

Evaluation of an Experimental Animal Model for Estimating the Pathogenicity and the Efficacy of Antibiotic Treatment in Bacterial Infection

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An experimental model of intracutaneous infection in the guinea pig, which was based on the study of MILES *et al.* (*Br. J. Exp. Path.* 38: 79-96, 1957) and MASKELL (*J. Med. Microbiol.* 14: 131-140, 1981), was used to investigate the pathogenicity and the effect of chemotherapy with lincomycin, using 36 strains of the genus *Bacteroides*. The pathogenicity was estimated by intracutaneous injection of 0.1-ml quantities of bacterial suspension into guinea pigs and the value was expressed as the number of inoculated organisms required to induce a skin lesion 10 mm in diameter 24 hours after injection. *Bacteroides fragilis* was, on average, three times as pathogenic as non-*B. fragilis* strains ($p < 0.1$). Two intramuscular injections of lincomycin were more effective than a single one against the intracutaneous infection, and the lower was the MIC value, the greater was the effect ($p < 0.001$). This experimental animal model was simple and convenient for estimating the pathogenicity and efficacy of antimicrobial agents. However, the skin lesions were often not clear if the bacteria with low virulence were inoculated and the induced infection is not natural.

Key words: *Bacteroides fragilis*; Pathogenicity; Chemotherapy; Experimental infection

INTRODUCTION

Many kinds of experimental animal model in bacterial infection have been developed for estimating the pathogenicity and the effect of antimicrobial treatment. We attempted to evaluate the method of intracutaneous infection in the guinea pig based on the those

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of MILES¹⁶⁾ and MASKELL¹⁵⁾ using the genus *Bacteroides*. *Bacteroides* species are frequently isolated from human clinical specimens and play an important role in anaerobic infection. It is well known that *Bacteroides fragilis* among the species of the genus *Bacteroides* is most frequently recognized as a causative pathogen in intraabdominal, postoperative, female genital and respiratory infections, and in bacteremia⁸⁾. *Bacteroides* species are the major components of the normal flora of the large intestine, and *B. fragilis* is a minor component⁹⁾¹⁷⁾, but it is more often isolated from clinical specimens and causes infections more frequently than non-*B. fragilis* strains. As a reason for the higher incidence of endogenous infections due to *B. fragilis* compared with non-*B. fragilis* species, several pathogenic factors have been proposed for *B. fragilis*³⁾⁴⁾¹⁰⁾¹¹⁾¹²⁾¹³⁾¹⁸⁾²¹⁾. In the present study, the pathogenicity of *B. fragilis* was compared with that of non-*B. fragilis* species by using intracutaneous infection in the guinea pig as an experimental model. The effect of the administration of an antibiotic, lincomycin, on infection with *B. fragilis* was also investigated. This paper describes the results of these studies with some discussion.

MATERIALS AND METHODS

Bacteria. Twenty-five strains of *B. fragilis* and 11 of non-*B. fragilis* strains were used; 23 were from clinical specimens such as pus, wound swabs, blood and ascitic fluid, one was *B. fragilis* G-2 provided by the Institute for Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan; and the remaining one was a reference strain, *B. fragilis* NCTC 9343. The non-*B. fragilis* species used consisted of 10 from clinical specimens (8 of *B. distasonis* and 2 of *B. thetaiotaomicron*) and one of *B. uniformis* G-3 from Gifu University.

Animals. Female Hartley strain guinea pigs weighing 450-550g were used in this study.

Preparation of suspensions for animal inoculation. The bacterial strains were cultured on a GAM agar medium (NISSUI, JAPAN) for two days at 37°C in an anaerobe box incubator (FORMA, USA) in an atmosphere of 10% carbon dioxide, 7% hydrogen and 83% nitrogen maintained at a humidity of 60%. The growth was scraped off with a platinum spatula and suspended densely in sterile saline (NaCl 0.8% w/v in water) in the anaerobe box. The number of bacteria in the suspensions was determined by the dilution culture method or with a standard curve prepared by photodensitometry. The original suspensions and 10-fold dilutions of both living bacteria and organisms which were killed by heating in a water bath at 80°C for 30 min were prepared for animal inoculation.

Intracutaneous inoculation in guinea pigs. The intracutaneous inoculation was carried out according to the method of MILES *et al.*¹⁶⁾. Guinea pigs were divided into groups of three, and the hair of the dorsal skin and flanks was shaved off before the injection. The bacterial suspension in 0.1-ml volumes was then randomly injected into the skin of each guinea pig with a tuberculin needle from 16 to 20 sites at about 2.5-cm

intervals.

Evaluation of intracutaneous pathogenicity. Pathogenicity was evaluated according to MASKELL's method¹⁵⁾ as follows. At 24 hours after the inoculation, a barium sulphate paste was applied for about 5 min on the shaved skin area of the guinea pig for complete depilation and then the diameter of the skin lesion at each inoculated site was measured with slide calipers. The pathogenicity of each *Bacteroides* species was expressed in terms of the number of bacteria in 0.1-ml capable of producing a lesion 10 mm in mean diameter. This value was termed the ED10, and for living and dead bacteria it was expressed as ED10 (L) and ED10 (D) respectively; these values were calculated from a graph on which were plotted the number of bacteria in the original suspension and the 10-fold dilutions injected and the diameter of the lesion produced by each strain. Pathogenicity was estimated on the assumption that the ED10 (L) value expresses the intracutaneous multiplication, persistence and invasiveness of the organisms, while the ED10 (D) value indicates the toxicity of the bacterial substances. Moreover, the ED10 (D)/ED10 (L) ratio (potency ratio) was thought to represent the intracutaneous multiplication or persistence of bacteria in the guinea pig. Thus, a low ED10 value means strong pathogenicity, and a potency ratio equal to or less than one (≤ 1) indicates lack of multiplication, while a potency ratio greater than one (> 1) means high multiplication and persistence of bacteria in guinea pig skin.

Antibiotic and the method of administration. Lincomycin (Upjohn) was employed in the current study. Lincomycin at 200 mg/kg was intramuscularly injected into each guinea pig in four groups, A-D, each group containing three guinea pigs. Group A was the control to which no antibiotic was given, group B was injected with lincomycin one hour before the *B. fragilis* inoculation, group C was given lincomycin six hours after the bacterial inoculation, and group D was treated with the antibiotic both one hour before and six hours after the inoculation of *B. fragilis*.

Measurement of the antibacterial activity and the tissue levels of lincomycin. The minimum inhibitory concentrations (MICs) of lincomycin for *B. fragilis* were measured by an agar dilution technique using GAM agar medium. The strains were grown for 24 hours in GAM broth (NISSUI) and further diluted in broth to provide 10^4 colony forming units when delivered to the surface of the GAM agar medium with a multipoint inoculator (SAKUMA, Japan). Doubling dilutions of lincomycin were prepared in 10 mM phosphate buffer (pH 7.0) to final concentrations of 100-0.05 $\mu\text{g/ml}$ in GAM agar plate. The plates were incubated in an anaerobe box incubator at 37°C for 24 hours, and then the MIC was defined as the lowest concentration resulting in the total suppression of growth. The levels of lincomycin in the blood and in the skin lesions of three guinea pigs administered 200 mg/kg intramuscularly were measured by the agar well diffusion assay method⁷⁾.

RESULTS

Comparison of the pathogenicity of B. fragilis and non-B. fragilis strains.

In the preliminary study the diameter of the skin lesion was approximately directly proportional to the number of the organisms inoculated ($r=0.75$). The pathogenicity of 11 strains of *B. fragilis* was compared with that of 11 non-*B. fragilis* strains. The results obtained are shown in Table 1, in which the lesions caused by living bacteria are seen to be generally larger than those caused by the dead organisms. The mean values of ED 10 (L) and ED 10 (D) for *B. fragilis* were approximately two or three times smaller than those for non-*B. fragilis* strains; these results may suggest stronger pathogenicity of *B. fragilis* than non-*B. fragilis* strains, but the differences were not statistically significant according to Student's *t*-test. The potency ratio of *B. fragilis* was greater than that of non-*B. fragilis* strains ($p<0.1$), suggesting that *B. fragilis* strains have a greater ability to persist or multiply in the guinea pig skin.

Tissue levels of lincomycin. Mean lincomycin levels in the serum and skin lesions of three guinea pigs are shown in Table 2. Peak levels of lincomycin in the serum and skin lesions reached 54 and 23.7 $\mu\text{g/ml}$, respectively, and generally exceeded the MICs used in the current study except for six resistant strains with MICs of ≥ 100 $\mu\text{g/ml}$.

The efficacy of lincomycin in intracutaneous infection in the guinea pig. Twenty-three strains of *B. fragilis* isolated from clinical specimens were used. The efficacy of lincomycin was expressed in terms of the diameter (mm) of the skin lesion 24 hours after the intracutaneous injection of 0.1-ml of a suspension with 10^9 living bacteria/ml. The results obtained are shown in Table 3. The skin lesions in the lincomycin treated groups B, C and D were smaller than those in the untreated group A ($p<0.01$), and

Table 1. Comparison of ED10 (D)*, ED10 (L) and ED10 (D): ED10 (L) (potency ratio) of *Bacteroides fragilis* and non-*B. fragilis* strains

	ED10 (D) ($\times 10^9$ cells)	ED10 (L) ($\times 10^9$ cells)	ED10 (D): ED10 (L)
<i>B. fragilis</i> (11 strains)	5.06 \pm 3.4 ⁺	2.23 \pm 2.4	3.89 \pm 3.07
Non- <i>B. fragilis</i> (11 strains)	10.3 \pm 8.1	6.45 \pm 7.5	2.35 \pm 1.60
Statistical analysis	NS	NS	$p<0.1$

* ED10 values are expressed as the number of organisms in 0.1-ml required to induce a mean lesion diameter of 10 mm in guinea pig skin; this value was calculated for living and dead organisms and termed ED10 (L) and ED10 (D) respectively. See Maskell (15) and Miles et al. (16).

+ Mean \pm SE

Table 2. Mean serum and skin lesion levels ($\mu\text{g/ml}$) of lincomycin after intramuscular injection of 200 mg/kg in three guinea pigs

Sample	Time after injection (hr)				
	1/2	1	2	4	6
Serum	54	36	22.2	9.1	4.0
Skin lesion	13.2	23.7	17.3	7.7	4.1

the effect especially strong in the lesions caused by lincomycin sensitive strains in group D ($p < 0.001$). The skin lesions due to highly resistant strains were also diminished significantly in groups C and D ($p < 0.05$). There was no significant difference between groups B and C or C and D by Student's *t*-test.

Table 3. Relative efficacy of lincomycin administration (200 mg/kg) in intradermal infection of guinea pig caused by injection of 10^8 *Bacteroides fragilis* cells in 0.1-ml

Lincomycin administration	MICs of lincomycin ($\mu\text{g/ml}$)		
	≥ 100 (6 strains)	1.56 - 6.25 (12 strains)	≤ 0.39 (5 strains)
A*	5.92 \pm 1.38 ⁺	6.76 \pm 1.46	6.58 \pm 0.70
B	4.48 \pm 0.77	3.96 \pm 1.00 ∇	4.46 \pm 0.84 ∇
C	4.03 \pm 0.76 \uparrow	3.88 \pm 0.84 ∇	4.06 \pm 0.25 ∇
D	3.85 \pm 0.67 \uparrow	4.03 \pm 0.87 ∇	3.22 \pm 0.74 θ , \uparrow

* A: Control (untreated).

B: Lincomycin was given one hr before bacterial inoculation.

C: Lincomycin was given six hrs after bacterial inoculation.

D: Lincomycin was given one hr before and six hrs after bacterial inoculation.

⁺ Mean diameter of the skin lesion (mm).

\uparrow Significantly different from A $p < 0.05$, $\nabla p < 0.01$, $\theta p < 0.01$

\uparrow Significantly different from B $p < 0.05$

DISCUSSION

The goal of this work was to estimate the pathogenicity of the *Bacteroides* species and to select the strains with strong pathogenicity for applying to further experimentally infected animal model, and also to evaluate the superiority or inferiority of the method of intracutaneous infection in guinea pig. The *Bacteroides* species are the major group of bacteria composing the resident bacterial flora in the intestinal tract, particularly in the large intestine. Non-*B. fragilis* strains such as *B. vulgatus*, *B. distasonis*, *B. thetaiotamicron* are more common than *B. fragilis*. However, *B. fragilis* is most frequently isolated from human specimens and clinical infections due to this organism are often encountered⁹⁾¹⁹⁾. As for the greater pathogenicity of *B. fragilis* strains, the capsular polysaccharide of *B. fragilis* has been considered to be a virulence factor by many investigators¹⁰⁾¹¹⁾¹²⁾¹³⁾¹⁴⁾¹⁸⁾²⁰⁾. On the other hand, it has also been reported that the capsule is not only characteristic of *B. fragilis*, but that it is also present rather commonly in non-*B. fragilis* strains except for *B. distasonis*¹⁸⁾. Superoxide dismutase production²¹⁾ and resistance to opsonin and to the bactericidal action of serum²⁾³⁾⁴⁾²⁰⁾ have been reported as other possible pathogenic factors of *B. fragilis*. The results of the present study showed that *B. fragilis* was about three times, on the average, as pathogenic as the non-*B. fragilis* strains. MASKELL¹⁵⁾, who used the same experimental model, reported that *B. fragilis* is 17 times as pathogenic as non-*B. fragilis* strains. As a reason for these differences in pathogenicity between *B. fragilis* and non-*fragilis* strains, it was assumed that *B. fragilis*

would be more persistent in the guinea pig skin. *B. fragilis* was consistently recovered from skin lesions 24 hours after inoculation of the bacteria into the guinea pig skin, while non-*B. fragilis* strains were not recovered in many cases in the present study.

The results of investigation on antibiotic therapy showed that two administrations of lincomycin, given intramuscularly one hour before and six hours after intracutaneous inoculation of the bacteria, were therapeutically most effective ($p < 0.001$). This finding seems to be in agreement with the results reported by EAGLE *et al.*⁵⁾⁶⁾ in a series of experiments. Even if the intracutaneous level of lincomycin was lower than the *in vitro* MIC required to kill the inoculated bacterial strain, this antibiotic showed a significant therapeutic effect. This apparent potentiation of the effect of lincomycin was thought to be a protective reaction by the neutrophils, macrophages and complements which were produced *in vivo* in response to inoculation of *B. fragilis*. In the majority of the inoculated foci, there were macroscopically observable abscess formation, and histologically many polynucleocytes were seen to have infiltrated to the skin along with some macrophages, lymphocytes, eosinophils and so on. These findings were observed uniformly in all groups of animals inoculated with living or dead suspensions of *B. fragilis* and non-*B. fragilis* strains. An advantage of the present experimental model is that the pathogenicity of many bacterial strains and the *in vivo* efficacy of the antibiotics can be tested with a small number of guinea pigs. On the other hand, the disadvantages are that the skin lesions are often not clear if small numbers of bacteria of low virulence are inoculated, physical reactions may possibly occur because of the inoculation of a large number (more than 10^7) of bacteria, and the induced infections are not natural.

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