Killing of α -haemolytic and non-haemolytic *Escherichia coli* strains in human serum and polymorphonuclear leucocytes

L. SIEGFRIED*, H. PUZOVÁ, M. KMEŤOVÁ and A. KERESTEŠOVÁ

Department of Microbiology, Faculty of Medicine, University of P. J. Šafárik, Šrobárova 57, 040 00 Košice, Czechoslovakia

Summary. The role of α -haemolysin (AH) in the resistance of *Escherichia coli* strains, isolated from patients with extra-intestinal diseases or diarrhoea, to the bactericidal activity of human serum and intracellular killing in polymorphonuclear leucocytes (PMNL) was investigated *in vitro*. Sets of α -haemolytic and non-haemolytic *E. coli* strains and sets of isogenic *E. coli* strains, which included wild-type α -haemolytic strains and derived strains with a reduced production of AH, were used. Compared with non-haemolytic strains, α -haemolytic strains were significantly more resistant to the bactericidal activity of 10% and 100% human serum and to intracellular killing in PMNL. Higher resistance to serum killing and to intracellular killing in PMNL was also found in wild-type α -haemolytic *E. coli* than in isogenic bacteria with reduced production of AH. These results provide evidence that production of AH in *E. coli* strains counteracts both the bactericidal activity of serum and intracellular killing in PMNL.

Introduction

From studies in recent years it appears that *Escherichia coli* strains isolated from cases of intestinal and extra-intestinal diseases are endowed with certain traits considered of importance for virulence. These include production of bacterial adhesins,¹ certain O group² and K antigens,³ expression of the siderophore aerobactin,⁴ production of colicin V,⁵ production of thermolabile and thermostable enterotoxins,⁶ verocytotoxin,⁷ cytotoxic necrotising factor,⁸ α -haemolysin⁹ (AH) and resistance to the bactericidal activity of serum¹⁰ and to phagocytosis.¹¹

In *E. coli*, it has been shown that several properties of the bacterial cell-wall complex function as determinants of resistance to the bactericidal effect of serum. Rough phenotypes are, generally, more sensitive to serum killing than are smooth strains.¹² Moreover, it has been shown that certain plasmids can increase the resistance of *E. coli* to serum killing, presumably via a mechanism that causes a modification in the bacterial surface.¹³ From several reports it is known that capsular antigens play an important role in virulence of bacteria, conferring serum resistance and inhibiting phagocytosis.^{14–16} Another factor influencing phagocytosis is AH. Gadeberg and Blom¹⁷

reported that AH may damage cells of the host immune system, i.e., human blood monocytes and polymorphonuclear leucocytes (PMNL). In our previous reports,^{18,19} we suggested that AH may contribute to the resistance of *E. coli* strains isolated from intestinal and extra-intestinal diseases to serum bactericidal effects and to phagocytosis. To establish if this is true, we have compared the response of groups of α -haemolytic and non-haemolytic *E. coli* strains, and sets of isogenic strains differing in AH production, to the bactericidal activity of human serum and intracellular killing in PMNL.

Materials and methods

Bacterial strains

A total of 80 clinical isolates of *E. coli* was studied—43 α -haemolytic and 37 non-haemolytic strains. They were isolated from faeces of infants with diarrhoea (51 isolates), or from extra-intestinal samples (29 isolates). Of these latter isolates, 13 were from pharyngeal exudate of infants with pharyngitis, eight from urine of infants with urinary tract infections and eight from mothers with vaginal discharge. The strains were serotyped with eight polyvalent and 52 monovalent O antisera (Imuna). Non-haemolytic mutant strains were derived from α -haemolytic *E. coli* strains by treatment with rifampicin.²⁰

Received 23 July 1991; accepted 29 Aug. 1991.

* Correspondence should be sent to: Dr L. Siegfried, Matušková 6, 040 11 Košice, Czechoslovakia.

Serum and PMNL

Blood from one healthy donor was obtained by venepuncture and allowed to clot for 15 min at room temperature. After centrifugation (1500 *g*) the serum was removed, fractionated into small volumes and stored at -70°C until needed.

PMNL for tests of intracellular killing of *E. coli* were obtained from fresh, unheparinised blood of healthy donors.

Quantification of α -haemolysin production

The test was a modification of the method of Tabouret *et al.*²¹ An overnight culture of bacteria (125 μl), grown in Todd-Hewitt Broth (Difco) containing 10 mM CaCl_2 , was inoculated into 5 ml of the same medium and incubated in a waterbath at 37°C with agitation for 4 h. Supernates were collected after centrifugation (10000 *g* for 10 min at 4°C), adjusted to pH 7.0–7.4 with 1 N NaOH and assayed for haemolytic activity in polystyrene microtitration plates. Two-fold dilutions (in 100- μl volumes) of culture supernate in Tris-buffered saline, pH 7.4, containing 0.01 M CaCl_2 were made; 50 μl of washed sheep erythrocytes (1% suspension) was added to each dilution and the mixtures were incubated at 37°C for 2 h with occasional shaking. After incubation, 0.1 ml of saline was added and unlysed erythrocytes were pelleted by centrifugation (1000 *g* for 2 min). Determination of 50% haemolysis was based on counting of unlysed erythrocytes in a Bürker chamber. The number of haemolytic units (HU50/ml) was expressed as the dilution corresponding to 50% haemolysis.

Serum bactericidal assay

The assay was a slight modification of a method previously described.²² Overnight cultures of *E. coli* grown at 37°C on blood agar, were harvested. The cell suspensions were adjusted in Hanks's Balanced Salts Solution (HBSS) to contain 2.5×10^4 cfu/ml. Microplates were employed for incubation of bacteria with serum. One well contained 0.05 ml of 10% or 100% serum. The control well contained 0.05 ml of HBSS instead of serum. The microplates were sealed with adhesive tape to prevent evaporation and placed on a roller (angle 45°) and rotated for 180 min. Samples (10 μl) were withdrawn after incubation for 60, 120 and 180 min at 37°C and spread on blood agar plates. The plates were incubated for 18 h at 37°C and the viable count was determined. Susceptibility of bacteria to serum bactericidal activity was expressed as percentage of bacteria surviving after 180 min in relation to the original count of bacteria found at 0 min in the controls. Susceptibility of bacteria to serum was estimated by the Benge method.²³ Strains were termed serum sensitive if the viable count dropped to 1% of the initial value and resistant if $> 90\%$ of organisms survived after 180 min. Strains that gave results

between these values were considered of intermediate sensitivity.

Intracellular killing in PMNL

The intracellular killing of bacteria by human PMNL was determined microscopically with a slightly modified acridine orange staining method.²⁴ Fresh human blood (0.2 ml) was placed on a sterile cover slip in a humidified plastic chamber and allowed to clot for 60 min at 37°C . Coagulated blood was removed by rinsing the cover slips gently in warm (37°C) HBSS. Bacteria pre-opsonised in 100% serum at 37°C for 15 min were placed on to the PMNL monolayer. After incubation for 60 min, the cover slips were removed, air dried and stained with acridine orange (1 mg/ml) for 1 min. In this assay, intracellular bacteria fluoresce green when viable and red if non-viable. The viability of organisms in a total of 100 PMNL was estimated. The percentage killed was the percentage of intracellular organisms that were non-viable (red), i.e.,

$$\text{percent killed} = \frac{\text{total number of non-viable bacteria in 100 PMNL}}{\text{total number of intracellular bacteria}} \times 100$$

Statistical analyses

The χ^2 test was used for statistical comparison of the proportion of serum resistant isolates in α -haemolytic versus non-haemolytic *E. coli*, and of the relationship of O type with isolate origin, AH and serum resistance. Differences in intracellular killing (α -haemolytic versus non-haemolytic isolates) were estimated by Student's *t* test (two-samples analysis). Statistical comparison of results in wild-type strains and isogenic derivatives was made by Student's paired *t* test. Values of $p < 0.05$ were regarded as significant.

Results

Association of O type with isolate origin, AH and serum resistance

Of the 80 *E. coli* strains, 29 were from extra-intestinal sources and 51 from faeces (table I); 60 strains were typable and belonged to 20 O serogroups. Of the 20 non-typable strains, four (13.8%) were from extra-intestinal sources and 16 (31.4%) from faeces. Serogroup O6 was significantly more frequent among strains from extra-intestinal sources than among those from faeces (24.1% versus 3.9%, $p < 0.01$). A marked, but not statistically significant, difference was in the presence of serogroup O55 among isolates from faecal specimens compared to those from extra-intestinal sources (23.5% versus 13.5%). Serogroup O26 was found with the same frequency among strains isolated

Table I. Association of *E. coli* O serogroups with isolate origin, α -haemolysin production and serum resistance

Serogroup	Source of isolates	Number of strains		
		Total	AH	R
02	E	1	1	1
	D	0	0	0
06	E	7	7	5
	D	2	1	1
08	E	2	1	1
	D	1	0	0
026	E	4	2	2
	D	7	4	2
055	E	4	4	3
	D	12	8	7
075	E	1	1	1
	D	1	0	0
Other O-antigenic type	E	6	3	2
	D	12	4	4
Non-typable isolates	E	4	3	3
	D	16	5	2

E, strains isolated from extra-intestinal sources; D, strains isolated from faeces in diarrhoeal diseases; AH, α -haemolysin production; R, serum resistance.

from both sources (13.7%). Production of AH was more frequent in serogroups O6, O26 and O55 than in other O-groups and non-typable isolates (72.2% *versus* 40.9%, $p < 0.01$). Serum resistance was more frequent in strains of serogroup O6 and O55 than in

strains of serogroup O26, other O-groups, and non-typable strains (64% *versus* 32.7%, $p < 0.05$).

Bactericidal activity of serum and PMNL against α -haemolytic and non-haemolytic E. coli strains

Results of killing of α -haemolytic and non-haemolytic *E. coli* in serum and PMNL are shown in table II. There were differences between the response of isolates to 10% and 100% serum. A greater proportion of α -haemolytic than non-haemolytic strains was serum resistant. When 10% serum was employed all the α -haemolytic isolates were resistant to serum killing, but only 84% of non-haemolytic strains ($\chi^2 = 7.54$, $p < 0.01$). When 100% serum was used, 63% of the α -haemolytic strains were resistant compared to 22% of the non-haemolytic strains ($\chi^2 = 13.69$, $p < 0.01$).

Response of wild-type α -haemolytic E. coli and their non-haemolytic derivatives to killing by serum and PMNL

After treatment with 100% serum for 180 min, the survival of non-haemolytic derivatives was markedly depressed compared with wild-type α -haemolytic isolates ($t = 4.62$, $p < 0.01$). Similarly, in wild-type α -haemolytic isolates compared to their non-haemolytic derivatives there was significantly higher resistance to intracellular killing in PMNL ($t = 4.88$, $p < 0.01$).

Table II. Susceptibility of α -haemolytic and non-haemolytic *E. coli* strains to the bactericidal activity of serum and intracellular killing in PMNL

Haemolytic status (number of strains)	In serum, number of strains that were						Percentage of intracellular killing in PMNL	
	R		I		S		Mean (SD)	Range
	10%	100%	10%	100%	10%	100%		
AH (43)	43	27	0	14	0	2	79.5 (8.6)	54-92
NH (37)	31	8	6	21	0	8	84.4 (6.9)	64-94

AH, α -haemolytic; NH, non-haemolytic; R, serum resistant; I, intermediate sensitivity; S, serum sensitive.

Table III. Characteristics of four pairs of isogenic *E. coli* strains

Strains nos.	Percentage survival in serum	Percentage intracellular killing	α -haemolysin production (HU50/ml)	Origin
688 AH ⁺	220	71	512	Diarrhoea
688 AH ⁻	0	83	< 2	Mut(Rif)*
694 AH ⁺	197	63.5	256	Diarrhoea
694 AH ⁻	3	74	< 2	Mut(Rif)
698 AH ⁺	124	72.5	128	Diarrhoea
698 AH ⁻	1	77	< 2	Mut(Rif)
699 AH ⁺	96	80	8	Diarrhoea
699 AH ⁻	2	90.5	< 2	Mut(Rif)

* Mutant strain selected by rifampicin.

Discussion

In this paper serum resistance and intracellular killing of α -haemolytic and non-haemolytic *E. coli* strains were compared. Not much has been known about the role of AH in resistance of *E. coli* to the bactericidal activity of human serum and intracellular killing in PMNL. As previously described, production of AH is associated with certain O antigens²⁵ and it is largely found in *E. coli* causing extra-intestinal infections,²⁶ rarely in isolates from healthy people.⁸ In earlier reports, Knipschildt²⁷ and Kauffman²⁸ demonstrated that haemolytic *E. coli* strains belonged mainly to O groups 2, 4 and 6. Hughes *et al.*²⁵ found 50% of *E. coli* strains of groups O18 and O75 were haemolytic. Moreover, they suggested an association of AH production and serum resistance with serogroups O6, O18 and O75. Our results showed that AH production and serum resistance were associated with O groups 6 and 55; the former dominated in extra-intestinal isolates and the latter in faecal isolates.

Several reports have shown that there are various virulence determinants at the cell-wall surface that may play certain roles in the resistance of *E. coli* strains to the bactericidal activity of human serum.^{15, 29, 30} Our results indicate that AH could be another factor increasing the serum resistance of *E. coli*. This suggestion arises not only from the significantly higher serum resistance found in α -haemolytic isolates than in non-haemolytic ones, but mainly from the significantly higher serum resistance found in wild-type α -haemolytic isolates than in isogenic derivatives with reduced production of AH. Hughes *et al.*²⁵ have suggested a possible role of AH in serum resistance.

Several recent reports have shown that treatment of phagocytes with AH reduces their ability to respond to chemotactic stimuli, to phagocytose bacteria and to kill them.^{31, 32} Moreover, Gadeberg and Larsen³³ found that α -haemolytic *E. coli* were toxic to

phagocytes and this cytotoxic effect corresponded with titres of AH production. However, experiments with pre-treated cells only incompletely indicate the significance of AH for the outcome of *E. coli* interaction with phagocytes. Results of this study support their findings; we found a lower level of killing of α -haemolytic *E. coli* strains in PMNL than of non-haemolytic ones. However, there were considerable differences in intracellular killing among isolates examined. This could be ascribed to different levels of AH produced by particular isolates (unpublished observation). To distinguish a role of AH in intracellular killing, four sets of isogenic strains differing in AH production were used. The results obtained with these strains clearly demonstrate that α -haemolytic *E. coli* were killed to a lesser extent than isogenic strains with reduced production of AH. These results support those of Gadeberg *et al.*³⁴ who also found less intracellular killing of wild-type α -haemolytic *E. coli* than of their non-haemolytic derivatives.

Taking into account the results obtained in this study and those previously reported,^{25, 24} we consider that AH contributes not only to the resistance of *E. coli* to intracellular killing, but also to resistance to the bactericidal activity of serum. The mechanism of increased serum resistance by AH production could be explained by the finding of Bhakdi *et al.*³⁵ who have suggested that serum albumin, lipoproteins and other unidentified plasma factors are involved in neutralising AH. Serum components involved in bacterial killing may be neutralised by interaction with AH thus leading to some failure of killing. Moreover, recent reports of Milford and Taylor³⁶ and Caprioli *et al.*^{8, 37} raise the question of possible effects of extracellular products of *E. coli* other than AH, e.g., verocytotoxin and cytotoxic necrotising factor closely associated with AH production, on phagocyte function. Further investigations are needed to elucidate this.

References

- Klemm P. Fimbrial adhesins of *Escherichia coli*. *Rev Infect Dis* 1985; **7**: 321–340.
- Ørskov F, Ørskov I. *Escherichia coli* O:H serotypes isolated from human blood. Prevalence of the K1 antigen with technical details of O and H antigenic determination. *APMIS Sect B* 1975; **83**: 595–600.
- Evans DJ, Evans DG, Höhne C *et al.* Hemolysin and K antigens in relation to serotype and hemagglutination type of *Escherichia coli* isolated from extraintestinal infections. *J Clin Microbiol* 1981; **13**: 171–178.
- Johnson JR, Moseley SL, Roberts PL, Stamm WE. Aerobactin and other virulence factor genes among strains of *Escherichia coli* causing urosepsis: association with patient characteristics. *Infect Immun* 1988; **56**: 405–412.
- Minshew BH, Jorgensen J, Counts GW, Falkov S. Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extra-intestinal *Escherichia coli* isolates. *Infect Immun* 1978; **20**: 50–54.
- Levine MM. *Escherichia coli* that cause diarrhoea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J Infect Dis* 1987; **155**: 377–389.
- Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev* 1989; **2**: 15–38.
- Caprioli A, Falbo V, Ruggeri FM *et al.* Cytotoxic necrotizing factor production by hemolytic strains of *Escherichia coli* causing extraintestinal infections. *J Clin Microbiol* 1987; **25**: 146–149.
- Cavalieri SJ, Bohach GA, Snyder IS. *Escherichia coli* α -hemolysin. Characteristics and probable role in pathogenicity. *Microbiol Rev* 1984; **48**: 326–343.
- Taylor BW. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol Rev* 1983; **47**: 46–83.
- Ørskov I, Ørskov F. *Escherichia coli* in extra-intestinal infections. *J Hyg* 1985; **95**: 551–575.
- Reynolds BL, Pruul H. Protective role of smooth lipopolysaccharide in the serum bactericidal reaction. *Infect Immun* 1971; **4**: 764–771.
- Moll A, Manning PA, Timmis KN. Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the *tra T* gene product, is responsible for plasmid-specified serum resistance in *Escherichia coli*. *Infect Immun* 1980; **28**: 359–367.
- Horwitz MA, Silverstein SC. Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. *J Clin Invest* 1980; **65**: 82–94.

15. Roberts IS, Saunders FK, Boulnois GJ. Bacterial capsules and interactions with complement and phagocytes. *Biochem Soc Trans* 1989; **17**: 462–464.
16. Leying H, Suerbaum S, Kroll H-P, Stahl D, Opferkuch W. The capsular polysaccharide is a major determinant of serum resistance in K-1-positive blood culture isolates of *Escherichia coli*. *Infect Immun* 1990; **58**: 222–227.
17. Gadeberg OV, Blom J. Morphological study of the *in vitro* cytotoxic effect of α -hemolytic *E. coli* bacteria and culture supernatants on human blood granulocytes and monocytes. *APMIS Sect B* 1986; **94**: 75–83.
18. Siegfried L, Puzová H, Hlaučová J, Ďurovičová J. Evaluation of phagocytosis of alpha-hemolytic strains of *Escherichia coli* using the fluorescence method. *Czech Epidemiol Mikrobiol Immunol* 1989; **38**: 204–208.
19. Siegfried L, Puzová H, Hlaučová J, Ďurovičová J, Kročková J, Ebert HH. Killing of seroresistant plasmid bearing *E. coli* strains in polymorphonuclear leukocytes. *Bratisl Lek Listy* 1990; **91**: 292–297.
20. Mitchell I de G, Kenworthy R. Attempted elimination of plasmid-determined hemolysin, K88 antigen and enterotoxin from *Escherichia coli* pathogenic for pigs. *J Appl Bacteriol* 1977; **42**: 207–212.
21. Tabouret M, De Rycke J, Audurier A, Poutrel B. Pathogenicity of *Listeria monocytogenes* isolates in immunocompromised mice in relation to listeriolysin production. *J Med Microbiol* 1991; **34**: 13–18.
22. Miler I, Vondráček J, Hromádková L. The bactericidal activity of sera of healthy neonates and of newborns with hyperbilirubinaemia to *Escherichia coli*. *Folia Microbiol* 1979; **24**: 143–152.
23. Bengt GR. Bactericidal activity of human serum against strains of *Klebsiella* from different sources. *J Med Microbiol* 1988; **27**: 11–15.
24. Pantazis CG, Kniker WT. Assessment of blood leukocyte microbial killing by using a new fluorochrome microassay. *RES* 1979; **26**: 155–170.
25. Hughes C, Phillips R, Roberts AP. Serum resistance among *Escherichia coli* strains causing urinary tract infection in relation to O type and the carriage of hemolysin, colicin, and antibiotic resistance determinants. *Infect Immun* 1982; **35**: 270–275.
26. Hughes C, Hacker J, Roberts AP, Goebel W. Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *Escherichia coli*. *Infect Immun* 1983; **39**: 546–551.
27. Knipschildt HE. Undersogelser over Coligruppens Serologi. Copenhagen, Nyt Nordisk Forlag, Arnold Busck. 1945: 1–156.
28. Kauffmann F. The serology of the coli group. *J Immunol* 1947; **57**: 71–100.
29. Timmis KN, Boulnois GJ, Bitter-Suermann D, Cabello FC. Surface components of *Escherichia coli* that mediate resistance to the bactericidal activities of serum and phagocytes. *Curr Top Microbiol Immunol* 1985; **118**: 197–218.
30. Stawski G, Nielsen L, Ørskov F, Ørskov I. Serum sensitivity of a diversity of *Escherichia coli* antigenic reference strains. Correlation with an LPS variation phenomenon. *APMIS* 1990; **98**: 828–838.
31. Cavalieri SJ, Snyder IS. Effect of *Escherichia coli* alpha-hemolysin on human peripheral leukocyte function *in vitro*. *Infect Immun* 1982; **37**: 966–974.
32. Pruett TL, Cheoweth DE, Fiegel VD, Nelson RD, Simmons RL. *Escherichia coli* and human neutrophils. *Arch Surg* 1985; **120**: 212–216.
33. Gadeberg OV, Larsen SO. *In vitro* cytotoxic effect of α -hemolytic *Escherichia coli* on human blood granulocytes. Correlation with size of α -hemolysin production. *APMIS* 1988; **96**: 337–341.
34. Gadeberg OV, Hacker J, Ørskov I. Role of α -hemolysin for the *in vitro* phagocytosis and intracellular killing of *Escherichia coli*. *Zentralbl Bakteriell* 1989; **271**: 205–213.
35. Bhakdi S, Greulich S, Muhly M *et al.* Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J Exp Med* 1989; **169**: 737–754.
36. Milford DV, Taylor CM. New insights into the haemolytic uremic syndromes. *Arch Dis Child* 1990; **65**: 713–715.
37. Caprioli A, Falbo V, Ruggeri FM, Minelli F, Ørskov I, Donelli G. Relationship between cytotoxic necrotizing factor production and serotype in hemolytic *Escherichia coli*. *J Clin Microbiol* 1989; **27**: 758–761.