THE QUANTITATIVE AND HISTOLOGICAL
DEMONSTRATION OF PATHOGENIC SYNERGY
BETWEEN *ESCHERICHIA COLI* AND
BACTEROIDES FRAGILIS IN GUINEA-PIG WOUNDS

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PLATE XLIV

THE concept of bacterial synergy is not new (Brewer and Meleney, 1926; Meleney, 1931; Altemeier, 1938; Hite, Locke and Hesseltine, 1949) and recent work suggests that aerobes and anaerobes exhibit pathogenic synergy in rats (Weinstein *et al.*, 1975) and jaundiced rabbits (Nielsen, Asnaes and Justensen, 1976). Ingham *et al.* (1977) demonstrated that a variety of species of obligate anaerobes interfered with phagocytosis measured *in vitro* by the uptake of *Proteus mirabilis* and other aerobic bacteria by human leucocytes; the inhibition was greatest with strains of *Bacteroides fragilis* and *Bacteroides melaninogenicus*, and the authors speculated that this supported the concept of pathogenic synergy between aerobic and anaerobic bacteria. A series of clinical trials at Addenbrooke's Hospital on the yields from operative swabs sent to the laboratory in Stuart's transport medium indicated that wounds from which mixed growths of aerobes and anaerobes could be cultured carried a 71% infection rate (Kelly and Warren, 1978). It was therefore decided to determine whether synergy could be demonstrated between *Escherichia coli* and *B. fragilis* in experimental wound infections in guinea-pigs.

MATERIALS AND METHODS

Organisms representative of the commonest aerobe and anaerobe found in the earlier clinical trials (Kelly and Warren, 1978) were *E. coli* strain NCTC9434, and *B. fragilis* strain NCTC9343. Lyophilised samples were obtained from the National Collection of Type Cultures and subcultured in Hartley's digest broth. The *E. coli* strain was incubated aerobically at 37°C for 18 h, and the *B. fragilis* anaerobically in a GasPak jar [BBL, supplied by Becton-Dickinson (UK) Ltd, York House, Empire Way, Wembley, Middlesex] at 37°C for 48 h. A single volume of approximately 100 ml of culture was obtained for each organism and 0.5-ml portions were dispensed in Teflon vials which were then snap-frozen and stored at -70°C. Under these conditions, the viability of the organisms stored in the ampoules was maintained at the original levels (*E. coli* 10⁸ and *B. fragilis* 10⁶ per ml); as a result, any combination of organisms could be prepared in the confidence that subsequent testing would verify the predicted values. For use, the contents were thawed rapidly by agitation of an ampoule in a 37°C waterbath and subsequently diluted in Hartley's digest broth to the required concentration. To secure uniformity, all of the experiments were done with bacteria taken from these ampoules and consequently from the two culture batches.

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Animal model. Female, albino Dunkin and Hartley (Redfern strain) guinea-pigs weighing 800–1000 g were used. Under neuroleptanalgesia with fentanyl, fluanazone and diazepam (Green, 1975), four measured 2-cm wounds were made through skin and fat but not muscle with a scalpel on the freshly shaven, povidone-iodine-washed suprascapular area of each animal. Each wound was inoculated with 0.1 ml of a dilution of bacterial suspension containing one or both organisms; the material was freshly prepared as described above, and delivered through the sterile plastic disposable nozzle of a calibrated Finnpiquette (Messrs Jenkin, Hemel Hempstead, Hertfordshire). The incisions were each closed with two sterile 12-mm Michel clips, and the operation site was covered with an adherent vapour-permeable plastic dressing ("Op-Site"; James and Watson, 1975).

The guinea-pigs were inspected daily for liveliness. Wound induration was measured by palpation, and rectal temperatures were taken.

On the 7th day the animals were given intraperitoneally an overdose of phenobarbitone sodium and killed by carbon-dioxide inhalation. Skin marking ink (Christoph and Sedwitz, 1965) was used to delineate blocks of tissue (4 × 2 cm) each containing one wound, and these were excised down to, but not through, the deep fascia. Each tissue block was weighed, placed in 10 ml sterile saline, homogenised in a Colworth stomacher (Sharpe and Jackson, 1972) and centrifuged; the viable bacterial content of the supernate from the tissue macerate ("wound homogenate") was then determined.

Bacterial viable counts. A four-plate technique (Kelly, 1977) was used to count the numbers of *E. coli* plus *B. fragilis* in the wound homogenates. After adding quantities of bacterial suspensions, quintuplicate 0.1-ml agar droplets enriched with 2% lysed horse-blood, with and without neomycin (2.5 µg per 0.1-ml droplet), were dispensed from a Colworth Droplette machine (Sharpe *et al.*, 1972) on to empty plastic Petri dishes. After the aerobic or anaerobic incubation of different plates, the enumeration of intradroplet microcolonies seen on the machine's viewing screen made it possible to obtain a count of each of the bacterial components of these mixtures. In addition, for each dilution, a sixth blood agar droplet could be placed (one per dish) on a blood agar plate warmed to 41°C. The molten droplet was quickly spread with a glass "L" spreader. After appropriate incubation, these "spread-plates" were used to confirm the identity of the intradroplet microcolonies counted on the viewer (Kelly, 1978). Aerobic and anaerobic bacterial biotypes were tested by subculture with the API disposable microfermentation systems 20A and 20E (API Laboratory Products Ltd, Philpot House, Rayleigh, Essex).

Histology. A few representative wound samples were submitted for histological examination. They were pegged out on cork, immersed in 10% formal saline (formalin 10% in aqueous sodium chloride solution 0.9%), and embedded in paraffin wax; 5- and 10-µm sections were cut and stained with Harris' haemotoxylin and eosin.

Experimental design. The initial experiments were undertaken to establish the minimal infective and lethal doses of the organisms. The effect of using a mixture of *E. coli* and *B. fragilis* was then tested and, as pathogenic synergy was readily demonstrable, it was further evaluated by measuring the results of altering the components of the challenge inocula, keeping the amount of one organism constant while varying the concentration of the other.

RESULTS

There were no deaths from anaesthesia nor any observed morbidity attributable to anaesthesia. No guinea-pigs were withdrawn from any of the experiments. Initial checks confirmed that the viable counts of *E. coli* and *B. fragilis* neither increased nor diminished when pure cultures, their mixtures, or wound homogenates were held for up to 4 h at 4°C, this being the maximal period that elapsed between killing a test animal and placing the droplets in the incubator. Four wounds were always used for each animal and, except for part of the first experiment with pure *E. coli*, each wound was inoculated

PATHOGENIC SYNERGY IN WOUNDS

FIG. 1.—Control wound. No organisms were introduced at operation. The guinea-pig was killed 1 week later. This wound is healing by first intention. Haematoxylin and eosin (HE). $\times 10$.

FIG. 2.—Wound contaminated with pure *Escherichia coli*, 9.0×10^4 cells, 1 week earlier. The wound is healing without inflammation. HE. $\times 10$.

FIG. 3.—Wound contaminated with pure *Bacteroides fragilis*, 9.3×10^4 cells, 1 week earlier. The wound is healing without inflammation. HE. $\times 10$.

FIG. 4.—Wound contaminated with a mixture of *E. coli* (4.5×10^4 cells) and *B. fragilis* (4.7×10^4 cells) 1 week earlier. This wound shows severe inflammation with copious pus. HE. $\times 10$.

with equal numbers of the same organisms. Guinea-pigs were found to be relatively susceptible to *E. coli* but not to *B. fragilis*. Inoculation of each of the four wounds with 0.1 ml of undiluted culture of *E. coli* (10^7 cells per wound) regularly caused septicaemia and death by 48 h, and pure cultures were recovered from all wound homogenates and heart blood in concentrations of 10^6 – 10^7 cells per ml; however, the 0.1-ml inocula of pure culture of *B. fragilis* (10^8 cells per ml) never caused death.

Studies with pure Escherichia coli

A series of experiments with decreasing total doses of *E. coli* in the four wounds on each guinea-pig showed that fatal *E. coli* septicaemia was generated by a total infective dose that exceeded $c.10^6$ cells. When four 0.1-ml inocula containing a total of 3×10^6 cells were added to the four wounds in each of two guinea-pigs (7.6×10^5 cells per wound), the animals recovered after an initial pyrexia (39 – 40°C lasting 1 day), and 1 week later each wound was moderately indurated (6–8 mm). Homogenates of these wounds grew the same *E. coli* (biotype confirmed by API 20E from spread-plate colonies) with a mean concentration of 1.2×10^6 (SD 0.53) cells per wound.

When total inocula of 3.6×10^5 cells of *E. coli* were similarly administered to three animals (9×10^4 cells per wound) there was no pyrexia, and minimal induration (2–5 mm) was clinically apparent in the healing incisions a week later (table I). The wound homogenates yielded pure cultures of *E. coli* with a mean concentration of 3.1×10^5 (SD 0.64) cells per wound. Wound challenges with fewer *E. coli* cells failed to produce any visible inflammation and the organism could not be retrieved from wound homogenates that contained normal skin flora (mainly *Staphylococcus albus*, 10^2 cells per wound). All the wounds inoculated with 9×10^4 *E. coli* appeared similar. The microscopical appearances of a control wound inoculated with 0.1 ml sterile nutrient broth

TABLE I

The effects of separate and combined bacterial challenges in wounds in guinea-pigs observed 1 week after challenge

Inoculum*		Clinical state	Temperature (°C)	Extent of wound induration (mm)	Number of bacteria present in wound homogenates†	
<i>E. coli</i>	<i>B. fragilis</i>				<i>E. coli</i>	<i>B. fragilis</i>
9.0 × 10 ⁴	...	Well	37.5	1	...‡	...
...	...	Well	37.5	2–5	3.1 × 10 ⁵	...
...	9.3 × 10 ⁴	Well	37.5	1–3	...‡	2.0 × 10 ⁸
4.5 × 10 ⁴	4.7 × 10 ⁴	Listless	39.0	10–30	1.5 × 10 ⁷	2.0 × 10 ⁷

* Number of cells per wound.

† Each figure represents the mean from 16 wounds in four guinea-pigs.

‡ *E. coli* were absent, but skin flora, mainly *S. albus* present (10^2 – 10^8 organisms).

... = Nil.

(fig. 1) are compared with those of a wound inoculated with 9×10^4 *E. coli* (fig. 2). The histological features typical of a clean incised wound healing by first intention are evident in fig. 1, and fig. 2 shows minimal inflammation that is visible only under high magnification and lies within the range acceptable in a clean wound. Thus the histological examination confirmed the clinical findings that this dose of *E. coli* had only a minimal effect on healing, and produced no pus.

Challenge inocula of 9×10^4 *E. coli* were therefore chosen for subsequent experiments in the knowledge that such a dose would cause a reproducible minimal skin lesion with no risk of septicaemia and death. After 1 week these wounds each yielded a total of 10^8 organisms, despite the clinical impression that they were healing with minimal inflammation.

Studies with pure Bacteroides fragilis

It proved impossible to kill any guinea-pig by inducing a bacteroides septicaemia even with challenges of 9.3×10^8 cells into each of the four incisions. This dose failed to cause the animals any overt distress, and they remained lively and apyrexial. At 1 week, moderate induration (5–9 mm) had developed across the wounds; wounds inoculated with fewer bacteria were neither indurated nor clinically infected. Homogenates of wounds that had been contaminated 1 week earlier continued to yield pure growths of the same *B. fragilis* (biotype confirmed by API 20A from the spread-plate colonies). Table II shows that the yield of *B. fragilis* in the homogenates decreased as the quantity in the original inoculum was reduced; nevertheless, with the higher infective doses, substantial numbers of the organisms could be isolated from what appeared to be uninflamed healing wounds. When the infective challenge was 9.3×10^4 cells per wound, cells of *B. fragilis* were just detectable in the wound homogenates, their numbers rising slightly above the general level of the commensal flora (10^3 cells per wound).

TABLE II

Variation in the yields of B. fragilis from homogenates of guinea-pig wounds contaminated 1 week earlier with a range of B. fragilis challenges

Number of bacteria in initial challenge inoculum	Whether wounds clinically infected	Mean concentration of <i>B. fragilis</i> in wound homogenate
10^8	+	10^8
10^7	—	10^5
10^6	—	10^4
10^5	—	10^3
10^4	—	10^3
10^3	—	...
10^2	—	...

+ = Infected; — = not infected; ... = not detected.

The histology of one such wound, inoculated with 9.3×10^4 cells, is shown in fig. 3. Again there was no visible inflammation and, even under high magnification, the appearances were indistinguishable from those of the cleanly incised control specimen (fig. 1).

Thus inocula containing 9.3×10^4 *B. fragilis* organisms were chosen for the initial experiments on bacterial synergy.

Studies with mixed inocula

It was decided to use a mixed inoculum containing the same *total* number of cells that had been used in the two foregoing experiments, i.e., *E. coli* (9×10^4 cells) and *B. fragilis* (9.3×10^4 cells). Accordingly, the mixture contained 4.5×10^4 *E. coli* plus 4.7×10^4 *B. fragilis* and gave a mixed challenge of 9.2×10^4 bacteria in 0.1 ml. When 16 wounds (four animals) were inoculated with this mixture, the guinea-pigs became listless, unwell and pyrexial ($38-40^\circ\text{C}$) by the 2nd day. After the 3rd day, marked induration of all the wounds became apparent and the malaise continued. At 1 week, just before being killed, the animals were still listless and pyrexial, and pus was just starting to point through the lines of the incisions. Very striking induration of the wounds was obvious. This induration ranged from 10–30 mm (mean 16, SE 3.7) across the wounds. The homogenates yielded *E. coli* 10^6-10^7 and *B. fragilis* 10^6-10^7 per wound, i.e., both organisms were present in approximately equal numbers.

The microscopical appearances of these wounds (fig. 4) and the previous wounds (figs. 1–3) were strikingly different. In the mixed infection (fig. 4) the changes associated with profound inflammation extended from the base to the surface of the wound, whose centre had been replaced by pus. Examination of the sections under high magnification confirmed these findings.

This series of experiments therefore shows unequivocally that, in the guinea-pig model, similar small infective doses of pure *E. coli* or pure *B. fragilis* failed to produce inflammation, whereas an inoculum of the same volume containing the same total number of organisms, half of which were *E. coli* and half *B. fragilis*, produced striking inflammation with the formation of pus. This is an experimental demonstration of pathogenic synergy *in vivo*.

The effect of the E. coli : B. fragilis ratio on pus formation

The effects of altering the ratio of *E. coli* to *B. fragilis* in the challenge inoculum were examined as follows. As 10^4 *E. coli* plus 10^4 *B. fragilis* produced copious pus, the amount of one organism in the inoculum was held at 10^4 while the concentration of the other was varied. Thus *E. coli* was held at 4.5×10^4 while the amount of *B. fragilis* varied from 10^8 to 0. The yields of both organisms from the wound homogenates are shown in fig. 5. When the numbers of *B. fragilis* in the challenge were held at 4.7×10^4 and the *E. coli* challenge was varied between 10^4 and 0, the results summarised in fig. 6 were obtained. The dose range of *E. coli* in this section was limited because higher concentrations produced septicaemia. Each point on the two graphs represents

the mean results of at least eight homogenised wounds. The standard error was approximately 0.5 log units for the higher values, but 1.5 log units for the low ones.

Both graphs show that pus was formed only when the inocula contained at least 10^3 *E. coli* and at least 10^4 *B. fragilis*. Once pus was formed, the numbers of bacteria in the initial inoculum appear to have had little effect on the numbers of bacteria subsequently obtained from the wound homogenates, which always lay between 10^7 and 10^8 . Furthermore, when pus was present, provided that the numbers of *E. coli* and *B. fragilis* exceeded the threshold values of 10^3 and 10^4 cells respectively, their ratio in the inoculum did not seem to affect their ultimate concentration in the wound, which always contained approximately equal numbers of aerobes and anaerobes.

As the dose of *B. fragilis* was reduced in mixed challenges, the yields of this organism from the wound homogenates dropped rapidly to zero, while the

FIG. 5.—Effects of altering the *E. coli* : *B. fragilis* ratio in the challenge inocula on the wound homogenate yields. Challenge inocula all contained *E. coli* (4.5×10^4 cells) while the amount of *B. fragilis* varied from 10^8 –0.

yields of *E. coli* (held constant in the inoculum at 4.5×10^4) remained steady at 10^6 cells. This was within the range of yields obtained when pure *E. coli* was administered alone. Thus, at lower doses in mixed challenges, *B. fragilis* had no effect. A similar phenomenon is depicted in fig. 6. As the dose of *E. coli* in the inoculum was reduced, with *B. fragilis* held constant at 4.7×10^4 cells,

FIG. 6.—Effects of altering the *E. coli* : *B. fragilis* ratio in the challenge inocula on the wound homogenate yields. Challenge inocula all contained *B. fragilis* (4.7×10^4 cells) while the amount of *E. coli* varied from 10^4 –0. Higher doses of *E. coli* resulted in early fatal septicaemia (see text).

the ultimate yield of *B. fragilis* from the wounds fell to 10^3 , which was within the range obtained when *B. fragilis* alone had been inoculated.

The weights of the wounds before homogenisation were in the range 2.5–10 g, those containing much pus being heavier. However, when the bacterial yields were related to these figures, the differences between the plots of values obtained from the absolute numbers of organisms and from the numbers of organisms per gram became smaller than the relative errors of the observations. For this reason the standardised figures are not given here.

The consequences of holding the "fixed-concentration organism" at a series of different values while the other bacterium was added through its range of 10^8 –0 were not explored, and experiments were not conducted to see whether the addition of *B. fragilis* to the infective inoculum might alter the LD50 of *E. coli*.

The results shown in figs. 5 and 6 for experiments with mixed challenges confirm the earlier finding with pure challenges that relatively large numbers of pathogens could be cultured repeatedly from the homogenates of contaminated wounds that were apparently healing well by first intention.

DISCUSSION

The series of experiments was planned to eliminate as many variables as practicable. Variations in the inocula were minimised by using portions from a single batch of frozen culture of each bacterium. After the initial experiments, all four wounds on each animal were inoculated with the same dose of organisms to avoid the problem of one wound affecting its fellows—a problem inherent in the use of different inocula for several incisions in the same animal. The wounds were excised and homogenised to reduce the sampling errors associated with the biopsy-smear techniques described by others (Robson and Heggors, 1969; Hamer *et al.*, 1975) at the expense of requiring the animals to be killed before assay. Neuroleptanalgesia (Green, 1975) proved to be completely safe and reliable, and is unlikely to have influenced the results.

Guinea-pigs are known to be fairly susceptible to challenge with pure *E. coli* (Smith, Conant and Willett, 1968) as are jaundiced rabbits (Nielsen *et al.*, 1976) but not rats (Krizek and Davis, 1965). Inoculations of pure bacteroides cultures produce no lesions in guinea-pigs, mice and rabbits (Weiss and Rettger, 1937; Hite *et al.*, 1949) although some liver abscesses were produced in a low proportion of jaundiced rabbits (Nielsen *et al.*, 1976). These findings were confirmed for guinea-pigs in the experimental model in the present study, and it was noted that pure cultures of the contaminating bacteria were later recovered from many of the wounds that had not formed pus and were apparently healing by first intention. This observation has been made before and it has been suggested that in dirty human wounds the concentration of organisms present may provide a useful index for deciding whether to proceed to delayed primary suture or not (Robson and Heggors, 1969).

The initial synergy experiment with 10^4 *E. coli* plus 10^4 *B. fragilis* produced macroscopically visible pus and showed quantitatively and histologically that

when small sub-infective inocula of each organism were mixed, the combination caused gross inflammation with pus from which both organisms could be obtained in much higher concentrations (10^7 – 10^8).

This phenomenon of pathogenic synergy was further evaluated in experiments that demonstrated that when pus had been formed, the wounds contained large and strikingly similar quantities of organisms (10^7 – 10^8). For both organisms in turn, as the concentration of one bacterium was reduced, a threshold was reached (10^3 *E. coli* or 10^4 *B. fragilis*) below which pus was not produced and the wound concentrations of the organisms that had been held constant in the original inoculum reverted to values (10^5 *E. coli* and 10^3 *B. fragilis*) similar to those obtained after contamination with the pure culture, while the concentration of the other organism fell towards zero.

In fig. 6 there is one pair of points (inoculum contained $10^{2.6}$ *E. coli* plus 4.7×10^4 *B. fragilis*) when enhancement of the numbers of both organisms in the wound homogenate occurred, but pus was not formed. Thus the term "synergy" may be used both for the enhancement of bacterial counts and also for the formation of macroscopically visible pus.

The observation that in all the cases of overt infection with pus formation both *E. coli* and *B. fragilis* were present in the wound homogenates in large and equal numbers might be considered as evidence for the existence of one-to-one pathogenic synergy. However, it is at variance with the generally accepted clinical view that "acute processes such as ruptured appendix, septic abortion, and early post-hysterectomy sepsis are predominantly associated with a mixed infection of aerobes and anaerobes, whereas once discrete abscesses develop, the incidence of pure anaerobic sepsis increases" (Gorbach and Bartlett, 1974). Nielsen *et al.* (1976), in reporting their results with experimental intravenous challenge of jaundiced rabbits with a mixture of *E. coli* and *B. fragilis*, implied that they expected, and therefore were not surprised to find, that culture of the liver abscesses would yield only *B. fragilis*. The mechanism of synergy has yet to be elucidated and may well depend on an inter-relationship between electrode potential (Eh), soluble factors and induced inhibition of leucocytic phagocytosis (Ingham *et al.*, 1977). Such mutual bacterial synergy has been described between *Fusiformis necrophorus* and *Corynebacterium pyogenes* in foot abscesses in sheep (Roberts, 1967*a* and *b*).

My guinea-pig wound model clearly possesses a considerable potential for use in further experiments that might (1) test further the above view of Gorbach and Bartlett, (2) investigate synergy with a range of different concentrations of different organisms, and (3) test the consequences of using either prophylactic, preoperative or postoperative antibiotic treatments with drugs specifically active against aerobes or anaerobes during the progress of mixed infections. It might also be possible to use the model to assess special forms of treatment such as hyperbaric oxygen, or to test the effect of different kinds of suture material on the progression of a septic wound infected with mixtures of aerobes and anaerobes that are commonly encountered, and cause considerable problems, in the surgical wards of our hospitals.

SUMMARY

Known numbers of standard strains of *Escherichia coli* and *Bacteroides fragilis* were inoculated together into surgical incisions in guinea-pigs. Histological and quantitative bacteriological proof is presented that pathogenic synergy occurred *in vivo* with copious pus formation when combinations of subinfective doses of each organism were inoculated. A further series of experiments showed that the amounts and ratios of *E. coli* and *B. fragilis* in the septic inocula were critical, and that the resultant pus contained equal numbers of both the aerobic and the anaerobic organisms. This model may be suitable for the experimental validation of short-term regimens of prophylactic antibiotics in surgery; the present results seem to have important therapeutic implications.

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