# Molecular Biology of Hematopoiesis 6

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# HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN HEREDITARY SPHEROCYTOSIS UNDER OXIDATIVE STRESS

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A broad spectrum of clinical manifestations in hereditary spherocytosis (HS) has been documented.

The aim of this study was to investigate a correlation between the development of an oxidative stress, during inflammatory or infectious stress, and the development of a hemolytic event in HS patients. We evaluated hematological and biochemical changes i) imposed by infection in 2 HS patients and in 2 HS patients who had undergone splenectomy (more than six months before) ii) in 5 HS patients immediately after splenectomy and iii) in 4 HS patients, more than six months after splenectomy. In both inflammatory and infectious stresses we found a reduction in RBCs concentration and in MCHC, a rise in MCV, and neutrophilic monocytic leukocytosis. The changes in RBCs seemed to correlate with neutrophils and monocytes values. To clarify this, we studied the same parameters and a few others in iiii) a group of 6 HS patients who had undergone splenectomy (more than six months) and compared the results with those presented by a healthy control, including 29 individuals with no HS and no infection.

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*Molecular Biology of Hematopoiesis 6*, edited by Abraham *et al.*
Kluwer Academic/Plenum Publishers, New York 1999

Our data suggest that inflammatory and infectious stress by inducing leukocytosis and leukocyte activation, may induce oxidative and proteolytic modifications upon RBCs, leading to an accelerated aging process, to a premature removal or even to a hemolytic event.

In Europe, Hereditary Spherocytosis (HS) is the most common congenital hemolytic anemia. A broad spectrum of clinical manifestations has been documented, which can range from mild to extremely severe. A set of mutations, namely in spectrin, ankirin and, band 3, may underlie HS.[1–3] The mode of transmission is dominant or recessive, with intermediate situations.

During its life span any normal red blood cell (RBC) is exposed to various insults and undergoes physical and chemical changes.[4–7] These modifications become more pronounced with cell age, and with the development of unusual physiologic circumstances, such as increased oxidative and proteolytic stress. In these circumstances, the accelerated and premature development of senescent modifications may trigger the premature removal of RBCs or even a hemolytic event. Cells, such as spherocytes, which develop intracellular defects early during their life span, are also removed from circulation at an earlier stage.

RBC senescence includes several modifications, namely a reduction in its metabolic activity, resulting from a general decreased enzymatic activity. The pathway for the removal of senescent or damaged RBCs involves the development of a senescent cell antigen immunologically related to band 3 protein,[8,9] which marks the RBC for death, by triggering the binding of specific auto-antiband 3 antibodies[10,11] and complement activation.[12] The modified antigenicity of band 3 may result from proteolytic cleavage, clustering or even by exposure of unusual epitopes.

Inflammatory and infectious diseases often present a rise in leukocytes, namely in neutrophils, and a reduction in total RBCs.[13,14] The exposure of circulating RBCs to oxygen metabolites and proteases, which are known to be produced by activated neutrophils, may account for some oxidative and proteolytic RBC damage, leading to an accelerated aging process and premature removal.[7,15–19] In the case of spherocytes presenting a destabilized membrane structure, with loss of membrane components, and with an abnormal reduced life span, the impact of an oxidative and proteolytic stress is probably enhanced, triggering a hemolytic event.

The aim of this study was to evaluate whether a correlation can be drawn between an inflammatory or an infectious stress and the development of a hemolytic event in HS patients. Splenectomy (SPL) was used as the model of inflammatory stress, and bacterial infection as the model of infectious stress. In searching for correlation we studied several hematological and biochemical parameters, that included: concentration of total WBC and of the several WBC types; concentration of RBCs; hematocrit (Ht); hemoglobin (Hb) concentration; hematimetric indexes; direct bilirrubin (DB) and total bilirrubin (TB). The same parameters and a few others were studied in HS patients who had undergone splenectomy several months before (more than 6 months) and compared to a healthy control, to investigate the impact of splenectomy in the spherocyte aging process and to compare it to the normal RBC aging. The other studied parameters included: membrane bound hemoglobin (%MBH); the band 3 profile (high molecular weight aggregates, monomer, and total proteolytic fragments) as a cumulative marker of RBC aging; the RBC glucose-6-phosphate dehydrogenase (G6PD) activity as an index of RBC age; lactoferrin (Lactof) concentration, a product of neutrophil activation, as a marker of leukocyte activation.

## MATERIALS AND METHODS

*Subjects.* We evaluated the referred hematological and biochemical parameters imposed by infection in 2 HS patients and in other 2 HS patients who had undergone splenectomy several months before (more than 6 months). The changes imposed by inflammatory stress induced immediately after surgical spleen removal were evaluated in 5 HS patients. The same parameters were studied in 4 HS patients, to evaluate the modifications imposed by the removal of the spleen (not less than 6 months before). To clarify the results obtained with the previous groups, we studied the same parameters and a few others in 6 HS patients, who had undergone splenectomy more than 6 months before and in 29 healthy individuals (with no HS and no infection).

*Assays.* Whole blood and plasma (EDTA as anticoagulant) and serum were used for hematological and biochemical procedures.

WBC and RBC count, Ht, Hb concentration, MCV, MCH, MCHC, RDW, and the WBC differential count were evaluated by a Ortho Counter. The morphology of the blood cells was evaluated in a Wright stained blood film.

To evaluate erythrocyte G6PD activity, RBCs were isolated after centrifugation on a double density gradient (Histopaque 1119 and 1077, Sigma), washed with phosphate buffered solution (PBS) pH 7.4 and lysed by thermic shock. The activity was then evaluated using a commercially available kit (Test-Combination G6PDH, Boehringer Mannheim), with modifications.[20] The G6PD activity was expressed as a function of hemolysate hemoglobin content (UI/g Hb), which was evaluated by a colorimetric method (Hb-Boehringer Mannheim).

RBC membrane ghosts were prepared by hypotonic lysis[21] using phenylmethylsulfonyl fluoride as a protease inhibitor (final concentration of 0.1 mM). Protein concentration of the prepared membranes was determined.[22]

%MBH was measured spectrophotometrically,[15] after dissociation of membrane components with Triton X-100 (5% in Dodge buffer) at 415 nm, and compared with membrane protein concentration.

To study band 3 profile RBC membranes were treated with an equal volume of a solubilization buffer (0.125 M Tris HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol) heat denatured and subjected to SDS PAGE (20 µg of protein/lane) using the discontinuous Laemmli system[23] (9% separating gel; 4,5% stacking gel). Membrane proteins were then electrophoretically transferred from SDS gels to nitrocellulose[24] (22 µm porosity). Additional reactive sites were blocked (3% gelatin and 0.1% Triton-X 100 in PBS pH 7.0/1 h). Monoclonal antibodies anti-human band 3, produced in mouse, recognizing an epitope located in the cytoplasmatic pole of the band 3 molecule (Sigma) were then added (dilution 1:3,000) and incubated for 4 h; the washing of the nitrocellulose was followed by the addition and incubation with anti-mouse IgG peroxidase-linked (Amersham) for 1 h (dilution 1:15,000). Both incubations were carried out at room temperature in PBS pH 7.0 containing 0.1% detergent and 0.5% gelatin. The washes used the same buffer without gelatin. The immunoblot was developed by the addition of hydrogen peroxide and horseradish peroxidase color developer reagent. The band 3 profile was quantified by densitometry (Cybertech CS1).

Plasma lactoferrin was evaluated by an enzyme immunoassay (Bioxytech lacto f enzyme immunoassay, Oxis International, Inc.).

*Statistics.* All measurements are expressed as mean value ± standard deviation. The calculations were performed using the StatView software. The significance of the

differences between each value presented by two groups was evaluated by the Student t-test and $P < 0.05$ was considered statistically significant. To draw graphs we used the Excel Microsoft.

## RESULTS

Figure 1a shows the percentual changes in some hematological and biochemical parameters, imposed by infectious stress in 2 HS-SPL patients and in 2 HS-no SPL patients. In both cases we observed a reduction in RBC concentration, which is accompanied by an increase in bilirrubins. These modifications are followed by an increase in WBC concentration. This rise is specially evident in HS-SPL patients and results mainly from a rise in neutrophils and less in monocytes. In HS-no SPL patients the WBC concentration rise is minor and results mainly from a rise in monocytes and less in neutrophils. Along with these changes the MCV rises and the MCHC reduces. There was an increase in the RDW and this rise was higher in HS-SPL patients than in HS-no SPL patients.

In Fig. 1b we present the percentual changes observed in the same hematological and biochemical parameters, imposed by inflammatory stress induced immediately after SPL in 5 HS patients. RBC modifications were similar to those we found for infectious stress, though with smaller values. As expected the removal of the spleen was followed by a reduction in bilirrubins. WBCs presented a rise similar to that imposed by infectious stress in HS-SPL patients. This rise is mainly due to the increase in monocytes and less in neutrophils.

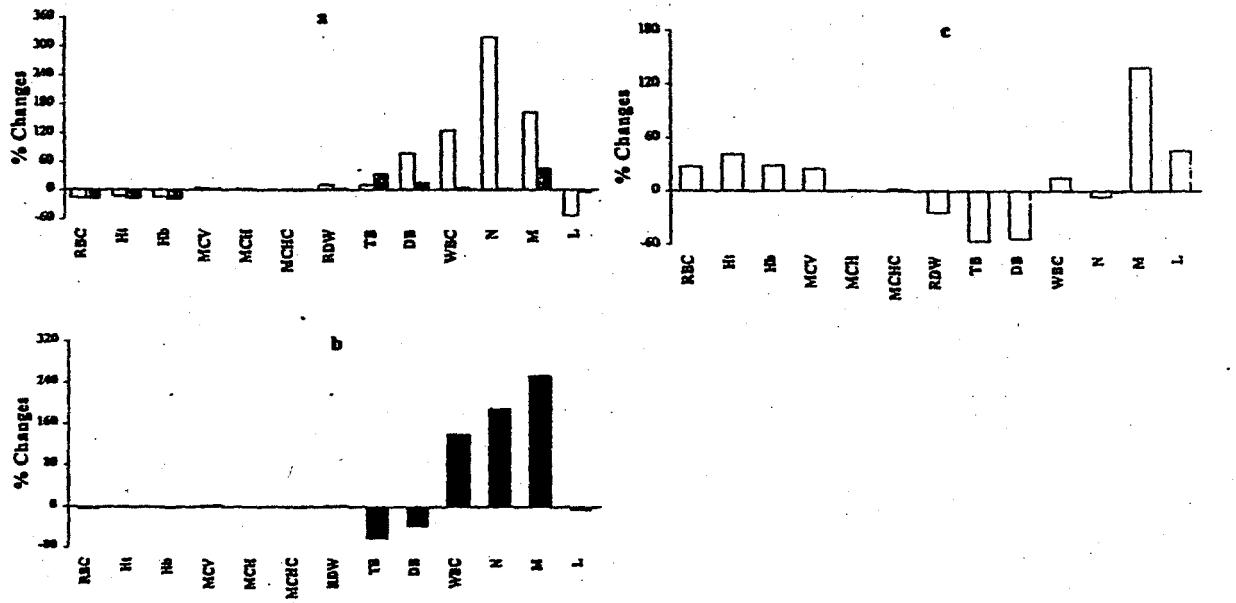


**Figure 1.** Changes (%) observed in hematological and biochemical parameters;

a) imposed by infectious stress in 2 HS patients who had undergone splenectomy (□) and in 2 HS patients who had not undergone splenectomy (●);
b) imposed by inflammatory stress, induced immediately after splenectomy, in 5 HS patients;
c) imposed by removal of the spleen (not less than 6 months after splenectomy), in 4 HS patients.

RBC ($\times 10^{12}$/l); Hb, MCHC (g/dl); Ht, RDW (%); MCV (fl); MCH (pg); TB, DB (mg/l); WBC, N, M, L ($\times 10^{9}$/l).

Figure 1c shows the impact of SPL (more than six months after splenectomy) in the same hematological and biochemical parameters, observed in 4 HS patients. We found a rise in RBC concentration and in the hematimetric indexes; the RDW and the bilirrubins presented a decrease. In WBCs we found a rise, resulting from an increase in lymphocytes and monocytes. To clarify the above changes, we evaluated in 6 HS-SPL patients the same parameters (Fig. 2a and Fig. 2b) and a few others, namely erythro-

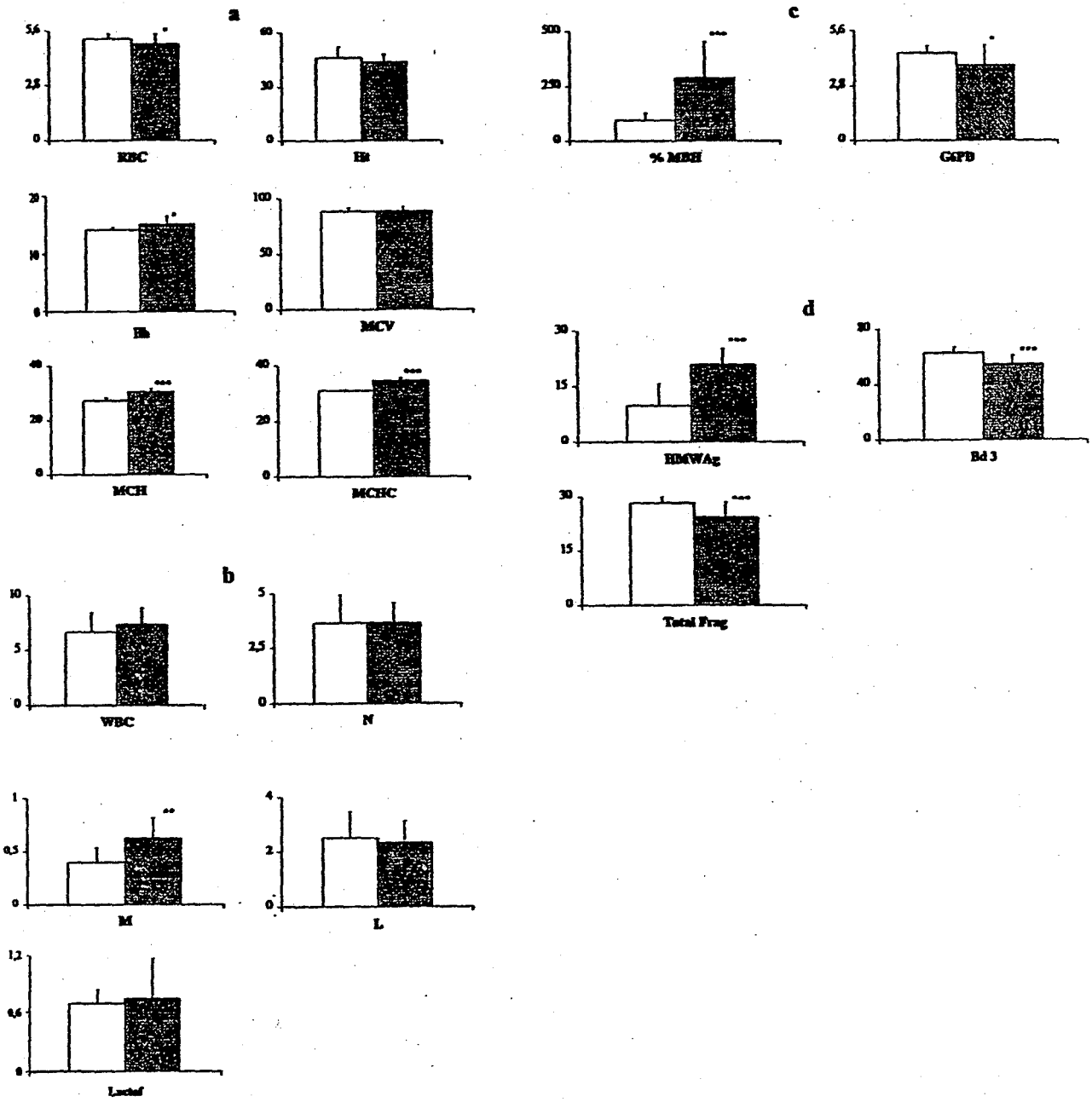


**Figure 2.** Hematological and biochemical parameters in HS patients who had undergone splenectomy (more than 6 months) and in a healthy control.
□ Controls (n = 29) ● Patients HS-SPL (n = 6)
$*P < 0.05$ $**P < 0.01$ $***P < 0.001$

a) RBC ($\times 10^{12}$/l); Ht (%); Hb, MCHC (g/dl); MCV (fl); MCH (pg);
b) WBC, N, M, L ($\times 10^{9}$/l); Lactof (µg/ml);
c) %MBH ($\times 10^{-4}$); G6PD (UI/g Hb);
d) HMWAg, Bd 3, Total Frag (relative %).

cyte G6PD activity and the %MBH (Fig. 2c); band 3 profile (Fig. 2d); and plasma lactoferrin concentration (Fig. 2b). The results were compared with those presented by a control including 29 healthy individuals (with no HS and no infection). We observed an evident improvement in RBC population though some of the characteristics of HS were still observed, namely a high MCHC. A significant reduction in erythrocyte G6PD, a significantly higher %MBH, and a different band 3 profile were also observed. Actually, HS-SPL patients presented a significant increase in high molecular weight aggregates (HMWAg) and a significant decrease in band 3 monomer (Bd 3) and in its proteolytic fragments (Total frag). Concerning leukocytes we found increased values for WBC concentration and a significantly higher value in monocyte concentration. Plasma lactoferrin presented no statistical significance, although presenting a higher value than that presented by the control.

## DISCUSSION

Hereditary spherocytes, on account of its membrane modifications, are more sensitive to oxidative and proteolytic stresses. During an inflammatory or an infectious process, activated leukocytes may be an important source of products, namely oxygen metabolites, and proteases. Either of these leukocyte activation products can damage the neighbouring RBCs, imposing oxidative and proteolytic lesions to RBCs, which may lead to an accelerated aging and removal process. Therefore, the impact of inflammatory or infectious stress in spherocytes is probably enhanced and may impose an even more accelerated aging and removal process, leading to a hemolytic event.

We showed (Fig. 1a) that the changes in the hematological and biochemical parameters studied, imposed by infectious stress in both HS-SPL and HS-no SPL patients, were similar. The reduction in RBC concentration was accompanied by a rise in bilirubins, revealing an increased RBC removal. Along with these changes we observed a rise in WBC concentration, which was specially evident in HS-SPL patients and resulted mainly from a rise in neutrophils and less in monocytes. HS-no SPL patients presented a minor rise in WBC concentration and resulted mainly from a rise in monocytes and less in neutrophils. These results suggest the involvement of these leukocytes in accelerated RBC aging, and premature removal. In addition, we showed that the MCV presented a rise and the MCHC a reduction in both HS patients, suggesting that molecular modifications occurred. As expected, there was an increase in the RDW and the rise was higher in HS-SPL patients. This is probably the result of the increase in RBCs presenting the referred molecular changes. The change in the RDW value of HS-no SPL patients was lower since the spleen will filter the RBCs presenting those molecular changes. In fact, the reduction in RBC concentration and the rise in TB was higher in HS-no SPL, denoting an enhanced splenic RBCs removal. It seems that the presence of the spleen, acting as a filter of damaged or senescent RBCs, renders the HS patients more susceptible to develop a hemolytic event during an inflammatory or an infectious stress.

The percentual changes (Fig. 1b) in the same hematological and biochemical parameters, imposed by inflammatory stress induced immediately after splenectomy in HS patients, were similar to those found for infectious stress, though with smaller values. As expected after the removal of the spleen, a reduction in bilirubin was observed denoting reduced RBC destruction.

HS-SPL patients, who had undergone splenectomy (not less than 6 months

before) presented the expected changes for RBC concentration and bilirrubins. Actually, the rise in RBC concentration and the reduction in bilirubin was followed by clinical and hematological improvement of the patients. The removal of the spleen, the filter of the damaged or senescent RBCs, seems to underlie these changes. However, we must expect that the absence of that filter may account for the accumulation of damaged RBCs. Contrary to the previous stressfull situations, in this case we observed a rise in WBCs, resulting from an increase in lymphocytes and monocytes. This suggests that the absence of the spleen may trigger a different mechanism to command the granulo-monocytopoiesis or even the immune system.

To clarify the above changes in RBCs, denoting accelerated RBC damage or senescence and the involvement of activated leukocytes in its development, we evaluated in HS-SPL patients and in a healthy control the same parameters and a few others, which could give us some information about the age of RBCs, about the amount of oxidative and proteolytic lesions suffered by RBCs during their life span and about leukocyte activation. Several months after splenectomy the HS patients presented an evident improvement in RBC concentration, showing that the absence of the spleen reduced the removal of damaged or senescent RBCs. We must note, however, that both MCH and MCHC maintained higher values when compared to the control, as it is characteristic of spherocytes. The reduction of RBC removal seems to be followed by the accumulation of older circulating RBCs, as shown by the significant decrease in erythrocyte G6PD activity (Fig. 2c). Strengthening this hypothesis we found also a significantly higher %MBH and a different band 3 profile (Fig. 2d). HS-SPL presented a significant increase in HMWAg and a significant decrease in band 3 monomer and in its proteolytic fragments. When compared this profile with that presented by the control, it appears that HMWAg might result from oxidation and crosslinking of band 3 monomer and its proteolytic fragments or even from a further proteolysis of band 3 followed by oxidation and crosslinking of proteolytic fragments. These results provide further evidence of the premature senescence of RBCs in these patients. We must emphasize that these changes for band 3 profile are similar to those found in patients under chronic stressfull situations, such as cardiovascular disease,[15] and similar to those found for RBC aging *in vitro*, i.e. exposed to activated neutrophils or neutrophilic elastase.[16] Concerning leukocytes, we found higher values for HS-SPL patients for all leukocyte types, though only for monocytes we did observe a significantly higher value when compared to the control (Fig. 2b). We must emphasize that monocytes are the WBC which present a higher rise in both inflammatory and infectious stress in HS-no SPL.

The destabilized membrane structure of spherocytes seems to underlie membrane vesiculation, premature aging and premature removal of the cells. Whenever an inflammatory or an infectious stress develops, a rise in leukocytes, mainly in neutrophils and monocytes occurs. It is known that leukocytes present a reduced deformability and that its activation may produce and release oxygen metabolites and proteases. The rise in leukocytes of reduced deformability may slow the blood flow and favour the interaction of the released leukocyte activation products with the surrounding cells, namely with the spherocytes. It seems, therefore, reasonable to assume that the change in blood flow and the easier interaction of leukocyte activation products with the circulating RBCs, is even more enhanced within the splenic microvasculature, favouring oxidative, and proteolytic damage of spherocytes. The oxidative and proteolytic lesions, by inducing splenic sequestration, may lead to premature removal of a higher number of spherocytes, or even to a hemolytic event.

In conclusion, our data show that inflammatory and infectious stress by inducing leukocytosis and eventually leukocyte activation, may underlie and trigger hemolytic events in HS patients. In addition, %MBH and band 3 profile may provide further evidence of the oxidative and proteolytic modifications imposed upon RBCs by leukocyte activation products.

## ACKNOWLEDGMENT

Supported in part by research grants from the "Fundação Gomes Teixeira", Universidade do Porto, and PRAXIS XXI n°2/2.1/SAU/1240/95 (JNICT).

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