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Chemotaxis and the inhibition of chemotaxis of human neutrophils in reponse to metal ions

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The possibility that three different types of metal ions, Cr(III), Co(II) and Ni(II), all relevant to metallic biomaterials, might influence chemotaxis has been investigated. Two new techniques, not previously used in biomaterials research involving the study of locomotor morphology of neutrophils and cell movement through agarose gels, were used in this study. Chromium ion concentrations from 0.05 to 10 p.p.m. and cobalt ion concentrations from 0.5 to 30 p.p.m. were not chemotactic for neutrophils under agarose and did not induce neutrophils to polarize. As neutrophils must take up a locomotor morphology before migrating, it is clear that cobalt and chromium ions in a protein-free environment are not chemotactic for human neutrophils. Concentrations of >2.5 p.p.m. nickel ions did induce neutrophils to polarize, probably a result of the nickel ions causing depolarization of the neutrophil plasma membrane.

1. Introduction

The host response to an implanted material may be regarded as a balance between inflammatory and repair processes [1–4]. The manner in which an implant may influence the various components of these reponses is a key factor in controlling the overall biocompatibility of the material in question. Many different variables will be involved, including those which are concerned with the material (bulk chemistry, surface chemistry, topography, rate of degradation, etc.) and those which are concerned with the host (age, species, location, general health, etc.).

When considering the implantation of a metallic biomaterial into tissue, there will inevitably be a release of metal from the surface, by one or more of several possible corrosion processes [5]. Once in the tissue, this metal, as ions, salts or organic complexes, may exert some influence on that tissue. Metal may bind to plasma proteins or to the membrane of cells [6], or may inhibit or activate intracellular enzymes [7]. There can be no doubt that the characteristics of the corrosion process and the biological activity of the released corrosion products are vitally important determinants of the biocompatibility of metals.

Much remains to be learnt, however, about the precise manner in which metals influence the cellular events in the host response, bearing in mind that several of the metals currently employed have a natural, albeit extremely low, presence in the tissue.

One of the ways in which metals could influence cellular behaviour is through chemotaxis. Chemotaxis is defined as the reaction by which the oriented and directional locomotion of cells or organisms is determined by substances in their environment. If cells move towards a stimulating substance the chemotaxis is positive, whereas if the movement is away from the stimulating substance it is negative.

Chemotaxis is important for all directional cellular movement and is a crucial factor in controlling the migration of inflammatory cells to the site of tissue injury. Thus, at the site of implantation, chemotaxis is an important contributory factor in the accumulation of cells such as neutrophils, macrophages and lymphocytes. Although it is substances generated within the tissue itself (e.g. C5a, C3a, leukotriene B4 and denatured proteins) that are primarily responsible for chemotaxis, exogenous substances released from an implant may have an effect, either enhancing or inhibiting, on this process. This paper addresses the question of whether three types of metals commonly used in surgical alloys (chromium, cobalt and nickel), all of which are known to be biologically active to some extent, could influence chemotaxis.

Most of the evidence demonstrating chemotaxis to a wide variety of substances has been obtained *in vitro*. Four types of assay have been employed.

1. The Boyden chamber, where cells migrate towards a chemotactic factor through a filter [8, 9].
2. Chemotaxis under agarose, where the cells in one well move towards a chemotactic factor in another well, through agarose [10, 11].
3. Visual assays involving direct observation of cell locomotion, using time-lapse cinematography [12, 13].
4. Neutrophil polarization assay, where the adoption of locomotor morphology (polarization) of neutrophils in an isotropic concentration of chemotactic factor is assessed [14].

Some studies have been made of chemotaxis by biomaterials, as reviewed recently [15], but almost exclusively by use of the Boyden chamber. This technique was developed to study chemotaxis with

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substances of high molecular weight such as casein, and it is not ideal for highly mobile metal ions. We report here, therefore, studies with the agarose and neutrophil polarization methods, which have not previously been used in the study of biomaterials.

2. Materials and methods

2.1. Materials

The medium used for the preparation of neutrophils was HBSS–MOPS. Hanks' balanced salt solution (HBSS) was prepared fresh every day from 10 × stock solution and sterile distilled water. Morpholinopropanesulphonic acid (MOPS; Sigma) was used at 10 mM concentration in HBSS.

The positive control used in these experiments was N-formyl–methionyl–leucyl–phenylalanine (FMLP), a bacterial chemotactic peptide (Sigma). Fresh peptide was prepared every day from a stock solution of FMLP at 10^{-8} M in dimethylsulphoxide, kept at −20° C.

The metal salts used in this study were cobalt (II) chloride, chromium (III) chloride and nickel (II) chloride (BDH Ltd). These were suspended at 1000 p.p.m. metal ion in 0.85% NaCl and diluted in physiological buffered saline (PBS; pH 7.4) to the appropriate concentration.

Agarose (ICN Immuno-biologicals) and × 10 minimal essential medium (MEM; Gibco) were used in this study.

2.2. Preparation of neutrophils

Neutrophils were isolated from fresh heparinized human blood obtained from healthy volunteers by mixing 2 vol blood with 1 vol 6% dextran. After 25 to 30 min the supernatant was layered on lymphocyte separation mixture (Ficoll-Hypaque, Pharamacia) and centrifuged at 1200 r.p.m. for 25 min. The pellet was transferred to a fresh conical-bottomed test tube and washed once in HBSS–MOPS. The remaining red blood cells were lysed by resuspending the pellet in 1 ml sterile distilled water for 30 sec, after which 10 ml HBSS–MOPS was added. This gave a population of > 95% round neutrophils.

2.3. Polarization assay

Neutrophils were adjusted to 1.25×10^6 cells ml^{-1}. 1 ml of the appropriate test solution was added to 1 ml neutrophil–HBSS–MOPS suspension, and incubated for 30 min at 37° C. 2 ml 2.5% glutaraldehyde was then added. After 10 min the fixed cells were centrifuged, washed twice and resuspended in the remaining drop of medium. Those fixed cells deviating from a spherical outline were scored as positive. A total of 300 cells were counted in each preparation and the number of polarized cells expressed as a percentage of this total.

In the positive control, where the neutrophils were suspended in 10^{-8} M FMLP, > 90% of the neutrophils were polarized. A negative control involving PBS alone always resulted in < 5% polarization (Figs 1a and b).

In order to examine whether the test metal solutions were able to inhibit the polarization response, a final concentration of 10^{-8} M FMLP was added to the metal chloride solutions and neutrophils were exposed to this mixed solution.

Since it has been shown that pH affects neutrophil polarization [16], the pH of each solution was measured and shown not to deviate from physiological value.

2.4. Agarose assay

Each experiment involved the use of 12 (60 mm × 15 mm) tissue culture plates. Agarose was prepared by adding 0.72 g agarose powder to 30 ml sterile distilled water and autoclaving for 10 to 15 min. Medium was prepared by mixing 6 ml × 10 MEM, 0.6 ml 7.5% solution bicarbonate, 23.4 ml sterile distilled water and 600 mg human serum albumin. The agarose was cooled to 60° C in a water bath and mixed with an equal volume of prewarmed medium. 5 ml of this agarose medium was delivered to each plate, set at room temperature and stored at 4° C. Wells were cut in the gel, using a Shandon cutter, facilitated by a vacuum line.

Eight series of three wells, 3 mm in diameter, 3 mm apart, were cut in each plate (Fig. 2). A template was used for this purpose. The pH was adjusted to 7.2 by incubating in 5% CO_2 for 15 to 30 min.

Neutrophils were resuspended at 2.5×10^7 cells ml^{-1}. 11.5 μl of the appropriate metal ion concentration was placed in the outer wells. Negative (PBS) and positive (FMLP) controls were included in each plate. The culture dishes were incubated for 2 h at 37° C in a humidified atmosphere containing 5% CO_2 in air.

The cells were fixed by flooding the plate with 3 ml methanol for 30 min followed by 3 ml formalin for 30 min. The gel was removed by floating the plate on a water bath at 60 to 90° C for 3 to 5 min. The plates were washed with water and stained with 3 ml 0.1% Wright's stain for 10 min, then air-dried.

Inhibition of chemotaxis was studied by adding 1 ml 2.5×10^6 cells ml^{-1} neutrophil suspension to 1 ml 2 × metal concentration, followed by incubation at 37° C for 30 min and centrifugation at 1200 r.p.m. for 5 min. The cells were then resuspended in 100 μl of the required metal concentration in PBS. 11.5 μl of this suspension was placed in the middle wells, with PBS in inner and FMLP in outer wells, respectively. FMLP was prepared at a concentration of 10^{-6} M. The higher concentration of FMLP was used in the agarose assay since FMLP must diffuse through the agarose before stimulating the neutrophils.

Chemotaxis was measured by placing the agarose plates on an overhead projector. The plates were projected with a magnification of 23 × on a screen. The distance that neutrophils had migrated from the central well was measured on the screen with a ruler.

3. Results

3.1. Neutrophil polarization

3.1.1. Chromium

The results with chromium, obtained with blood from two sources, are given in Table I. No neutrophil polarization occurred up to a concentration of 2.5 p.p.m. Cr(III). At 1 p.p.m., neutrophils were observed to aggregate. This phenomenon was even more apparent

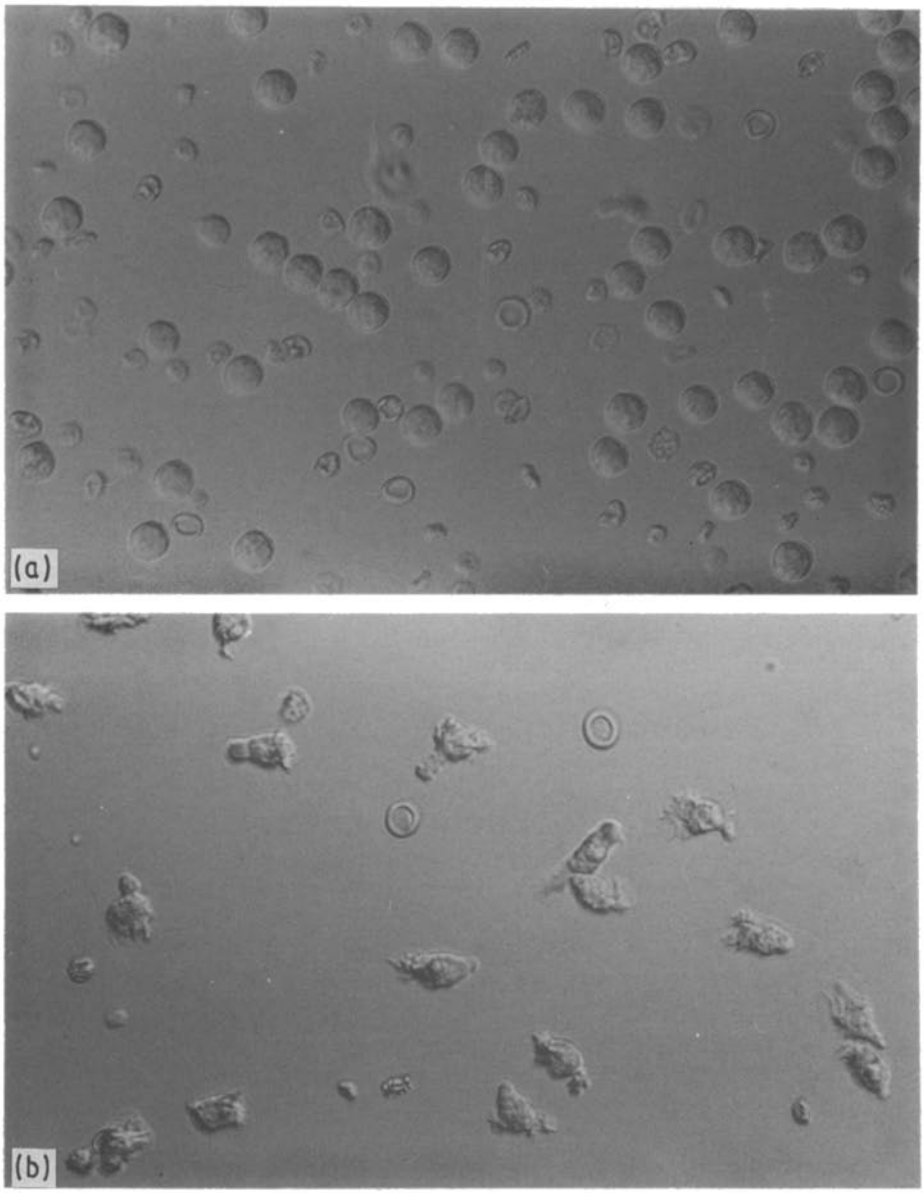

Figure 1 Neutrophils suspended for 30 min at 37° C in (a) HBSS–MOPS and (b) 10^{-8} M FMLP (both × 425).

at 5 and 10 p.p.m. (Fig. 3). At these levels a small percentage of the neutrophils deviated from a spherical outline, but they did not show typical polarized morphology.

3.1.2. Cobalt

As seen from Table II, no neutrophil polarization occurred up to 20 p.p.m. cobalt. At 20 and 30 p.p.m., significant cell aggregation was seen, with a small number of cells deviating from a spherical outline. As for chromium these neutrophils did not show a typical polarized morphology.

TABLE I Neutrophil polarization response to Cr(III)

Cr(III) (p.p.m.)	Polarization (%)	
	Blood source A	Blood source B
10.0	*	*
5.0	6	10
2.5	6	4
1.0	3	1
0.5	1	2
0.25	2	2
0.125	2	1
0.05	2	1
0.025	1	1
0.005	2	0
PBS	2	1
FMLP	94	92

*Cells aggregate.

TABLE II Neutrophil polarization response to Co(II)

Co(II) (p.p.m.)	Polarization (%)	
	Blood source C	Blood source D
30.0	*	*
20.0	5	13
10.0	2	5
5.0	1	2
2.5	2	3
1.0		4
0.5	2	3
0.25	4	3
0.125	2	
0.1		4
0.05	2	
0.01		4
0.005	2	
PBS	3	4
FMLP	94	95

*Cells aggregate.

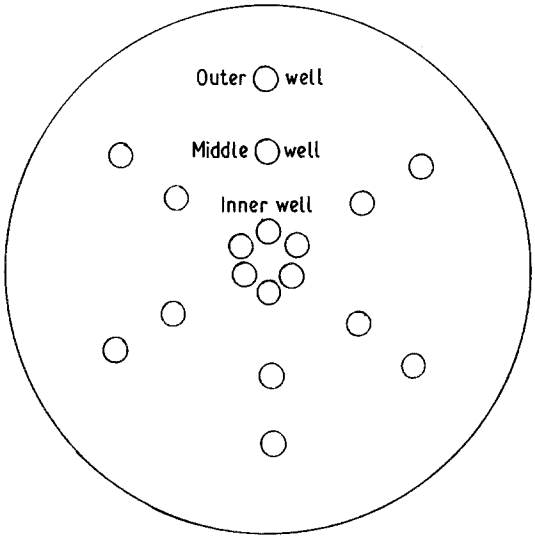

Figure 2 Template for agarose assay.

3.1.3. Nickel

Table III demonstrates some variability with nickel in relation to blood from different volunteers. At 2.5 p.p.m. there is a slight increase in the percentage of polarized neutrophils. The number of polarized neutrophils gradually increases up to a nickel ion concentration of 50 p.p.m. High concentrations of nickel ions did not produce any aggregation of the cells.

In the inhibition experiments, the presence of 10^{-8} M FMLP produced 99% polarization. Concentrations up to 10 p.p.m. Cr(III), 30 p.p.m. Co(II) and 50 p.p.m. Ni(II) did not reduce this percentage of cells polarizing in response to the FMLP (Table IV).

3.2. Agarose tests

3.2.1. Chromium

Chromium ion concentrations ranging from 0.05 to 10 p.p.m. did not cause neutrophils to migrate either towards or away from the metal ions (Fig. 4). In the inhibition experiments, at a concentration of < 1 p.p.m., chromium did not inhibit chemotaxis to 10^{-6} M FMLP, but at 2.5 p.p.m. chemotaxis was inhibited by 56% (Figs 5 and 6).

3.2.2. Cobalt

Cobalt in the range 0.5 to 30 p.p.m. did not cause neutrophil migration. At concentrations of < 10 p.p.m. no inhibition of chemotaxis to FMLP was seen, but in the presence of a visible precipitate at greater concentration, neutrophil migration was significantly inhibited (Figs 7 and 8).

3.2.3. Nickel

Nickel did not cause neutrophil migration when present in the concentration range 0.1 to 50 p.p.m. At a concentration of < 2.5 p.p.m. nickel did not inhibit chemotaxis to FMLP, but this migration was progressively inhibited by higher concentration up to 50 p.p.m. (Figs 9 and 10).

4. Discussion

Chromium ion concentrations from 0.05 to 10 p.p.m.

TABLE III Neutrophil polarization response to Ni(II)

Ni(II) (p.p.m.)	Polarization (%)			
	Blood source E	Blood source F	Blood source G	Blood source H
50.0	8	19	8	20
40.0	8	16	5	18
30.0	8	14	5	15
20.0	7	9	5	7
10.0	8	4	7	6
5.0	9	5	4	–
2.5	6	5	4	4
0.5	5	3	0	4
0.05	2	1	1	5
0.005	2	1		
PBS	3	1	1	3
FMLP	93	99	95	90

TABLE IV Neutrophil polarization response to FMLP in the presence of Cr(III), Co(II) or Ni(II)

Cr(III) concentration (p.p.m.)	Polarization (%)	Co(II) concentration (p.p.m.)	Polarization (%)	Ni(II) concentration (p.p.m.)	Polarization (%)
10	99	30	96	50	94
5	99	20	95	30	98
2.5	98	10	91	20	96
1	96	5	93	10	96
0.5	95	2.5	96	5	95
0.25	95	0.5	97	2.5	97
0.125	94	0.25	96	1	97
0.05	99	0.125	95	0.5	95
0.025	99	0.05	94	0.25	94
0.005	97	0.005	98	0.1	95
0	99	0	99	0	96
PBS	1	PBS	1	PBS	0

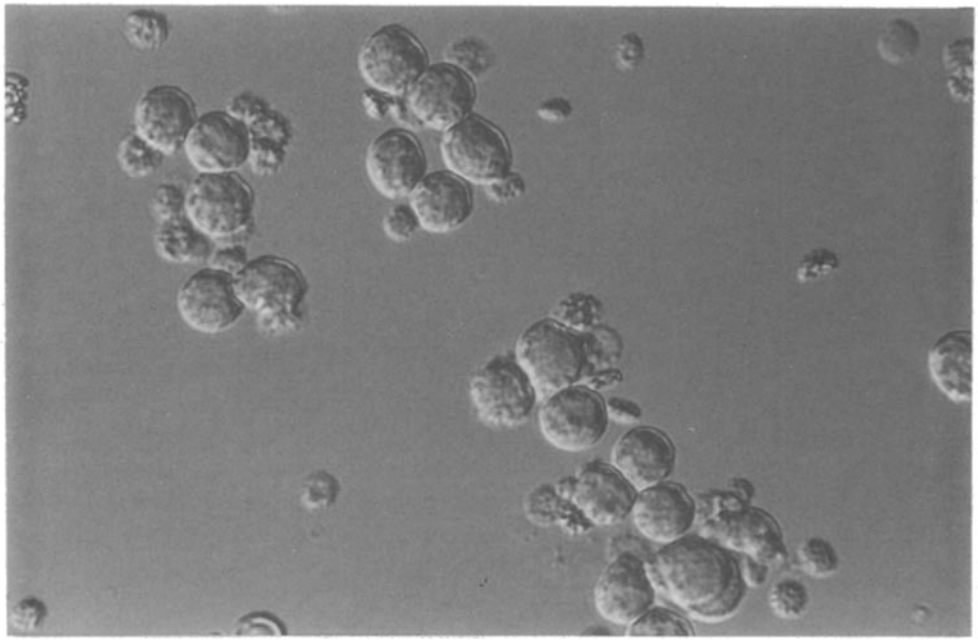

Figure 3 Neutrophils incubated with 10 p.p.m. chromium ions (× 425).

and cobalt ion concentrations from 0.5 to 30 p.p.m. were not chemotactic for neutrophils under agarose and did not induce neutrophils to polarize. As neutrophils must take up a locomotor morphology before migrating, it is clear that cobalt and chromium ions in a protein-free environment are not chemotactic for human neutrophils.

Concentrations of > 2.5 p.p.m. nickel ions induce neutrophil polarization (Table III). Since the degree of polarization differs in different experiments, and furthermore nickel ions at 10 to 50 p.p.m. do not induce, but inhibit, chemotaxis under agarose (Figs 9 and 10), we suggest that nickel ions are not chemotactic for human neutrophils and that the polarization response noted above is a result of the nickel ions causing depolarization of the neutrophil plasma membrane. Neutrophils from different individuals may have different threshold values. This would explain why the degree of neutrophil polarization differs in replicate experiments. Potassium ions have previously been noted to induce neutrophil polarization without invoking chemotaxis [17]. The results show that nickel ions start to inhibit chemotaxis under agarose at a concentration of between 2.5 and 10 p.p.m. A concentration of nickel ions is reached, between 10 and 30 p.p.m., above which no further increase in inhibition of chemotaxis occurs. Thus, the process that the nickel ions are inhibiting appears to be saturable. This inhibition of chemotaxis is not due to nickel ions inhibiting neutrophils from taking up a locomotor morphology (Tables III and IV). Nickel ions are well known to act as calcium ion antagonists by blocking a wide variety of calcium ion-dependent processes [18]. Therefore, we suggest that nickel ions could inhibit chemotaxis under agarose by inhibiting calcium ion-dependent contractile events.

Cobalt ion concentrations of 0.5 to 10 p.p.m. did not inhibit neutrophil chemotaxis under agarose to 10^{-6} M FMLP (Figs 7 and 8). Chemotaxis for the majority of neutrophils was completely inhibited by those concentrations of cobalt ions (20 and 30 p.p.m.)

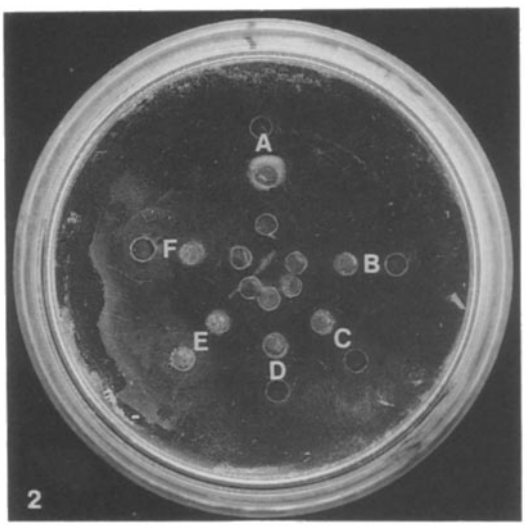

Figure 4 Neutrophil chemotaxis to chromium ions, (A) 0 (+ve control), (B) 0 (−ve control), (C) 5, (D) 2.5, (E) 0.5 and (F) 0.25 p.p.m. (× 1.4).

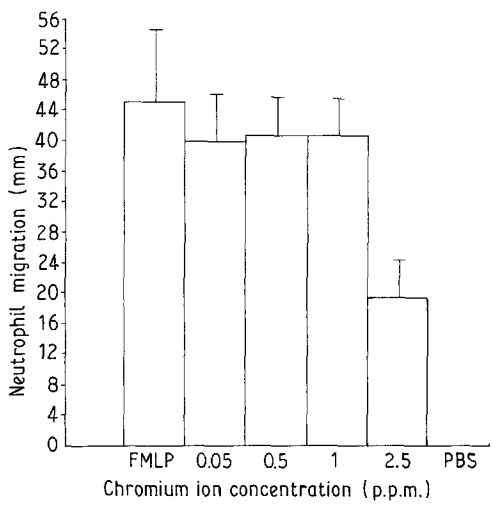

Figure 5 Mean ± e.s.e. of six replicate plates to examine inhibition of chemotaxis by chromium ions.

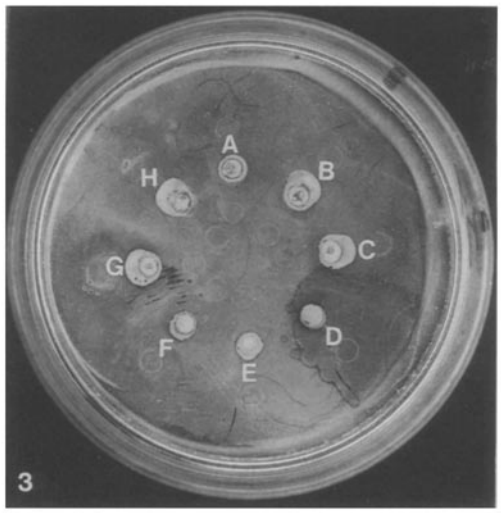

Figure 6 Neutrophil chemotaxis to 10^{-6} M FMLP after neutrophils had been incubated at 37° C for 30 min with chromium ions at (A) 0 (− ve control), (B) 0.05, (C) 0 (+ ve control), (D) 10, (E) 5, (F) 2.5 (G) 0.5 and (H) 0.25 p.p.m. (× 1.4).

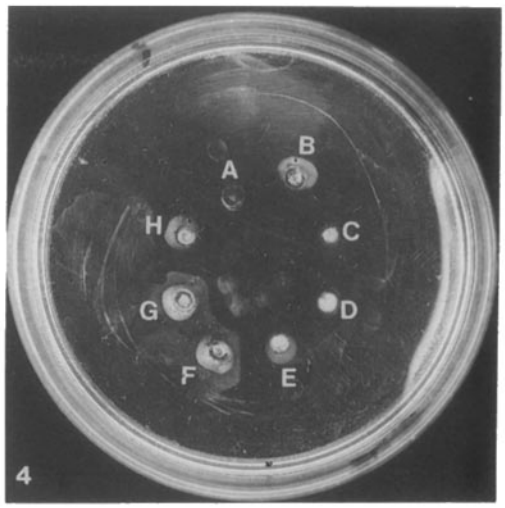

Figure 8 Neutrophil chemotaxis to 10^{-6} M FMLP after neutrophils had been incubated at 37° C for 30 min with cobalt particles at (A) 0 (− ve control), (B) 0 (+ ve control), (C) 30, (D) 20, (E) 10, (F) 5, (G) 2.5 and (H) 0.5 p.p.m. (× 1.4).

where a precipitate was visible. These cobalt ion concentrations also caused neutrophil aggregation. Current investigations indicate that cobalt, phosphate precipitate is responsible for neutrophil aggregation and inhibition of chemotaxis under agarose. Neutrophil aggregation may itself be responsible for the inhibition of chemotaxis or may be a coincident secondary event due to the toxic effect that the precipitate has on the neutrophil. Indeed, Haston and Shields [19] have previously remarked on neutrophil aggregation being a result of the toxic effect of substances on neutrophils.

Chromium ion concentrations of 0.05 to 1 p.p.m. did not inhibit neutrophil chemotaxis under agarose to 10^{-6} M FMLP. Chemotaxis was inhibited by 56% at 2.5 p.p.m. (Figs 5 and 6). Neutrophil aggregation was noted at > 1 p.p.m. chromium. The 20 p.p.m. chromium ion stock solution was noted to be cloudy, indicating the presence of a precipitate. As for cobalt, the chromium ion precipitate may be responsible for the observed neutrophil aggregation and inhibition of chemotaxis.

For all concentrations of cobalt and chromium ions, in the presence of 10^{-8} M FMLP > 90% of neutrophils polarized (Table IV). At high concentrations of

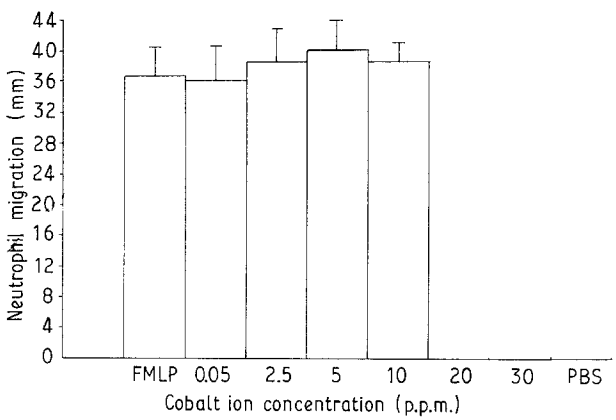

Figure 7 Mean ± e.s.e. of six replicate plates to examine inhibition of chemotaxis by cobalt ions.

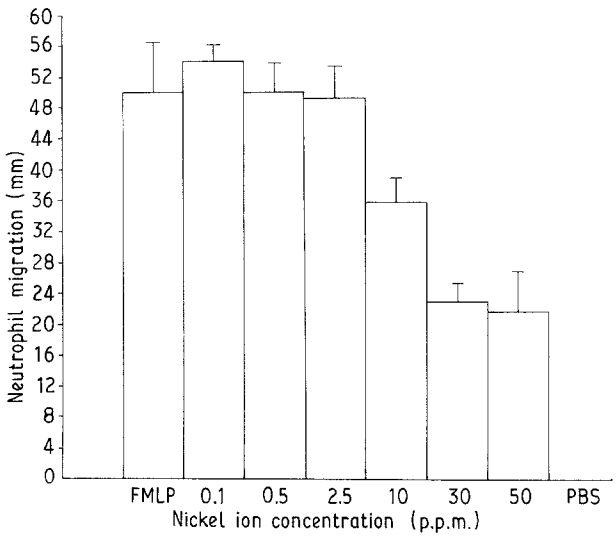

Figure 9 Mean ± e.s.e. of six replicate plates to examine inhibition of chemotaxis by nickel ions.

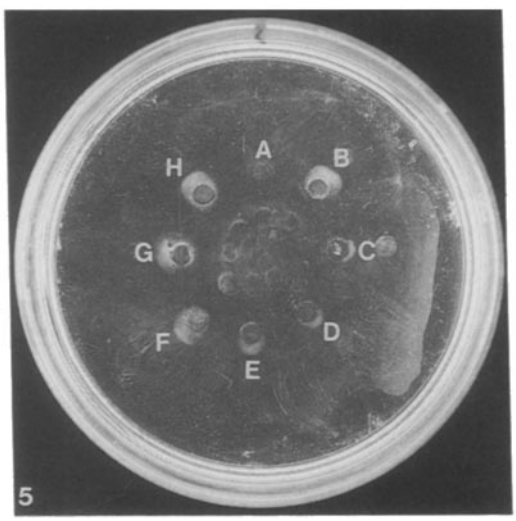

Figure 10 Neutrophil chemotaxis to 10^{-6} M FMLP after neutrophils had been incubated at 37° C for 30 min with nickel ions at (A) 0 (− ve control), (B) 0 (+ ve control), (C) 50, (D) 30, (E) 10, (F) 2.5, (G) 0.5 and (H) 0.1 p.p.m. (× 1.4).

metal ions where neutrophil aggregation occurs, those neutrophils that were viable polarized. Thus, inhibition of chemotaxis was not due to an inhibition of neutrophil polarization.

5. Conclusions

Chromium ion concentrations (0.05 to 10 p.p.m.), nickel ion concentrations (0.1 to 50 p.p.m.) and cobalt ion concentrations (0.5 to 10 p.p.m.) are not chemotactic for human peripheral blood neutrophils under these experimental conditions.

High concentrations of chromium ions ($\geqslant$ 2.5 p.p.m.) and cobalt ions ($\geqslant$ 30 p.p.m.) in the presence of a precipitate inhibited chemotaxis to the bacterial chemotactic peptide FMLP.

For nickel ion concentrations between 2.5 and 50 p.p.m., chemotaxis is inhibited. This may be due to nickel ions inhibiting calcium ion-dependent contractile events.

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