POLYMORPHONUCLEAR LEUKOCYTE CHEMOTAXIS BY MIXED ANAEROBIC AND AEROBIC BACTERIA

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SUMMARY. The induction of chemotactic activity of polymorphonuclear leukocytes (PMNL) by anaerobic and aerobic bacteria alone or in combination was evaluated. Washed cells as well as the supernate of *Proteus mirabilis* were chemotactic for leukocytes. The supernate of cultures of two strains of *Bacteroides fragilis* contained small amounts of chemotactic factors. No chemotactic factors were released from the non-fragilis *Bacteroides* strains. The supernates of cultures of anaerobic bacteria were capable of inhibiting chemotaxis of leukocytes to the chemotactic factors of *P. mirabilis*. *P. mirabilis* and two strains of *B. fragilis* generated chemotactic factors in serum but none of the other *Bacteroides* spp. tested were able to induce serum chemotactic factors.

INTRODUCTION

The initial response of the body to invasion by pathogenic microorganisms and the events in the first few hours are critical in determining the outcome of infection. Neutrophil leukocytes play an important role in this early response because one of their functions is directed migration toward a specific location in the body, a phenomenon called chemotaxis. Chemotaxis follows the recognition by the neutrophil of chemical substances and ensures that leukocytes accumulate where they are needed. Subsequent phagocytosis forms a first line of defence. However, bacterial products have been shown to inhibit specific neutrophil functions such as phagocytosis and killing (Scharmann, Jacob and Portendörfer, 1976; Baehni *et al.*, 1979). In previous investigations the presence of some but not all anaerobic bacteria inhibited the killing of aerobic bacteria (Namavar *et al.*, 1983).

The purpose of this study was to determine whether the presence of anaerobic bacteria affected the chemotactic stimulus of an aerobic species because a negative influence on the attraction of neutrophils would increase the likelihood of infection becoming established. Chemotaxis may be induced by the release of substances from certain bacteria or by bacterial activation of serum chemotactic factors. We assessed both mechanisms by in-vitro experiments.

MATERIALS AND METHODS

Bacteria. The following anaerobes were used: *Bacteroides gingivalis* strain W83, *B. asaccharolyticus* strain VPI 4199, *B. loeschei* strain ATCC 15930 (formerly *B. melaninogenicus* ss. *melaninogenicus*), *B. fragilis* strain 1503, and *B. fragilis* strain 5MB. The aerobic strain in all experiments was *Proteus mirabilis* strain P154. All bacteria used in this study were clinical isolates with the exception of *B. asaccharolyticus* strain VPI 4199, which was isolated from human faeces. Anaerobes were maintained by weekly subculture on 5% horse-blood-agar plates (Oxoid No. 2) supplemented with haemin 5 µg/ml (BDH) and menadione 2 µg/ml (Merck). *P. mirabilis* was maintained on Dorset egg slopes and subcultured before use on CLED-agar plates (Oxoid).

Preparation of supernates and cell suspensions. The broth normally used itself exerted a chemotactic influence on human PMNL. Therefore, to assess the chemotactic response of leukocytes towards bacteria, a medium was tested which had previously been described for measuring the chemiluminescence response of leukocytes (Namavar *et al.*, 1983). This was a modification of a minimal medium for growth of *B. fragilis* (Varel and Bryant, 1974). All the bacterial strains used grew sufficiently in this medium and it did not itself induce chemotaxis. Anaerobes were incubated for 48 h at 37°C in an anaerobic jar in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10%. *P. mirabilis* was incubated overnight at 37°C in a shaking water bath. The numbers of anaerobic and aerobic bacteria were determined by optical density and adjusted to 1×10^9 cfu/ml. Culture supernates were prepared by centrifugation (1300 g, 15 min, 20°C) and filter sterilised through 0.45-µm membrane filters (Millipore Corp., Bedford, MA). Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in modified minimal medium to give a concentration of 1×10^9 cfu/ml. Both cells and supernates were tested for chemotactic factors.

Preparation of leukocytes. PMNL were separated from heparinised blood of healthy adults by dextran sedimentation. The leukocytes were washed once in TC medium 199 containing 2 mM L-glutamine and resuspended in the same medium at a concentration of 1×10^7 leukocytes/ml. The pH of the medium was adjusted to 7.4 with 7.5% NaHCO₃ solution.

Induction of chemotactic factor in serum. Bacterial pellets were suspended in 1 ml of pooled human serum diluted 1 in 2 in TC medium 199 and incubated at 37°C for 30 min in a shaking water bath to generate chemotactic factors in the serum. Bacteria were separated from the suspension by centrifugation (1300 g, 15 min, 4°C). In some experiments activated serum was then incubated at 56°C for 30 min.

Chemotactic assay. Agarose (Type 1: low EEO, Sigma) was dissolved in sterile distilled water at a concentration of 2.4% (w/v) by heating for 30 min in a boiling water bath. After cooling in a 56°C water bath, the agarose was mixed with an equal volume of prewarmed (56°C) buffer medium. This consisted of 20 ml of TC medium 199 (10× concentrated), 2 ml of 7.5% sodium bicarbonate, 20 ml of inactivated new-born calf serum (NBCS) and 58 ml of sterile distilled water. A 10-ml volume of the agarose medium was delivered into each 60 mm × 15 mm tissue-culture plate (No. 3002, Falcon, CA). Four series of three wells 3 mm in diameter and 3 mm apart were made in the agarose gel with a punch in a straight row (Nelson, Quie and Simmons, 1975). The plugs of agarose were removed and accumulated fluid soaked up with tissue paper. The centre well of each three-well series received 10 µl of leukocyte suspension (1×10^7 /ml), the outer well 10 µl of chemotactic factor and the inner well 10 µl of control medium. The plates were incubated at 37°C in a humidified atmosphere containing CO₂ 5% in air. After incubation for 3 h, the plates were flooded with 5 ml of methanol for 30 min, then with 4% formalin for 30 min. The agarose was removed and the cells stained with Wright's stain. Each migration figure was magnified 25 times under a microscope and chemotaxis was measured by subtracting the spontaneous migration from the directed migration. The chemotaxis of the supernate or the washed cells of *P. mirabilis* was considered to be 100%.

Statistical analysis of the data was performed using analysis of variants and Student's t-test.

RESULTS

Chemotactic factor of P. mirabilis

Fig. 1 shows the mean chemotactic activity of the neutrophils from 10 different healthy donors when induced by the supernate and washed cells of *P. mirabilis* cultures. Both induced chemotaxis equally. Chemotactic factors produced by *P. mirabilis* were a property of viable cells; heat killed (60 min, 70°C) and formalin killed (0.5% v/v) cells did not produce chemotactic factors. The heat-treated supernate did not show a significant difference in chemotactic stimulation from the unheated supernate. These results suggest that the chemotactic factors of *P. mirabilis* are extracellular and heat stable.

FIG. 1.—Chemotactic activities of PMNL induced by A) supernate, B) supernate heated for 30 min at 100°C, C) viable cells, D) killed cells of *P. mirabilis*. Values are expressed as mean percentage $\pm$ standard deviation of six experiments.

FIG. 2.—Chemotactic activities of PMNL induced by supernates (■) and washed cells (▨) of *Bacteroides* spp. and *P. mirabilis*. Values are expressed as mean percentage $\pm$ standard deviation of 10 experiments.

Chemotactic factor of anaerobes

The mean chemotactic activities of neutrophils when stimulated by the supernates and by the washed cells of *Bacteroides* spp. and *P. mirabilis* are compared in fig. 2. The supernate and the washed cells of *P. mirabilis* produced far more powerful chemotactic factors than did those of *Bacteroides* spp. Neither the supernates nor the washed cells of the *Bacteroides* spp. tested produced potent chemotactic factors, although two strains of *B. fragilis* (1503 and 5MB) produced small amounts. Chemotactic stimulation by anaerobes was significantly lower than that by *P. mirabilis* ($P < 0.01$).

Induction of chemotaxis by combinations of anaerobes and P. mirabilis

The effects of combining the supernates and the washed cells of *P. mirabilis* and *Bacteroides* spp. on the chemotactic activity of PMNL are shown in figs. 3 and 4. The supernates of all *Bacteroides* spp. reduced significantly the chemotactic activity of PMNL towards *P. mirabilis* supernate (fig. 3) ($P < 0.05$). No significant differences

TABLE I
PMN leukocyte chemotactic activity generated in pooled human serum after incubation with anaerobic and aerobic bacteria

Bacterial species	Percentage chemotactic activity* in	
	Activated serum	Activated serum heated for 30 min at 56°C
<i>P. mirabilis</i> P154	100	75 ± 3.5
<i>B. gingivalis</i> W83	14 ± 2.4	12 ± 3.6
<i>B. asaccharolyticus</i> 4199	15 ± 7.0	13 ± 6.8
<i>B. loeschei</i> 15930	10 ± 2.1	10 ± 1.7
<i>B. fragilis</i> 1503	45 ± 4.5	38 ± 7.0
<i>B. fragilis</i> 5MB	55 ± 7.0	45 ± 3.5
serum control	13 ± 2.1	10 ± 3.6

* Values are expressed as mean percentage ± standard deviation of six experiments; activity with activated serum and *P. mirabilis* = 100%.

were observed between anaerobes in inhibiting chemotactic stimulation by *P. mirabilis*. Using the same experimental procedures we found no significant differences when combinations of washed cells of *P. mirabilis* and *Bacteroides* spp. were compared with washed cells of *P. mirabilis* alone (fig. 4).

Induction of chemotactic factors in serum by anaerobes and *P. mirabilis*

The capacity of anaerobic and aerobic bacteria to generate chemotactic factors in serum is shown in table 1. *P. mirabilis* and two strains of *B. fragilis* (1503 and 5MB) generated chemotactic factors in serum. However, serum activated by these *B. fragilis* strains showed much less chemotactic activity than serum activated by *P. mirabilis*. None of the other *Bacteroides* spp. tested were capable of inducing serum chemotactic

FIG. 3.—Chemotactic activities of PMNL induced by the supernate of *P. mirabilis* alone or in combination with supernates of *Bacteroides* spp. Supernate of *P. mirabilis* was diluted 1 in 2 in control medium (■) and supernates of *Bacteroides* spp. were diluted 1 in 2 in the supernate of *P. mirabilis* (▨). Values are expressed as mean percentage ± standard deviation of 10 experiments.

FIG. 4.—Chemotactic activities of PMNL induced by washed cells of *P. mirabilis* alone or in combination with washed cells of different *Bacteroides* spp. Washed cells of *P. mirabilis* were diluted 1 in 2 in control medium (■) and washed cells of *Bacteroides* spp. were diluted 1 in 2 in the washed cells of *P. mirabilis* (▨). Values are expressed as mean percentage ± standard deviation of 10 experiments.

factors. When serum-activated chemotactic factors induced by *P. mirabilis* and *B. fragilis* strains were heated for 30 min at 56°C, the chemotactic activity of the serum was reduced but did not disappear.

DISCUSSION

Mixtures of anaerobic and aerobic bacteria are commonly implicated in various infections, e.g., post operative wound sepsis and pleuro-pulmonary infection (Gorbach and Bartlett, 1974; Mayrand and McBride, 1980). Animal studies have shown that the combination of anaerobes and aerobes produces sepsis which cannot be produced by the same doses of either component individually (Kelly, 1978; Onderdonk *et al.*, 1979). Recent work has shown that anaerobic bacteria inhibit phagocytosis and killing of aerobic bacteria (Ingham *et al.*, 1977 and 1981; Jones and Gemell, 1982; Namavar *et al.*, 1983). This paper is the first report on the chemotactic activity of PMNL induced by mixed anaerobic and aerobic bacteria.

Cooked meat medium, trypticase soy broth and BM medium (van Steenberg, Vlaanderen and De Graaff, 1981) used for growth of aerobes were themselves chemotactic to PMNL. Therefore, it was not possible to test the chemotactic stimulus of the supernates of anaerobes grown in these media. There are reports indicating that a wide variety of agents which are found in growth media are chemotactic, e.g., peptides (Showell *et al.*, 1976), lectins (van Epps and Tung, 1977) and proteins, denaturated proteins and polypeptides (Wilkinson, 1974). The medium used in our experiments overcame this problem. Chemotactic factors of *P. mirabilis* were extracellular and heat stable. None of the *Bacteroides* spp. tested stimulated chemotaxis except the supernates of two strains of *B. fragilis* that did so moderately. This is in keeping with the reports of Adamu and Sperry (1981) that a slight to moderate amount of chemotaxis was induced by the supernate, the outer membrane and the lipopolysaccharide of *B. fragilis*. In contrast to our results, Sundqvist and Johansson (1980) reported that *B. melaninogenicus* and *B. asaccharolyticus* had a high capacity to induce chemotaxis by PMNL. On the other hand, Van Dyke *et al.* (1982) found that *B. gingivalis* and *B. asaccharolyticus* and some other obligate anaerobes did not produce chemotactic factors.

The supernates of anaerobes significantly inhibited chemotaxis of PMNL by *P. mirabilis*. Washed cells of anaerobic bacteria, in contrast to their supernates, had no effect on chemotactic attraction of *P. mirabilis* for PMNL. Interestingly, in a previous study (Namavar *et al.*, 1983) we found that the supernates of some but not all anaerobes inhibited the killing of *P. mirabilis* by PMNL, whereas washed cells did not have such an effect. Of the anaerobes tested in the previous study, the supernate of a *B. gingivalis* strain showed the greatest inhibition. However, all anaerobes tested had equal effects on the inhibition of PMNL chemotaxis induced by *P. mirabilis*. The exact mechanism whereby the supernates of anaerobes interfere with the attraction of *P. mirabilis* for PMNL is not clear. It would seem that this is not a direct effect on PMNL, because their random migration is unaffected. Chemotactic factors generated in serum by *B. fragilis* strains were significantly less than those generated by *P. mirabilis*. Heating the serum activated by *P. mirabilis* or the two strains of *B. fragilis* slightly reduced the chemotactic effect of the serum. Wright and Gallin (1975) have shown that the fifth component of complement is stable at 56°C for 30 min. In our

study the heat-stable serum chemotactic activity may be a cleavage product of C5. This, however, has not been studied.

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