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# A lipid chemotactic factor from anaerobic coryneform bacteria including *Corynebacterium parvum* with activity for macrophages and monocytes

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**Summary.** A lipid with chemoattractant (chemotactic) activity for mouse and guinea-pig macrophages and for human blood monocytes is released by anaerobic coryneform bacteria (including *Corynebacterium parvum*). The active lipid is associated with fibrillar structures which lie on the outside of the bacterial cell and are released spontaneously during growth. The lipid can also be extracted easily by a number of methods. The fibrils are loosely associated with a capsule-like structure composed largely of polysaccharide. Purification of the active lipid was achieved by chloroform–methanol extraction of the whole organisms yielding a chloroform-soluble fraction attracting mononuclear phagocytes at concentrations around 10 μg/ml. The infra-red spectrum of this material showed lipid but no peptide or sugar. Thin-layer chromatography yielded twelve spots of which three had chemoattractant properties. The most active of these gave staining reactions consistent with the presence of phospholipid, the other two probably contained free fatty acids and triglycerides. Thin-layer electrophoresis also yielded an active phosphorus-containing spot. Saturated fatty acids of chain lengths found in the anaerobic coryneforms had weak monocyte-attractant activity. As the active material was progressively purified, its activity as a monocyte attractant weakened.

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## INTRODUCTION

In previous publications (Wilkinson, O'Neill, McInroy, Cater and Roberts, 1973a; Wilkinson, O'Neill and Wapshaw, 1973b) we reported that the anaerobic coryneform bacteria produce a factor which attracts macrophages *in vitro* in a micropore filter system. This group of bacteria is most familiar under the general designation '*Corynebacterium parvum*' but in fact it is a complex group of bacteria containing many strains. A serological classification of these organisms has been proposed (Johnson and Cummins, 1972) and more recently they have been reclassified in the genus *Propionibacteriaceae* in *Bergey's Manual of Determinative Bacteriology* (1974). The anaerobic coryneforms have excited especial interest because of a variety of biological effects, among them induction of tumour regression (Woodruff and Boak, 1966), immunological adjuvant effects (Neveu, Branallec and Biozzi, 1964; O'Neill, Henderson and White, 1973) and enhancement of particle clearance from the circulation (Halpern, Prévot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Decreusefond 1964; O'Neill *et al.*, 1973). It has been thought that some of these effects might be directly due to stimulation of macrophages, thus a chemoattractant effect on macrophages might be an essential preliminary for biological activity of these organisms *in vivo*.

We suggested earlier that the chemoattractant factor might be a protein (Wilkinson *et al.*, 1973b).

In the present communication we describe more recent experiments designed to identify the factor which show that the major activity is in fact associated with a lipid fraction which is loosely attached to the bacterial surface and is released during growth. A brief report to this effect has appeared elsewhere (Russell, McInroy, Wilkinson and White, 1975). There has been little interest in lipids as leucocyte chemoattractants. Some decades ago scattered reports of such activity in fatty acids and lipids appeared (Häbler and Weber, 1930) but these used inaccurate methods for measuring leucocyte migration. Recently Lynn and his colleagues (Turner, Campbell and Lynn, 1975) have suggested that unsaturated lipids during oxidation may be chemotactic for neutrophils. Lipids have a variety of other effects on phagocytic cells. They have been reported to enhance particle clearance (Biozzi, Stiffel and Mouton, 1963) and to cause changes in macrophage morphology and behaviour (Stuart, 1970). Fauve and Hevin (1974) have recently isolated a lipid fraction from a number of bacterial species including *C. parvum* which they reported to have immunopotentiating effects and to cause tumour regression, effects which the authors ascribe largely to an action on macrophages. Indeed, the idea that lipids may cause tumours to regress is by no means a new one (Murphy, 1924). The characteristics of the lipid chemoattractant isolated from the anaerobic coryneform bacteria are described below.

## MATERIALS AND METHODS

*Bacterial strains and culture methods*

The organisms used in these experiments were representative strains from each of the major serological groups of Johnson and Cummins (1972). The source of these organisms, their characteristics and methods for their culture have been described previously (O'Neill *et al.*, 1973). They were grown routinely in thioglycollate medium U.S.P. (Oxoid) but where an alternative medium was required in control experiments, 1 per cent glucose broth was used. Organisms were harvested by centrifugation, killed by heating at 56° for 30 min, washed twice in 0·9 per cent NaCl and lyophilized. Culture-medium filtrate was prepared by passing the centrifuged supernatant first through a membrane filter (Millpore, Bedford, Massachusetts) of 3 $\mu$m pore size followed by one of 0·22 $\mu$m pore size. The pH of the filtrate was adjusted to 7·2 and it was then tested for chemoattractant activity either immediately at 1 in 100 dilution or alternatively following lyophilization.

Since *Propionibacterium avidum* 4982 caused higher macrophage migration relative to the negative control than other strains and has previously been shown to have high activity in enhancing carbon clearance (O'Neill *et al.*, 1973), this organism was selected for isolation of the chemoattractant factor. Despite its name *Propionibacterium avidum* is classified among the anaerobic coryneforms and shares similar biological properties to organisms designated '*Corynebacterium parvum*' as used by other workers.

*Cells for migration tests*

Peritoneal macrophages were obtained from mice or guinea-pigs by injection intraperitoneally of 2 mg oyster glycogen (B.D.H.) in 0·2 ml per mouse or 10 mg glycogen in 1 ml per guinea-pig. The cells were harvested 4 days later, washed once and finally resuspended in Gey's solution (Wilkinson, 1974) at $10^6$ cells/ml. Human monocytes and neutrophils were obtained from freshly collected heparinized blood. The red blood cells were sedimented for 1 h using dextran of molecular weight 110,000 in 0·9 per cent NaCl (Fisons, Loughborough) in the ratio of 1 ml dextran per 10 ml blood. The leucocyte-rich plasma was withdrawn and layered onto Ficoll–Triosil 440 (Pharmacia, Uppsala, and Nyegaard, Oslo) separating mixture and spun for 30 min at 500 *g*. The separated monocytes and neutrophils were collected washed once and resuspended in Gey's solution at $10^6$ cells/ml.

*Migration tests*

The modified Boyden technique has been described previously in detail (Zigmond and Hirsch, 1973; Wilkinson, 1974). This method allows cells in an upper chamber to migrate through the pores of a membrane filter in a gradient of test substance diffusing from a lower chamber. Negative controls contained Gey's solution only, whereas positive controls contained casein (Merck, Darmstadt) 1 mg/ml in the lower chamber. Duplicate chambers containing each control were set up in all experiments. Micropore filters (Millipore, Bedford, Massachusetts, and Sartorius, Göttingen, Germany) (8 $\mu$m or 12 $\mu$m pore size) were used for tests involving macrophages and monocytes (incubation time 130 min); neutrophil migration was assayed using 3 $\mu$m pore size filters and an incubation time of 75 min. Only direct

chemoattraction by the test substances was assayed. The effects of factors generated in serum by contact with anaerobic coryneforms were not investigated further since we have reported on these previously (Wilkinson *et al.*, 1973b). Cell migration was measured by estimating the distance in micrometres migrated by the leading front of cells by the method of Zigmond and Hirsch (1973). The results in the tables are the means of ten such readings per test. Tests were considered positive only if the mean migration of the cells towards the test substance exceeded that towards the negative control by 10 $\mu$m or more. This difference is usually statistically significant.

*Bacterial growth curves*

These were measured in duplicate, starting separate cultures on fourteen consecutive days. On day 15 the cultures were spun down, washed twice using 0·9 per cent NaCl and resuspended in the original volume for nephelometric estimation. Organisms were lyophilized and tested for chemoattractant activity at 1 mg/ml. Supernatants from the daily stages of the growth curves were tested at 1 in 100 dilution. Duplicate experimental readings of optical density and migration distances were averaged and means quoted.

*Bacterial staining and electron microscopy*

Alcian blue and Alcian green (Gurr) were used as 1 per cent aqueous preparations. Heat-fixed smears were stained for 2 min, rinsed with water, counterstained with dilute fuchsin for 10 s, rinsed again and examined under oil immersion. To stain for sulphated polysaccharide, Alcian blue was made up as a 1 per cent solution in 0·2 N KCl–HCl buffer, pH 1·8. Smears were stained for 2 min and rinsed with buffer, then counterstained as before.

For electron microscopy, saline suspensions of organisms from solid and liquid cultures were applied to carbon-coated copper grids and were negatively stained using ammonium molybdate (1 per cent). Specimens were examined using a Siemens Elmiskop 1A at 80 kV with double condenser illumination. An instrumental magnification of $\times$ 30,000 was obtained with an objective aperture of 80 $\mu$m.

*Culture filtrate fractionation*

Crude protein fractions were prepared from culture filtrate by precipitation with 50 per cent saturated ammonium sulphate and centrifugation at 5000 *g* followed by lyophilization. Crude polysaccharide was precipitated from filtrates by adding cold ethanol to 70 per cent by volume and standing at 4° overnight. The precipitate was removed by centrifugation at 5000 *g* and was lyophilized. Crude lipid fractions were prepared by extracting lyophilized filtrates using methanol/chloroform (2:1 by volume) for 24 h with agitation, by the method of Bligh and Dyer (1959). Lipid from the chloroform phase was concentrated by rotary evaporation at 35° under partial vaccum.

*Gel-filtration of crude protein*

Crude protein (400 mg in 1 ml eluant) was applied to a 27 $\times$ 1 cm column of Sephadex G-25, G-100 or G-200 (Pharmacia). Blue dextran 2000 (Pharmacia, Uppsala) and cytochrome C (Sigma Chemical Company, London) were used as markers for void volume and molecular weight respectively and were detected spectrophotometrically at 254 and 550 nm respectively. Eluants used were Gey's solution or 6 M urea at 48 cm operating head pressure. Fractions of 0·5 ml were collected and tested for activity, those in 6 M urea first being dialysed against Gey's solution for 24 h.

*Lipid extraction of whole lyophilized organisms*

One gram of lyophilized organisms from a 5-day culture was suspended in 20 ml 0·9 per cent NaCl and was twice extracted using chloroform–methanol–water mixtures for 24 h with agitation by the method of Bligh and Dyer (1959). After centrifugation the post-extraction supernatants were combined and partitioned into two phases, one chloroform, the other methanol–water by adding 35 per cent of the total volume each of chloroform and of water. The lower chloroform phase was brought to dryness by rotary evaporation at 35° under vacuum. The residue was stored in freshly distilled chloroform–methanol (2:1) at $-20°$.

*Ultra-violet (u.v.) and infra-red (i.r.) spectroscopy*

Samples for u.v. spectroscopy were suspended in chloroform. Automatic scans of the spectrum from 190 nm were carried out using a Unicam SP 1800 (Pye) recording spectrophotometer with quartz cells of 1 cm path-length. Samples for i.r. spectroscopy were either incorporated into or smeared onto KBr discs. Spectra from 4000 $cm^{-1}$ to 700 $cm^{-1}$ were

read using a grating i.r. spectrophotometer (Perkin-Elmer Model 237).

*Thin-layer chromatography (T.L.C.) and thin-layer electrophoresis (T.L.E.)*

Thin-layer plates were coated with silica gel (B.D.H.) as absorbent. Lipid material was spotted onto plates in quantitities of approximately 20 μg. The chromatographic solvent system used was chloroform–methanol–water (65:35:5). After development and drying, spots were detected either by being directly visible or by fluorescence under a u.v. source using Rhodamine 6G (B.D.H.). Phosphate was detected by the method of Vaskovsky and Kostetsky (1968) and free amino groups by the ninhydrin method (Marinetti, 1964). Tests for chemoattractant activity were carried out on material scraped from areas parallel with indicator spots which were untouched by detection reagents. Negative control samples for these tests were obtained from two areas of differing Rf value. Thin-layer electrophoresis was carried out using silica gel-coated plates in a pyridine (10 per cent) acetic acid (0·4 per cent) buffer system, pH 6·0, run at 500 V and 7 mA for 6 h. The plates were then dried and spots detected as for thin-layer chromatography.

## RESULTS

### Comparative studies of the migration of mononuclear phagocytes from various sources towards *P. avidum* or casein

Our previous experiments on the chemoattractant effects of anaerobic coryneforms (Wilkinson *et al.*, 1973a, b) had been done using guinea-pig or mouse macrophages. However, in our experience these cells give variable results from day to day in chemotaxis tests. The present study showed that human blood monocytes were attracted by the coryneforms to about the same extent as other mononuclear phagocytes but that they give much more reproducible results from day to day than experimentally induced macrophages both with coryneforms and other chemoattractants. Table 1 shows that all cell types tested gave a similar dose–response curve to casein, a protein routinely used as the positive control in cell migration tests in this laboratory, with a single optimum at 1 mg casein per ml. Macrophages and monocytes tested against *P. avidum* gave a dose–response curve unlike that to casein since this had two optima, at 1 mg organisms per ml and 10 μg organisms per ml. Human blood neutrophils, however, showed only a very small response to *P.*

**Table 1.** Chemoattractant effect of killed *P. avidum* 4982 and casein suspensions on mouse and guinea-pig peritoneal macrophages and human blood leucocytes

| Agent under test | Mouse peritoneal macrophages | Guinea-pig peritoneal macrophages | Human blood monocytes | Human blood neutrophils |
|---|---|---|---|---|
| *P. avidum* | | | | |
| 10 mg/ml | 20 | 36 | 37 | 20 |
| 1 mg/ml | 68 | 74 | 69 | 31 |
| 100 μg/ml | 47 | 50 | 50 | 27 |
| 10 μg/ml | 55 | 53 | 59 | 20 |
| 1 μg/ml | 29 | 35 | 30 | 21 |
| Casein | | | | |
| 10 mg/ml | 57 | 79 | 71 | 74 |
| 5 mg/ml | 83 | 85 | 74 | 98 |
| 1 mg/ml | 98 | 102 | 109 | 118 |
| 500 μg/ml | 57 | 90 | 88 | 93 |
| 250 μg/ml | 35 | 51 | 42 | 47 |
| 10 μg/ml | 28 | 34 | 37 | 21 |
| Negative control (Gey's solution) | 26 | 35 | 33 | 21 |

Figures quoted are mean migration distances in micrometres through filters of appropriate pore size (see Materials and Methods section).

*avidum* with a single optimum at 1 mg organisms per ml.

**Presence of the chemoattractant factor(s) in bacterial filtrates: relation to bacterial growth curve**

An experiment was designed to establish the period during bacterial growth at which production of the chemoattractant factor was optimal and to determine whether the factor could be isolated from the organisms themselves and whether it was released into the culture medium. Fig. 1 shows growth kinetics of the organisms in thioglycollate medium as determined by nephelometric measurement at times up to day 15 post-inoculation. This followed a typical bacterial growth curve pattern. The logarithmic phase lasted from day 2 till day 6, levelling off to a peak at day 9 after which the optical density (OD) decreased again to the level seen at day 5.

Samples of organisms taken at 48 hourly intervals during the growth cycle were tested at 1 mg/ml for their ability to attract blood monocytes. The attractant activity which was already present on day 4 showed an initial decrease to day 6, a subsequent increase to a peak at day 8 and thereafter declined.

When samples of culture medium filtrate at a 1 in 100 dilution were tested in the same way (Fig. 1), they showed a peak of chemoattractant activity in the filtrate coincident with the late logarithmic phase of growth of the organisms (day 6) and also a later peak towards day 14 of the growth. These peaks coincided with decreased chemoattractant activity of the organisms themselves, suggesting that the active material was first associated with the bacterial cells which then released it into the medium during late logarithmic phase. This would then be followed by synthesis of more material present on the organisms in large quantities, which would then again be released into the medium. Activity of the culture medium decreased unexpectedly between day 6 and day 10 suggesting either that the factor already present had a short half-life or that autolysis of older cells in the culture caused a transient release of excessive quantities of factors which inhibited cell migration.

Similar growth curve studies were done using other strains namely *C. anaerobium* 6134, *C. anaerobium* 578, *C. parvum* 1383 and *C. parvum* 2683. The study on *P. avidum* 4982 was also repeated using 1 per cent glucose broth as an alternative growth medium. In each case the pattern resembled that described above with slight variations in the growth rate of the particular strain and the level of activity produced.

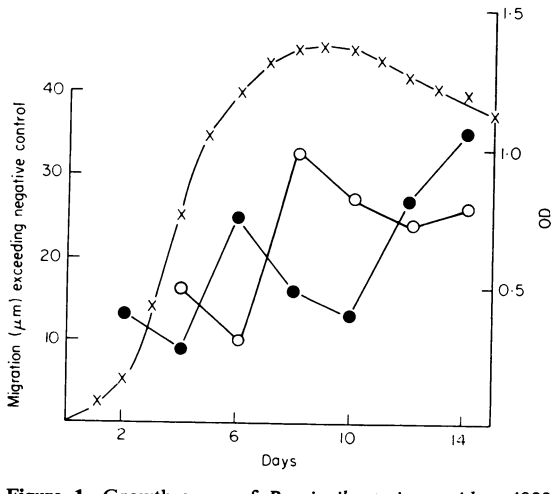


**Figure 1.** Growth curve of *Propionibacterium avidum* 4982 in thioglycollate medium and the chemoattractant activity for blood monocytes of the washed organisms and of the culture filtrates at various stages of growth. (×) Optical density; (●) culture filtrate activity; (○) bacterial activity. All points on this graph are mean values for duplicate experiments.

**Morphological examination of the organisms**

As growth curve studies gave strong evidence that chemoattractant material was released from the organism in late logarithmic phase, the organisms were examined directly by microscopy to determine whether visible morphological evidence for release of material at this stage of growth could be obtained. Conventional light microscopy showed that organisms in logarithmic phase possessed what appeared to be a capsular layer of material barely visible using Gram's stain but which stained with Alcian blue, a stain with a high affinity for acid polysaccharides (Fig. 2). This became reduced in amount or was absent in older cultures. The material did not stain at pH values below 2·0, a characteristic which makes it unlikely to be sulphated polysaccharide (Pearse, 1961), nor did it stain with Alcian green. When the organisms were stained with Alcian blue and the background with Indian ink it was seen that there were unstained patches amid the Alcian blue-positive material suggesting the presence of some additional non-polysaccharide material. Smears of organisms stained with Nigrosin, Sudan black or Sudan III

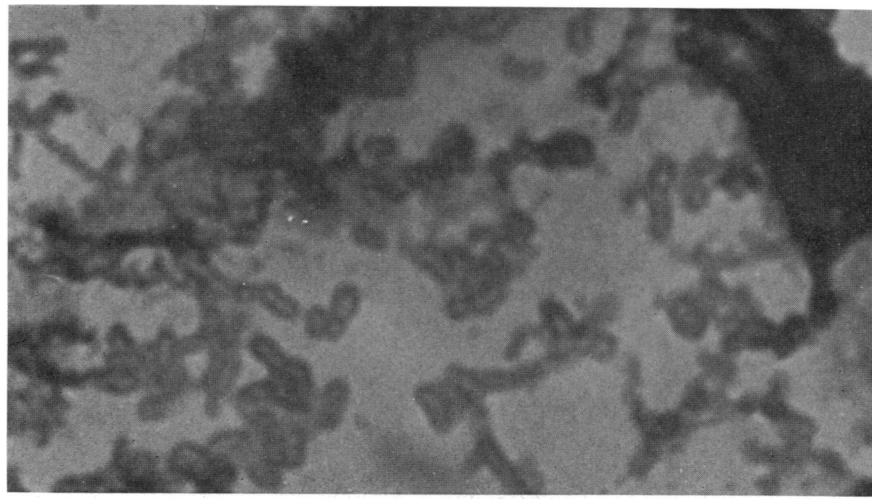

**Figure 2.** Light photomicrograph of *P. avidum* 4982 (2 day culture) stained with Alcian blue. Note that the bacterial cells have not taken up the stain but that each cell is surrounded by a dark rim, indicating staining of capsular polysaccharide. (Magnification × 3120).

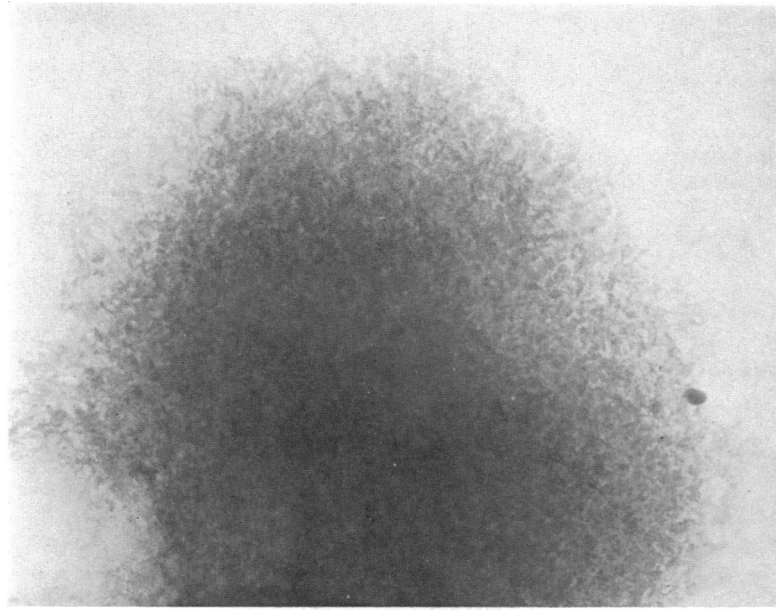

**Figure 3.** Electron micrograph of *P. avidum* 4892 as visualized by negative stain. The organism is in early logarithmic phase of growth. The entire surface of the organism is covered by a loose ill-defined fibrillar material. (Magnification × 115,500.)

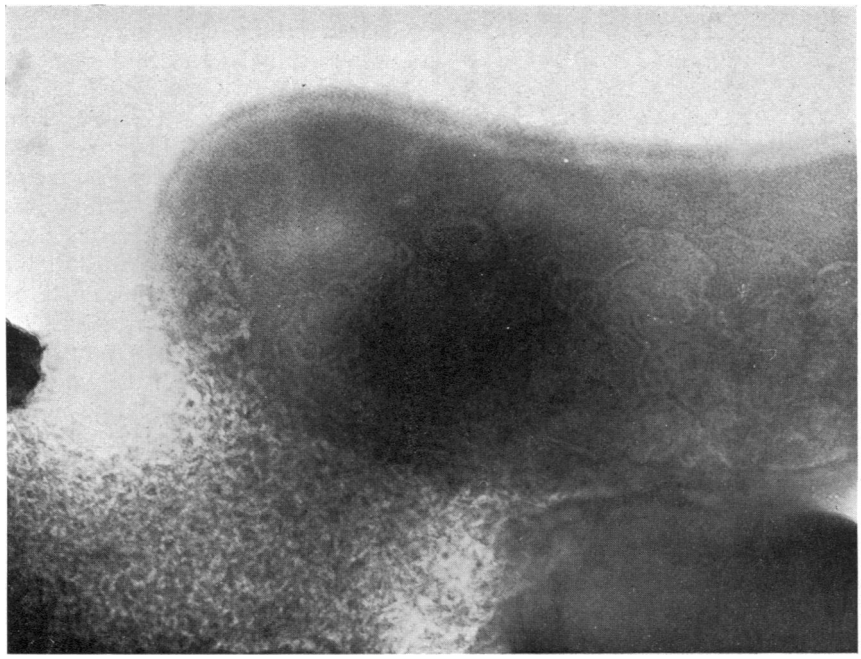

**Figure 4.** Electron micrograph of negatively stained *P. avidum* 4982 in late logarithmic phase showing: (a) free fibrillar material following release from the surface of the organism (bottom left); (b) release in progress (centre); and (c) the exposed undersurface of the bacterium following complete release of the material (along top). (Magnification × 81,000.)

(lipid stains) showed an overall mottled appearance caused by uneven staining. More even staining was achieved using unfixed organisms. These were mixed with stain, washed and observed under a coverslip as wet preparations. This staining behaviour indicated the presence of lipid material on the surface of the organism in addition to the polysaccharide.

Electron microscopy using negative staining techniques was used to explore the ultrastructural nature of the surface material. This showed quite clearly that the surface of the organism was covered by a fibrillar or spaghetti-like substance (Fig. 3) which was apparently loosely attached to the cell surface and which became released from the organism in late logarithmic phase (Fig. 4). On release the fibrils became less densely packed and increased in diameter, and occasionally formed doughnut shapes. Fibrils attached to the cell surface were of the order of 50 Å in diameter while some of those which had been shed increased in diameter to a maximum of 250 Å. Release of the capsular material seemed to occur on its reaching an unstable state in which the capsular extremity became disrupted and material broke free into the medium. The cell-surface revealed after release of the fibrillar material was amorphous, an appearance which would be consistent with the presence of polysaccharide—possibly the same material as had been observed under light microscopy using Alcian blue stain. Occasionally, parts of the lower smooth unstructured surface layer were also released into the medium. Electron microscopy of the culture liquid itself showed both free fibrillar material and also fibrillar material with amorphous material attached to it.

These findings suggest that the anaerobic corynebacteria possess a capsule composed of polysaccharide together with a non-polysaccharide substance which has a fibrillar appearance under electron microscopy. This capsular material is easily detached and is released during growth. As shown below, on release of this material, the culture medium becomes active as a chemoattractant for mononuclear phagocytes.

### Chemoattractant properties of culture medium filtrate and its crude fractions

For the initial attempts to isolate the chemoattractant

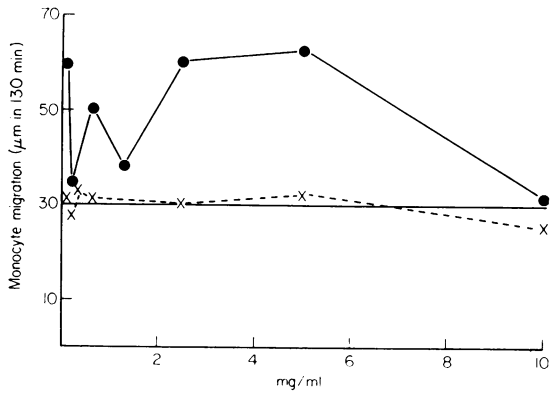


**Figure 5.** Dose–response curve for monocyte migration towards lyophilized filtrates from unused thioglycollate medium and 6-day culture of *P. avidum* 4982. (×) Control filtrate activity; (●) *P. avidum* culture filtrate activity; (—) negative control level.

factor from the culture medium filtrate, filtrates taken at 6 days after inoculation were chosen. As a preliminary step, a lyophilized sample of culture medium filtrate was tested in parallel with a lyophilized sample of unused medium as control in dose–response tests. From Fig. 5 it can be seen that the control filtrate had little or no attractant effect on monocytes. At a dose above 10 mg/ml it was inhibitory to migration. The filtrate of the culture in which bacteria had been grown was also inhibitory at this dose. The culture filtrate tested at 5 mg/ml, 2·5 mg/ml, 625 μg/ml and 78 μg/ml caused increased monocyte migration. It was inactive at three intermediate concentrations (156 μg/ml, 312 μg/ml and 1·25 mg/ml). This complex dose–response curve suggests the presence either of several forms of the same chemoattractant or of several chemoattractant substances with different effective optima. Most defined protein chemoattractants produce simple dose–response curves having one single optimum concentration.

Crude 'lipid', 'polysaccharide' and 'protein' fractions from culture filtrates were all found to attract monocytes more strongly than the negative control (Table 2) and the 'lipid' extract gave a marginally higher result than the others. None was as active as the unfractionated culture filtrate. Chromatographic fractionation of the crude 'protein' fraction was attempted using Sephadex G-200 with Gey's solution as eluant. This produced several active fractions and although the pattern of activity was polydisperse, the active fractions emerged after the cytochrome c peak denoting a molecular weight of less than 13,000. Other fractionation attempts using Sephadex G-25 and G-100 with either Gey's solution or 6 M urea as eluants were unsuccessful since activity occurred along the whole elution profile and some activity was retained in the column. This polydisperse behaviour was unlike that expected for a typical soluble protein and suggested the presence of a high proportion of hydrophobic groups such as might be present were lipid bound to the protein.

### Auto-release of active material from lyophilized killed organisms after reconstitution

Suspensions of lyophilized killed organisms were reconstituted at a concentration of 1 mg/ml and were stored at 4° in Gey's solution for up to 5 days, thus allowing spontaneous release of chemoattractant material into the suspending medium. The supernatants from these suspensions showed increasing

**Table 2.** Monocyte chemoattractant activity of crude fractions from *P. avidum* 4982 culture filtrate

| | Mean distance (micrometres) migrated by human blood monocytes through 8 μm pore size filters in 130 min |
|---|---|
| Negative control (Gey's solution) | 33 |
| Positive control (casein 1 mg/ml) | 103 |
| Culture medium control (1 mg/ml) | 37 |
| *P. avidum* culture filtrate (1 mg/ml) | 62 |
| Crude 'protein' fraction (1 mg/ml) | 45 |
| Crude 'polysaccharide' fraction (1 mg/ml) | 46 |
| Crude 'lipid' fraction (1 mg/ml) | 51 |

**Table 3.** Monocyte chemoattractant activity of supernatants from killed *P. avidum* 4982 suspensions (1 mg/ml) stored at 4°

| | Mean distance (micrometres) migrated by human blood monocytes into 8 μm pore size filter in 130 min |
|---|---|
| Negative control (Gey's solution) | 37 |
| Positive control (casein 1 mg/ml) | 98 |
| Supernatant from unstored organisms | 58 |
| Supernatant from organisms stored 1 day | 61 |
| Supernatant from organisms stored 2 days | 67 |
| Supernatant from organisms stored 3 days | 69 |
| Supernatant from organisms stored 4 days | 75 |
| Supernatant from organisms stored 5 days | 72 |
| Whole organisms unstored and washed | 49 |
| Whole organisms stored 5 days and washed | 35 |
| Supernatant from organisms stored 5 days, washed and stored a further 2 days | 48 |

Washed organisms were washed four times in 0·9 per cent saline.

chemoattractant activity with increasing time of storage up to a maximum value at day 4 (Table 3), Lyophilized organisms freshly reconstituted immediately before testing showed chemoattractant activity. Washing four times in saline did not entirely remove this activity. Lyophilized organisms which had been reconstituted for 5 days, when washed four times, did lose their chemoattractant activity. On standing for a further 2 days, the fresh suspension medium became weakly active indicating the release of further small amounts of chemoattractant.

It was noted that the organisms clumped more readily after storage in the above manner and also that waxy leaflets visible to the naked eye formed on the surface of the storage liquids.

### Chemoattractant activity of lyophilized organisms and their extracts

From the polydisperse behaviour of the active fraction on gel filtration and from its general characteristics (solubility, etc.), it seemed possible that it might be a lipid or a lipid complex. It was therefore decided to extract material from whole killed *P. avidum* 4982 using a chloroform–methanol–water extraction method. The first attempt (Table 4, expt 1) produced a waxy brown extract from the chloroform phase which was highly attractant for monocytes at both 1 μg/ml and 10 μg/ml but not at higher concentrations. The second attempt using larger quantities yielded similar results, this extract being active down to 1 μg/ml also and being monocyte specific. Whole organisms previously active for monocytes when diluted to 10 μg/ml and for neutrophils at 1 mg/ml lost their activity following treatment by the lipid extraction process. A similar result was obtained using several other strains of anaerobic coryneforms: *C. parvum* 1383 (Table 4, expt 2), *C. anaerobium* 6134, *C. anaerobium* 578 and *C. parvum* 2683. With some strains the organisms were actually inhibitory to migration following extraction of the lipid. Following extraction, the organisms retained Gram positivity but electron microscopy at this stage showed a lack of surface fibrillar material. These findings support an association of the chemoattractant with the surface material.

The chloroform solubility of the monocyte-specific factor suggested that it was partially or wholly lipid in composition. This lipid extract withstood heating to 80° for 30 min without loss of activity. Its melting point was 56°. Material—possibly a different factor—with slight neutrophil activity separated with the aqueous phase of the extract. This material contained both carbohydrate and protein as estimated by infra-red spectroscopy. Crude lipid fractions of culture filtrates and of supernatants from stored bacterial suspensions also had activity but the residues remaining from these after lipid extraction had reduced activity. A trichloroacetic acid extraction of the organism's polysaccharide antigens using the method of Cummins (1975) gave a white crystalline powder on lyophiliza-

**Table 4.** Chemoattractant activity of *P. avidum* 4982, *C. parvum* 1383 and fractions, obtained by lipid extraction

| Experiment 1 *P. avidum* 4982 | Human blood monocytes | Human blood neutrophils |
|---|---|---|
| Negative control (Gey's solution) | 43 | n.t. |
| Positive control (casein 1 mg/ml) | 128 | n.t. |
| Lipid extract from chloroform phase | | |
| 1 mg/ml | 32 | n.t. |
| 100 μg/ml | 44 | n.t. |
| 10 μg/ml | 121 | n.t. |
| 1 μg/ml | 87 | n.t. |

| Experiment 2 | Human blood monocytes | | Human blood neutrophils | |
|---|---|---|---|---|
| | *P. avidum* 4982 | *C. parvum* 1383 | *P. avidum* 4982 | *C. parvum* 1383 |
| Negative control (Gey's solution) | 55 | 53 | 33 | 28 |
| Positive control (casein 1 mg/ml) | 75 | 85 | 80 | 71 |
| Whole organisms pre-extraction | | | | |
| 1 mg/ml | 73 | 79 | 44 | 43 |
| 100 μg/ml | 80 | 80 | 37 | 33 |
| 10 μg/ml | 70 | 79 | 31 | 33 |
| 1 μg/ml | 57 | 62 | 32 | 32 |
| Whole organisms post-extraction | | | | |
| 1 mg/ml | 53 | 62 | 30 | 34 |
| 100 μg/ml | 59 | 62 | 30 | 30 |
| 10 μg/ml | 55 | 57 | 34 | 30 |
| Lipid extract (chloroform phase) | | | | |
| 1 mg/ml | 62 | n.t. | 36 | n.t. |
| 100 μg/ml | 73 | 70 | 39 | 30 |
| 10 μg/ml | 66 | 71 | 30 | 27 |
| 1 μg/ml | 68 | 59 | 31 | 30 |
| 0·1 μg/ml | 60 | 58 | n.t. | 29 |
| Lipid extract (methanol/water phase) | | | | |
| 500 μg/ml | 73 | 40 | 43 | 30 |
| 50 μg/ml | 67 | 69 | 38 | 27 |
| 5 μg/ml | 68 | 63 | 30 | 34 |

Figures quoted are mean migration distances in micrometres through filters of appropriate pore size (see Materials and Methods section).
n.t. = Not tested.

tion. This material when examined by infra-red spectroscopy did not show presence of any lipid. This material had chemoattractant activity for either monocytes or neutrophils within the range 1 mg/ml to 1 μg/ml.

### Ultra-violet and infra-red spectroscopic analysis of the lipid extract

The ultra-violet spectrum of the extracted lipid material suspended in chloroform showed three absorption maxima at 247 nm, 310 nm and 398 nm, that at 310 nm being broader and more intense than the others. There were no specific peaks at 280 nm or 260 nm which would have suggested the presence of protein or nucleic acids. The infra-red spectrum (Fig. 6) also supported the absence of protein in not having the characteristic absorption bands for peptide or amide at 1650 $cm^{-1}$ and 1550 $cm^{-1}$. Low absorption at 3300 $cm^{-1}$ signified the lack of detectable carbohydrate. Strong absorption bands characteristic of $CH_2$ groups were present at 2920 $cm^{-1}$, 2850 $cm^{-1}$ and 1470 $cm^{-1}$. Bands at 1713 $cm^{-1}$ and 1280 $cm^{-1}$ signified carboxylic acid groups while another at 1735 $cm^{-1}$ denoted the presence of ester

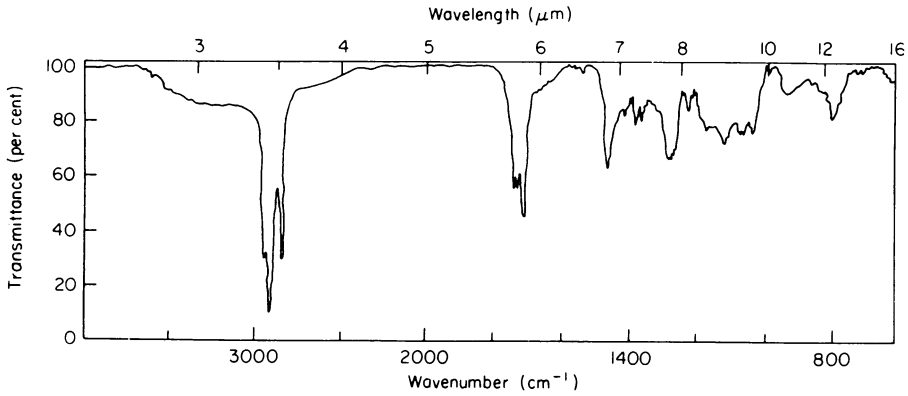


**Figure 6.** Infra-red spectrum of the chloroform-soluble lipid extract from *P. avidum* 4982.

bonds. No high wavenumber peaks corresponding to unsaturated lipids were present. These results provide strong evidence that the active material is largely lipid.

### Thin-layer chromatography (TLC) and thin-layer electrophoresis (TLE) of lipid extract

Using TLC the brown lipid material was fractionated into twelve separate component spots. Individual spots were scraped off the plates and tested for chemoattractant activity. Table 5 shows the chemoattractant activities of the whole organisms, the lipid extract, and its fractions. Crude lipid extract produced a chemoattractant effect and was monocyte specific. Of the separated fractions, a dark spot at Rf 0·18 showed the strongest monocyte attractant activity. This spot which was visible without staining gave a positive reaction for the presence of a free amino group and also for the presence of phosphate. These reactions together with the Rf values are consistent with a phospholipid identity. The TLC fractions with Rf values 0·78, 0·95 and 0·98 showed

**Table 5.** Chemoattractant activity for blood monocytes and neutrophils of *P. avidum* 4982 and its TLC separated fractions

| | Human blood monocytes | Human blood neutrophils |
|---|---|---|
| Negative control (Gey's solution) | 39 | 20 |
| Positive control (casein 1 mg/ml) | 114 | 116 |
| Whole organisms pre-lipid extraction (1 mg/ml) | 81 | 25 |
| Whole organisms post-lipid extraction (1 mg/ml) | 47 | 20 |
| Crude lipid extract (10 μg/ml) | 96 | 21 |
| Crude lipid extract (1 μg/ml) | 48 | 25 |
| TLC negative control Rf 0·2 | 39 | 21 |
| TLC negative control Rf 0·8 | 40 | 21 |
| TLC spot Rf 0·04 | 38 | 24 |
| TLC spot Rf 0·18 | 64 | 28 |
| TLC spot Rf 0·78 | 52 | 29 |
| TLC spot Rf 0·95 | 50 | 31 |
| TLC spot Rf 0·98 | 48 | 19 |

Figures quoted are mean migration distances in micrometres through filters of appropriate pore size (see Materials and Methods section).

moderate monocyte-attractant activity. These high Rf value spots occurred at chromatographic positions where one would expect to isolate free fatty acids and triglycerides. All fractions showed negligible effects on neutrophil migration. The remaining eight fractions at a wide range of Rf values did not show any chemoattractant activity for monocytes or neutrophils.

Using TLE a dark spot giving similar reactions to that at Rf 0·18 on TLC was separated. The spot moved towards the anode immediately behind a visible reddish spot, possibly pigment in identity. A phosphate-positive but free amino-group negative spot moved towards the cathode. Only the dark spot which moved towards the anode showed any significant chemoattractant activity and this activity was not monocyte-specific.

### Chemoattractant effects of lipids of known identity

Pentadecanoic, heptadecanoic, myristic, palmitic and linoleic acids are known to occur in anaerobic coryneforms. These were therefore obtained commercially (Sigma), purified and tested as chemoattractants. They all showed some effect on attracting both monocytes and neutrophils. The activity observed was usually of low to moderate strength paralleling that seen with *P. avidum* lipid extract TLC fractions. The active concentrations of these lipids varied from test to test between 200 $\mu$g/ml and 0·01 $\mu$g/ml but they are poorly soluble in aqueous solutions so the effective concentrations could not be determined accurately. The major fatty acid occurring in the anaerobic coryneforms is the iso-form of pentadecanoic acid (Etemadi, 1963). This could not be obtained commercially but iso-stearic acid resembles it closely. Iso-stearic acid and stearic acid were compared as chemoattractants but no difference was found between them, both being moderately attractant.

### Is the locomotor response of human blood monocytes towards the lipid fraction from *P. avidum* truly chemotactic?

Monocytes show two types of locomotor response to chemoattractants: (a) enhanced random migration (chemokinesis) and (b) directed migration (chemotaxis). It is possible to distinguish between these two types of locomotion by observation of cell migration at varying concentrations of chemoattractant and in the presence and absence of concentration gradients (Zigmond and Hirsch, 1973). Tests of this type were done using as chemoattractant the lipid extract which partitioned with the chloroform phase. Table 6 shows the migration distances covered by monocytes in one such test. Migration increased with increasing absolute concentration of chemoattractant in the absence of a gradient (along the diagonal, top left to bottom right). Figures for migration in a positive gradient are shown above the diagonal, those in a negative gradient below the diagonal. Influence of the gradient on migration (chemotaxis) was assessed by comparing the migration obtained experimentally with the migration figures which would be expected if cells migrated randomly and did not respond to a gradient (figures in parentheses). These figures can be calculated as described by Zigmond and Hirsch (1973). Comparing these figures with those obtained experimentally, it can be seen that they differ sufficiently to suggest that where a gradient is present, the cells are not migrating randomly. In a positive gradient, migration is higher than expected; in a negative gradient, lower. The trend was similar but not as sharp in a duplicate experiment. These findings suggest that the cells are showing a true chemotactic response to the lipid extract and that this lipid may therefore legitimately be described as a 'chemotactic factor'. This requires further confirmation.

**Table 6.** Locomotor response of human monocytes in presence and in absence of chemoattractant gradients of chloroform-soluble lipid from *P. avidum* 4982

| Dilution above filter | Dilution of lipid below filter: 0 | $5\times10^{-7}$ | $10^{-6}$ |
|---|---|---|---|
| 0 | 35 | 50 (39) | 56 (45) |
| $5\times10^{-7}$ | 42 (50) | 54 | 45 (52) |
| $10^{-6}$ | 37 (50) | 47 (52) | 50 |

Figures without parentheses are mean distances in micrometres migrated by monocytes into 8 $\mu$m poresize filters in 130 min. Figures within parentheses are migration distances in micrometres predicted on the basis of random migration by the method of Zigmond and Hirsch (1973). Dilutions of lipid were prepared from a 20 mg/ml stock solution.

## DISCUSSION

The experiments described in this paper have shown that a lipid fraction isolated from anaerobic coryneform bacteria acts as a chemoattractant for mononuclear phagocytes at low doses, and in the absence of polysaccharide or protein as detectable by infra-red spectroscopy. It is highly probable that this lipid is associated with a capsular material which shows a fibrillar structure in the electron microscope and is released from the organisms during growth.

Observation of a capsule-like structure on the anaerobic coryneforms was rather surprising as, to our knowledge, no reports that *P. avidum* or other members of this group are capsulated, exist in the literature. Alcian blue staining suggested that the capsule structure was acid polysaccharide in composition and was present on all young cultures. Further staining with lipophilic dyes suggested the presence of a lipid component. Electron microscope examination using negative staining showed superficial structures on the surface of the organisms, visible in relief, which resembled the lipid fibrillar structures seen on mycobacteria (Gordon and White, 1971), Nocardia and *Corynebacterium diphtheriae* (Russell, unpublished observations). On release this material had an appearance similar to endotoxin from Gram-negative organisms as visualized by negative staining (Russell, unpublished observations). However, the diameter of the fibrils and the depth at which they occurred in the surface layer were unlike those seen in the organisms mentioned above since the fibrils of the anaerobic coryneforms are very superficial whereas those in mycobacteria are intrinsic to the cell wall. Furthermore the diameter of the fibrils of the anaerobic coryneforms is approximately 50 Å whereas those on mycobacteria are over twice as thick (Gordon and White, 1971). Its superficial position may explain the ease with which the fibrillar material leaves the surface of the anaerobic coryneform bacteria. The amorphous surface exposed following the release of the fibrillar material is consistent with the presence of polysaccharide. Occasional release of parts of this amorphous layer attached to the fibrillar material suggests that binding between these two layers is present at certain points. Release of the capsular material was usually maximal in the late logarithmic phase of growth and paralleled release of chemoattractant activity suggesting a strong relationship between the two. Killed organisms released the majority of their chemoattractant material gradually over a period of 4 days.

We originally thought that the chemoattractant was a protein (Wilkinson *et al.*, 1973b) since the activity of culture supernatants was reduced somewhat by treatment with proteases. Our present study suggests major activity for lipid. However, it is possible that this lipid might be partly associated with protein in the whole organisms, or alternatively that there are several chemotactic factors. Considering the complex nature of bacterial cells, it is probably simplistic to think that a single factor is responsible for their chemoattractant effects. In the case of *Staphylococcus aureus* we have shown that multiple factors are certainly involved (Russell, Wilkinson, McInroy, McKay, McCartney and Arbuthnott, 1976). Nevertheless, the anaerobic coryneforms show strongest activity at stages in growth when the superficial lipid is most easily demonstrable and it seems likely, especially given the easy accessibility of this loosely attached material for interaction with phagocytes, that this lipid forms the major chemoattractant released from these organisms. The activity of this superficial lipid is supported by (a) retention of Gram-positivity of organisms following extraction of the active material, suggesting that this extraction does not disrupt the structural integrity of the cell, (b) loss of activity when the fibrillar material disappears from the cell surface, and (c) spontaneous release of activity from growing cells into the culture medium or from killed cells into the storage medium. As the lipid material was taken through purification steps, its monocyte attractant activity became somewhat reduced. It also began to show slight activity for neutrophils. This could be due to removal of an inhibitor of neutrophil migration or to a different interaction of the crude factor than of the purified factor with the phagocyte membrane.

The active lipid has not been characterized with certainty but was associated with TLC spots which probably contained phospholipid, free fatty acids and triglyceride. Furthermore, commercially obtained saturated fatty acids known to occur in the anaerobic coryneforms were active when tested as chemoattractants. The activity of purified lipids of low molecular weight is rather low and does not compare with that of the better-known protein or peptide chemoattractants used in many chemotactic studies. It is possible that for optimal activation of locomotor

responses in phagocytes these lipids require attachment to a macromolecular carrier. Possibly this is why activity drops as the lipid is purified. The lipids presumably penetrate the lipid bilayer of the phagocyte membrane and it may be that several such molecules linked to a carrier and penetrating in proximity to one another activate the cell more effectively than the same molecules would if dispersed. There has been a recent report by Turner *et al.* (1975) of chemoattractant activity of lipids for neutrophils. These workers suggested that this activity occurred on oxidation of multi-unsaturated fatty acids. Our unpublished studies would lead us to agree that unsaturated lipids may have such activity but other studies suggest that such lipids are uncommon in anaerobic coryneforms and it seems that this cannot be the whole or correct explanation for the activity of these bacteria.

The work of Fauve and Hevin (1974) on the immunopotentiating effects of phospholipid fractions from a wide range of bacteria suggests that many bacterial species possess lipids capable of interacting with the membranes of phagocytes or lymphocytes and activating their functions in immune responses. We feel that the unique feature of the lipid from *C. parvum* and other anaerobic coryneforms which may distinguish it (them) from the lipids of other bacteria is that in the anaerobic coryneforms this biologically active lipid is superficially placed and easily released and thus possibly able to exert its effects *in vivo* much more easily than molecules from other bacteria which require disruption of the bacterial cell before they can be made available to cells of the immune system. Further studies are now required to determine whether the fibrillar material from the anaerobic coryneforms possesses the full range of biological activities of the whole organisms other than chemoattraction. The identity of the chemoattractant with the phospholipid of Fauve and Hevin (1974), extracted by a similar method, also requires to be established.

On purifying the coryneform lipid we observed that its capacity to attract mononuclear phagocytes was reduced. As discussed above, it is possible that for maximal activity such lipids require to be associated with or stabilized by other molecules or to be attached to a particle such as the bacterial cell. If this is so, then the search for a pure substance representing the biologically active principle of the whole bacteria may not necessarily be practicable or desirable.

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