Two Distinct Categories of Warm Autoantibody Reactivity With Age-Fractionated Red Cells

By Donald R. Branch, Ira A. Shulman, Anita L. Sy Siok Hian, and Lawrence D. Petz

Using age-fractionated erythrocytes, warm autoantibodies can be classified into two distinct categories, depending on their reactivity with reticulocyte-enriched (younger) or reticulocyte-poor (older) red cell fractions. The strength of the direct antiglobulin test (DAT) on the age-fractionated red cells of 24 patients indicated that 19 (79%) had an IgG warm autoantibody that reacted preferentially with older red blood cells. In 7 of these 19 patients (37%), the DAT was negative using reticulocyte-enriched red cell fractions. We have termed this preferential reactivity of warm autoantibodies with older red cells as type I. Five of the 24 patients studied (21%) had an IgG warm autoantibody that demonstrated no preference for young or older red cells. We have termed this pattern of warm autoantibody reactivity as type II. All 5 patients having type II warm autoantibodies had severe anemia. In contrast, 6 of 19 patients having type I warm autoantibody did not have clinical evidence of anemia when tested, and 11 of the 19 had only slight to moderate anemia. Additionally, our results using type I warm autoantibody raise questions regarding the blood group specificity of warm autoantibodies. The antigen recognized by type I warm autoantibody may be a cryptantigen. Rh specificity or relative Rh specificity, often associated with warm autoantibodies, may simply be a coincidental finding.

UNTIL 1953, warm autoantibodies were generally considered to be "nonspecific," reacting with all human red cells tested.[1] However, in that year, Wiener and coworkers[2,3] suggested that these autoantibodies might be directed against the "nucleus of the Rh-hr substance," as some similarities to Rh alloantibodies had been observed. Shortly thereafter, it was discovered that some warm autoantibodies could be subdivided into two groups; one that reacted with the rare Rh phenotype –D– and the other that did not.[4] In 1963, Weiner and Vos, based on reactivity using –D– and the rare Rh_{null} red cells, concluded that most warm autoantibodies had a specificity within the complex Rh blood group system.[5] Since then, many investigators have confirmed these findings and have attempted to classify warm autoantibodies based on their reactivity with various Rh phenotypes, including –D– and Rh_{null},[6–8] or by showing a preferential reactivity or "relative specificity" when using certain Rh phenotypes.[8]

Using density gradient centrifugation for red cell age-fractionation, we have discovered that warm autoantibodies can also be classified according to their reactivity with reticulocyte-enriched or reticulocyte-poor red cell fractions. Many warm autoantibodies react less strongly or do not react at all with reticulocyte-enriched red cell fractions. This finding raises questions of the blood group specificity of these warm autoantibodies.

MATERIALS AND METHODS

Isolation of Reticulocyte-Rich and Reticulocyte-Poor Red Cells

Whole blood from patients suspected of having warm antibody autoimmune hemolytic anemia (AIHA) or from normal donors was collected into EDTA anticoagulant and fractionated on density gradients comprised of Percoll and Renografin-60, as has been previously described.[9,10] For certain testing using patients' sera or eluates, the red cells of one patient with reticulocytosis who had a nonimmunologic hemolytic anemia and a negative direct antiglobulin test were fractionated to obtain a highly reticulocyte-enriched red cell population.

Serologic Studies

Direct and indirect antiglobulin tests were performed using licensed commercial antisera and standard serologic techniques.[11] Antiglobulin test titrations were performed using serial doubling dilutions of the antisera, as previously described.[11] The apparent specificity or "relative specificity" of the warm autoantibodies was determined by tests using the patient's serum or antibody eluted from autologous red cells with panels of phenotyped red cells and/or by titration against R_1R_1 (CDe/CDe), R_2R_2 (cDE/cDE), and rr (cde/cde) red cells.[11] Grading and scoring of reactions was done in the manner of Petz and Branch.[11] Eluates were prepared using chloroform, as previously described.[12] Tests using –D– and Rh_{null} red cells were kindly performed by Virginia Vengelen-Tyler of the Los Angeles Red Cross Blood Services.

RESULTS

The results of direct antiglobulin tests (DAT) on age-fractionated red cells from 24 patients are shown in Table 1. Based on differences of at least 10 in DAT titration scores and/or a negative DAT of reticulocyte-enriched compared to reticulocyte-poor red cell fractions, the antibody in 19 (79%) of the patients reacted preferentially with reticulocyte-poor red cell fractions

From the Department of Clinical and Experimental Immunology, City of Hope National Medical Center, Duarte, CA, and the Blood Bank and Transfusion Service, Los Angeles County-University of Southern California Medical Center, Los Angeles, CA.

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Address reprint requests to Donald R. Branch, MT(ASCP)SBB, Department of Clinical and Experimental Immunology, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010.

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Table 1. Results of Direct Antiglobulin Tests Using Patients' Age-Fractionated Red Cells

Patient	Direct Antiglobulin Test Titration Score		Reticulocyte Count (%)	
	RR	RP	RR	RP
Type I				
1	9	65	81	0.4
2	25	41	12.4	1.5
3	0	15	5.9	0
4	18	58	65.9	3.4
5	0	47	93	1.0
6	0	25	26.7	0
7	8	21	9.0	0
8	11	43	8.0	0
9	20	64	23.6	<0.1
10	0	14	54	1.6
11	40	55	30.4	0.3
12	0	44	30.7	0.2
13	0	8	54.8	0.5
14	48	60	19.4	0.5
15	19	36	71.8	2.0
16	55	81	34.1	0.8
17	38	49	59	3.3
18	0	4	41.4	2.3
19	24	50	38.2	2.1
Type II				
20	54	53	>98	17.4
21	60	68	75.6	1.6
22	59	67	>98	10.2
23	39	42	60.3	12.5
24	33	42	90.5	10.8

RR, Reticulocyte-enriched patients' red cells; RP, reticulocyte-poor patients' red cells.

and was classified as type I. Further, when using the reticulocyte-enriched fraction, the DAT was negative in 7 patients (37%); the mean reticulocyte count of the reticulocyte-enriched red cell fraction for the 19 patients was 40%. Additionally, in all 5 patients tested who had both IgG and complement (C3d) sensitizing their red cells, titration studies using anticomplement reagents also demonstrated a similar type I pattern of reactivity (data not shown).

The red cells of 5 of 24 patients (21%) reacted equally well in the DAT using either reticulocyte-enriched or reticulocyte-poor red blood cells and were classified as having type II warm autoantibody. In order to be more confident that this observation was not a result of failure of adequate reticulocyte-enrichment, a reticulocyte count of >60% in the reticulocyte-enriched fraction was arbitrarily considered necessary for a patient to be classifiable as having type II warm autoantibody. In fact, this group of patients averaged 84.5% reticulocytes in the enriched red cell fractions. Two patients could not be classified as having either type I or type II warm autoantibody because of inadequate reticulocyte enrichment (data not shown). These patients were not further evaluated.

Table 2 illustrates that patients with type I warm autoantibody had slight to moderate anemia on presentation, with a mean hemoglobin of 10.2 g/dl with mild reticulocytosis (mean = 6.2%). Six of these patients (32%) did not have laboratory evidence of hemolysis when tested, although 2 of these had a previous history of hemolytic anemia. Only 2 patients (10.5%) with type I warm autoantibody had severe anemia, with hemoglobin values of 5.3 and 4.9 g/dl, respectively. In contrast, patients having type II warm autoantibody presented with severe anemia (mean hemoglobin = 6.4 g/dl) and greatly elevated reticulocyte counts (mean = 23.4%).

When serum autoantibody was present, tests using allogeneic age-fractionated red cells showed that, if a type I pattern was found by DAT titration scores using the patients' red cells, the pattern of reactivity of the serum autoantibody was similar (Table 3). Likewise, when red cell eluates from three patients having type I warm autoantibody sensitizing their red cells were tested against allogeneic age-fractionated red cells, a type I pattern of reactivity was obtained. Conversely, when serum or red cell eluate from one patient having type II warm autoantibody sensitizing his red cells was tested against age-fractionated allogeneic red cells, a similar type II pattern was obtained (data not shown).

In patients classified as type I, the autoantibody in the serum and eluate often had specificity within the Rh system: in 5 of 8 patients (63%), the serum specificity was anti-e in 2, "relative specificity" anti-e in 2, and "relative specificity" anti-Rh:27 (cE) in 1; in 3 of 11 patients (27%), the eluate specificity was anti-e in 2 and "relative specificity" anti-e in 1. In 2 patients classified as type II, the autoantibody in serum and eluate had no apparent blood group specificity.

Serum autoantibody and red cell eluates from 2

Table 2. Relative Clinical Significance of Type I or Type II Warm Autoantibody

Warm Autoantibody Category	Reactivity* Using Age-Fractionated RBC	Number of Patients	Hemoglobin† (g/dl)	Reticulocyte-Counts† Using Unseparated Red Cells
Type I	Preference for reticulocyte-poor red blood cells	19	10.2 ± 2.9	6.2% ± 6.2%
Type II	No preference	5	6.4 ± 2.4	23.4% ± 17.1%

*Determined by direct antiglobulin test titration.

†Mean ± standard deviation.

Table 3. Reactivity of Type I Serum Autoantibody

	Indirect Antiglobulin Test (IAT)			
	Allogeneic Reticulocyte-Enriched RBC Fraction* (54.9%)		Allogeneic Reticulocyte-Poor RBC Fraction* (3.1%)	
Patient	IAT	IAT Titration Score	IAT	IAT Titration Score
1	0	0	1½+	9
4	0	0	1½+	6
9	0	0	2+	13

*Prepared from a blood sample with reticulocyte count of 9.6% and negative direct antiglobulin test.

patients having type I and 1 patient having type II warm autoantibody were tested against –D– or Rh_{null} red cells (Table 4). In type I, the serum autoantibody reacted weakly with –D– cells and failed to react with Rh_{null} red cells; red cell eluates were less reactive with Rh_{null} red cells than with –D– red cells. In type II, the serum and eluate reacted equally well with –D– and Rh_{null} red cells.

DISCUSSION

Using age-fractionated red cells, we found that warm autoantibodies can be classified as either type I or type II based on their reactivity with younger reticulocyte-enriched (lower buoyant density) and/or older reticulocyte-poor (higher buoyant density) red cell fractions. These differences in warm autoantibody reactivity may be clinically important. All patients with type II warm autoantibodies had severe anemia. In contrast, patients who had type I warm autoantibodies generally had a mild anemia or, in some instances, no clinical evidence of hemolysis.

Although it has been reported that the Rho(D) antigen expression is weaker on reticulocytes compared to older red cell fractions,[15] we have not found this slight reduction in antigen strength to markedly interfere with Rho(D) antigen detection using an indirect antiglobulin test (IAT). Indeed, we have previously reported that highly reticulocyte-enriched red cell fractions may be accurately typed using alloantisera of all major blood group systems, including ABO, Lewis, P, Rh, Duffy, Kidd, Kell, and MNSsU.[10] Thus, it would appear that the membrane properties of reticulocytes do not markedly interfere with the binding of alloantibodies to corresponding antigens or with the detection of these antigens using IAT. Therefore, the detection of type I warm autoantibody binding to autoantigens present on younger red cells would not be expected to be impaired unless the antigen sites were inaccessible or of very low density. In contrast, type II warm autoantibodies appear to recognize a different antigenic determinant, since these autoantibodies react equally well with highly reticulocyte-enriched red cells.

Table 4. Correlation Between Category of Autoantibody and Rh Specificity

		Reactivity of Warm Autoantibody			
		Serum Versus		Eluate Versus	
Patient	Autoantibody Type	-D-	Rh_{null}	-D-	Rh_{null}
1	I	1+	0	3+	2+
4	I	1+	0	3+	2+
20	II	3+	3+	4+	4+

Although the Rh antigens appear to be adequately expressed on reticulocyte-enriched red cells, our data indicate that 5 of 8 type I serum autoantibodies tested had either clear-cut or "relative" Rh specificity. These findings support the suggestion that Rh autoantibodies and alloantibodies recognize different antigenic determinants.[13,14]

Also, studies in three patients indicate that type I warm autoantibody either fails to react, or reacts less strongly, with Rh_{null} red cells than does type II warm autoantibody. This pattern of reactivity is generally interpreted as indicating Rh specificity. However, this observed decreased reactivity of type I warm autoantibody with Rh_{null} cells may be unrelated to a lack of Rh antigens on these cells. Since Rh_{null} red cells are thought to have defective membrane components,[7] there may be a membrane abnormality of these red cells not necessarily related to Rh blood group antigens that renders them less reactive to type I warm autoantibodies. Indeed, Rh_{null} red cells are known to have other abnormally expressed red cell antigens, in addition to the Rh antigens.[7] Alternatively, since Rh_{null} individuals have hemolytic anemia, their blood may contain relatively more young red cells. The weak reactions of type I warm autoantibodies with Rh_{null} cells could, thus, be in part due to cell age.

A number of possible mechanisms could explain the observed serologic reactivity of type I warm autoantibody. This antibody may recognize an, as yet, unidentified red cell antigen, possibly a cryptantigen closely associated with the Rh peptide but not yet fully expressed on very young red cells. Further, we would like to hypothesize that type I warm autoantibody may represent augmented production of the physiologic autoantibody reported to be responsible for the normal immune-mediated clearance of senescent red cells.[16–19]

The findings reported in this article may provide an additional means for determining the clinical significance of warm autoantibodies and aid in establishing the prognosis of patients having warm antibody AIHA. In addition, further study of type I and type II warm autoantibodies may provide additional information regarding the specificities of warm autoantibodies and may provide further insights into the mechanisms of warm antibody AIHA.

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