-	-	5	10
	4.5	-	-	7	27
	3.5	-	-	12	29
	2.5	-	-	14	30
	1.5	-	-	25	30
	-	-	-	-	-
P5	8.8	5	32	3	12
	8.5	6	28	3	12
	8.0	8	-	3	17
	7.5	11	-	4	17
	7.0	-	-	4	18
	6.0	-	-	11	20
	5.0	-	-	-	-
	-	-	-	-	-
H209	8.8	5	20	3	10
	8.5	6	-	4	20
	8.0	-	-	5	23
	7.5	-	-	-	-

-, Few or no deaths occurred in these groups.

WAF111, were lower than counts from the corresponding groups at 3.5 h. When mice given the Hly⁻ derivatives with FeSO₄ were nearing death at 13–16 h after inoculation, the numbers of organisms in their peritoneal washings were about 10 times higher than the numbers in the peritoneal washings of the mice dying 3.5 h after inoculation with the Hly⁺ derivative WAF107; the numbers in their lungs and blood were about 1000 times higher than the numbers in those tissues of the latter mice. The numbers of organisms at this time in the tissues of the mice given the Hly⁻ derivatives without FeSO₄ were much lower. So were the numbers in the mice given the Hly⁺ derivative WAF111 without FeSO₄, but these mice were dying whereas those given the Hly⁻ derivatives without FeSO₄ appeared normal.

Inoculation of mice with cultures of E. coli strains possessing or not possessing 'wild' Hly plasmids

The median mortality in 15 groups of 12 mice inoculated i.p. with 3×10^8 viable organisms of *E. coli* strain H209, to which the Hly plasmids of 15 epidemiologically unrelated strains of pig enteropathogenic *E. coli* belonging to seven different serotypes had been transferred, was 11 (range 7–12); all except 24 of these 162 mice died within 6 h of inoculation. By contrast, only three mice died in a group of 12 given the same dose of H209. One died 8–18 h and the other two died 18–24 h after inoculation.

Different doses of cultures of *E. coli* H209 and of the human invasive strain MW, of derivatives of these two strains to which the Hly plasmid of the pig enteropathogenic P102 had been transferred, of the pig enteropathogen P5 and of an Hly⁻ derivative of it, were inoculated i.p. into groups of 10 mice. The Hly⁺ form was more lethal than the Hly⁻ form by a factor of about 3 in the case of H209 and about 10 in the case of P5; there was little difference in lethality between the two forms of MW. In mice pre-treated with FeSO₄, the lethality of the Hly⁺ and Hly⁻ forms of each strain was about the same; it was greater than in the mice not given FeSO₄, especially in the case of MW and P5. As in the studies with the derivatives of *E. coli* J198, the

Table 5. Counts on tissues of mice when near to death having been inoculated i.p. with different doses of Hly⁺ and Hly⁻ forms of *E. coli* MW

Five mice given each treatment were killed and the numbers of MW organisms in peritoneal washings, lung and blood counted; the median for each set of counts is shown.

Pre-treatment with FeSO ₄	log ₁₀ (No. of viable organisms inoculated)	Hly status	Survival time (h) of mice*	log ₁₀ [No. of organisms (g material) ⁻¹] in†:		
				Peritoneal washings	Lung	Blood
No	8.5	+	2.5	8.3 (8.5)	7.0 (7.0)	7.3 (7.6)
		-	9.0	9.4	8.8	9.2
	7.5	+	4.0	8.0 (8.0)	7.4 (6.4)	7.5 (7.2)
		-	11.0	9.5	8.5	8.6
	6.5	+	7.3	8.3 (8.0)	6.9 (6.0)	7.3 (6.5)
		-	17.5	10.0	9.0	8.6
Yes	8.5	+	2.0	8.8 (8.5)	7.0 (7.4)	7.3 (8.0)
		-	7.5	9.9	8.8	9.0
	6.5	+	4.5	8.3 (7.3)	7.0 (6.6)	7.0 (6.6)
		-	9.5	9.9	8.9	9.0
	4.5	+	10.0	8.8 (9.7)	6.0 (7.0)	6.3 (6.4)
		-	17.0	10.0	9.0	8.0
	2.5	+	12.5	8.6 (9.5)	6.3 (5.6)	6.0 (5.7)
		-	22.0	10.3	9.0	7.8

* Survival times recorded as in Table 4.

† The figures in parentheses are the results for a group of five mice identically treated except that they were given the Hly⁻ form and examined at the stated time rather than when they were near to death.

disease was more acute in the mice inoculated with the Hly⁺ forms than in the mice inoculated with the corresponding Hly⁻ forms, the survival times of the former being much shorter than those of the latter (Table 4).

Numbers of organisms in tissues of mice near death following i.p. inoculation with E. coli MW or its Hly⁺ derivative

Irrespective of whether or not they had been pre-treated with FeSO₄, the numbers of organisms in the peritoneal washings, lungs and blood of mice near death following inoculation with different doses of the Hly⁺ derivative of *E. coli* MW were at least 10 times lower than they were in mice near death following i.p. inoculation with similar doses of the Hly⁻ MW (Table 5). When the mice given any particular dose of the Hly⁺ form were near death, the corresponding group given the Hly⁻ form looked reasonably healthy. Yet, in those pre-treated with FeSO₄, the numbers of organisms in the peritoneal washings, lungs and blood of the Hly⁻ group at that time were usually as high as they were in the Hly⁺ group. This was also the case for mice not pre-treated with FeSO₄ in the case of the peritoneal washings and of the lungs and blood of those given the highest inoculation dose; the numbers of organisms in the lungs and blood of those given the lower doses were somewhat higher than they were in the mice given the Hly⁻ form.

DISCUSSION

The experiments with bacteria-free preparations of the WAF set of strains, differing only in respect of whether or not they contained functioning genes for haemolysin production, established the validity of the findings of earlier studies (Smith & Halls, 1967) and of the present study employing 'wild' Hly plasmids, i.e. that haemolysin is toxic for mice to the extent that it may be lethal in high dosage. Mice that died soon after inoculation did not exhibit any evidence of haemoglobinuria, frequently noted in animals that succumbed later, indicating that mass destruction of erythrocytes was not the main manner whereby haemolysin exerted its lethal action.

In assessing the role of haemolysin in the disease produced by i.p. inoculation of mice with living organisms, the following observations have also to be taken into consideration. (1) Mice given the Hly⁺ WAF strains and the strains possessing 'wild' Hly plasmids died much sooner than the mice given the corresponding Hly⁻ forms of these strains. This was so even in mice that had been pre-treated with FeSO₄, a procedure which rendered them equally susceptible to the lethal activity of both the Hly⁺ and Hly⁻ forms of a strain. (2) Mice dying from infection with the Hly⁺ forms had much lower numbers of organisms in their tissues than did mice dying from infection with the corresponding Hly⁻ forms. The numbers of organisms in the tissues of FeSO₄-treated mice dying from infection with Hly⁺ forms were no higher than they were at that particular time in mice given the corresponding Hly⁻ forms; those in the lungs and blood were often lower. Yet the latter mice appeared reasonably healthy. The numbers of organisms in their tissues increased to a much greater extent before they became gravely ill. (3) Not only were bacteria-free preparations of WAF111 [which Welch & Falkow (1984) and ourselves found to produce much less haemolysin *in vitro* than the other Hly⁺ derivative of J198, WAF107] less toxic for mice than similar preparations of WAF107, but mice inoculated with live cultures of WAF111 were less susceptible to fatal infection and survived for much longer than mice inoculated with WAF107. (4) The clinical and pathological signs in mice infected with Hly⁺ organisms resembled those in mice given haemolysin preparations, the haemoglobinuria often noted in mice given the Hly⁺ organisms, indicating that the organisms were producing haemolysin *in vivo*; these signs were not noted in mice given Hly⁻ organisms. These observations are compatible with the following hypothesis. After inoculation, Hly⁺ organisms multiply and produce haemolysin. When sufficient has been liberated the host dies. Haemolysin is the most toxic product of the organisms. Consequently, inoculated Hly⁻ organisms have to multiply to a much greater extent than the Hly⁺ organisms before they cause the death of the host. Whether or not haemolysin also increased the severity of the disease in our mouse model by making iron more readily available to the invading organisms by lysing erythrocytes, as propounded by Linggood & Ingram (1982), is not known – our experiments were not designed to throw light on this aspect of the infection. There was no evidence that it was so in the experiments we performed with the WAF derivatives or with the highest dose of MW. However, in the mice given the two lower doses of MW without FeSO₄ and examined when those given the Hly⁺ form were dying, the numbers of organisms in the lungs and blood, but not the peritoneal washings, of those given the Hly⁺ form did tend to be higher than in those given the corresponding dose of the Hly⁻ form.

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