The role of *Escherichia coli* haemolysin in the pathogenic synergy of colonic bacteria in subcutaneous abscess formation in mice

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Summary. The growth of nine species of colonic bacteria—*Escherichia coli*, *Enterococcus faecalis*, *Bacteroides ovatus*, *Fusobacterium varium*, *Clostridium perfringens*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Staphylococcus aureus* and *Bifidobacterium adolescentis*—was examined after concomitant injection to form experimental subcutaneous abscesses in mice. Injection of a mixture of c. 10^5 cfu of each of the first five strains (*E. coli*, *Ent. faecalis*, *B. ovatus*, *F. varium* and *C. perfringens*) resulted in abscess formation in all mice tested when the *E. coli* strain was haemolytic. *E. coli* and *B. ovatus* multiplied and reached a maximum population of c. 10^8 cfu/abscess. When non-haemolytic *E. coli* was used, injection of $\geq 10^7$ cfu was required for abscess formation. The inclusion of partially purified *E. coli* haemolysin (125 HU₅₀) with c. 10^5 cfu of bacteria including non-haemolytic *E. coli* resulted in abscess formation in most mice tested. These results indicate that *E. coli* haemolysin is one factor that may potentiate pathogenic synergy among colonic bacteria especially between *E. coli* and *B. ovatus*, during abscess formation.

Introduction

A wide variety of aerobic and anaerobic bacteria has been isolated from infected foci, such as abscesses, caused by colonic flora. *Escherichia coli* and members of the fragilis group of *Bacteroides* are detected most frequently.^{1,2} Since these bacteria usually predominate in the colonic flora, their frequent isolation is to be expected. However, the reasons for the selective survival of *E. coli* and *B. fragilis* in such foci of infection are not clear. Many investigations have reported pathogenic synergy among aerobic and anaerobic bacteria.^{3–5} Verweij-van Vught *et al.*⁶ found synergy between *E. coli* and *B. fragilis* in abscess formation, and showed that the intensity of synergic activity was dependent on *E. coli* although the factors involved were not identified. In this study, the role of haemolysin produced by *E. coli* in the pathogenic synergy of nine bacterial species predominant in the colonic microflora was examined in a subcutaneous abscess model in mice.

Materials and methods

Bacterial species

Bacteria used were representative strains of seven predominant bacterial species of the human colon—*E.*

coli ATCC 25922 (haemolytic) and ATCC 11775 (non-haemolytic), *Klebsiella pneumoniae* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Enterococcus faecalis* IFO 12969, *B. ovatus* SU 39, *Fusobacterium varium* ATCC 8501 and *Bifidobacterium adolescentis* ATCC 15703—and two species resident in small numbers—*Staphylococcus aureus* ATCC 25923 and *Clostridium perfringens* VPI 5201. The last two bacteria are recognised pathogens but are rarely found in lesions caused by colonic flora. Bacteria were obtained from the American Type Culture Collection (ATCC), the Institute for Fermentation Osaka, Japan (IFO), the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA (VPI); *B. ovatus* was from our own laboratory (SU, Shiga University).

Inocula

The test strains were inoculated into 5 ml of Gifu anaerobe medium (GAM) broth (Nissui Seiyaku Co. Ltd, Tokyo) and incubated anaerobically in an atmosphere of CO₂ 10%, N₂ 90% at 37°C for 18–20 h. The culture was then centrifuged at 4000 *g* for 10–15 min at 5°C and the resulting sediment was suspended in 5 ml of suspending medium so that the density was (5×10^8) – (1×10^9) cfu/ml (c. McFarland tube no. 3–4); 0.5–1.0-ml samples of the cell suspension of each test strain were pooled to form the mixtures. Mice were inoculated with 0.1 ml of the original mixture or its appropriate dilution.

The suspending medium contained (/L) Trypticase Peptone (BBL) 10 g, Proteose Peptone No. 3 (Difco) 10 g

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Yeast Extract (BBL) 2 g, chondroitin sulphate sodium salt (Tokyo Kasei, Tokyo) 1 g, haemin (equine, type III; Sigma) 0.01 g, glucose 1 g, glycogen (oyster) 3 g, NaCl 3 g, Na₂HPO₄ (anhydrous) 5 g, KH₂PO₄ (anhydrous) 2 g, L-cysteine HCl-H₂O 0.5 g, Tween 80 0.25 g, and agar 1.5 g (all supplied by Nacalai Tesque, Tokyo). The pH of the medium was adjusted to 7.0 with NaOH 10% w/v and HCl 10% v/v.

Preparation of partially purified haemolysin

Haemolysin was prepared according to the method of Ushijima *et al.*⁷ From a fresh culture of *E. coli* ATCC 25922 in Heart Infusion Broth (Nissui, Tokyo) supplemented with glucose (Nacalai Tesque) 0.1% w/v and Tween 80, 0.05% w/v, 2 ml was inoculated into 200 ml of the same broth—c. $(1-5) \times 10^6$ cfu/ml. After aerobic incubation for 4–6 h at 37°C with occasional shaking, cells in the late logarithmic to early stationary growth phase were produced. Next NaN₃ (Nacalai Tesque) 0.02% w/v was added to the culture and the preparation was centrifuged at 16 000 *g* for 30 min at 0–2°C. The supernate was passed through a membrane filter (0.45- μ m pore) and 50–60 g of polyethylene glycol (PEG) 4000 (Nacalai Tesque) were added to the filtrate. After the PEG had dissolved, the preparation was held for 60 min in an ice bath with occasional shaking. The solution was centrifuged at 16 000 *g* for 30 min at 0–2°C and the supernate was removed carefully. The precipitate was dissolved in 2 ml of 0.15 M NaCl supplemented with 0.01 M CaCl₂.⁸ The absence of viable *E. coli* was checked by spreading 0.1 ml of the haemolysin solution on nutrient-agar plates. The haemolysin preparation was stored until needed at below –20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the partially purified *E. coli* haemolysin was performed according to the method of Laemmli⁹ with slight modification. Briefly, a separation gel of acrylamide 7.5% w/v containing 0.375 M Tris-HCl, pH 8.8, and SDS 0.1% w/v was prepared from a stock solution of acrylamide 30% w/v and N, N-bis-methylene acrylamide 1% w/v. A plate gel 10 cm long, 13 cm wide and 1 mm deep was prepared and polymerised chemically by the addition of tetramethylethylenediamine and ammonium persulphate 0.025% v/v. A stacking gel (c. 1 cm length) of acrylamide 3% w/v containing 0.125 M Tris-HCl, pH 6.8, and SDS 0.1% w/v was polymerised as for the separation gel. The samples contained 0.0625 M Tris-HCl, pH 6.8, SDS 2% w/v, glycerol 10% v/v, 2-mercaptoethanol 5% v/v and bromophenol blue 0.001% w/v; they were immersed in boiling water for 1.5 min and then 80 μ l of the sample was applied to the injection port of the gel. Electrophoresis was performed at 10 mA/gel until bromophenol blue had passed through the stacking gel and then at 25 mA until the dye reached the bottom of the gel (about 4 h). The proteins in the gel

were stained for 30 min with Coomassie Brilliant Blue 0.25% w/v in methanol:acetic acid:water 5:1:5 v:v:v. The gel was destained by repeated washing in methanol:acetic acid:water. Mol.-wt markers used were egg albumin (45 Kda), bovine serum albumin (66 Kda), rabbit muscle phosphorylase B (97.4 Kda), β -galactosidase (116 Kda), and myosin (205 Kda).

Subcutaneous abscess formation in mice

Specific pathogen-free mice (Institute of Cancer Research, USA) weighing 20–25 g were given subcutaneous (s.c.) injections of 0.1 ml of the bacterial mixtures about 1 cm from the midline in the shaved lower back. To determine the minimum dose required to produce an abscess in at least 50% of the mice inoculated, progressive 10-fold dilutions of cell suspension containing various combinations of bacterial species were injected s.c.

The concentration of each bacterial species in the inoculum was quantified by viable counts on a range of selective media that allowed good growth of only a single species.^{10,11} The basal medium used in the preparation of the selective media for *E. coli*, *K. pneumoniae* and *B. ovatus* were semi-defined but those for the other six species were non-defined. The main constituents of the selective media were as follows: *E. coli* (ALPPL: ammonium sulphate-linolate-propionate-penicillin-lincomycin); *K. pneumoniae* (APLC: ammonium sulphate-penicillin-lincomycin-clindamycin); *P. vulgaris* (PLVFC: propionate - linolate - vancomycin - furazolidone - colimycin); *Ent. faecalis* (AP: azide-propionate); *S. aureus* (SPP: sodium chloride-propionate-polymyxin); *B. ovatus* (BFAG: ammonium sulphate-gentamicin); *F. varium* (GTNRL: gentamicin - trimethoprim - neomycin-rifampicin-lino-late); *Bif. adolescentis* (PPL: polymyxin-propionate-linolate); and *C. perfringens* (TGPAC: trimethoprim-gentamicin-propionate-azide-cycloserine). Cultures were incubated in air or in an anaerobic jar (CO₂ 10%, N₂ 90%) for 2–5 days depending on the species involved. Colonies were then counted and the viable counts (cfu/ml) were calculated.

Effect on subcutaneous abscess formation of concomitant injection of partially purified *E. coli* haemolysin with live bacteria

Mice were given s.c. injections containing 0.05 ml of native *E. coli* haemolysin (125 HU₅₀) and 0.1 ml of a mixture of five bacteria—*E. coli* ATCC 11775 (non-haemolytic), *Ent. faecalis*, *B. ovatus*, *F. varium* and *C. perfringens*, each in a concentration of c. 10^6 cfu/ml. Haemolysin inactivated by autoclaving at 120°C for 10 min was used as a control.

Viable count of each bacterial species in abscess material

When well developed abscesses were formed, i.e., 8–10 days after injection of inocula with non-haemolytic *E.*

coli and 12–14 days after inocula with haemolytic *E. coli*, mice were killed by ether inhalation. The skin was cleaned with alcohol 80% and allowed to dry, then the whole abscess (3–4 mm diameter) was excised aseptically and homogenised in 2 ml of saline with a teflon homogeniser. The homogenate was diluted and 0.1 ml of appropriate dilution was inoculated on to a series of selective media^{10,11} which were incubated as described earlier.

Results

Abscess forming ability of haemolytic and non-haemolytic E. coli with other bacteria

When an inoculum containing *c.* 5×10^5 cfu of haemolytic *E. coli*, *B. ovatus*, *Ent. faecalis*, *F. varium* and *C. perfringens* was injected into mice, abscesses were formed (table). However, when the non-haemolytic strain of *E. coli* was tested with the same four species, a concentration of 10^7 cfu of each was required to produce an abscess (table). In experiments with *E. coli* haemolysin (125 HU₅₀) injected concomitantly with an inoculum containing the non-haemolytic *E. coli* strain, abscesses formed with inocula containing only *c.* 5×10^5 bacteria (fig. 1). When heat inactivated haemolysin was used under the same experimental conditions, no abscesses were formed.

Population of each bacterial species in abscesses

In abscesses produced by injection of five bacterial species including non-haemolytic *E. coli*, the number of *E. coli*, *Ent. faecalis* and *B. ovatus* remained fairly constant. Numbers of both *F. varium* and *C. perfringens* tended to decrease gradually (fig. 2C, D). However, with inocula

Table. Influence of the presence of haemolytic *E. coli* and inoculum size on subcutaneous abscess formation in mice

Bacteria in inoculum	Inoculum size (log cfu)			
<i>E. coli</i> ATCC 11775	...	...	6.36	7.30‡
<i>E. coli</i> ATCC 25922	6.41*	5.41†	...	...
<i>E. faecalis</i> IFO 12969	5.45	5.45	6.54	7.54
<i>F. varium</i> ATCC 8501	5.60	5.60	6.58	7.56
<i>B. ovatus</i> SU 39	5.26	5.26	6.75	7.45
<i>C. perfringens</i> VPI 5201	5.16	5.16	6.60	7.24
Number of mice with abscess	4	3	0	5
Number of mice tested	4	4	5	5

The population of each species in the resultant abscesses is shown in fig. 2A*, B† and D‡.

Fig. 1. Population of each species in abscesses formed by concomitant injection of five bacterial species—(2) non-haemolytic *E. coli* ATCC 11775; (3) *B. ovatus*; (4) *Ent. faecalis*; (5) *C. perfringens*; (6) *F. varium*—and partially purified *E. coli* haemolysin. Each result represents a mean of two mice and vertical lines indicate standard deviations (SD).

containing haemolytic *E. coli* (fig. 2A, B, E) and with those containing non-haemolytic *E. coli* and partially purified *E. coli* haemolysin (fig. 1), the numbers of *B. ovatus* and *E. coli* increased. The final number of *B. ovatus* was apparently influenced by the *E. coli* inoculum size. Greater increases in numbers of both *E. coli* and *B. ovatus* were seen when the original inoculum contained a lower number of *E. coli*. The number of *Ent. faecalis* (fig. 2A–E) remained fairly constant while the concentrations of *F. varium* and *C. perfringens* decreased rapidly. When nine bacterial species, each in a concentration of *c.* 10^5 cfu, were injected in mixed culture (fig. 2E), *E. coli* and *B. ovatus* again increased rapidly *Ent. faecalis* and *K. pneumoniae* persisted at levels similar to those at zero time, but *F. varium*, *C. perfringens*, *S. aureus*, *P. vulgaris* and *Bif. adolescentis* decreased rapidly and were not detected in abscesses 14 days after injection.

Properties of purified E. coli haemolysin

The properties of the partially purified *E. coli* haemolysin in this study are similar to those

Fig. 2. Population of each species in abscesses formed by concomitant injection of five (2A–D) and nine (2E) bacterial species—(1) *E. coli* ATCC 25922; (2) *E. coli* ATCC 11775; (3) *B. ovatus*; (4) *Ent. faecalis*; (5) *C. perfringens*; (6) *F. varium*; (7) *K. pneumoniae*; (8) *S. aureus*; (9) *P. vulgaris*; (10) *Bif. adolescentis*. Each result represents a mean value obtained from two mice and vertical lines indicate standard deviations.

described by Bhakdi *et al.*¹² The preparation contained 2500 HU₅₀/ml and heating of the haemolysin at 56°C for 60 min resulted in loss of $\geq 90\%$ of haemolytic activity, i.e., the preparation was thermolabile.¹³ SDS-PAGE of the haemolysin showed a single protein band of *c.* 103 Kda.

Discussion

In 1985, Verweij-van Vught *et al.*⁶ demonstrated bacterial synergy between *E. coli* and *B. fragilis* in subcutaneous abscess formation in mice and found evidence that this pathogenic synergy was observed in only one of the two combinations of bacteria and was dependent on the properties of *E. coli*. However, no information regarding the possible pathogenic mechanisms involved was presented. The results of this investigation indicate that *E. coli* haemolysin is a factor potentiating pathogenic synergy among several of the predominant bacterial species of the human colonic flora, especially between *E. coli* and *B. ovatus* (a species of the fragilis group of *Bacteroides*) during subcutaneous abscess formation in mice.

Concomitant injection of *E. coli* haemolysin reduced the number of non-haemolytic *E. coli* required for abscess formation from *c.* 10⁷ cfu to 10³ cfu, the latter being the same level as that needed for the haemolytic strain. The haemolysin of *E. coli* is leucotoxic⁸ and may impair the host's cellular defence mechanisms.

The size of the *B. ovatus* population in the abscesses seemed to be determined by the number of haemolytic *E. coli* injected concomitantly. An explanation for this could be that the prolific increase of *E. coli* resulted in the production of large

quantities of haemolysin which damaged the host defence mechanisms, and also provided a suitable environment for the growth of *B. ovatus* by lowering the redox potential.¹⁴ However, even in these conditions *Ent. faecalis*, and *K. pneumoniae* populations remained stable after injection. In the case of other bacterial species injected with the pathogenic mixture, i.e., *S. aureus*, *P. vulgaris*, *Bif. adolescentis*, *C. perfringens* and *F. varium*, numbers decreased steadily until most had disappeared from the site of injection. The rapid decrease in numbers of these bacteria may be largely due to host defence activities, and partly to impairment of proliferation by antagonistic effects of co-existing bacteria.¹⁵

Haemolytic *E. coli* and *B. ovatus* grew prolifically when *c.* 10⁵ cfu of each were injected with equal numbers of either three or seven other species of colonic flora. Ushijima¹⁶ has defined these bacteria as strong abscess formers (SAFB). When non-haemolytic instead of haemolytic cells of *E. coli* were used, an injection of at least 10⁷ cfu was required for abscess formation. In these cases, almost none of the bacteria injected showed an appreciable population increase during the experimental period and Ushijima¹⁶ has defined such bacteria as weak abscess formers (WAFB).

In human faeces, *E. coli* is the predominant aerobic/facultative species and members of the *fragilis* group of *Bacteroides* are the most common anaerobes.¹⁷ Smith¹⁸ reported that haemolytic *E. coli* were found in 18% of human faeces; we found them in 43% of a group of healthy adults (unpublished data). The prevalence of these haemolytic strains may partly account for the high risk of abscess formation of the SAFB type, especially in cases where the host is compromised by various factors.

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