

Small quantities of erythrocyte bound immunoglobulins and autoimmune haemolysis

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SUMMARY Enzyme linked and radioimmune direct antiglobulin tests (DAGTs) were used to assess red cell bound IgG, IgA, and IgM in 585 patients referred to an immunohaematology reference centre. One hundred and fifty eight patients with ≤ 200 mol IgG and small amounts of IgA and IgM coating their red cells were studied in detail. The presence of autoimmune haemolysis was determined from the clinical, haematological, and biochemical findings; it occurred in at least 25% of the 158 patients, the degree varying widely. There was a highly significant association between small increases in cell bound immunoglobulins and the presence of autoimmune haemolysis. Immunoglobulins of IgG, IgA, and IgM classes could produce autoimmune haemolysis when the classical agglutination DAGTs were negative; the IgA and IgM were usually found in association with IgG. The haemolytic effect was enhanced by the presence of complement and combinations of immunoglobulin classes on the red cells.

Autoimmune haemolysis is often associated with comparatively few positive serological findings, and the direct antiglobulin test (DAGT), using agglutination techniques, may be negative,¹ one reason being that although significant amounts of immunoglobulins are bound to the red cells, the amounts are too low to be detected by classical methods.¹ DAGTs, using enzyme linked² and radioimmune techniques,³ have been introduced into routine use in this centre. These tests are of similar sensitivity in that they both detect immunoglobulin at levels found on the red cells of normal subjects; the enzyme linked test is used to detect IgG, IgA, and IgM and the radioimmune assay to quantitate IgG.

In the present study the occurrence of autoimmune haemolysis was assessed in patients with only small quantities of immunoglobulins coating their red cells.

Material and methods

The results of the serological investigations on all patients referred to our immunohaematology reference centre between January 1 1984 and December 31 1985 were examined. Samples were sent because of suspected or previous autoimmune haemolysis or because of difficulties caused by auto-antibodies when grouping or cross matching. Five hundred and eighty five patients with a wide variety

of conditions were seen during this period. Details of the investigations have been reported previously.^{2,4} Quantitative DAGT results were obtained for IgG only, usually by radioimmune assay, but in seven instances values were calculated from the result of the enzyme linked test using a correlation between the two techniques ($r = 0.956$).² Cell bound IgG was considered to be raised if it was above 100 mol/red cell.³ The enzyme linked tests, which assessed IgA and IgM as well as IgG, were considered to be positive when the optical density using patients' cells exceeded that of pooled 0 cells by 0.2²; in the case of IgG the test became positive at about 110 mol/red cell.

One hundred and fifty eight patients had ≤ 200 of IgG/red cell and were selected for detailed study—this was irrespective of the IgA and IgM coating. The enzyme linked test showed that the amounts of IgA and IgM were also small and were rarely detected by the standard agglutination DAGT. The presence of haemolysis was determined from the clinical findings and results of laboratory investigations.⁵ These investigations included the haemoglobin concentration, reticulocyte count, blood film appearance, the presence of marrow erythroid hyperplasia, red cell lifespan, and measurement of serum haptoglobins, bilirubin and lactic dehydrogenase. The serological profile was not allowed to prejudice the decision.

The results of the enzyme linked DAGTs were used to examine the relation between small increases in red

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cell bound immunoglobulins (as judged by a positive test for IgG, IgA, or IgM) and the presence of haemolysis. A χ^2 test⁶ was carried out and the confidence interval for the difference between the population proportions was calculated from the standard error of the difference.⁷

Results

The one hundred and fifty eight patients were divided into three main groups depending on the certainty with which autoimmune haemolysis could be ascribed. Patients with confirmed autoimmune haemolysis (irrespective of its severity) were placed in group 1 and those in whom haemolysis was absent or unlikely in group 3. Group 2 contained cases in which the quantity and strength of available information did not permit a firm decision, but suggested that haemolysis was probable or possible. Group 3 included several patients undergoing routine follow up for previously diagnosed autoimmune haemolysis. Table 1 shows these groups and the results of the agglutination, radioimmune, and enzyme linked DAGTs. Subgroupings were made on the serological nature of the autoantibodies present.⁴ The strength of these autoantibodies varied; the reactions tended to be

strong in patients with florid cold agglutinin disease, but were usually weak when warm reacting, particularly in patients in group 3 in whom haemolysis was absent or unlikely. "Associated disorders" (table 1) refers to those conditions where there is a predisposition for autoimmune haemolysis to develop,⁴ and "chronic disorders" to those in which a characteristic "sideropenic anaemia with reticuloendothelial disorder" is commonly found⁸; the same types of disorder often feature in both categories. In the present study these conditions included acute and chronic infections, collagen and other immune based diseases (rheumatoid arthritis, systemic lupus erythematosus, scleroderma, ulcerative colitis, diabetes mellitus), neoplasia (acute myeloid and lymphocytic leukaemias, chronic lymphocytic leukaemia, non-Hodgkin's lymphoma and carcinomata), myelodysplastic states, tissue disruption (chronic inflammation, trauma) and pregnancy.

Statistical analysis showed a highly significant association between small increases in cell bound IgG, IgA, and IgM and the presence of haemolysis when patients in groups 1 and 3 were compared. There were also significant differences between groups 2 and 3, but these were not found between groups 1 and 2 (table 2).

Table 1 *Haemolytic state, serological findings, and results of direct antiglobulin tests*

<i>Patient group (No of patients)</i>	<i>No of patients with positive* agglutination DAGTs and nature of cell coating (No tested)</i>	<i>No of patients with raised* cell bound IgG using radioimmune DAGT (No tested)</i>	<i>No of patients with positive* enzyme linked DAGTs and nature of cell coating (No tested)</i>	<i>Comments</i>
1 Haemolysis confirmed:				
Patients with warm autoimmune haemolysis (20)	C3d 6; IgG + C3d 7 (20)	15 (20)	IgG 8; \$ IgM 1; IgG + IgM 2; IgG + IgA 1 (18)	} 16 patients had disorders known to predispose towards development of autoimmune haemolysis (mostly chronic lymphocytic leukaemia and non-Hodgkin's lymphoma); patients with paroxysmal cold haemoglobinuria 5 years or younger \$ Monomeric IgM
Patients with cold autoimmune haemolysis				
Cold agglutinin disease (9) Paroxysmal cold haemoglobinuria (4)	C3d 9 (9) IgG 1; C3d 3; (4)	4 (9) 3 (4)	IgM 4 (9) 0 (2)	
Patients with mixed autoimmune haemolysis (6)	C3d 2; IgG + C3d 4 (6)	6 (6)	IgG 2; IgG + IgM 3; IgG + IgA 1 (6)	
2 Haemolysis probable or possible				
Patients with warm auto/pan antibodies (31)	IgG 9; C3d 6; IgG + C3d 3 (31)	19 (29)	IgG 13; IgM 1; IgG + IgA 2; IgG + IgM 1 (29)	} Complicated cases; often strong clinical suspicion of haemolysis, most patients suffering from chronic disorders, 3 had acute myeloid leukaemia; severity of illness often made assessment of haemolysis difficult
Patients with cold auto/pan agglutinins (14)	C3d 8; IgG + C3d 1 (14)	3 (12)	IgG 2; IgM 1; IgG + IgA + IgM 1 (13)	
3 Haemolysis absent or unlikely				
Patients with warm auto/pan antibodies (19)	IgG 1; C3d 5; IgG + C3d 2 (19)	6 (16)	IgG 5; IgA 1 (18)	} Patients referred with a history of previous autoimmune haemolysis, for investigation of possible haemolysis, or following grouping or cross matching difficulties
Patients with cold auto/pan agglutinins (21)	C3d 12 (21)	4 (21)	IgM 3; IgA 1 (21)	
Patients with warm and cold auto/pan antibodies (16)	IgG 1; C3d 3; IgG + C3d 4 (16)	9 (16)	IgG 8 (14)	
Patients with no auto/pan antibodies (18)	IgG 1; C4 1 (18)	5 (18)	IgG 2; IgM 1 (16)	

*Negative results or normal concentrations can be derived from No tested.

Table 2 Statistical analysis comparing haemolytic state and small increases in cell bound IgG, IgA, and IgM, judged by positive enzyme linked DAGTs

Patient group	χ^2	pvalue	Confidence interval (%)
1 v 3	11.45	<0.001	0.06 to 0.54 (99)
2 v 3	5.12	<0.025	0.01 to 0.39 (99)
1 v 2	1.85	>0.1	

1 degree of freedom.

Discussion

The patients included in the present study form an important group, accounting for 27% of new cases referred to our immunohaematology laboratory. Previous reports have tended to select patients with obvious haemolysis and a negative agglutination DAGT^{1,9-12}; these cases represent only the extreme of a spectrum of complicated clinical disorders, and to put them into perspective we looked for autoimmune haemolysis in all patients in whom the red cells were coated with ≤ 200 mol IgG/cell, a value chosen to be at the borderline of sensitivity for the agglutination DAGT. Table 1 shows that haemolysis is common in such patients, certainly occurring in 25% (group 1) and probably in many more, if group 2 patients are considered. It also shows that the agglutination DAGT does not provide sufficient information to assist in the diagnosis and clinical management, and it emphasises the need to use the more sensitive tests. At their present stage of development we feel that both radioimmune and enzyme linked techniques are needed; they give similar results in individual cases, though slight variations around 100 mol IgG/red cell may show as apparently discrepant results (table 1).

The study had many problems. The degree of haemolysis varied considerably between patients: there was often difficulty in determining its presence, particularly if mild or compensated, or in patients with chronic disorders or serious conditions where the effect of blood loss, treatment, and blood transfusion had to be considered. In these cases haemolysis, even if present, was not the main clinical problem, and often only a limited number of the investigations listed in the material and methods section were carried out. Certain laboratory results must be considered with caution—low haemoglobin concentrations may be due to other causes, and normal haptoglobin concentrations and absence of reticulocytosis do not exclude haemolysis.⁵ Measurement of red cell survival (a definitive assessment of haemolysis) was rarely carried out. A “best decision” into which group to place the patient had therefore often to be made on limited data—hence the need

for group 2 (table 1). The statistical analysis (table 2), however, indicates that this does not affect the overall findings of the study, group 2 showing a significant difference from group 3 but not from group 1.

There was a highly significant association between small increases in cell bound immunoglobulins and the presence of autoimmune haemolysis (table 2). Correlation, however, was not absolute, the development of haemolysis depending on a complexity of interrelated factors. In patients with paroxysmal cold haemoglobinuria and cold agglutinin disease (table 1), small quantities of cold autoantibodies (of IgG and IgM classes, respectively) are known to activate complement, resulting in florid intravascular haemolysis; the cells remaining are coated with C3d.¹³

In other instances (table 1) increased amounts of IgG were present on the red cells of patients with active autoimmune haemolysis, even though the agglutination DAGT for IgG was negative; these represent the classical “Coombs’s negative” type of case.¹⁹ Although the rate of haemolysis generally corresponds to the amount of red cell bound IgG when larger quantities are entailed,¹⁴ the present findings show that the association is much more variable with small amounts, and other factors are clearly important: small quantities of IgG are known to activate complement,¹⁵ the effect of IgG being increased by the binding of C3 components, which significantly reduce the number of IgG molecules necessary to initiate haemolysis¹⁶; such cases are included in group 1 (table 1). Also shown (table 1) are examples where immunoglobulins of IgA and IgM classes helped to cause haemolysis, usually in association with IgG. Combinations of immunoglobulin classes, which are often detected when sensitive enzyme linked DAGTs are used, can act synergistically²; their presence is often associated with severe haemolysis.¹⁷ We consider that multiple immunoglobulin coating and the augmentation by complement were particularly important in producing haemolysis in the patients studied here (group 1, table 1).

Other factors known to be important in the development of haemolysis include IgG subclass and mononuclear phagocyte activity.¹³ No conclusions regarding IgG subclass could be drawn in the present study as the antisera currently available rarely give satisfactory results with small amounts of cell bound IgG. IgG3 is considered to be particularly liable to cause “Coombs’s negative” autoimmune haemolysis,¹⁸ the Fc receptors on mononuclear phagocytes having a higher affinity for IgG3 than IgG1.¹⁹ We have seen, however, a healthy 19 year old blood donor with 2300 mol IgG3/red cell and no evidence of haemolysis. Tests for mononuclear phagocyte activity do not readily lend themselves to routine use and insufficient results were available for the present

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investigation. In previous reports enhanced function^{20,21} and increased numbers of Fc receptors²² have been found in patients with autoimmune haemolysis; conversely, haemolysis may not be evident in patients with impaired reticuloendothelial function, even though their cells are coated with large amounts of IgG.²³

Other points of interest arose from the present study. Many patients were suffering from chronic disorders. The associated anaemia is known to have a haemolytic element,⁸ and the findings (table 1) suggest that increased cell bound immunoglobulins, as well as the increased reticuloendothelial function reported previously,⁸ may be responsible. Clearance of circulating immune complexes is a physiological function of red cells,²⁴ and increased amounts of cell bound immunoglobulins may be due to immune complexes rather than to autoantibodies; this phenomenon has been particularly noted in patients with rheumatoid arthritis and systemic lupus erythematosus.²⁵ Where patients with similar disorders in our series were investigated, however, and in another report of patients with systemic lupus erythematosus,¹⁵ red cell bound IgG was confirmed to be autoantibody by elution and re-adsorption on to normal cells. In several patients, particularly those in whom haemolysis was absent or unlikely (groups 3, table 1), increased plasma immunoglobulins may have produced non-specific adsorption on to the red cells. This finding has been described previously, serum concentrations sometimes being high enough to cause a positive agglutination DAGT.²⁶ How non-specific adsorption of IgG or binding of immune complexes affect erythrocyte survival is not known.

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