Biochimica et Biophysica Acta 904 (1987) 330–336
Elsevier

BBA 73767

The effect of sodium periodate treatment on the modulation of the sodium pump in low-potassium type (LK) sheep red cells by the L antigen

Janet M. Stein, J. Clive Ellory and Elizabeth M. Tucker

AFRC Institute of Animal Physiology and Genetics Research, Babraham, Physiological Laboratory, Cambridge, and University Laboratory of Physiology, Oxford (U.K.)

(Received 21 May 1987)

Key words: Sodium pump; L antigen; Antigen, anti-L; Sodium periodate; Neuraminidase; (Sheep red cell)

1. The action of sodium periodate and neuraminidase on active and passive K^+ transport in low-potassium type (LK) sheep red cells was investigated in relation to the contribution of the L_p and L_l antigens. 2. Active K^+ transport in LK sheep red cells was not affected by treatment with sodium periodate (2 mM), or with neuraminidase. 3. Passive K^+ transport in LK sheep red cells was increased by sodium periodate treatment in a concentration-dependent manner. The increase was not Cl^- dependent, and so differed from the increased passive K^+ uptake resulting from *N*-ethylmaleimide treatment. 4. HK sheep red cells treated with sodium periodate showed small increases in passive K^+ uptake, and *N*-ethylmaleimide treatment used sequentially with sodium periodate resulted in further small increases in passive K^+ uptake. 5. In LK sheep red cells the stimulation of active K^+ transport by anti-L was impaired by 50% in cells treated with sodium periodate (2 mM) and was slightly lowered in cells treated with neuraminidase. 6. In LK sheep red cells inhibition of passive K^+ transport by anti-L was not impaired by sodium periodate treatment (2 mM), or by neuraminidase treatment.

Introduction

LK (low-potassium type) sheep red cells possess the blood group antigen L which has a major role in K^+ transport regulation [1]. There is evidence that the L antigen has two specificities, L_p which is concerned with inhibition of the sodium pump, and L_l which affects ouabain-insensitive (passive) K^+ uptake; L_l is also identified by its role in complement mediated lysis. Anti-L serum, prepared by immunizing HK (high-potassium type) sheep with red cells of LK sheep can combine with both L_p and L_l antigens with the effects of stimulating active and inhibiting passive K^+ transport, respectively [2]. Although the L antigen is known to have an effect on membrane transport, little is known of its biochemical composition [3].

Several human blood group antigens contain a polysaccharide component, with characteristic sugar residues and linkages which have been investigated using enzymic digestion and chemical modification [4,5]. Interestingly, sodium periodate selectively inactivates certain blood group receptors in human red cells [6].

The present work shows that neuraminidase treatment of LK sheep red cells resulted in a small loss of L_p antigen function; L_l antigen function was unaffected. Sodium periodate treatment impaired the function of the L_p antigen but not of the L_l antigen. Sodium periodate treatment also caused increased passive K^+ uptake and this was not related to L-antigen/antibody binding.

Correspondence (present address): J.M. Stein, Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

Materials and Methods

Materials

Sodium *m*-periodate and *N*-ethylmaleimide were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Neuraminidase was from Cambridge Bioscience, Cambridge. ^{86}Rb chloride was from Amersham International, Bucks., U.K.

All other chemicals were Analar grade or the purest available.

Methods

Heparinised blood from homozygous LK and HK sheep was used [7]. Red cells were washed 3 times with buffered saline composed of 145 mM NaCl/15 mM 4-morpholinepropane sulphonic acid (Mops)/5 mM glucose (pH 7.4), and the buffy coat removed.

Anion replacement Washed red cells were divided into two portions and resuspended in either Cl^--containing buffer (pH 7.4) (see above), or NO_3^--containing buffer composed of 145 mM $NaNO_3$/15 mM Mops/5 mM glucose (pH 7.4). After three washes in the appropriate buffer the red cells were resuspended at 10% haematocrit in the same buffer for 30 min at 37 °C, washed once and incubated in fresh buffer for a further 30 min at 37 °C. The cells were then washed three times with either Cl^-- or NO_3^--containing buffer before use [8].

K^+ influx. Washed red cells were resuspended to 5% haematocrit in either Cl^--containing or NO_3^--containing buffered saline (pH 7.4) (see above), containing KCl (5 mM) or KNO_3 (5 mM) as appropriate. K^+ influx was measured using rubidium as a tracer for potassium, and the red cells were incubated for 30 min at 37 °C in the presence and absence of ouabain (0.2 mM). The red cells were washed according to Dunham and Ellory [8], and ouabain-sensitive (active) and ouabain-insensitive (passive) K^+ influx calculated in mmol/l red cells per h.

Neuraminidase treatment. Washed red cells were suspended at 30% haematocrit in buffered saline (see above) which also contained neuraminidase (42 mU/ml) $CaCl_2$ (8 mM) and bovine serum albumin (0.5%), incubated at 37 °C for either 1 or 3 h as indicated in Table I, and then washed twice in buffered saline before use.

Microelectrophoresis. The electrophoretic mobility of red cells was measured as described in Bangham et al. [9].

Sodium periodate treatment Since sodium periodate attacks vicinal hydroxyl groups, glucose was omitted from the modified buffers used. These were either 145 mM NaCl/15 mM Mops, (pH 7.0), or 145 mM $NaNO_3$/15 mM Mops (pH 7.0) Washed red cells were resuspended in the appropriate modified buffer (pH 7.0), washed twice then resuspended in the same buffer containing sodium periodate (2–5 mM as specified in text) for 20 min at 18 °C. The incubation was stopped by the addition of 3% (w/v) trisodium citrate and the red cells washed three times in normal glucose-containing saline (pH 7.4) as described above

N-Ethylmaleimide treatment Washed red cells were suspended at 10% haematocrit for 15 min at 37 °C in glucose-containing buffered saline (pH 7.4) with *N*-ethylmaleimide (2 mM) obtained by diluting a stock solution of *N*-ethylmaleimide in dimethylsulphoxide (DMSO). The red cells were then washed three times in fresh glucose-containing buffered saline (pH 7.4). Control incubations with DMSO alone showed no significant effect on K^+ uptake.

Preparation of anti-L serum An HK sheep was immunised with LK sheep red cells, and anti-serum was prepared [7].

Absorptions. Washed packed red cells were mixed with an equal volume of antiserum, left for 30 min at room temperature, centrifuged, the absorbed serum was removed, added to fresh packed red cells, and the absorption repeated. Three absorptions were performed.

Results

Neuraminidase treatment. Effect on L antigen

LK sheep red cells treated with neuraminidase and then sensitised with anti-L showed a small but consistent fall in anti-L-stimulated active K^+ uptake compared with untreated red cells (Table I). In four out of six separate experiments this effect of neuraminidase treatment was significant (at least $P < 0.05$ using a Student's *t*-test of the interaction for each experiment, indicated by * in Table I, last column). An overall comparison utilising the individual mean differences for the

TABLE I

EFFECT OF NEURAMINIDASE TREATMENT OF LK SHEEP RED CELLS ON ANTI-L-STIMULATED TRANSPORT

LK sheep red cells were incubated with neuraminidase (see Methods), washed in buffered saline (pH 7 4), and resuspended to 5% hematocrit Sensitization with anti-L red cells (0 5 ml) were incubated with anti-L (0 1 ml) for 30 min at 37 °C, centrifuged and the supernatants were discarded Buffered saline, (pH 7 4) (1 ml), containing KCl (5 mM), ^{86}Rb, with or without ouabain (0 2 mM) was added, and K^+ uptake determined as in Methods Values are mean ± S E of three separate incubations. * $P < 0.05$

Expt No	Neuraminidase treatment (h)	Active K^+ uptake (mmol/l cells per h)					
		untreated			neuraminidase-treated		
		control	anti-L	increase	control	anti-L	increase
1	3	0 298 ± 0.007	1 009 ± 0 009	0 711 ± 0 011	0 282 ± 0 002	0 89 ± 0 005	0 608 ± 0 005 *
2	3	0 251 ± 0 01	0 998 ± 0 012	0 747 ± 0 016	0 267 ± 0.005	0 903 ± 0 003	0 636 ± 0 006 *
3	1	0 089 ± 0 018	0 634 ± 0 003	0 545 ± 0 018	0 093 ± 0.006	0 551 ± 0 005	0 458 ± 0 008 *
4	1	0 149 ± 0 005	0 701 ± 0 016	0 552 ± 0 017	0 146 ± 0.015	0 678 ± 0 03	0 532 ± 0 033
5	1	0 114 ± 0 005	0 634 ± 0 039	0 52 ± 0 039	0 114 ± 0 007	0 565 ± 0 007	0 451 ± 0 01
6	1	0 106 ± 0 018	0 68 ± 0 006	0 574 ± 0 019	0.112 ± 0 004	0 535 ± 0 006	0 423 ± 0 007 *

six experiments (irrespective of the time of incubation with neuraminidase) by Wilcoxon's signed rank test was also significant ($P < 0.05$). In the same experiments the anti-L inhibition of passive K^+ uptake was not significantly altered by neuraminidase treatment. Both active and passive K^+ uptake under control (i.e., no anti-L) conditions were unchanged in red cells treated with neuraminidase compared with untreated red cells.

The efficacy of neuraminidase treatment in these experiments was confirmed by electrophoretic mobility measurements of the red cells. The zeta potential of LK sheep red cells (untreated) in three separate experiments was 14 17 ± 0.05 mV (mean ± S.E.). Digestion with neuraminidase for 1 h resulted in a reduction of the surface charge to 5.94 ± 0.47 mV (i.e., 41% of the control value), and digestion for 3 h gave a value of 4.44 (31% of control value).

Sodium periodate treatment of red cells

(a) Effect on L antigen. In a preliminary experiment LK sheep red cells were treated with varied concentrations of sodium periodate (1–5 mM),

TABLE II

EFFECT OF SODIUM PERIODATE TREATMENT OF LK SHEEP RED CELLS ON ANTI-L-STIMULATED ACTIVE AND PASSIVE K^+ UPTAKE

LK sheep red cells were treated with sodium periodate (2 mM) as described in Methods Sensitisation with anti-L and K^+ uptake measurements were as in the Table I legend Values are mean ± S E of three separate incubations (a), active K^+ transport, (b), passive K^+ transport Fractional pump stimulation is defined as active K uptake in anti-L treated cells/active K uptake in control cells

(a) Active K^+ uptake (mmol/l cells per h)

untreated				sodium periodate-treated			
control	anti-L	increase	fractional stimulation	control	anti-L	increase	fractional stimulation
0 089 ± 0 018	0 634 ± 0 003	0 545 ± 0 018	7 1	0 084 ± 0 011	0 388 ± 0 033	0 304 ± 0 035	4 6

(b) Passive K^+ uptake (mmol/l cells per h)

untreated			sodium periodate-treated		
control	anti-L	decrease	control	anti-L	decrease
0 264 ± 0 003	0 097 ± 0.001	0 167 ± 0 004	0 941 ± 0 008	0 777 ± 0 02	0 164 ± 0 022

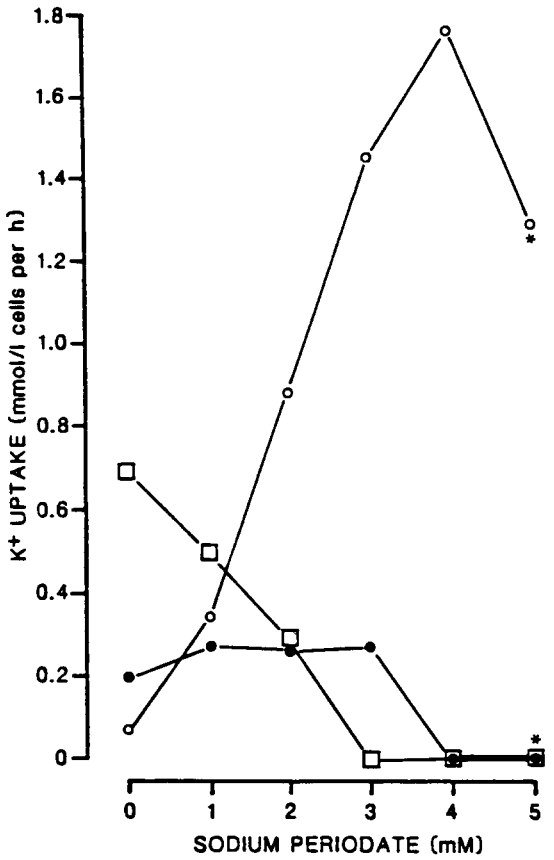

Fig. 1 Effect of sodium periodate treatment of LK sheep red cells on anti-L-stimulated K^+ uptake and passive K^+ uptake Red cells were treated with various concentrations of sodium periodate, washed three times (see Methods), sensitised with anti-L as in Table I legend, and K^+ uptake was measured Each point is the mean of duplicate incubations ●, active K^+ uptake, □, anti-L stimulated K^+ uptake, ○, passive K^+ uptake. * indicates some haemolysis

washed and then sensitized with anti-L (Fig. 1). Active K^+ uptake was unaffected in red cells treated with sodium periodate at concentrations 1–3 mM, and was abolished at 4 mM. In contrast, the stimulation of ouabain-sensitive (active) K^+ uptake by anti-L decreased progressively as the sodium periodate concentration increased, and was absent in red cells treated with 3 mM and 4 mM sodium periodate. Passive K^+ uptake increased as the sodium periodate concentration increased and some inhibition of passive K^+ uptake by anti-L could be detected in red cells treated with sodium periodate (1–5 mM). Red cells treated with 5 mM sodium periodate showed some haemolysis.

On the basis of the above tests we used sodium periodate (2 mM) to treat red cells in the experiments to be described; at this concentration the effect of anti-L on active K^+ uptake was reduced, control levels (i.e., no anti-L) of active K^+ uptake were unchanged, and there was no red cell haemolysis. A typical experiment in which LK sheep red cells were treated with sodium periodate and then sensitised with anti-L is summarized in Table IIa (active K^+ uptake) and IIb (passive K^+ uptake).

In periodate-treated red cells the effect of anti-L on active K^+ transport was reduced (Table IIa), giving a fractional stimulation value of 4.6 compared with 7.1 in untreated cells. From four separate experiments the anti-L-stimulated active K^+ uptake (mean ± S.E.) was 0.294 ± 0.015 mmol/l cells per h in periodate-treated cells compared with 0.624 ± 0.044 mmol/l cells per h in untreated cells, whereas control active K^+ uptake was unaffected (0.155 ± 0.041 mmol/l cells per h in periodate treated and 0.152 ± 0.023 mmol/l cells per h in untreated cells). This shows that periodate treatment has decreased the effect of anti-L on the L_p antigen directly, rather than acting nonspecifically on active K^+ transport. Inhibition of passive K^+ uptake by anti-L was apparently unimpaired in periodate treated LK sheep red cells (Table IIb). From pooled data of four separate experiments, inhibition of passive K^+ uptake by anti-L in sodium periodate-treated red cells (mean ± S.E.) was 0.164 ± 0.027 mmol/l cells per h compared with 0.107 ± 0.026 mmol/l cells per h in untreated cells. Clearly, the L_1 antigen as defined by its effect on passive K^+ uptake survived sodium periodate attack, but it is not evident whether the apparent increase is significant, since the anti-L_1 effect depends critically on cell volume [10]

It was not possible to use complement-mediated lysis to determine whether the L_1 antigen was destroyed by sodium periodate treatment, because sheep red cells treated in this way lysed spontaneously in the presence of complement. However, it was possible to test for L_1 indirectly by absorption tests. Anti-L was absorbed three times with (a) LK sheep red cells treated with sodium periodate, (b) untreated LK sheep red cells or, (c) HK sheep red cells treated with sodium periodate, and then tested serologically against normal LK sheep

red cells. Following absorption by the LK cells, both treated and untreated, no lytic anti-L activity remained, whereas the anti-L absorbed by the HK cells contained lytic activity. The same absorbed antisera were also tested on normal LK sheep red cells for stimulation of active K^+ uptake There was no stimulation from the sera absorbed by treated and untreated LK cells, whereas the sera absorbed by the HK cells still stimulated active transport. From this it could be concluded that at least some L_p and L_1 antigens had survived periodate treatment.

(b) Effect on passive uptake Both K^+ and Na^+ uptake are increased in LK red cells treated with sodium periodate, and show linear concentration dependence as a function of external cation concentration [11]. At an extracellular K^+ concentration of 5 mM, passive K^+ uptake in LK cells treated with sodium periodate (2 mM) was increased to 0.731 ± 0.08 mmol/l cells per h compared with $0.106 \pm 0\,008$ mmol/l cells per h in untreated cells (mean $\pm$ S.E.; data of six separate experiments). We used *N*-ethylmaleimide treatment, in combination with sodium periodate, to characterise these changes in K^+ uptake further.

LK sheep red cells with anions Cl^- or NO_3^- substituted (see Methods section) were treated with *N*-ethylmaleimide and sodium periodate, either separately or in sequence (Table III). Passive K^+ uptake in *N*-ethylmaleimide-treated cells was increased, and was Cl^- dependent, confirming what Lauf and Theg [12] and Lauf found [13], whereas the increased K^+ uptake in sodium periodate-treated cells showed no Cl^- dependency. Cl^--substituted LK sheep red cells treated with sodium periodate, washed then treated with *N*-ethylmaleimide showed increased passive K^+ uptake compared with NO_3^--substituted cells, the increment was about the same as the increased Cl^--dependent K^+ flux in *N*-ethylmaleimide-treated cells, and gives partial support to the idea that the transport system modified by *N*-ethylmaleimide remains unaffected by treatment of LK sheep red cells with sodium periodate In contrast, LK sheep red cells treated first with *N*-ethylmaleimide, washed, then treated with sodium periodate (Table III, final column) showed greatly increased passive K^+ uptake, which was not Cl^- dependent Red cells treated by this latter procedure often haemolysed.

HK sheep red cells in Cl^--containing buffer were subjected to the four treatments used in Table III. Table IV summarises data for HK sheep red cells (four experiments) and LK sheep red cells (seven experiments), and compares the increased passive K^+ flux with control (untreated) cells. HK sheep red cells treated with *N*-ethylmaleimide showed a negligible increase in passive K^+ uptake, confirming the findings of Lauf and Theg [12] and Lauf [13]. Surprisingly, HK sheep red cells treated with *N*-ethylmaleimide in combination with sodium periodate showed passive K^+ uptake that was always greater than that from sodium periodate treatment alone.

Discussion

Leaving aside nonspecific effects on leak pathways, enzymic or chemical attack on mediated active or passive K^+ transport in LK sheep or goat red cells can act at one of several points (a) directly on the K^+ transport system, (b) on K^+

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE AND SODIUM PERIODATE TREATMENT OF LK SHEEP RED CELLS ON PASSIVE K^+ UPTAKE

Red cells were anion substituted with Cl^- or NO_3^- (see methods) then treated with *N*-ethylmaleimide (NEM, 2 mM) and sodium periodate (2 mM) either separately or in sequence and compared with controls Each treatment was followed by three washes (see Methods) Values are means $\pm$ S E of triplicate incubations

Passive K^+ uptake (mmol/l cells per h)					
	control	NEM	sodium periodate	sodium periodate followed by NEM	NEM followed by sodium periodate
Cl^--substituted	$0\,109 \pm 0\,006$	$0\,622 \pm 0\,006$	$1\,013 \pm 0.020$	1.910 ± 0.010	$3\,160 \pm 0\,033$
NO_3^--substituted	$0\,053 \pm 0\,007$	$0\,051 \pm 0\,003$	$0\,889 \pm 0.018$	$1\,111 \pm 0\,018$	$3\,564 \pm 0\,038$

TABLE IV

EFFECT OF *N*-ETHYLMALEIMIDE AND SODIUM PERIODATE TREATMENT OF HK AND LK SHEEP RED CELLS ON PASSIVE K^+ UPTAKE

HK and LK sheep red cells were treated with *N*-ethylmaleimide (NEM, 2 mM) and sodium periodate (2 mM) either separately or in sequence Each treatment was followed by three washes (see Methods) Each experiment was carried out with triplicate incubations Values are means ± S.E of four separate experiments with HK sheep red cells and seven experiments with LK sheep red cells

Passive K^+ uptake (mmol/l cells per h)					
K type	control	NEM	sodium periodate	sodium periodate followed by NEM	NEM followed by sodium periodate
HK	0.075 ± 0 008	0 131 ± 0 008	0 470 ± 0 090	0 868 ± 0 088	1 364 ± 0 272
LK	0 104 ± 0 01	0 448 ± 0 054	0 893 ± 0 094	1 529 ± 0 166	3 102 ± 0 162

flux/antigen interaction in the absence of antibody, (c) on K^+ flux regulated by antigen sensitized by antibody, (d) on antigen/antibody binding.

LK sheep red cells treated with a proteolytic enzyme, trypsin [14] showed impaired absorption of anti-L, and a reduced response of K^+ pump stimulation by anti-L (i.e., conditions d and c). LK goat red cells treated with trypsin [8] showed stimulation in active K^+ transport in the absence of anti-L (i.e., condition b). We have used the enzyme neuraminidase to assess the nature of the polysaccharide components of L antigens of LK sheep red cells.

Neuraminidase specifically removes sialic acid residues from glycoproteins or glycolipids, and in earlier work [14] LK red cells treated with this enzyme showed no effect on absorption of anti-L or of the stimulating effect of anti-L on active K^+ transport. In the present work we have found that neuraminidase treatment of LK sheep red cells resulted in a small though statistically significant fall in the stimulation of active K^+ uptake by added anti-L (i.e., conditions c or d) which accompanied a major loss (60–70%) of the red cell surface charge. This suggests that even if sialic acid residues are present on the L_p antigen they are either shielded from neuraminidase attack or are not critically involved in antigen/anti-L binding and stimulation of active K^+ uptake by anti-L.

Chemical attack on LK sheep red cell membranes used sodium periodate. The conditions were a compromise whereby we attempted to modify selectively vicinal hydroxyl groups of the putative carbohydrate portion of the antigen without causing too drastic an increase in passive K^+ transport, by oxidation of other membrane groups, principally sulphydryl groups (see later). Oxidation of LK sheep red cells with sodium periodate (2 mM) did not affect normal sodium pump activity but did cause a 50% reduction in the stimulatory effect of anti-L on active K^+ uptake (Table IIa). A possible explanation for this may be oxidation of a carbohydrate residue concerned with the Lp antigen (i.e., conditions c or d), although further investigations will be necessary to determine whether L_p antigen/anti-L binding or L_p antigen/active K^+ transport interaction is responsible for the fall in activity.

The composition of the L_1 antigen is less clear, since the effect of anti-L on passive transport survived periodate treatment (Table IIb), and even showed enhanced activity. This represents direct chemical evidence for differences between L_p and L_1.

In addition to its effects on the L_p antigen, sodium periodate increased passive K^+ uptake in LK and HK sheep red cells. A comparison between the effects of sodium periodate treatment and of *N*-ethylmaleimide treatment is helpful, since the reactions of *N*-ethylmaleimide are well characterised. *N*-Ethylmaleimide is an irreversible monofunctional alkylating reagent, reacting with sulphydryl groups of spectrin and other defined proteins in human red cell membranes [15], and affecting passive ion transport in LK sheep red cells [12,13] and human red cells [16]. The reactions of sodium periodate differ from *N*-ethylmaleimide in that sulphydryl groups can be converted to several oxidation states [17]. Thus, per-

iodate treatment of human red cells caused disulphide bond formation, and was reversible by dithiothreitol [18].

In the present investigation the large increase in passive K^+ uptake caused by sodium periodate treatment of LK red cells was not Cl^- dependent; treatment of HK cells resulted in a similar, rather smaller effect. The absence of a saturable flux for both Na^+ and K^+ in periodate-treated cells [11] also suggested some unspecific membrane oxidation by sodium periodate. It is probable that the changes we observed in passive K^+ uptake in sheep red cells are similar to the results of Heller et al. [18] under more stringent conditions of oxidation.

The effect of *N*-ethylmaleimide treatment of LK sheep red cells is to cause an increase in Cl^--dependent passive K^+ uptake which is unaffected by pretreatment with periodate (Table III). This clearly suggests that *N*-ethylmaleimide and sodium periodate react with different populations of membrane proteins, emphasising likely differences in sulphydryl group effects of these two reagents. The marked increase in passive K^+ uptake which finally followed from treatment of LK sheep red cells with *N*-ethylmaleimide and then sodium periodate was not Cl^- dependent and may be the expression of generalised membrane damage following extensive chemical attack.

Acknowledgements

We thank Andrew Hall for advice and discussion throughout this work. We also thank Nigel Miller for performing the microelectrophoresis of red cells and David Brown for advice on statistics. The work was supported by an AFRC Link Grant.

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