Identification by cell electrophoresis of a subpopulation of polymorphonuclear cells which is increased in patients with rheumatoid arthritis and certain other rheumatological disorders

K A BROWN,¹ J D PERRY,² C BLACK,³ AND D C DUMONDE¹

From the ¹Department of Immunology, United Medical and Dental Schools, St Thomas's Campus, London; the ²Department of Rheumatology, The London Hospital; and the ³Department of Rheumatology, West Middlesex University Hospital

SUMMARY Two and occasionally three electrophoretic subpopulations of polymorphonuclear cells (PMNs) were identified in the blood of normal healthy subjects and patients with rheumatoid arthritis (RA). Most of the PMNs from both groups of subjects were found in the population with the highest surface charge; the remainder being in the other distributions, which were collectively termed the slow cell population. There was a significant increase in the percentage of rheumatoid PMNs (mean 42%) ascribed to the slow population when compared with PMNs from normal subjects (mean 17%). A similar increase in the slow cell population was also seen in patients with Felty's syndrome (mean 38%) and scleroderma (mean 51%), but not in patients with Behçet's syndrome (mean 18%). Synovial fluid aspirated from the knee joints of patients with RA contained PMNs with the lowest surface charge. With nylon fibre as an adherence substrate cells of a low surface charge were found to be more adherent than those of a high surface charge. An alteration in the electrophoretic distribution of PMNs may represent changes that are related to the expression of functionally related membrane ionogenic groups.

The polymorphonuclear cell (PMN) is the major infiltrating leucocyte in the synovial fluid in inflammatory joints of patients with rheumatoid arthritis (RA). Within this environment the extracellular release of lysosomal enzymes¹ and oxygen radicals² from PMNs may contribute to changes that underlie the pathogenesis of joint damage.

Because the half life of the synovial fluid PMN in RA is approximately four hours and the daily turnover of PMNs in a rheumatoid knee joint has been estimated at over one million cells,³ there must be a continued extravasation of PMNs from the circulation into the joint in order to maintain the high local concentration of these cells. The question arises of whether such PMNs represent a selected subpopulation of cells whose functional characteristics favour diapedesis into the rheumatoid joint.

Cell electrophoresis permits the indirect measure-

ment of surface ionogenic groups, whose expression may reflect the status of a cell's functional activity. The maturation stages of the human PMNs in the bone marrow are associated with a decrease in electrophoretic mobility (EPM),⁴ whereas treatment of macrophages with agents that reduce the surface charge results in an enhancement of phagocytosis.^{5,6}

Earlier studies had shown that the surface charge of PMNs from a small number of patients with RA was lower than that of PMNs from normal subjects⁷ and that cells of a high surface charge were less adherent than those with a low surface charge.⁸ It was the aim of the present study to confirm and extend those findings by analysing PMNs from a larger number of patients not only with RA but also with other selected rheumatological disorders. Furthermore, investigations were undertaken to assess the adherent properties of the electrophoretic subpopulations of rheumatoid PMNs, and to compare the surface charge of synovial fluid PMNs with that of blood PMNs from patients with RA.

Accepted for publication 28 October 1987.

Correspondence to Dr K A Brown, Department of Immunology, The Rayne Institute, St Thomas's Hospital, London SE1 7EH.

Patients and methods

PATIENTS AND CONTROLS

Blood was collected from 53 patients with definite or classical RA⁹ and seven patients with Felty's syndrome, all of whom had neutropenia (mean $1.41 \times 10^9/l$). In addition, blood samples were obtained from 12 patients who satisfied the American Rheumatism Association criteria for scleroderma¹⁰ and 11 patients with Behçet's syndrome who fulfilled the criteria of Mason and Barnes.¹¹ The control panel consisted of 69 blood samples taken from 46 healthy hospital personnel. Eleven of the normal subjects donated blood on two to five occasions during the study.

SEPARATION OF PMNS

The method was a modification of that of Dioguardi *et al.*¹² Twenty millilitres of heparinised blood (10 U/ml) was diluted with 140 ml of 0.83% ammonium chloride and allowed to stand for 10 minutes at room temperature. After centrifuging at 450 g for 10 minutes the supernatant was discarded and the NH_4Cl lysis stage repeated. The PMN pellet from the second lysis was washed three times by slow centrifugation (55 g for 10 minutes) in calcium and magnesium free Hank's balanced salt solution. This slow centrifugation separated the large dense PMNs from the smaller less dense lymphocytes, which remained in suspension. The absence of divalent cations in the medium prevented any spontaneous aggregation of the PMNs and greatly increased the yield of cells. The final cell pellet was adjusted to 1×10^6 cells/ml. Cell viability as shown by exclusion of the trypan blue was always >96%, and the purity of PMNs was approximately 90%. Once separated, the PMNs were subjected to electrophoretic analysis.

For the isolation of PMNs from the synovial fluid of patients with RA the fluid aspirated from knee joints in the course of treatment was incubated with 75 U/ml lyalase for 30 minutes at 37°C before dilution with ammonium chloride.

CELL ELECTROPHORESIS

A detailed description of the apparatus used for electrophoretic analysis and its application in the measurement of surface charge has been given elsewhere.¹³ PMNs were suspended at 1×10^6 cells/ml in Medium 199 containing 20 nmol/l HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; pH 7.2), and electrophoretic measurements were made on 1 ml samples in a cylindrical electrophoretic assembly (manufactured by Rank Bros, Bottisham, Cambridge). Both electrode and capillary chambers were kept at a constant 24°C and

contained Medium 199, pH 7.2. A polarising voltage of 60 V produced a 4 mA current through the capillary, resulting in a field strength of 8.0 V.cm^{-1} .

For each determination 100 PMNs were scored and the EPM, expressed as $\mu\text{m.cm.s}^{-1}.\text{V}^{-1}$, calculated from the following equation:

$$\text{EPM} (\mu\text{m.cm.s}^{-1}.\text{V}^{-1}) = \frac{\text{distance migrated (25 } \mu\text{m)}}{\text{time(s)/field strength (V.cm}^{-1}\text{)}}$$

If the distance migrated by the cells and the field strength applied are known the only variable needed to calculate the EPM is the migration time of the cells, to an accuracy of $1/40$ s, over this fixed distance. By reversing the polarity each cell was timed over the same distance but in the opposite direction and mean values accepted from pairs of timings with less than 5% variation. Results were subjected to probit analysis to test for departures from normal distribution.¹⁴ Subpopulations of cells with distinct differences in surface charge were distinguished from plots of probits against EPM values. Deviations from linearity implied the presence of more than one population of cells.

Fig. 1 Electrophoretic mobility distributions and probit analyses of PMNs from (a) a typical normal healthy subject and (b) a patient with RA.

ADHERENCE ASSAY

Nylon fibre which had been washed on three separate occasions with 0.1 M HCl, distilled H₂O and Medium 199 containing 10% fetal calf serum was gently packed into the barrel of a 10 ml plastic syringe to a depth of one inch from the narrow orifice. Ten million PMNs suspended in medium 199 with 10% fetal calf serum were added to the column (narrow orifice being closed) and incubated for 30 minutes at 37°C. The non-adherent PMNs were eluted by several washes with medium 199.

STATISTICAL METHODS

Student's *t* test was used to determine the significance of differences between the means of two groups.

Results

Fig. 1 shows a typical EPM histogram of PMNs isolated from the blood of a normal subject and a patient with RA. Probit analysis of the different speeds of migrating PMNs identified two and occasionally three distinct electrophoretic subpopulations. Those PMNs with a high surface charge (i.e., cells contained in the histogram blocks nearest

the vertical axis) are referred to as population I (EPM >0.90 $\mu\text{m.cm.s}^{-1}.\text{V}^{-1}$) and the others, in decreasing order of surface charge, as population II (EPM 0.80–0.89 $\mu\text{m.cm.s}^{-1}.\text{V}^{-1}$), and population III (EPM <0.80 $\mu\text{m.cm.s}^{-1}.\text{V}^{-1}$). For normal subjects the EPM and percentage distribution of cells within these subpopulations showed only minor fluctuations as demonstrated by repeated tests on six volunteers over a 20 month period, two of which are shown in Table 1. Table 2 shows the results of

Table 1 Longitudinal study of the electrophoretic distribution of blood PMNs from two normal healthy subjects

Subject	Months	EPM ($\mu\text{m.cm.s}^{-1}.\text{V}^{-1}$)		
		Population I	Population II	Population III
A	1	0.91 (90)*	0.85 (8)	0.78 (2)
	2	0.92 (90)	0.84 (10)	
	8	0.90 (85)	0.81 (12)	0.80 (3)
	20	0.91 (84)	0.83 (11)	0.80 (5)
B	1	0.91 (85)	0.82 (7)	0.79 (8)
	11	0.90 (85)	0.85 (9)	0.80 (6)
	17	0.91 (84)	0.86 (11)	0.80 (5)

*Numbers in brackets denote percentage of cells ascribed to that population.

Fig. 2 Comparison of the distribution of the slow population of PMNs from normal subjects with that for patients with RA, Felty's syndrome, scleroderma, and Behçet's syndrome.

Table 2 Distribution of electrophoretic subpopulations of PMNs in the blood of normal healthy subjects and patients with RA

Subjects	Electrophoretic subpopulations					
	I		II		III	
	EPM*	%	EPM	%	EPM	%
Normals (n=69)	0.91 (0.01)	83 (10)	0.84 (0.02)	13 (17)	0.77 (0.03)	4 (5)
RA (n=53)	0.91 (0.01)	58 (19)	0.83 (0.02)	31 (15)	0.76 (0.03)	11 (10)

*EPM values are expressed as $\mu\text{m.cm.s}^{-1}.\text{V}^{-1}$ (standard deviation).

measuring surface charge distribution on PMNs isolated from the blood of 53 rheumatoid patients and 69 normal samples. There was a significant decrease in the representation of population I ('fast' cells) in the RA group (prevalence 58%; $p<0.001$). Correspondingly, the representation of populations II and III ('slow' cells) was increased in patients with RA (prevalence 42%; $p<0.001$). Although the EPM values of individual subpopulations in RA corresponded with those of normal subjects, two thirds (35/53) of rheumatoid samples contained a percentage of 'slow' cells (i.e., with EPM <0.90) higher than two standard deviations above the normal mean (i.e. 30%), while this was seen only in 10/69 normal samples.

To ascertain if the increase in the slow population was a general consequence of inflammation rather than an observation peculiar to RA, electrophoretic determinations were performed on PMNs isolated from the blood of patients with other rheumatological disorders. Fig. 2 shows that the slow population was increased in the blood of patients with RA

(mean 42%; $p<0.001$), Felty's syndrome (mean 38%; $p<0.001$), and scleroderma (mean 51%; $p<0.001$) but not in patients with Behçet's syndrome (mean 18%) when compared with normal subjects (mean 17%).

Electrophoretic determinations were also performed on synovial fluid PMNs isolated from patients with RA. Of the 13 samples examined, only two presented an EPM profile similar to that seen with blood PMNs from normal healthy subjects (Table 3). Interestingly, both of these aspirates were obtained from the left and right knee joints of a patient with RA during one visit to the clinic. Overall, the percentage of synovial fluid PMNs ascribed to population I was significantly less (mean 31%; $p<0.001$) than that of blood PMNs obtained from any of the patient groups. Many of the synovial fluid PMNs often possessed the lowest EPM recorded in this study.

When blood PMNs from patients with RA were applied to nylon fibre columns and subsequently eluted it was the cells of a low surface charge that

Table 3 Electrophoretic analysis of synovial fluid PMNs obtained from knee aspirates of 12 patients with RA

Patient No	Percentage Distribution of PMNs for the following EPMs [*] :				
	0.91-0.93	0.80-0.89	0.70-0.79	0.60-0.69	0.50-0.59
1 (L) [†]	82	16	2		
(R)	77	20	3		
2	39	53	8		
3	18	20	62		
4	24	0	25	31	20
5	20	0	73	7	
6	8	0	80	12	
7	38	0	62		
8	13	43	27	19	
9	30	53	17		
10	17	0	30	53	
11	14	0	39	47	
12	22	0	78		
Mean (SD)	31 (23)	16 (21)	39 (28)	13 (19)	

*EPM units are $\mu\text{m.cm.s}^{-1}\text{V}^{-1}$.

[†]For patient No 1 PMNs were obtained from the left (L) and right (R) knee joints.

Table 4 Electrophoretic profile of PMNs from four patients with RA before and after passage through nylon fibre columns

Patient No	1		2		3		4	
Electrophoretic population	I	II+III	I	II+III	I	II+III	I	II+III
Percentage PMNs								
Before column	51	49	44	46	69	31	73	27
After column	92	8	88	12	97	3	87	13

were preferentially retained (Table 4). These results suggest that PMNs of a low EPM are more adherent than cells of a higher surface charge.

Discussion

This study has shown that blood PMNs of normal healthy subjects and patients with rheumatological disorders often consist of at least two electrophoretic subpopulations with most of the cells residing in the population of a high surface charge. For normal healthy subjects the EPM values and percentage distribution of cells ascribed to these subpopulations showed little fluctuation over several months. Although the EPMs of the subpopulations in RA were identical to those shown from normal subjects, they often contained significantly more cells of a low surface charge, which were selectively retained on nylon fibre columns. This increase in the slow population was not confined to RA as shown by the similar finding with PMNs from patients with Felty's syndrome and scleroderma but not with PMNs from patients with Behçet's syndrome.

From previous demonstrations that a decrease in cell surface charge is associated with an increase in adherence⁵⁻⁷ the present data could be interpreted as suggesting that the adhesiveness of RA PMNs is greater than that of control PMNs. We recently showed, however, that there was no difference between the adherence properties of RA and control PMNs to cultured porcine endothelium.¹⁵ The apparent anomaly of these observations is probably related to a difference in experimental conditions. In the adherence studies most experiments were undertaken with serum present in the culture media, whereas all electrophoretic determinations were undertaken in the absence of serum. When the adherence assay was performed without serum the small number of blood PMNs attaching to endothelium made it difficult to evaluate any significant changes between the normal and RA groups. Interestingly, the adherence of RA synovial fluid PMNs to endothelium in the absence of serum was greater than that of matched blood PMNs, and in the present study many of the synovial fluid PMNs possessed the lowest recorded surface charge.

Scanning electron microscopy of blood PMNs from patients with RA has shown morphological characteristics distinct from those of normal PMNs,¹⁶ and such changes may reflect an alteration in the expression of membrane glycoproteins, whose ionogenic groups determine the electronegative nature of the cell surface. The very low EPM of the synovial fluid PMNs may represent a loss of membrane associated, *N*-acetylneuraminic acid containing glycoproteins, whose decreased expression relates to the impaired chemotaxis and phagocytosis

of synovial fluid PMNs.^{17,18} Although previous reports have shown that a decrease in surface charge increases the phagocytic activity of macrophages,^{5,6} there may not be a single direct relation between a lowering of the EPM and an enhancement of cell function. It is conceivable that an augmentation or impairment of the functional activity of phagocytic cells depends upon the extent of PMN charge reduction. Experiments are in progress to examine this possibility.

Blood PMNs are known to consist of a circulating and marginating pool of approximately equal distribution,¹⁹ the circulating PMNs being located in the axial stream, and marginating PMNs in contact with vascular endothelium. The slow population identified in this study may contain a proportion of marginating PMNs which are not firmly bound to the vascular wall. Hence an increase of the slow population could represent an increase in the marginating : circulating PMN ratios. The leucopenia associated with Felty's syndrome is believed to be a result of an increase in the number of marginating cells,^{20,21} though as far as we are aware there have been no reports of a disturbance between the circulating and marginating PMN pools in patients with RA and scleroderma.

That the slow population was increased in PMNs from patients with scleroderma was unexpected in that, as far as we are aware, these cells have not been accorded an active role in the pathology of this disease. Serum samples from patients with scleroderma are cytotoxic for cultured vascular endothelium,^{22,23} and as a protease dependent mechanism has been described²⁴ it is plausible that some of the agents inducing cytotoxic activity, which may include the reactive oxygen metabolites, originate from PMNs. Support for this view stems from the recent demonstration that the chemiluminescence of PMNs from patients with scleroderma is greater than that of controls.²⁵ Thus in scleroderma an increase in the slow population may be associated with a particular stage of activation that is not seen in patients with Behçet's syndrome, where the PMNs display an in vitro chemotactic hyperresponsiveness^{26,27} but an impaired migration through skin abrasions into chambers in vivo.²⁷

A decrease in surface charge may result from the acquisition of more electropositively charged groups on the membrane, the loss of ionogenic groups containing *N*-acetylneuraminic acid, or their masking by circulating soluble factors. The last possibility was investigated in the accompanying paper.²⁸

References

- 1 Weissman G. Activation of neutrophils and the lesions of rheumatoid arthritis. *J Lab Clin Med* 1982; **100**: 322-33.

- 2 Burkhardt H, Schwingel M, Menninger H, McCartney M W, Tscheche H. Oxygen radicals as effectors of cartilage destruction. Direct degradative effect on matrix components and indirect action via activation of latent collagenase from polymorphonuclear leukocytes. *Arthritis Rheum* 1986; **29**: 379-87.
- 3 Hollingsworth J W, Siegel E R, Creasey W A. Granulocyte survival in synovial exudate of patients with rheumatoid arthritis and other inflammatory joint disease. *Yale J Biol Med* 1967; **39**: 289-96.
- 4 Lichtman M A, Weed R I. Alteration of the cell periphery during granulocyte maturation: relationship to cell function. *Blood* 1972; **39**: 301-16.
- 5 Weiss L, Mayhew E, Ulrich K. The effect of neuraminidase on the phagocyte process in human monocytes. *Lab Invest* 1966; **15**: 1304-9.
- 6 Nagura H, Asai J, Katsumata Y, Kajima K. Role of electric surface charge of cell membrane in phagocytosis. *Acta Pathol Jpn* 1973; **23**: 279-90.
- 7 Brown K A, Collins A J, Holborow E J. Cell electrophoretic analysis of lymphocytes and polymorphonuclear cells from patients with rheumatoid arthritis. *Lancet* 1977; **i**: 114-7.
- 8 Howe G B, Fordham J N, Brown K A, Currey H L F. Polymorphonuclear cell function in rheumatoid arthritis and in Felty's syndrome. *Ann Rheum Dis* 1981; **40**: 370-5.
- 9 Ropes M W, Bennett G A, Cobb S, Jacox R, Jessar R A. Revision of diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 1958; **9**: 175-6.
- 10 Subcommittee for Scleroderma Criteria of the American Rheumatism Association. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; **23**: 581-90.
- 11 Mason R M, Barnes C G. Behçet's syndrome with arthritis. *Ann Rheum Dis* 1969; **28**: 95-103.
- 12 Dioguardi N, Agostini A, Fiorella G, Lomanto B. Characterisation of lactic dehydrogenase of normal human granulocytes. *J Lab Clin Med* 1963; **61**: 713-23.
- 13 Preece A W, Light P A. The macrophage electrophoretic mobility (MEM) test for malignant disease. Further clinical investigations and studies on macrophage slowing factors. *Clin Exp Immunol* 1974; **18**: 543-52.
- 14 Brown K A, Embling P H F, Perry J D, Holborow E J. Electrophoretic behaviour of blood and synovial fluid lymphocytes in rheumatoid arthritis. *Clin Exp Immunol* 1979; **36**: 272-8.
- 15 Sheehan N J, Brown K A, Perry J D, Chasty R C, Yates D A H, Dumonde D C. Adherence of rheumatoid polymorphonuclear cells (PMNs) to cultured endothelial cell monolayers. *Ann Rheum Dis* 1987; **46**: 93-7.
- 16 McCarthy D A, Holburn C M, Pell B K, Moore S R, Kirk A P, Perry J D. Scanning electron microscopy of rheumatoid arthritis peripheral blood polymorphonuclear leucocytes. *Ann Rheum Dis* 1986; **45**: 899-910.
- 17 Turner R A, Schumacher H R, Myers A R. Phagocytic function of polymorphonuclear leucocytes in rheumatic disease. *J Clin Invest* 1973; **52**: 1632-5.
- 18 Kemp S A, Brown S, Brooks P M, Neoh S H. Migration of blood and synovial fluid neutrophils obtained from patients with rheumatoid arthritis. *Clin Exp Immunol* 1980; **39**: 240-6.
- 19 Athens J W, Raab S O, Haab O P, et al. Leukokinetic studies. III. The distribution of granulocytes in the blood of normal subjects. *J Clin Invest* 1961; **40**: 159-64.
- 20 Vincent P C, Levi J A, Macqueen A. The mechanism of neutropenia in Felty's syndrome. *Br J Haematol* 1974; **27**: 463-75.
- 21 Joyce R A, Boggs D R, Chervenick P A, Lalezavi P. Neutrophil kinetics in Felty's syndrome. *Am J Med* 1980; **69**: 695-702.
- 22 Cohen S, Johnson A R, Hurd E. Cytotoxicity of sera from patients with scleroderma. *Arthritis Rheum* 1983; **26**: 170-8.
- 23 Shanahan Jr W A, Korn J H. Cytotoxic activity of sera from scleroderma and other connective tissue diseases. *Arthritis Rheum* 1982; **25**: 1391-5.
- 24 Kahalch M B, Leroy E C. Endothelial cell injury in scleroderma. A protease mechanism. *J Lab Clin Med* 1983; **101**: 553-60.
- 25 Kovacs I B, Thomas R H M, Mackay A R, Rustin M H A, Kirby J D T. Increased chemiluminescence of polymorphonuclear leucocytes from patients with progressive systemic sclerosis. *Clin Sci* 1986; **70**: 257-61.
- 26 Matsumura N, Mizushima Y. Leucocyte movement and colchicine treatment in Behçet's disease. *Lancet* 1975; **ii**: 813.
- 27 James R W, Walker J R, Smith M J H. Abnormal polymorphonuclear leucocyte chemotaxis in Behçet's syndrome. *Ann Rheum Dis* 1979; **38**: 219-21.
- 28 Brown K A, McCarthy D, Perry J D, Dumonde D C. Reduction of the surface charge of blood polymorphonuclear cells by rheumatoid sera and heat induced aggregated human IgG (HAGG). *Ann Rheum Dis* 1988; **47**: 000-0.