The pathology of autoimmune haemolytic anaemia

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Introduction

Autoimmune haemolytic anaemia (AIHA) can be defined as a reduced haemoglobin concentration, resulting from a shortened red cell lifespan, caused by autoantibodies directed against antigens on the patient's erythrocytes. Although AIHA is usually considered to be a well defined clinical syndrome, the more closely cases are examined the more obvious it becomes that severe anaemia is one extreme of a fundamental disturbance of immune homeostasis; at the other end of the spectrum are those in whom erythrocyte autoantibodies are the only abnormality found. The degree of haemolysis can vary considerably and as it often occurs in the absence of anaemia, we prefer the term autoimmune haemolysis (AIH). Detecting its presence often causes enormous difficulties, particularly if the condition is mild or in patients with chronic disorders or serious conditions where the effects of haemorrhage, treatment, and blood transfusion, as well as other causes of anaemia, have to be considered. In such circumstances AIH is not the main problem and confirmatory investigations will probably not be carried out. The

above points should be borne in mind when considering published findings which have so far tended to favour reports of patients with obvious haemolysis and those cases with interesting disease or drug associations.

For this article, we have selected some aspects of the pathology of AIH which are considered to be of current interest, namely classification, the concept of secondary AIH, incidence, and factors which influence red cell destruction. We have related them to our own experiences of 1834 patients referred either because AIH was suspected or because erythrocyte autoantibodies were causing difficulties in blood grouping and cross-matching. The patients were seen over a 10 year period from 1 January 1982 to 31 December 1991 and were drawn from a population of roughly 4.7 million people living in an area of 16 500 km².

Classification and the concept of secondary autoimmune haemolysis

The 1834 patients were classified according to serology and disease association (table 1).^{1,2} As well as the traditional warm and cold autoantibody types, a separate category of mixed AIH is included where both warm and cold autoantibodies are present and their reactions are such that both are deemed capable of causing haemolysis.³ The distribution of cases by serological group is shown in table 2.

The concept of secondary AIH is complicated. It may mean that the association with another disease occurs more often than can be explained by chance alone (for example, lymphoid neoplasms, systemic lupus erythematosus, *Mycoplasma* infections); or that the AIH resolves when the associated disease is corrected (for example, some ovarian neoplasms); or that the two conditions are different parts of a complex immunologically mediated multisystem disorder (such as ulcerative colitis); or that there may be a combination of these factors. In other situations it can be more difficult to judge whether the occasional association of AIH with a relatively common disorder, such as acute myeloid leukaemia, pernicious anaemia, cirrhosis, carcinoma, rheumatoid arthritis, thyroid disease, diabetes mellitus and certain infections, is clinically important or whether this should be

Table 1 Classification of 1834 patients with AIH, based on serological findings and disease association

Condition	Warm AIH	Cold AIH	PCH	Mixed AIH
Idiopathic AIH	617	344	11	82
Drug related AIH	140	0	0	0
Neoplasia				
Lymphoid neoplasms				
Chronic lymphocytic leukaemia	63	13	0	0
Acute lymphoblastic leukaemia	1	4	0	0
Non-Hodgkin's lymphoma	32	22	0	8
Angioimmunoblastic lymphadenopathy	1	1	0	0
Myeloma	4	1	0	0
Macroglobulinaemia	2	3	0	0
Thymoma	1	0	0	0
Hodgkin's disease	10	4	0	4
Ovarian tumours	4	0	0	0
Carcinoma (non-ovarian)	67	23	0	6
Acute non-lymphoid leukaemias	5	4	0	0
Chronic myeloid leukaemia	5	1	0	1
Myelofibrosis	12	0	0	3
Myelodysplastic syndromes	23	4	0	3
Other neoplasms	3	2	0	0
Infection				
Pneumonia— <i>Mycoplasma</i>	0	29	0	1
Pneumonia—"viral" and unspecified	2	13	0	1
Infectious mononucleosis	0	6	0	0
Infectious hepatitis	1	1	0	0
Tuberculosis	0	1	0	0
Syphilis	0	0	1	0
Infection—unspecified	13	11	7	3
Collagen diseases				
Systemic lupus erythematosus	17	8	0	3
Rheumatoid arthritis	34	9	0	3
Polyarteritis nodosa	1	0	0	0
Other immune based and miscellaneous disorders				
Ulcerative colitis	19	1	0	1
Thyroiditis	3	1	0	1
Myxoedema	1	0	0	0
Chronic active hepatitis	2	0	0	0
Pernicious anaemia	7	1	0	0
Diabetes mellitus	8	3	0	0
Sarcoidosis	1	0	0	0
Myasthenia gravis	1	0	0	0
AIH associated with pregnancy	22	15	0	1
AIH associated with chronic renal failure	29	12	0	6

PCH: paroxysmal cold haemoglobinuria.

Table 2 Serological distribution of 1834 patients with AIH

Autoantibody type	Number (%)
Warm	1151 (62.8)
Cold	
Cold agglutinin disease	537 (29.3)
PCH	19 (1.0)
Mixed (warm + cold)	127 (6.9)

Incidence of AIH per 100 000 of population at risk, related to age at presentation. The early childhood peak of cold AIH (arrow) is largely due to patients with PCH.

regarded as a chance occurrence. These considerations (and the accuracy of diagnosis on the referral form) should be borne in mind when considering table 1, which classes 43% of the cases as secondary. One recently recognised association is that of the myelodysplastic syndromes (table 1⁴); now that these disorders have been more clearly defined,⁵ the association is increasingly being recognised. Myelodysplastic syndromes did not feature in a large series of patients with AIH in 1981,¹ but in 1991 13% of all samples tested were from individuals with this diagnosis.

Incidence

The incidence of AIH per 100 000 of the population at risk, related to age at presentation, was calculated for the 1834 patients (table 1). The results show that, except for a childhood peak of cold AIH, there is a general increase in incidence throughout life, with a dramatic rise occurring after 50 (figure). The figures equate with incidences of about 1 in 25 000, overall, and 1 in 41 000 for warm AIH; the latter is greater than the usually quoted minimal annual incidence of 1 in 80 000 for this group.⁶ The difference is probably due to improvement in recognising mild and compensated forms. Table 3 gives the serological profiles of the children and adolescents and shows that the early childhood peak of cold AIH (figure) is largely due to patients with paroxysmal cold haemoglobinuria (PCH) caused by Donath-Landsteiner antibodies. Eleven of the 19 patients with this diagnosis were under 5 years old (table 3). AIH is more common in females, even when sex differences in the general population are taken into account; the male:female ratio in our patients was 1:1.3.

Table 3 AIH in childhood and adolescence

Age (years)	Warm AIH	Cold AIH	PCH*	Mixed AIH
0-4	8	7	11	1
5-9	2	9	1	0
10-15	6	1	0	1

*Secondary in seven patients ("Infection—unspecified" in table 1)

Factors influencing immune mediated red cell destruction

In over 80% of patients with AIH erythrocyte destruction is extravascular and involves red cells coated with antibody or C3b, or both, reacting with mononuclear phagocytes via specific receptors. Less frequently, haemolysis is intravascular and results from complement being fully activated or, more controversially, through the coated erythrocytes interacting with receptors on lymphoid cells or granulocytes. Receptors consist of heterogeneous groups of glycoproteins; in the case of IgG, for example, three distinct biochemical entities have been defined with differing properties and effector cell expression⁷—FcγRI is found only on mononuclear phagocytes, but FcγRII and FcγRIII are also present on granulocytes and certain lymphocyte fractions.⁸ Many factors interact in a complex manner to induce red cell destruction. We intend to consider the activity of the mononuclear phagocyte and other cellular systems, the characteristics of the target antigens, the autoantibody response and complement in this article.

THE MONONUCLEAR PHAGOCYTE SYSTEM

Immune haemolysis is induced by attachment of bound immunoglobulin or complement to specific macrophage receptors; adherence leads to erythrocyte damage by antibody dependent cellular cytotoxicity (ADCC) and by erythrophagocytosis, incomplete engulfment leaving a non-phagocytosed portion which becomes a rigid spherocyte and is prematurely destroyed in the spleen. Whether ADCC or erythrophagocytosis is the prime mechanism remains to be determined, though both cause considerable haemolysis in vivo. The amount of cell bound antibody seems to be important; with low amounts erythrophagocytosis becomes predominant, the level at which this occurs being much lower for IgG3 antibodies than for IgG1. Assessment of mononuclear phagocyte-red cell interactions is rarely carried out in patients with AIH as a routine. Most previous investigations have used peripheral blood monocytes (rather than the tissue macrophages which have the major role in vivo) and have shown that red cells from patients with active warm type AIH had increased adherence, rosetting, and phagocytosis per monocyte compared with those from normal subjects. The reactions were further enhanced where complement was detected on the red cells in addition to IgG.

The degree of mononuclear phagocyte activation has an important effect on red cell destruction. Increased activity occurs in certain conditions, including AIHA and various infections⁹; in AIHA, autologous monocytes showed enhanced rosetting and erythrophagocytosis compared with normal monocytes exposed to the same IgG coated red cells. The most consistent increase in activity was seen when the red cells were coated with both IgG and complement. AIHA has been reported following viral infections, vaccination, and immunisation, and one might speculate that these events tipped the balance to overt hae-

molysis in patients who had small quantities of IgG already on their cells by increasing mononuclear phagocyte activity.⁹ In vitro an increase in rosette formation has been noted during the course of viral infections, and in experimental studies increased clearance of IgG sensitised red cells was found in animals infected with Bacille Calmette-Guérin.⁹

Decreased mononuclear phagocyte activity can also occur. For example, 15% of patients taking methyl dopa developed IgG autoantibodies to red cells yet only 0.5% showed active haemolysis.⁹ Studies using radiolabelled red cells suggested that methyl dopa suppressed mononuclear phagocyte function in most patients and that where this did not occur, the cells were lysed. When patients with methyl dopa induced autoantibodies were followed up sequentially, significant in vitro monocyte erythrophagocytosis was seen only during periods of haemolysis. In another example, antibody coated red cells, which were rapidly destroyed in all normal subjects, survived normally in some patients with systemic lupus erythematosus (SLE); it was suggested that immune complexes (DNA:anti-DNA) were saturating the macrophage Fc receptors and this would explain why certain individuals with SLE whose red cells are coated with autoantibody do not experience haemolysis.⁹ A reduction in the number of monocyte Fc γ receptors following glucocorticoid treatment has been reported in patients with AIH and in normal volunteers. In the former group rosette formation, which was evident in untreated patients, was usually absent in those taking more than 30 mg of prednisolone a day.²

IMMUNE DESTRUCTION OF ERYTHROCYTES

MEDIATED BY LYMPHOID CELLS AND GRANULOCYTES

Although the role of lymphoid cells and granulocytes in immune mediated haemolysis is controversial,² there have been suggestions that in patients with AIH significant red cell destruction can be caused by lymphocytes and, compared with normal individuals, that such patients have more aggressive killer cell activity. In vitro, killer or null cells, which possess receptors for IgG and C3, can induce ADCC which is almost as efficient as that promoted by monocytes and is similarly more effectively mediated by IgG3 antibodies than by IgG1.

Neutrophil granulocytes also carry Fc receptors for IgG, although they normally show little cytotoxicity towards antibody coated red cells. However, considerable activity may be seen in granulocytes during infections and this may in part explain the increased severity of AIH at these times. Granulocyte erythrophagocytosis can be quite prominent in patients with PCH.²

THE TARGET ANTIGENS

The quantity and distribution of the target antigens also have an important effect on immune mediated haemolysis, and recent studies in patients with warm type AIHA have shown a wide variety of antigen sites on the red cell membrane. Serological specificity within the Rh system is common and in a recent

investigation, eluted autoantibodies from more than 25% of cases reacted with the same polypeptides as those identified with specific anti-Rh monoclonal antibodies.¹⁰ In other studies the autoantibodies reacted with band 3, the main membrane glycoprotein,¹¹ or had anti-phospholipid activity.¹² In cases showing anti-Gerbich specificity the antigenic determinants were identified on the β sialoglycoproteins,^{13,14} while autoantibodies showing anti-Sc1 specificity reacted with a membrane antigen of 60–65 kilodaltons.¹⁵ There have been similar findings in canine AIHA where autoantibodies recognised a combination of 29 and 42 kilodalton peptides, possibly equivalent to the human Rh complex, as well as an autoantigen having the same molecular mass as band 3.¹⁶

Exposure of the T cryptantigens can occur as a result of microbial infection; this leads to polyagglutinability of the red cells and, in some cases, to severe intravascular haemolysis due to the interaction with natural anti-T causing complement activation.¹⁷

Antigen depression is occasionally found in patients with AIHA; in such cases the autoantibodies can masquerade as alloantibodies. This is a rare phenomenon but seems to occur most frequently in relation to the Kell¹⁸ and Gerbich^{14,19} blood group systems. Depression of the Rh antigens has also been reported^{18,20}; large amounts of autoantibody were demonstrated in the serum but the direct antiglobulin test was negative. The autoantibody seemed to have destroyed most of the red cells and only the small proportion with a reduced Rh antigen status could survive in vivo. The antigen depression, which was transient, was thought to represent a protective mechanism against haemolysis.^{18,20} Patients with cold haemagglutinin disease due to anti-I or i red cells coated solely with C3d,g have a normal lifespan. The erythrocytes are protected against premature destruction because the complement binding and Ii antigen sites are in close proximity. Bound C3b, which can effect red cell destruction, is rapidly converted to C3d,g, which sterically inhibits the attachment of more autoantibody and hence fixation of further active C3b on to the cells. The result is a dual red cell survival curve in most patients, a population of rapidly destroyed cells being present in conjunction with one having a normal lifespan. C3d,g protection does not extend to cases where the autoantibodies have other specificities.²

AMOUNT OF ANTIBODY BOUND TO THE RED CELLS AND EFFECT OF IMMUNOGLOBULIN CLASS AND SUBCLASS

The immune response in AIH is more complex than commonly supposed, and multiple immunoglobulin classes and subclasses, as well as complement components, are frequently bound to the red cells.^{21,22} To assess the role and interrelations of individual factors, studies must be performed using sensitive methods and strictly defined population groups. In one such investigation, where only non-complement binding IgG1 autoantibodies were

Table 4 Pattern of IgG subclasses eluted from red cells in 426 samples from 304 patients with warm autoantibodies

IgG subclasses	Number
1	273
2	1
3	5
1 + 2	31
1 + 3	31
1 + 4	22
2 + 4	1
1 + 2 + 3	18
1 + 2 + 4	18
1 + 3 + 4	6
1 + 2 + 3 + 4	20

Table 5 Immunoglobulin classes bound to red cells in 590 samples from 404 patients with warm autoantibodies

Immunoglobulins	Number
IgG	372
IgG + IgM	137
IgG + IgA	34
IgG + IgM + IgA	47

present, haemolysis was determined predominantly by the quantity of antibody coating the cells—the larger the amount the greater the degree of haemolysis, with a haemolytic threshold at about 1000 molecules of IgG per cell.²³

The distribution and effects of IgG subclasses in AIH were studied in concentrated red cell eluates from 304 patients on 426 occasions.²² Multiple subclasses were found in 147 (34.5%) of the samples (table 4), a higher incidence than in previous reports.²⁴ This was probably because examining eluates is more sensitive than testing the red cells directly.²² IgG1 was by far the most common, being present in 419 (98%) samples overall and in 273 out of the 279 where only one subclass was identified (table 4). This pattern of results precluded comparing individual subclasses, but did permit examination of the effects of multiple versus single subclass coating. As multiple IgG subclasses were significantly associated with the presence of multiple immunoglobulin classes (which have a haemolytic effect in their own right²¹), further analyses were carried out using samples where IgG alone coated the red cells. In these circumstances multiple subclasses were significantly associated with higher amounts of cell bound IgG (> 800 mol/red blood cell) and more cases had haptoglobin concentration of < 0.1 g/l. Reduced haptoglobin concentration were not significantly associated with the presence of IgG3 unless the cases with multiple immunoglobulins were included in the analysis. Studies which reported that overt AIH occurred in virtually all cases where IgG3 was

present,^{24 25} did not take into account any effects due to associations with multiple immunoglobulin classes and subclasses.

The IgG subclasses have different interactions with mononuclear phagocytes which are thought to be the result of their varying affinity for Fc receptors, rather than there being specific receptors for each subclass.⁹ IgG3 antibodies have a higher affinity for mononuclear phagocyte receptors than IgG1 antibodies, with IgG2 and IgG4 binding much less efficiently still. This can be shown in vitro where monocyte reactions with IgG3 coated erythrocytes are much more rapid than with IgG1, rosette formation is greater, and fewer molecules are required to initiate erythrophagocytosis.

The effects of immunoglobulin class in warm type AIH were investigated in 590 samples from 404 patients.²¹ Using the very sensitive enzyme linked antiglobulin test, the red cells of 218 (37%) samples were found to be coated with increased amounts of IgM or IgA in addition to IgG (table 5). Compared with cases where red cells were coated with IgG alone, the presence of multiple immunoglobulins was significantly, associated with cases where larger amounts of IgG (> 800 mol/red blood cell), multiple IgG subclasses, IgG3, and C3d were bound to the cells, and where haptoglobin concentrations were < 0.1 g/l. The latter association was still significant when cells were coated with low concentrations of IgG (< 400 mol/red blood cell) of a single subclass (IgG1 of 98% of cases), and was not due to the presence of cell bound complement. The study concluded that multiple immunoglobulin coating, even when undetected by agglutination methods, was a major cause of haemolysis. Although it was part of a more generalised autoimmune response and acted with other factors such as the quantity of bound IgG, the IgG subclass pattern and complement, it also had an important haemolytic effect in its own right.²¹

Cases of severe AIHA have been reported where IgA was the sole immunoglobulin bound to the red cells, but in our experience using enzyme-linked antiglobulin tests, other immunoglobulin classes have usually also been present in subagglutinating amounts. IgA class autoantibodies probably induce haemolysis via Fc receptors in the same way as IgG. Specific receptors for IgA have been demonstrated on monocytes,^{24 26} and spherocytosis has been reported in patients with IgA coated red cells, splenic sequestration occurring when such cells were labelled with ⁵¹Cr and used for survival studies.²⁵ In vitro, IgA antibodies have been shown to augment the effect of IgG induced ADCC.²⁶

It is still controversial whether IgM autoantibodies can cause immune mediated haemolysis other than by activating complement, and if they do, how this is possible, as macrophages are said to lack specific receptors for IgM.⁹ Nevertheless, there are reports of AIHA that seemed to be associated with non-complement activating IgM autoantibodies,²⁷ and we have felt for some time that IgM coating acts

Table 6 Patients with warm type red cell autoantibodies: relation between presence of IgM coating and serum haptoglobin concentration

	Immunoglobulins coating red cells	
	IgG + IgM	IgG
Proportion with haptoglobins < 0.1 g/l	0.44	0.09
Number of samples in total population	18	150
χ^2		14.5*
95% confidence interval for difference between proportions		0.12 to 0.58

*highly significant ($p < 0.0005$) at 1 degree of freedom

synergistically with IgG in effecting red cell destruction.

To provide further information on this point, we examined the records of the 1834 patients (table 1) and selected those with only warm autoantibodies and where the red cells were coated with < 800 mol/red blood cell of IgG1, but not with other IgG subclasses, complement, or IgA. Cohorts were distinguished by the presence or absence of cell bound IgM and analysed for their association with haptoglobin concentrations of < 0.1 g/l and ≥ 0.5 g/l by a χ^2 procedure described previously.^{21, 22} One hundred and sixty eight cases were included in the study; their distribution is shown in table 6, together with the results of the statistical analysis. The significantly higher ($p < 0.0005$) proportion of cases in the IgG + IgM group with haptoglobin concentrations of < 0.1 g/l, compared with the IgG group, provides substantial evidence for warm IgM autoantibodies being able to induce red cell destruction independently of any effect through complement. Elucidating the mechanisms of such haemolysis will be an important and fascinating area for future investigations.

THE ROLE OF COMPLEMENT

Complement mediated haemolysis is usually extravascular. Activation is often triggered, but regulatory inactivators operate at several points on the complement cascade causing inhibition, usually at the level of C3, so that red cells circulate with C3b or iC3b bound on their surface. Destruction is then by phagocytosis (or through the production of spherocytes) and occurs mostly in the liver macrophages. Although the binding of C3b coated cells to macrophage CR1 or CR3 receptors is very efficient, maximal adherence and phagocytic capacities (the former considerably greater than the latter) limit the amount of destruction. Many erythrocytes are engulfed immediately, but others slowly return to the circulation due to cleavage of the fixed C3b by naturally occurring regulatory factors to produce C3d,g (which is further reduced in vitro to C3d). Macrophages do not react efficiently with C3d,g in vivo and, as already mentioned, the red cells may now survive normally, even though the direct antiglobulin test is strongly positive. Erythrocytes coated solely with C3b do not undergo ADCC, but complement coating significantly reduces the number of IgG molecules needed to initiate this process, the augmentation persisting after degradation of C3b to C3d.²⁸

There is some evidence that red cell bound complement has a quantitative effect in relation to haemolysis. This is difficult to assess in vitro as the C3b coated cells have either been destroyed or the C3b converted to C3d,g. Nevertheless, it is thought that about 550–800 bound C3b molecules are required to activate the hepatic clearance mechanism, and in one study most patients with more than 1100 C3d molecules per red cell had an associated haemolytic anaemia²⁹—presumably because of a proportional relation with the number of C3b molecules originally bound to the cells.

Intravascular haemolysis is rare and only occurs if activation of red cell bound complement proceeds to completion: it is seen in less than 20% of patients with AIHA, but is the most efficient method of red cell destruction and can produce severe anaemia. Antibodies which trigger this process are mostly of IgM class, although it can also be caused by some warm IgG1 and IgG3 antibodies; the classic IgG example, however, is the cold-reacting Donath-Landsteiner antibody. In spite of IgM class antibodies being better at activating complement than IgG, those found in the cold agglutinin syndrome are far less efficient than Donath-Landsteiner antibodies. As a result, severe intravascular haemolysis, a common feature in PCH, is not obvious in most patients with cold haemagglutinin disease.⁹ This may be due to differences in affinity of the two types of antibody; cold reacting IgM autoantibodies rapidly leave the red cells when the temperature increases to 37°C (the optimal temperature for complement activation), whereas Donath-Landsteiner antibodies stay bound for longer and are thus better able to trigger activation.⁹ Intravascular haemolysis occurs about 10 times more frequently in children and this seems to parallel an increase in AIHA secondary to infection. Possibly, autoantibodies stimulated by viruses or bacteria are more efficient at activating complement, or perhaps the pathogens make the red cell more susceptible to its action.⁹ Infection is an important factor in the development of PCH,² and it is of interest that although this type of haemolysis is rare, accounting for only about 1% of all our patients (table 1), in children 26% of cases were in this group (table 3).

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