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# Elution of antispectrin antibodies from red cells in homozygous β-thalassaemia

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## SUMMARY

It is well-established that autoantibodies against red cell membrane antigens, such as spectrin are present in plasma of humans and animals. It is shown here that antispectrin IgG antibodies can be eluted from the red cells of patients with β-thalassaemia intermedia. It is suggested that these antibodies could play a part in the increased rate of destruction of red cells in homozygous β-thalassaemia, either by reacting with spectrin which is abnormally exposed on the red cell surface or by a cross-reaction with a different membrane component. No rise was found in serum antispectrin levels in β-thalassaemia; anti-red cell membrane IgG levels were slightly raised, but there was considerable overlap between the levels in patients and in normals.

No autoantibody could be found in eluates from cells of normal subjects or patients with sickle cell anaemia.

**Keywords** antispectrin antibodies red cells β-thalassaemia.

## INTRODUCTION

The anaemia of β-thalassaemia results from ineffective erythropoiesis in the marrow together with a shortened red cell (RBC) life span of those cells that reach the circulation (Wickramasinghe, 1975; Weatherall & Clegg, 1981). In sickle cell disease the anaemia is mainly due to excessive haemolysis (Weatherall & Clegg, 1981). Both ineffective erythropoiesis and haemolysis involve the recognition and subsequent phagocytosis of erythroid cells by macrophages but the processes which underlie these cellular interactions remain largely unknown. One of the possible mechanisms which needs to be explored further is that the phagocytosis is mediated by antibody. There is now a considerable body of evidence that the plasma of both humans and animals contains low levels of natural antibodies against self-antigens. These antigens are either soluble proteins in the plasma, such as albumin, thyroglobulin and transferrin (Guilbert, Dighiero & Avrameas, 1982), or they are hidden membrane proteins of cells such as thymocytes (Martin & Martin, 1975), lymphocytes (Rogentine & Plocinik, 1974) and erythrocytes (Winchester *et al.*, 1975; Lutz & Wipf, 1982). The role of these autoantibodies is unknown but it has been suggested that they may be far more widespread than has so far been identified and that they may participate in the elimination of damaged and aged tissue (Guilbert *et al.*, 1982) and in self-tolerance mechanisms (Hellstrom & Hellstrom, 1972). In the case of autoantibodies reacting with the RBC membrane glycoprotein, band 3, it has been suggested that they play a role in destruction of senescent RBC (Kay, 1984). Although the primary function of

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these autoantibodies may be related to physiological homeostatic mechanisms, they may also play a secondary role in the destruction of abnormal RBC in disease.

We have recently established an *in vitro* system based on mouse cells for the study of processes participating in the recognition of defective erythroblasts by macrophages (Wiener & Wickramasinghe, 1982; 1983a). Using this system, we have demonstrated that immunoglobulins, such as anti-spectrin, mediate the interaction between defective erythroblasts and macrophages (Wiener & Wickramasinghe 1983b; Wiener, 1985). Moreover, IgG-antibodies seem to play a role in the *in vitro* recognition of thalassaemic RBC by normal human macrophages (Wiener & Wickramasinghe, 1985). For these reasons, it was of interest to investigate whether antibodies with specificity for spectrin or for other membrane components could be demonstrated to be present on the surface of RBC in thalassaemia and sickle cell anaemia and also whether serum levels of these antibodies are raised in these diseases.

## MATERIALS AND METHODS

*Subjects.* The subjects studied consisted of (1) 10 haematologically normal children aged 2–17 years (mean 9·3 years) who were admitted for tonsillectomy, (2) 13 healthy adult volunteers aged over 18 years, (3) nine splenectomized patients aged 6–18 years (mean 11.3 years) and three non-splenectomized patients aged 2–6 years with homozygous $\beta$-thalassaemia and the clinical syndrome of thalassaemia major, (4) three splenectomized patients and one non-splenectomized patient with homozygous $\beta$-thalassaemia and the clinical syndrome of thalassaemia intermedia aged 15–29 years and (5) eight adults with sickle cell anaemia aged 18–51 years. All of the patients with $\beta$-thalassaemia major were receiving regular blood transfusions. The patients with thalassaemia intermedia had not been transfused for 4 months or more prior to obtaining blood for the present study. All but two of the patients with sickle cell anaemia had received blood transfusions in the past but not during the preceding 4 months.

*Antibody elution from RBC.* Antibodies were eluted from 1ml of RBC using the choloroform method of Branch *et al.* (1982).

*Preparation of RBC ghosts.* Ghosts were prepared from group O RBC using the method of Steck *et al.* (1970). For use as antigen, liposomes were prepared by sonication of the ghosts with an MSE (100 W) sonicator used at maximum amplitude for 2 min; the protein concentration was adjusted to 7mg/ml.

*Preparation of spectrin and $^{125}$I-spectrin.* Spectrin was obtained from the RBC ghosts by the O°C extraction method and separated from actin by gel-filtration on a Sepharose-4B column (Pharmacia Fine Chemicals, Milton-Keynes, UK) (Shotton, Burke & Branton, 1979). SDS-polyacrylamide gel electrophoresis of the final product failed to reveal any protein contaminants. Spectrin was labelled with $^{125}$I (Amersham International) using Iodo-beads (Pierce Chemical Company, Chester, Cheshire, UK), to a specific activity of $1 \times 10^4$ ct min$^{-1}$ $\mu$g$^{-1}$ protein.

*Estimation of serum anti-spectrin and anti-RBC membrane IgG concentrations.* The enzyme-linked immunosorbent assay (ELISA assay) (Voller, Bidwell & Bartlett, 1979) was performed using spectrin or sonicated RBC membranes adsorbed to microtitre plates (Wahlgren *et al.*, 1983). Alkaline phosphatase-conjugated goat anti-human IgG (Sigma Chemical Co., St Louis, MO, USA) served as the second antibody. For the anti-membrane antibodies, samples were treated in duplicate and the titration curve for each test serum was compared with that of a standard serum from a normal person. The anti-RBC membrane IgG concentration in the standard serum was defined as 1 unit and the relative concentrations in the test sera expressed as a multiple of this. For the antispectrin antibodies, the activity in test sera was defined in relation to that of a standard serum with an antispectrin content of 0·42 $\mu$g/ml (see below). The antispectrin antibodies were not detected in the assay for antimembrane antibodies, presumably because spectrin was not present on the surface of the sonicated membranes.

*Antispectrin content of standard serum.* In order to obtain an estimate of the concentration of antibody in the standard anti-spectrin serum, the ELISA assay was calibrated using a known amount of IgG antibody. For this purpose, a purified $^{125}$I-labelled monoclonal IgG human blood

group antibody, anti-Rh(D), (Melamed *et al.*, 1985) was used. The absorbance at 405 nm was determined using cells coated with 36 μg anti-Rh(D)/ml of red cells. In order to avoid red cell lysis, the assay was carried out in a medium which was isotonic for red cells and at a pH of 7·0. The quantitative assessment of the antispectrin content was then carried out by adding varying concentrations of a known amount of spectrin bound to Sepharose 4B to the standard serum and the amount of anti-spectrin bound at equilibrium determined using the calibration for conversion of absorbance at 405 nm to micrograms of IgG. Standard Scatchard analysis was then carried out to give the total amount of antibody present in the serum (0·42 μg/ml).

*Absorption of serum with spectrin.* Aliquots of the standard serum diluted 1:4 with PBS containing 0·5% Tween 20 and 1% bovine serum albumin (BSA) were absorbed with different amounts of $^{125}$I-spectrin-Sepharose 4B (Pharmacia Fine Chemicals, Milton-Keynes, UK) during incubation at room temperature for 2 h. Similarly, control absorptions of the serum were performed with bovine muscle actin, human transferrin, human albumin and human haemoglobin (Sigma Chemical Co., St Louis, MO, USA).

## RESULTS

*Specificity of assay for anti-spectrin IgG*

In order to establish the specificity of the anti-spectrin IgG, the standard serum was absorbed with different concentrations of spectrin linked to Sepharose and subsequently assayed for anti-spectrin IgG. The absorption of the sera with spectrin caused a dose-dependent inhibition of its anti-spectrin activity which became undetectable when 55 pM spectrin was employed (Fig. 1). Absorptions of the same sera with haemoglobin, transferrin, albumin and actin failed to affect their anti-spectrin activity.

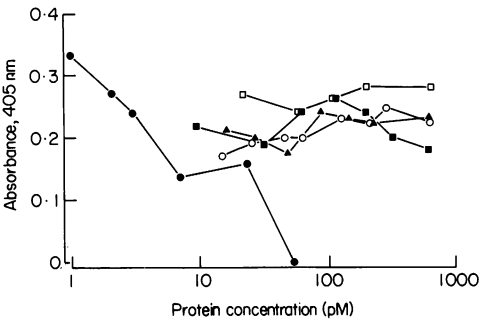


**Fig. 1.** Demonstration of the specificity of the anti-spectrin IgG assay. Aliquots of 1:4 dilutions of the standard serum were absorbed with spectrin or other specific proteins in various concentrations and subsequently assayed for anti-spectrin IgG. (●) spectrin; (□) haemoglobin; (■) actin; (○) albumin; (▲) transferrin.

*Anti-spectrin and anti-RBC membrane IgG in eluates from RBC*

Eluates were prepared from RBC obtained from the four patients with β-thalassaemia intermedia, four patients with sickle cell anaemia and three normal people, none of whom had been transfused during the preceding 4 months and therefore had only autologous red cells in their circulation. Anti-spectrin activity was detected only in the eluates from the four patients with thalassaemia. The amount of anti-spectrin eluted from 1ml of RBC varied between 0·02 and 0·42 μg, this being equivalent to 16–320 molecules of antibody/red cell (Table 1).

The lowest level of anti-spectrin IgG was obtained in the RBC eluate from patient MG who had an intact spleen. No correlation was found between the level of anti-spectrin IgG in the RBC-eluate and serum of the same patient. The serum anti-spectrin IgG of patients SM and MG were within the observed range in normal age-matched individuals, while those of MR and GC were slightly below and above the age-matched control range respectively (Table 1, Fig. 1).

None of the RBC eluates showed any anti-RBC membrane IgG.

**Table 1.** Antispectrin IgG in the serum and red cell eluates of patients with β-thalassaemia intermedia

| Patient | Age (years) | Spleen | anti-spectrin IgG (μg/ml) serum | red cell eluate |
|---|---|---|---|---|
| MR | 15 | – | 0·37 | 0·42 |
| SM | 21 | – | 0·16 | 0·08 |
| MG | 15 | + | 0·60 | 0·02 |
| GC | 29 | – | 1·09 | 0·08 |

*Anti-spectrin and anti-RBC membrane IgG in the sera of normal subjects and patients*

Anti-spectrin antibody was found in the sera of all the normal subjects, the children as a group having approximately twice the amount ($P < 0{\cdot}01$) found in adults; the geometric mean value in children was 0·95 μg/ml (observed range, 0·45–1·8 μg/ml) and in adults it was 0·38 μg/ml (observed range, 0·25–0·66 μg/ml) (Fig. 2). On the other hand, there was no essential difference in anti-RBC membrane antibody levels between the children and the adults (Fig. 3).

In children with β-thalassaemia major, the only abnormality found was a small rise in anti-RBC membrane levels ($P < 0{\cdot}05$), the geometric mean being 3·7 units compared to 2·7 units in the age-matched controls (Fig. 3). There was no difference in anti-spectrin levels between the normal and the thalassaemic children (Fig. 2).

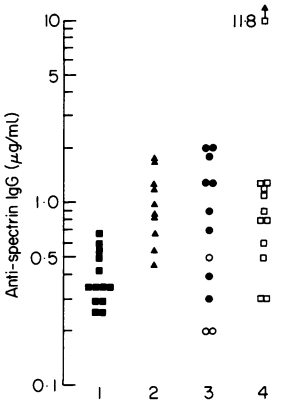


**Fig. 2.** The concentration of anti spectrin IgG in the sera of the individuals studied. (1) normal adults; (2) normal children; (3) β-thalassaemia major; (●) splenectomized patients; (○) non-splenectomized patients; (4) patients with sickle cell anaemia.

The adults with sickle cell anaemia showed a different pattern. In these patients it was the anti-spectrin levels which were slightly raised ($P < 0{\cdot}01$), the geometric mean value being 0·76 μg/ml compared to 0·38 μg/ml in the normals, although there was considerable overlap between the two groups (Fig. 2); on the other hand, the anti-RBC membrane antibodies in six of the eight patients were no different from normal values; two of the patients had considerably raised levels of 20 and 30 units, respectively (Fig. 3).

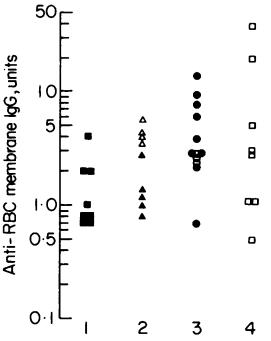


**Fig. 3.** The concentration of anti-RBC membrane IgG in the sera of the individuals studied. The values for anti-RBC membrane are expressed as units (1 unit equals the anti-RBC-membrane IgG in the standard serum). (1) normal adults; (2) normal children; (3) β-thalassaemia major; (●) splenectomized patients, (○) non-splenectomized patients; (4) patients with sickle cell anaemia.

## DISCUSSION

In the present study, we have made quantitative estimates of the amount of anti-spectrin and anti-RBC membrane IgG antibodies in the serum of patients with β-thalassaemia and sickle cell anaemia and also looked for the presence of these antibodies on the surface of the red cells. An ELISA assay was used for detecting the antibodies and in the case of antispectrin, it was possible to make a quantitative estimate of the amount present by calibrating the assay using red cells coated with a known amount of the blood group IgG antibody, anti-Rh(D). The antimembrane antibodies were arbitrarily defined as those that would combine with liposomes derived by sonication of RBC membranes. The most significant finding was the presence of substantial amounts of anti-spectrin on the RBC of patients with β-thalassaemia intermedia. The amount obtained by chloroform elution was such that each RBC was coated with at least 16–320 molecules of antibody if it were evenly distributed throughout the RBC population. The number is certainly greater than this as elution of antibody is never complete (Hughes-Jones, Gardner & Telford, 1963). It is possible that only a subset of RBC were coated with antibody and hence antibody density on these cells would have been greater still. A density of 16–320 molecules per cell is more than adequate to bring about phagocytosis, especially if it is of the $IgG\text{-}_1$ and $IgG\text{-}_3$ isotypes (van der Meulen *et al.*, 1978). A density of only 10 molecules per cell was found to bring about the slow destruction of anti-Rh(D) coated RBC (Mollison & Hughes-Jones, 1967).

It is a reasonable assumption that the antibody eluted from the cells in homozygotes for β-thalassaemia was the same as the autoantibody found in the plasma of these patients, although identifying properties such as isotype subclass were not investigated.

In the normal RBC, spectrin forms part of the cytoskeleton and is located on the cytoplasmic surface of the membrane (Marchesi, 1983). The recovery of antispectrin from thalassaemic cells therefore implies either that spectrin has become exposed on the outer surface of the cells or that the antibody is cross-reacting with an epitope on another membrane molecule which is similar to the epitope present on spectrin. If spectrin is exposed on the outer surface of the RBC, then it may be a consequence of the abnormal membrane architecture found in β-thalassaemia (Kahane, Shifter & Rachmilewitz, 1978). On the other hand, reaction with a membrane constituent other than spectrin must be considered a possibility, even though no reaction was found with haemoglobin, albumin, transferrin or actin. For instance, it has been found that anti-actin autoantibodies also react with tubulin and thyroglobulin (Guilbert *et al.*, 1982). Dighiero *et al.* (1983) have suggested that certain autoantibodies may recognise a conformational determinant, such as an α-helix.

No anti-membrane antibody could be detected in the eluates from any of the cells. It is possible that antibody was present in such low concentrations that it was below the sensitivity limit of the test or alternatively, the antibodies may not have been extracted by the chloroform procedure.

No consistent change was seen in serum levels of antispectrin or antimembrane antibodies in either homozygous β-thalassaemia or sickle cell disease. Despite the finding of antispectrin on the thalassaemic cells, the serum levels were essentially within the observed range in normal individuals and there was only a very small increase in antimembrane levels. In contrast to this, it was the antispectrin levels which were found to be slightly raised in sickle cell anaemia and anti-membrane levels were normal, except in two cases. It should be emphasized however that there was a considerable overlap in the range of concentrations seen in the normal and disease groups (Figs 2 & 3). When it is considered that in the secondary alloimmune response to an antigen or in autoimmune diseases there is a rise of one or more orders of magnitude in serum antibody concentrations, the interpretation of the results presented here is essentially that there is no significant autoimmune response to RBC membrane components resulting from the excessive destruction of erythroid cells in these two diseases. These findings are similar to that of Cox *et al.* (1977) who also observed no increase in autoantibody levels after antigenic stimulation with bromelin-treated RBC in mice. This unresponsiveness is in keeping with the suggestion that the primary role of these autoantibodies is different from the predominant role of antibodies in bringing about destruction of foreign antigens (Poncet *et al.*, 1985). If this argument is correct, then it follows that the destruction of thalassaemic cells by antispectrin is a fortuitous function of the autoantibody normally present in the plasma and haemolysis is brought about by virtue of the abnormal exposure of spectrin or a cross-reacting antigen on the surface of the RBC.

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## REFERENCES

BRANCH, D.R., SIOK, A.L.S.S. HIAN & PETZ, L.D. (1982) A new elution procedure using chloroform, a non-inflammable organic solvent. Technical note. *Vox. Sang.* **42,** 46.

COX, K.O., BADDAMS, H. & EVANS, A. (1977) Studies of the antigenicity and immunogenicity of bromelin pretreated red blood cells. *Aust. J. exp. biol. med. Sci.* **55,** 27.

DIGHIERO, G., LYMBERI, P., MAZIE, J.-C., ROUYRE, S., BUTLER-BROWN, G.S., WHALEN, R.G. & AVRAMEAS, S. (1983) Murine hybridomas secreting natural monoclonal antibodies reacting with self antigens. *J. Immunol.* **131,** 2267.

GUILBERT, B., DIGHIERO, G. & AVRAMEAS, S. (1982) Naturally occurring auto-antibodies against nine common antigens in human sera. I. Detection, isolation and characterization. *J. Immunol.* **128,** 2779.

HELLSTROM, I. & HELLSTROM, K.E. (1972) Can 'blocking' serum factors protect against autoimmunity. *Nature*, **240,** 471.

HUGHES-JONES, N.C., GARDNER, B. & TELFORD, R. (1963) Comparison of various methods of dissociation of anti-D using $^{131}$I-labelled antibody. *Vox. Sang.* **8,** 531.

KAHANE, L., SHIFTER, A. & RACHMILEWITZ, E.A. (1978) Cross-linking of red blood cell membrane proteins induced by oxidative stress in β-thalassaemia. *FEBS Lett.* **85,** 267.

KAY, M.M.B. (1984) Localization of senescent cell antigen on band 3. *Proc. natn. Acad. Sci. USA.* **81,** 5753.

LUTZ, H.U. & WIPF, G. (1982) Naturally occurring autoantibodies to skeletal proteins from human red blood cells. *J. Immunol.* **128,** 1695.

MARCHESI, V.T. (1983) The red cell membrane skeleton: recent progress. *Blood*, **61,** 1.

MARTIN, W.J. & MARTIN, S.E. (1975) Thymus reactive IgM autoantibodies in normal mouse sera. *Nature*, **254,** 716.

MELAMED, M.D., GORDON, J., LEY, S.J., EDGAR, D. & HUGHES-JONES, N.C. (1985) Senescence of a human lymphoblastoid clone producing anti-Rhesus (D). *Eur. J. Immunol.* **15,** 742.

MOLLISON, P.L. & HUGHES-JONES, N.C. (1967) Clearance of Rh-positive cells by low concentrations of Rh antibody. *Immunology*, **12,** 63.

PONCET, P., KOCHER, H.P., PAGES, J., JATON, J.-C, & BUSSARD, A.E. (1985) Monoclonal autoantibodies against mouse red blood cells: a family of structurally restricted molecules. *Mol. Immunol.* **22,** 541.

ROGENTINE, G.N. & PLOCINIK, B.A. (1974) Carbohydrate inhibition studies of the naturally occurring human antibody to neuramidase-treated lymphocytes. *J. Immunol.* **113,** 848.

STECK, T.L., WEINSTEIN, R.S., STRAUS, J.H. & WALLACH, D.E.M. (1970) Inside-out red cell membrane vesicles: preparation and purification. *Science*, **168,** 255.

SHOTTON, D.M., BURKE, B.F. & BRANTON, D. (1979) The molecular structure of human erythrocyte spectrin: biophysical and electron microscope studies. *J. Mol. Biol.* **131,** 303.

VAN DER MEULEN, F.W., VAN DER HART, M., FLEER, A., VAN DEM BORNE, A.E.G. K.R., ENGLEFRIET, C.P. & VAN LOGHEM, J.J. (1978) The role of

adherance to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies. *Br. J. Haematol.* **38,** 541.

VOLLER, A., BIDWELL, D.E. & BARTLETT, A. (1979) The enzyme-linked immunosorbent assay (ELISA): A guide with abstracts of microplate applications. *Dynatech Europe,* Guernsey.

WAHLGREN, M., BERZINS, K., PERLMANN, P. & BJORKMAN, A. (1983) Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of micro-ELISA. *Clin. exp. Immunol.* **54,** 127.

WEATHERALL, D.J. & CLEGG, J.B. (1981) The Thalassaemia Syndromes, 3rd edn. Blackwell, UK.

WICKRAMASINGHE, S.N. (1975) Human bone marrow. Blackwell, UK.

WIENER, E. (1985) A possible role for antibodies against spectrin in the interaction between erythroblasts and macropages *in vitro*. *Br. J. exp. Pathol.* **66,** 17.

WIENER, E. & WICKRAMASINGHE, S.N. (1982) The interaction of DMSO-induced murine Friend leukaemia erythroblasts with cultured syngeneic mouse peritoneal macrophages: an *in vitro* model for the study of ineffective erythropoiesis. *Br. J. exp. Pathol.* **63,** 568.

WIENER, E. & WICKRAMASINGHE, S.N. (1983a) Impaired protein synthesis in erythroblasts enhances their phagocytosis by macrophages. *Br. J. Haematol.* **53,** 117.

WIENER, E. & WICKRAMASINGHE, S.N. (1983b) Interaction between erythroblasts and macrophages *in vitro*: effect of neuraminidase-treatment of erythroblasts and the role of serum factors. *Br. J. Haematol.* **55,** 369.

WIENER, E. & WICKRAMASINGHE, S.N. (1985) The *in vitro* interaction between red cells from patients with homozygous β-thalassaemia and normal human macrophages. *Exp. Haematol.* **13,** 401.

WINCHESTER, R.J., SHU MAN FU, WINFIELD, J.B. & KUNKEL, H.G. (1975) Immunofluorescent studies on antibodies directed to a membrane structure present in lymphocytes and erythrocytes. *J. Immunol.* **114,** 410.