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Role of Anaerobic Coryneforms in Specific and Non-specific Immunological Reactions

II. PRODUCTION OF A CHEMOTACTIC FACTOR SPECIFIC FOR MACROPHAGES*

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Summary. A chemotactic factor has been isolated from cultures and culture filtrates of many members of the group of anaerobic coryneform bacteria which includes various strains of *Corynebacterium parvum*. This factor attracts guinea-pig and mouse peritoneal exudate macrophages specifically and fails to attract either human blood neutrophils or guinea-pig peritoneal exudate neutrophils. Its activity is serum-independent. It is non-dialysable and destroyed by boiling. There is strong, positive correlation between the capacity of individual strains of anaerobic coryneforms to produce this macrophage chemotactic factor and the capacity of the same micro-organisms, when injected *in vivo* into mice, to enhance the clearance of carbon from the circulation.

INTRODUCTION

Anaerobic corynebacteria, which include a variety of different strains loosely grouped together under the name *Corynebacterium parvum*, have excited considerable interest in recent years because they markedly influence the course of specific and non-specific immune responses. Thus they act as powerful stimulators of the reticuloendothelial system as judged by the capacity of previously injected animals to clear foreign particles from the blood stream (Halpern, Prévot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Decreuseford, 1964); they act as adjuvants to enhance humoral and cell-mediated responses (Neveu, Branellac and Biozzi, 1964; Pinckard, Weir and McBride, 1967, 1968) and they modify the immunological responses to tumours and accelerate tumour rejection (Halpern, Biozzi, Stiffel and Mouton, 1966; Woodruff and Boak, 1966; Smith and Woodruff, 1968; Fisher, Grace and Mannick, 1970). It has been observed (Pinckard, Weir and McBride, 1968) that the local response to injection of such bacteria takes the form of a macrophage granuloma.

The taxonomic position of the anaerobic coryneforms is still uncertain, many workers proposing the transfer to the genus *Propionibacterium* (Douglas and Gunter, 1946; Moore and Cato, 1963; Johnson and Cummins, 1972). Such anaerobic coryneforms have recently been divided into four distinct groups on the basis of cell wall analysis, serological relation-

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ships and deoxyribonucleic acid similarities (Johnson and Cummins, 1972) and we are grateful to Professor Cummins for serotyping and grouping the organisms used in this study.

The capacity of these bacteria to stimulate chemotaxis of macrophages *in vitro* has not been previously studied (although it has been clearly shown that they produce macrophage granulomata and enhance reticuloendothelial activity *in vivo*). Pyogenic bacteria such as *Staphylococcus aureus* or *Escherichia coli* attract neutrophils strongly, either by producing cytotaxins, which act directly on the cells, or by producing cytotaxigens, which are substances with no direct effect on cells but with the capacity to activate serum mediators —usually complement—so that chemotactic factors are produced from the serum (Keller and Sorkin, 1967; Ward, Lepow and Newman, 1968). The activity of *Mycobacterium tuberculosis* in attracting macrophages formed the subject of a previous paper from this laboratory (Symon, McKay and Wilkinson, 1972). It was shown that heat-killed mycobacteria did not attract macrophages directly, but that whole mycobacteria and protein fractions from them, when incubated with guinea-pig plasma, generated a macrophage cytotaxin from the plasma. This factor had the characteristics which suggested that it was derived from complement. In the present paper we have studied the chemotactic activity of anaerobic coryneforms and propionibacteria and we report the presence in these organisms of a strongly active macrophage-specific cytotaxin.

MATERIALS AND METHODS

Preparation of organisms and test solutions

The organisms studied were those from the groups of anaerobic coryneforms and propionibacteria classified by Johnson and Cummins (1972).

All organisms were obtained from sources listed in the previous paper (O'Neill, Henderson and White, 1973) and grown under the conditions described there. The bacteria were harvested and washed three times in saline and each batch was finally resuspended in Gey's solution at a concentration of 1500×10^6 organisms per ml—determined using Brown's opacity tube number two—and stored at 4° for at least 5 days.

For each organism the following preparations were set up:

1. Test organism at a concentration of 1500×10^6/ml in Gey's solution pH 7·2.
2. Test organism at a concentration of 1500×10^4/ml in Gey's solution pH 7·2.
3. Test organism at a concentration of 1500×10^4/ml in Gey's solution pH 7·2 plus 10 per cent human serum or guinea-pig plasma.

Preparations 2 and 3 were incubated at 37° for 30 minutes followed by heating at 56° for 30 minutes to inactivate complement.

Gey's solution, casein guinea-pig plasma and human serum were prepared as previously described (Maroni, Symon and Wilkinson, 1972). Plasma was used as a source of cytotaxins in the macrophage experiments and serum was used in the neutrophil experiments for reasons described previously (Symon, McKay and Wilkinson, 1972).

Neutrophils were obtained from normal heparinized human blood after Dextran sedimentation by adding 2 ml of Dextran (Dextraven 110) to 20 ml of whole blood in a siliconized container. After 1 hour, the leucocyte-rich supernatant was removed and centrifuged to deposit the neutrophils which were then washed three times in Gey's solution.

Macrophages were obtained from the peritoneal cavity of normal healthy guinea-pigs 4

days after an i.p. injection of 20 ml of liquid paraffin oil (B.P.) The cells were then washed three times in Gey's solution. Both neutrophils and macrophages were finally suspended in Gey's solution at the volume required for each test.

Because the distance migrated by the cells, rather than the number of cells which had migrated through the filter, was taken as a measure of activity using the 'leading-front method' (see below), the concentration of cells used in the chemotaxis chamber was not critical. All the test solutions were made up to a final volume of 3·6 ml and the pH adjusted to between 7·2 and 7·4.

Chemotaxis method

The method was as previously described by Wilkinson (1971), except that chemotaxis was measured not by counting the number of migrating cells per high power field, but as the distance which cells travelled through a Micropore filter in a given time. This method was described by Sally Zigmond, Rockefeller University, New York (personal communication), but has not previously been adapted to the measurement of macrophage chemotaxis. Cells were allowed to migrate for an appropriate time (75 minutes for neutrophils in 3-μm filters; 130 minutes for macrophages in 8-μm filters). After fixation, staining and clarification of the filters, cell migration was quantified as follows:

Using the 40× objective of a light microscope the distance from the top of the filter to the furthest two cells both in the same focal plane was measured with the micrometer on the fine adjustment. Isolated single cells which migrated further than this were ignored. This distance was measured for five fields on each filter and the overall mean was calculated for that filter. All tests were done in triplicate and the figures in Tables 1 and 2 refer to mean migration in μm for all three filters. Variations in readings for individual filters were rarely larger than 10–15 per cent of this mean. Thus the chemotactic activity of each test substance was expressed by the distance in μm which the cells had migrated towards it through the filter in a fixed period of time. We propose the term 'Leading-Front Method' to describe this technique of determining chemotactic activity.

RESULTS

THE CHEMOTACTIC ACTIVITY OF THE ANAEROBIC CORYNEFORMS AND PROPIONIBACTERIA FOR GUINEA-PIG MACROPHAGES

Table 1 shows the chemotactic activities of a wide range of anaerobic coryneforms and propionibacteria for guinea-pig macrophages. The most striking and important finding from these experiments is that many of these organisms exert a direct chemotactic effect on macrophages in the absence of plasma. Some strains such as *Corynebacterium liquefaciens* 814, *C. diphtheroides* 2764 and *C. anaerobium* 578, show maximal chemotactic activity at the higher test concentration of 1500×10^6 organisms per ml, whereas other organisms such as *C. parvum* 1383, *C. parvum* 3085 and *Propionibacterium avidum* are less chemotactic at high concentration but exert a powerful macrophage cytotactic effect only after dilution 100-fold to a concentration of 1500×10^4 organism per ml. Preliminary results show that paraffin-oil induced mouse peritoneal exudate macrophages respond chemotactically to this cytotaxin in the same way as guinea-pig macrophages.

Bacteria from all of the four groups, *Corynebacterium acnes* (group I), *C. acnes* (group II), *C. granulosum* (group III) and *C. avidum* (group IV) were shown to produce highly active

macrophage cytotaxins. On the other hand, none of the four strains tested from the classical propionibacterium group had any chemotactic activity for macrophages at any dilution.

In addition to this direct chemotactic effect, some strains of anaerobic corynebacteria had the capacity to activate chemotactic factors in plasma. However, generally speaking, this indirect activity was not as strong as the direct chemotactic activity of these organisms (Table 1).

TABLE 1

CHEMOTACTIC ACTIVITY OF ANAEROBIC CORYNEFORMS AND PROPIONIBACTERIA FOR GUINEA-PIG PERITONEAL MACROPHAGES

Strain	Chemotactic migration of macrophages Distance (μm) migrated through an 8-μm filter in 130 minutes towards bacterial suspension at a concentration of:		
	1500×10^6 org./ml	1500×10^4 org./ml	1500×10^4 org./ml + 10% guinea-pig plasma
	(Mean migration to the nearest 10 μm) for three filters		
Group I *C. acnes* group			
C. parvum 0208	20	40	50
C. parvum 1383	20	120	60
C. liquefaciens 814	120	30	20
C. acnes 737	60	50	50
C. parvum 3085	30	90	50
Group II. *C. acnes* group			
C. parvum 10390	30	20	20
C. diphtheriodes 2764	100	10	10
C. anaerobium 578	90	40	60
Group III. *C. granulosum* group			
C. parvum 10387	60	40	120
C. parvum A	30	50	30
C. parvum B	40	20	80
C. parvum C	30	60	30
C. granulosum 0507	40	40	40
Group IV. *C. avidum* group			
P. avidum 0575	30	90	60
P. avidum 4982	40	120	40
P. avidum 0589	30	90	60
Classical propionibacteria			
P. freudenreichii	30	30	30
P. jensenii	20	NT	NT
P. rubrum	20	10	20
P. arabinosum	30	20	30
Negative control Gey's solution	20		
Positive control Casein 5 mg/ml	140		

NT = not tested.

THE CHEMOTACTIC ACTIVITY OF THE ANAEROBIC CORYNEBACTERIA AND PROPIONIBACTERIA FOR HUMAN NEUTROPHILS

Table 2 shows the chemotactic activity of the same groups of organisms for human neutrophils. As can be seen, no direct chemotactic activity was found with any of the strains tested. Tests with guinea-pig peritoneal exudate neutrophils taken after intraperitoneal injection of glycogen revealed that guinea-pig peritoneal neutrophils, like

human blood neutrophils, were unresponsive to the cytotaxin produced by the anaerobic corynebacteria. Thus, the cytotactic activity of the anaerobic corynebacteria is highly macrophage specific. However, neutrophil chemotactic activity was generated on incubation of many strains of these bacteria with serum (Table 2). We have not investigated the relationship of this serum activity to complement.

TABLE 2

CHEMOTACTIC ACTIVITY OF ANAEROBIC CORYNEFORMS AND PROPIONIBACTERIA FOR HUMAN BLOOD NEUTROPHILS

Strain	Chemotactic migration of macrophages Distance (μm) migrated through a 3-μm filter in 75 minutes towards bacterial suspension at a concentration of:		
	1500×10^6 org./ml	1500×10^4 org./ml	1500×10^4 org./ml + 10% human serum
	(Mean migration to the nearest 10 μm) for three filters		
Group I *C. acnes* group			
C. parvum 0208	30	20	110
C. parvum 1383	20	20	50
C. liquefaciens 814	20	10	40
C. acnes 737	30	20	90
C. parvum 3035	20	20	70
Group II *C. acnes* group			
C. parvum 10390	30	20	110
C. diphtheriodes 2764	20	10	40
C. anaerobium 578	30	20	90
Group III *C. granulosum* group			
C. parvum 10387	30	20	120
C. parvum A	30	20	70
C. parvum B	NT	NT	NT
C. parvum C	30	20	80
C. granulosum 0507	30	20	90
Group IV *C. avidum* group			
P. avidum 0575	30	20	70
P. avidum 4982	20	20	60
P. avidum 0589	20	20	60
Classical propionibacteria			
P. freudenreichii	30	20	70
P. jensenii	20	20	40
P. rubrum	10	20	30
P. arabinosum	20	20	70
Negative control Gey's solution	20		
Positive control Casein 5 mg/ml	109		

NT = not tested.

Having established the production of a macrophage-specific cytotaxin by anaerobic cornyeforms, we next carried out experiments to determine some of its basic characteristics such as the dose at which it was effective, its time of appearance in relation to the curve of bacterial growth and its physical properties. For these experiments, one organism, *C. anaerobium* 578 was taken as a representative for the whole group in terms of macrophage chemotactic activity.

DOSE-RESPONSE OF MACROPHAGES TOWARDS THE MACROPHAGE CYTOTAXIN

The effect of dilution on the macrophage chemotactic activity of a suspension of *C. anaerobium* 578 is shown in Fig. 1. As can be seen the organisms are highly active at the

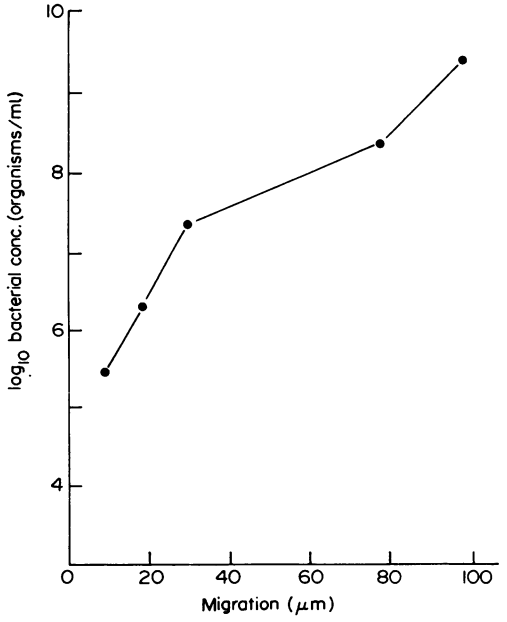

FIG. 1. Dose-response curve for migration of guinea-pig peritoneal macrophages to *C. anaerobium* 578 at varying concentrations.

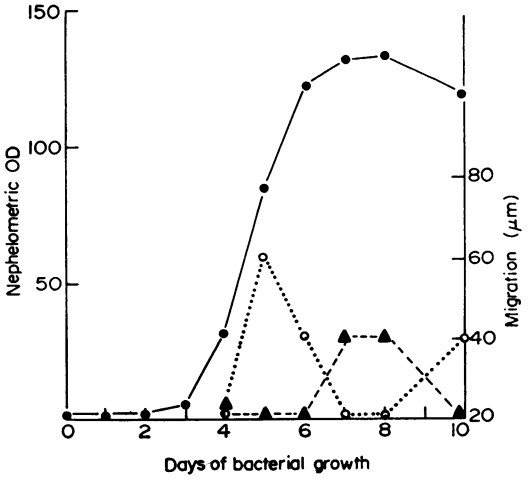

FIG 2. Macrophage chemotactic activity of cell suspensions and culture filtrates of *C. anaerobium* 578 collected on different days of growth. Growth curve (●) measured nephelometrically (ordinate on left). The scale (ordinate on right) for migration of macrophages towards cell suspensions (○) and culture filtrates (▲) of *C. anaerobium* 578 begins at 20 μm which is the distance migrated by macrophages in the absence of a chemotactic stimulus (see Table 1). Note that chemotactic activity appears during the logarithmic phase of bacterial growth.

initial concentration of 1500 per 10^6 organisms per ml and at a concentration of 1500×10^5 organism per ml (1/10 dilution). The chemotactic activity then decreases rapidly when the cells are further diluted to 1500×10^4 organisms per ml (1/100 dilution) and is completely lost at both 1500×10^3 (1/1000 dilution) and 300×10^3 (1/5000 dilution).

CHEMOTACTIC ACTIVITY OF THE ANAEROBIC CORYNEBACTERIA IN RELATION TO BACTERIAL GROWTH

Fig. 2 illustrates the growth curve obtained for *C. anaerobium* 578, and the results of testing both cell suspensions and culture filtrate for macrophage cytotactic activity at different days of growth. The cell suspensions were prepared by centrifuging the culture

TABLE 3

EFFECT OF HEAT AND DIALYSIS ON THE CHEMOTACTIC ACTIVITY OF *C. anaerobium* 578

Treatment given and preparation tested (all organisms at 1500×10^6/ml	Chemotactic migration of macrophages Distance (μm) migrated (to nearest 10 μm)		
Untreated *C. Anaerobium* 578	90		
Heated *C. anaerobium* 578			
56° for 30 minutes	80		
100° for 30 minutes	30		
	Undialysed	Residual material in dialysis sac	Dialysate (Gey's solution)
C. anaerobium 578	100	70	30
Medium from culture of *C. anaerobium* 578	20	90	20
Sterile medium	20	30	20

and resuspending the cells at a concentration of 1500×10^6 organism per ml. The results indicate that the factor is produced during exponential growth, is present in the stationary phase and can be detected in culture filtrates.

PHYSICAL PROPERTIES OF THE CYTOTACTIC FACTOR

Preliminary experiments are in progress on the physicochemical characterization of the macrophage cytotaxin and we report here some of the preliminary findings on its physical properties. Table 3 shows the activity of cytotaxin preparations derived from both whole cells and from culture filtrates, after heat and dialysis treatment. The results show that the chemotactic factor is stable to heating at 56° for 30 minutes but that activity is markedly reduced after heating at 100° for 30 minutes. On dialysis against Gey's solution cell suspensions of *C. anaerobium* 578 at 1500×10^6 organisms per ml maintain their high activity. Very little chemotactic activity is lost from the material in the sac and we conclude that this activity is largely non-dialysable. Untreated culture filtrates of *C. anaerobium* 578 did not show chemotactic activity. This was due to the presence of low molecular-weight toxic materials in the culture medium. On dialysis against Gey's solution to

remove such materials, the culture filtrates became highly chemotactically active. The chemotactic factor is stable on storage at 4° but there is some evidence that storage at −20° reduces the activity.

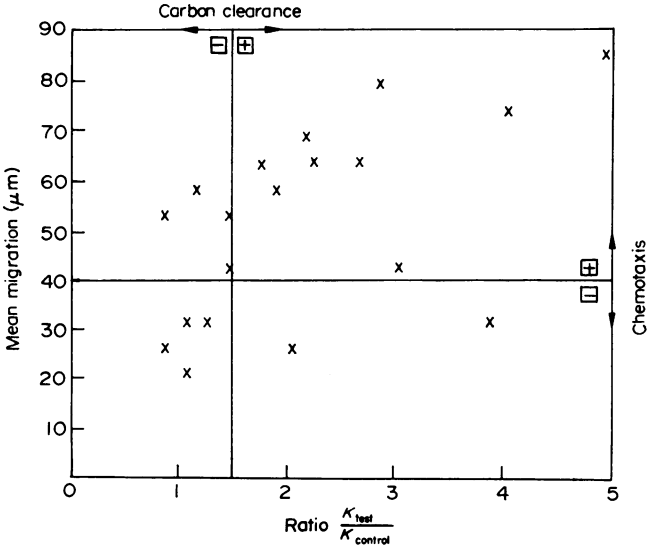

FIG. 3. Correlation of macrophage chemotactic activity of nineteen strains of anaerobic coryneforms and propionibacteria (see Table 1) with carbon clearance (K values) for the same organisms (see Table 3 of O'Neill *et al.*, 1973). Organisms to the left of the vertical line are considered inactive in carbon clearance ($\frac{K_t}{K_c} < 1{\cdot}5$), those to the right are active. Organisms below the horizontal line are negative in macrophage chemotaxis (mean migration < 35 μm), those above the line are positive.

Note that fifteen out of nineteen strains are either (a) active in both carbon clearance and chemotaxis (upper right, eleven organisms) or (b) inactive in both carbon clearance and chemotaxis (lower left, four organisms).

DISCUSSION

The most important finding from these experiments is that anaerobic corynebacteria and propionibacteria produce a factor with a strong, direct chemotactic activity for macrophages. This factor is produced during growth of the organisms and is present in culture filtrates. These facts suggest that the chemotactic substance is secreted from the bacteria as a product of the cell's metabolism. The production of the factor is independent of the medium on which the organism is grown, whether liquid or solid.

The only previous report of a well-defined bacterial factor with a chemotactic activity for macrophages is that of Ward (1968) who described a peptide derived from *Streptococcus pneumoniae* which was active for both neutrophils and macrophages. The cytotaxin from the anaerobic corynebacteria differs from this in that it is highly specific for macrophages alone. Neither human peripheral blood neutrophils nor guinea-pig peritoneal exudate neutrophils respond to this factor. It is thus unique among bacterial cytotaxins described to date in its macrophage specificity and its further characterization will be of considerable importance in the understanding of macrophage-specific chemotaxis.

The chemotactic activity of the anaerobic coryneforms is not restricted to any one of the taxonomic groups proposed by Johnson and Cummins (1972), as highly active cytotaxins are produced by strains from all four groups, *Corynebacterium acnes* (group I), *C. acnes* (group II), *C. granulosum* (group III) and *C. avidum* (group IV). Thus these bacteria are closely related not only serologically and biochemically but also by virtue of the fact that they produce factors with similar chemotactic activities for macrophages. All of the organisms that produced a macrophage chemotactic factor were derived from human sources. None of the classical propionibacteria, derived from dairy products and not included in the above four groups, showed any chemotactic activity. We have tested too few of these organisms to draw any conclusions about their taxonomic relationship with the anaerobic corynebacteria.

Purification and characterization of the macrophage chemotactic factor is in progress. Preliminary experiments have shown that the factor is stable to heating at 56° for 30 minutes but that the activity is markedly reduced after heating at 100° for 30 minutes. The substance is also non-dialysable and therefore a macromolecule. These characteristics suggest that the factor may be protein in nature, but much further work is required on the biochemical purification and characterization of the factor before any generalization can be made about it.

The anaerobic corynebacteria also stimulate the reticuloendothelial system *in vivo*, shown by intense proliferation of hepatic Kupffer cells as seen histologically and by a marked increase in the ability of animals previously injected with these organisms to remove foreign particles (gelatin stabilized carbon) from the blood stream (O'Neill *et al.*, 1973). We have found that the organisms active in macrophage chemotaxis (shown in Fig. 3) are essentially the same group of organisms as those described by O'Neill *et al.* (1973) which are active as reticuloendothelial stimulants in the mouse as judged by enhancement of carbon clearance. The macrophage chemotactic factor may well be the same as the factor responsible for reticuloendothelial stimulation. This relationship will require thorough examination in the near future. If it holds good, it would imply that similar cell surface receptors are involved in recognition of foreign particles by fixed reticuloendothelial cells as in the response of motile macrophages to chemotactic agents.

The chemotactic factor described here which is present in culture filtrates and acts directly on macrophages differs from the factor produced by *Mycobacterium tuberculosis* and described earlier by us (Symon *et al.*, 1972). That factor, which was also a protein derived from culture filtrates, acted indirectly by activating complement, or a similar enzyme system in plasma. In those experiments, heat-killed mycobacteria were used and the present work describes a factor derived from living anaerobic coryneforms. Both mycobacteria and anaerobic coryneforms are active in causing elevation of serum antibody levels although the action of mycobacteria, at least in the guinea-pig and chicken, is the stronger. We have speculated elsewhere (Symon *et al.*, 1972) that bacterial chemotactic factors for macrophages may play an important rôle in the adjuvant activity of those bacteria since they are important for the formation of a local macrophage granuloma. Such granuloma formation is possibly a prerequisite for adjuvant activity of mycobacteria and related organisms (Suter and White, 1954; Wilkinson and White, 1966; Wilkinson, 1966). Furthermore, if such bacterial factors stimulate macrophages into the burst of metabolic activity which has been shown to accompany particle trapping and ingestion (Karnovsky, Simmons, Glass, Shafer and d'Arcy Hart, 1970) they may also enhance the uptake of antigen by macrophages or macrophage-like cells in an adjuvant granuloma.

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