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Role of α-Hemolysin for the in vitro Phagocytosis and Intracellular Killing of Escherichia coli

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Abstract

The role of α-hemolysin for the elimination of *Escherichia coli* by phagocytes *in vitro* was investigated using sets of isogenic strains which included wild-type α-hemolytic strains, derived strains with a reduced production of α-hemolysin and derived nonhemolytic strains. Phagocytosis and intracellular killing of the bacteria by human blood granulocytes or monocytes were measured using growth inhibition techniques. α-hemolytic strains were phagocytosed and killed to a lesser extent than isogenic strains with a reduced production of α-hemolysin and isogenic nonhemolytic strains. The results obtained with granulocytes were similar to those obtained with monocytes although the elimination of bacteria by monocytes was less than that by granulocytes. These results strongly suggest that production of α-hemolysin is a means by which *E. coli* counteracts the activity of phagocytes by injuring these cells with the toxin.

Zusammenfassung

Die Rolle von α-Hämolysin bei der in vitro-Eliminierung von *Escherichia coli* durch Phagozyten wurde unter Verwendung isogener Stämme einschließlich von α-hämolysierenden Wildstämmen, davon abstammenden Stämmen mit reduzierter α-Hämolysin-Bildung und davon abstammenden nicht hämolysierenden Stämmen untersucht. Phagozytose und intrazelluläre Abtötung der Bakterien durch Granulozyten oder Monozyten im menschlichen Blut wurden unter Verwendung von Wachstums-Hemmtechniken gemessen. α-hämolysierende Stämme wurden in geringerem Maße als isogene Stämme mit einer geringeren Hämolysin-Bildung und isogene nicht hämolysierende Stämme phagozytiert und abgetötet. Die mit Granulozyten erzielten Ergebnisse waren den bei Monozyten ähnlich, obwohl die Bakterienelimination durch Monozyten geringer war als durch Granulozyten. Diese Ergebnisse deuten stark darauf hin, daß die Bildung von α-Hämolysin ein Mittel ist, mit dem *E. coli* der Aktivität der Phagozyten durch Schädigung dieser Zellen mit dem Toxin entgegenwirkt.

Introduction

It is generally agreed that α-hemolysin is one of the virulence factors of *Escherichia coli* (for a review see 21). Several recent reports have shown that treatment of phagocytic and other cells with *E. coli* α-hemolysin reduces the functional abilities and viability of these cells *in vitro* (3, 4, 5, 7, 8, 16, 22, 23). In addition, we have shown that α-hemolytic (Hly^+(α)) *E. coli* organisms are cytotoxic to various degrees *in vitro* towards human blood granulocytes, monocytes and monocyte-derived macrophages in the presence of fresh plasma and that the cytotoxic effect is probably mediated by secreted α-hemolysin (9–13 and *Gadeberg, O. V.* and *J. M. Rhodes*, unpublished observations). Nonhemolytic (Hly^-) *E. coli* are not cytotoxic (12). These results indicate that α-hemolysin may be a tool by which *E. coli* counteracts the activity of phagocytes.

The purpose of the present work was to investigate, in experiments exposing sets of isogenic *E. coli* organisms to fresh phagocytes, whether the ability to produce α-hemolysin plays a role for the extent to which these bacteria are phagocytosed and killed.

Materials and Methods

Fractionation of blood leukocytes. Blood was collected from healthy adults and suspensions of mononuclear cells and purified granulocytes were prepared as described earlier (9). Plasma was centrifuged and filtered to remove platelets.

Bacteria. A total of 27 O : K : H serotyped *E. coli* strains were used (Table 1). 19 strains originated from the International Escherichia Centre (WHO), Statens Seruminstitut, and 8 strains from the Institut für Genetik und Mikrobiologie, University of Würzburg. 9 were Hly^+(α) wild-type strains and originated from humans with various infections. 8 were nonhemolytic mutant strains derived from the α-hemolytic strains by treatment with ethylmethane sulfonate (18) or rifampicin (20). One was a spontaneous α-hemolytic revertant strain (C1497-80 Hly^+(α)R) derived from the nonhemolytic mutant strain C1497-80 Hly^-. One was an α-hemolytic mutant strain (C459-81 Hly^+(α)M) with a reduced production of α-hemolysin derived from strain C459-81 Hly^+(α) by treatment with ethylmethane sulfonate. The wild-type α-hemolytic strains 536 and 764 originated from a patient with urinary tract infection (15) and from the faeces of a healthy person (2), respectively. Strains 536-31 and 764-2 were spontaneous nonhemolytic mutants derived from strains 536 and 764, respectively. The strains were transformed with the cloning vector pACYC 184 (6) or with one of the α-hemolysin encoding recombinant plasmids pANN 5311 (which was derived from the chromosome of strain 764 (2)) or pANN 202-312 (which was derived from the plasmid pHly 152 (14)) by a modified $CaCl_2$ procedure (17).

The α-hemolytic status of the strains was determined as described previously (11).

The bacteria were cultured in ox-meat broth, washed in Eagle's modified minimal essential medium pH 7.4 (MEM) and the bacterial concentration was adjusted by photometry as described earlier (9). In experiments with transformed strains a concentration of 20 μg chloramphenicol per ml was maintained throughout all procedures.

Measurement of susceptibility to the bactericidal activity of plasma. To circumvent any disturbing influences of plasma only strains resistant to the bactericidal activity of fresh human plasma were used. The susceptibility of the strains to plasma (40%) was measured as described earlier (13).

Tests for adhesins. All strains were tested for mannose-sensitive and mannose-resistant adhesins using techniques described previously (26). All isogenic strains except one set were similar with respect to these adhesins: Strain C829-81 Hly^- did not possess mannose-resistant adhesins whereas strain C829-81 Hly^+(α) did.

Table 1. Characteristics of 11 sets of isogenic *E. coli* strains

Strains	Serotype	α-Hemolysin (HU^{a}_{50}/ml)[ae]	Origin
C1611-80 $Hly^{+}(\alpha)$ C1611-80 Hly^{-}	O6:K13:H1	1206 (820–1900) <2	Dia Mut(E)
C1792-80 $Hly^{+}(\alpha)$ C1792-80 Hly^{-}	O6:K2:H31	140 (64–210) <2	Sep. Mut(E)
C385-81 $Hly^{+}(\alpha)$ C385-81 Hly^{-}	O75:K95:H5	440 (340–520) <2	UTI Mut(E)
C400-81 $Hly^{+}(\alpha)$ C400-81 Hly^{-}	O18:K5:H⁻	60 (45–80) <2	UTI Mut(E)
C829-81 $Hly^{+}(\alpha)$ C829-81 Hly^{-}	O6:K2:H1	230 (220–230) <2	Dia Mut(E)
C281-82 $Hly^{+}(\alpha)$ C281-82 Hly^{-}	O6:K13:H⁻	975 (880–1150) <2	UTI Mut(R)
Test K13 $Hly^{+}(\alpha)$ Test K13 Hly^{-}	O6:K13:H1	316 (210–440) <2	UTI Mut(E)
C1497-80 $Hly^{+}(\alpha)$ C1497-80 $Hly^{+}(\alpha)$ R C1497-80 Hly^{-}	O75:K5:H5	121 (85–240) 120 (76–240) <2	Dia rev. Mut(E)
C459-81 $Hly^{+}(\alpha)$ C459-81 $Hly^{+}(\alpha)$ M	O25:K1:H1	1702 (800–3800) 3 (2–4)	Sep. Mut(E)
536 pACYC184 $Hly^{+}(\alpha)$ 536-31 pANN5311 $Hly^{+}(\alpha)$ 536-31 pANN202-312 $Hly^{+}(\alpha)$ 536-31 pACYC184 Hly^{-}	O6:K15:H31	523 (380–720) 200 (140–260) 160 (140–180) <2	UTI[b] Tra Tra Mut(S)[b]
764 pACYC184 $Hly^{+}(\alpha)$ 764-2 pANN5311 $Hly^{+}(\alpha)$ 764-2 pANN202-312 $Hly^{+}(\alpha)$ 764-2 pACYC184 Hly^{-}	O18:K5:H⁻	64 (52–76) 58 (52–64) 28 (27–28) <2	Faec[b] Tra Tra Mut(S)[b]

[a] Hemolytic units 50, see text. The figures of the column are geometric means and ranges
$Hly^{+}(\alpha)$: α-hemolytic. Hly^{-}: Nonhemolytic
Dia: Diarrheal disease. Sep: Septicemia. UTI: Urinary tract infection. Faec.: Faeces from healthy person
Mut(E) and Mut(R): Mutant strain induced by ethylmethane sulfonate or rifampicin, respectively, see text
Mut(S): Spontaneously induced mutant strain
Rev: Revertant strain
[b] Transformed with the cloning vector pACYC184
Tra: Transformed strain, see text

Quantitative test for α-hemolysin. The production of standardized culture supernatants and the measurement of the concentration of α-hemolysin (HU_{50}/ml) was carried out as described earlier (10, 11).

Measurement of phagocytosis of E. coli in vitro. These experiments were carried out according to a modification of the technique of *van Furth* et al. (28). 0.5 ml granulocytes

(2×10^7/ml) was incubated with 0.1 ml bacteria (5×10^7/ml) and 0.4 ml fresh autologous plasma at 37 °C on a roller (30 revolutions per min). Control tubes without granulocytes were incubated simultaneously. A 0.4 ml sample was removed after 15 and 30 min and mixed with 3.6 ml ice-cold MEM to prevent further phagocytosis. The samples were then centrifuged at 100 G for 5 min to sediment the granulocytes and appropriate 10-fold dilutions of the supernatant were made in MEM and spread on agar plates. After incubation overnight the extracellular bacterial concentration (EBC) was estimated by counting the colony forming units (CFU). The extent of phagocytosis was expressed as a ratio: The bacterial concentration after incubation in these absence of phagocytes divided by EBC after incubation in the presence of phagocytes. In order to exclude the possibility that bactericidal cell products might have caused extracellular killing of the bacteria, incubation media originating from 30 min phagocytosis experiments were tested for bactericidal activity: The media were centrifuged (1000 G, 5 min) and sterilized by filtration (Millipore). 0.9 ml of filtrate was then mixed with 0.1 ml bacteria (5×10^7/ml) and incubated on a roller as described above for 30 min. Controls in which bacteria were mixed with MEM containing 40% plasma, were also incubated. The bacterial concentration after incubation was estimated as described above. No bactericidal activity was observed.

Measurement of intracellular killing of E. coli in vitro. These experiments were carried out according to a modification of the technique of *Steigbigel* et al. (24). 0.5 ml granulocyte or mononuclear cell suspension (10^7 or 4×10^7/ml, respectively) was incubated with 0.1 ml bacteria (10^8/ml) and 0.4 ml fresh autologous plasma on a roller as described above. Control tubes without leukocytes were incubated simultaneously. A 0.1 ml sample was removed after 2 and 4 h and mixed with 0.6 ml 37 °C bacterial culture supernatant containing 2010 HU_{50} α-hemolysin/ml (produced by *E. coli* strain C459-81 Hly^+ (α)). After 5 min 9.3 ml redistilled water was added and the sample was placed on ice for 10 min. This hemolysin-water treatment effectively disrupted the leucocytes and did not affect the viability of the bacteria (*Gadeberg, O. V.*, unpublished observation). Appropriate 10-fold dilutions of the samples were prepared in MEM and spread on agar plates. After incubation overnight total (intracellular and extracellular) bacterial concentrations (TBC) were estimated by counting the CFU. The extent of intracellular killing was expressed as a ratio: The bacterial concentration after incubation in the absence of phagocytes divided by TBC after incubation in the presence of phagocytes. To exclude extracellular killing by bactericidal cell products incubation media originating from 4 h killing experiments were tested for bactericidal activity as described above except that incubation was continued for 4 h. No bactericidal activity was found.

Results

Phagocytosis and intracellular killing of isogenic E. coli in vitro

The reduction of the extracellular and total bacterial concentrations of *E. coli* caused by human blood granulocytes varied greatly between the 27 strains tested including the nonhemolytic strains (Table 2).

Within each of the 11 sets of isogenic strains the extracellular bacterial concentrations at 15 and 30 min and the total bacterial concentrations at 2 and 4 h of wild-type Hly^+(α) strains were reduced to a lesser extent than those of derived strains with a reduced production of α-hemolysin (strain sets C459-81, 536 and 764) and those of derived nonhemolytic strains (e.g. strain sets C1611-80, C1792-80 and C385-81).

However, the difference between isogenic strains with respect to the reduction of the bacterial concentrations varied substantially (Table 2, Figs. 1 and 2). Even the difference between Hly^+(α) strains which produced about equal amounts of α-hemolysin and their Hly^- counterparts varied significantly (strain sets C1792-80 and C1497-80).

Table 2. *In vitro* phagocytosis and intracellular killing of isogenic strains of *E. coli* by human granulocytes

Strains	Reduction of EBC[a]	Reduction of TBC[b]
C1611-80 $Hly^+(\alpha)/Hly^-$	8.2/46.2	7.4/1630
C1792-80 $Hly^+(\alpha)/Hly^-$	29.4/87.0	18.1/3850
C385-81 $Hly^+(\alpha)/Hly^-$	8.2/21.2	575/7380
C400-81 $Hly^+(\alpha)/Hly^-$	2.1/3.8	11.8/24.0
C829-81 $Hly^+(\alpha)/Hly^-$	3.8/80.0	6.5/3020
C281-82 $Hly^+(\alpha)/Hly^-$	1.3/34.6	44.1/3200
Test K13 $Hly^+(\alpha)/Hly^-$	5.6/52.7	20.3/714
C1497-80 $Hly^+(\alpha)/Hly^+(\alpha)R/Hly^-$	1.9/2.2/2.4	3.8/5.7/10.3
C459-81 $Hly^+(\alpha)/Hly^+(\alpha)$ M	14.8/39.2	46.3/3200
536 pACYC184 $Hly^+(\alpha)$/ 536-31 pANN5311 $Hly^+(\alpha)$/ 536-31 pANN202-312 $Hly^+(\alpha)$/ 536-31 pACYC184 Hly^-	6.0/7.2/9.1/11.5	40.5/43.6/68.5/323
764 pACYC184 $Hly^+(\alpha)$/ 764-2 pANN5311 $Hly^+(\alpha)$/ 764-2 pANN202-312 $Hly^+(\alpha)$/ 764-2 pACYC184 Hly^-	1.9/2.8/2.7/4.1	1.9/5.9/7.9/9.3

[a] Bacterial concentration after incubation in the absence of granulocytes divided by extracellular bacterial concentration (EBC) after incubation in the presence of granulocytes for 30 min. The results obtained after incubation for 15 min correspond closely to those shown, the figures being smaller. The average bacterial concentration before incubation was 5.1 (range 2.4–8.5) $\times 10^6$/ml. The technique used was modified from *van Furth* et al. (28), see text. Phagocyte: bacteria ratio 2:1.

[b] Bacterial concentration after incubation in the absence of granulocytes divided by total (extracellular + intracellular) bacterial concentration (TBC) after incubation in the presence of granulocytes for 4 h. The results obtained after incubation for 2 h correspond closely to those shown, the figures being smaller. The average bacterial concentration before incubation was 0.83 (range 0.43–1.3) $\times 10^7$/ml. The technique used was modified from *Steigbigel* et al. (24), see text. Phagocyte: bacteria ratio 1:2.

Other abbreviations: See Table 1.

Similar results were obtained using human blood monocytes. However, generally the capacity of monocytes to reduce bacterial concentrations of *E. coli* was significantly smaller than that of granulocytes (monocyte data not shown).

Discussion

Various features of *E. coli*, including the amount and character of the K-antigens and the nature of the O-antigens and outer membrane proteins, play a significant role in the extent to which these bacteria are phagocytosed (1, 19, 25).

Recent reports (4, 5, 22, 23) have shown that granulocytes after exposure to *E. coli* α-hemolysin in non-leucocidal concentrations exhibit depressed functional capacities, e.g. ability to respond to chemotactic stimuli, to phagocytose and to kill microorgan-

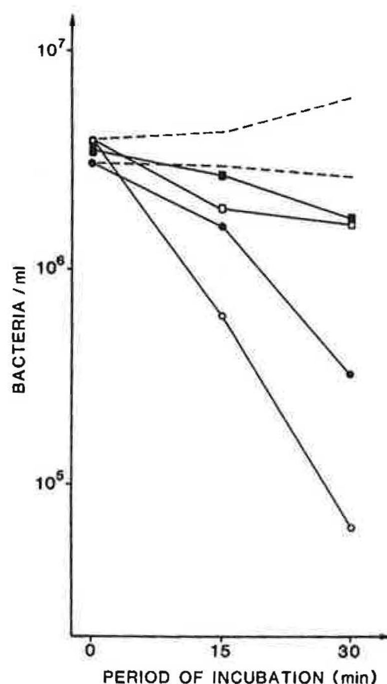

Fig. 1

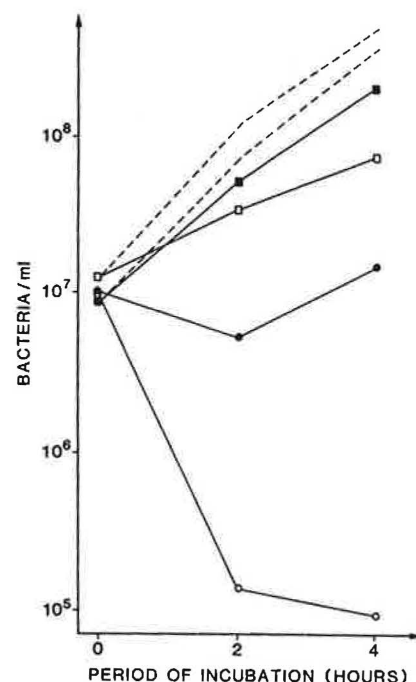

Fig. 2

Fig. 1. Example showing the *in vitro* phagocytosis of two sets of isogenic *E. coli* strains by human granulocytes: In the case of the C1611-81 set the extracellular bacterial concentration of the Hly⁻ strain (– ○ –) was reduced more than that of the Hly⁺(α) strain (– ● –). In the case of the C400-81 set there was little difference between the reduction of the extracellular bacterial concentration of the Hly⁻ (– □ –) and that of the Hly⁺ (α) (– ■ –) strain. In the absence of granulocytes the concentrations of bacteria were found to be within the range indicated by the dotted lines.

Fig. 2. Example showing the *in vitro* intracellular killing of two sets of isogenic *E. coli* strains by human granulocytes. In the case of the C1792-80 set the total (intracellular and extracellular) bacterial concentration of the Hly⁻ strain (– ○ –) was reduced much more than that of the Hly⁺ (α) strain (– ● –). In the case of the C1497-80 set there was little difference between the reduction of the total bacterial concentration of the Hly⁻ (– □ –) and that of the Hly⁺ (α) (– ■ –) strain. In the absence of granulocytes, the concentrations of bacteria were found to be within the range indicated by the dotted lines.

isms. We have obtained similar results with granulocytes pretreated with *E. coli* culture supernatants containing α-hemolysin (*Gadeberg, O. V.*, unpublished observations). Moreover, Hly⁺(α) *E. coli* organisms are toxic to phagocytes to degrees which correspond to the magnitude of their production of α-hemolysin (13). These data indicate that the ability to produce α-hemolysin is an additional feature by which *E. coli* may oppose the activity of phagocytes by injuring these cells. However, experiments with pretreated phagocytes only incompletely elucidate the significance of α-hemolysin for

the outcome of the interaction of *E. coli* with these cells because the effect of other bacterial characteristics have not been taken into consideration in these experiments.

We have addressed the subject in a different way, i.e. by incubating isogenic strains of *E. coli*, supposed to differ only with respect to the ability to produce α-hemolysin, with fresh phagocytes in the presence of fresh autologous plasma. The results obtained with several sets of isogenic strains are in complete agreement. Irrespective of whether mutant or transformed strains were used, $Hly^{+}(\alpha)$ strains were phagocytosed and killed to a lesser extent than isogenic strains with reduced or no production of α-hemolysin.

Since other characteristics apart from the α-hemolytic trait influence the outcome of the phagocyte-bacteria interaction (illustrated in this study by the large differences between the nonhemolytic strains), the significance of the α-hemolytic trait for this outcome varies with the other features of the microorganisms. Thus, if a strain possesses other well developed factors by which attack by phagocytes are counteracted, α-hemolysin may play a minor additional role. If, however, such factors are not expressed or only weakly expressed in a strain the effect of α-hemolysin will be more dominant. This may explain why e.g. strain sets C1497-80 and C1792-80, the $Hly^{+}(\alpha)$ strains of which produced about the same amount of α-hemolysin (Table 1), behaved rather differently when incubated with the phagocytes. The $Hly^{+}(\alpha)$ and Hly^{-} strains of the C1497-80 set were phagocytosed and killed to only a small extent and the difference between them in this respect was slight. In contrast, both the $Hly^{+}(\alpha)$ and Hly^{-} strains of the C1792-80 set were phagocytosed and killed. However, this occurred to a much lesser extent in the $Hly^{+}(\alpha)$ strain compared with its Hly^{-} counterpart.

The varying significance of the α-hemolytic trait for the elimination of different strains of *E. coli* by phagocytes indicated by our results may well be relevant for observations made by *van den Bosch* et al. (27). They reported that the production of α-hemolysin was a decisive virulence factor in most of the mouse pathogenic *E. coli* strains called group II, whereas this was not the case with strains belonging to the highly virulent group III, which had a more general virulence for mice.

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