ORIGINAL ARTICLE

ERYTHROPHAGOCYTOSIS ASSAY IN PATIENTS WITH AUTOIMMUNE HEMOLYTIC ANEMIA

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Abstract   The aim of this paper is to evaluate the erythrophagocytosis assay (EA) in patients with autoimmune hemolytic anemia (AIHA). Direct antiglobulin test (DAT), indirect antiglobulin test (IAT) and EA were performed in blood samples from 46 patients with presumed AIHA. The EA was carried out incubating patients' erythrocytes and peripheral blood monocytes. A total of 200 monocytes were analysed to determine the percentage of active phagocytic cells (% APC). In 9 of these patients the applied treatment was evaluated by DAT, IAT and EA. In 14 transfusion requirements, the compatibility tests and EA were performed. For EA, patients' monocytes were incubated with erythrocytes from previously selected units sensitized with patients' sera. The % of APC was 32.1 ± 1.7 in 35 patients with positive DAT and 17.8 ± 1.3 in 11 patients with negative DAT. This last value was significantly higher than that with negative controls (3.7 ± 0.3)(p ≤ 0.01). As regards the applied treatment, patients with a successful response (n=6) showed a significant decrease in the initial % APC (31.8 ± 1.6 to 15.3 ± 2.4; p ≤ 0.05) while DAT and IAT remained positive. In those patients who required blood transfusion the compatibility tests were positive with all the units to be transfused, whereas the % APC varied for each one. Blood units were selected according to the lower % APC.

Key words: erythrophagocytosis, active phagocytic cells, autoimmune hemolytic anemia

Resumen   ***Ensayo de eritrofagocitosis en pacientes con anemia hemolítica autoinmune***. El objetivo del presente trabajo es evaluar el ensayo de eritrofagocitosis (EE) en pacientes con anemia hemolítica autoinmune (AHAI). En 46 pacientes con diagnóstico presuntivo de AHAI se realizó la prueba de Coombs Directa (PCD), búsqueda de anticuerpos libres en el suero (PCI) y el ensayo de eritrofagocitosis (EE). Esta prueba funcional fue llevada a cabo incubando eritrocitos de cada paciente con monocitos de sangre periférica. Se analizaron 200 monocitos y se determinó el porcentaje de células fagocíticas activas (CFA). En 9 de estos pacientes se evaluó el tratamiento aplicado a través de la PCD, PCI y el EE. En 14 pacientes que requirieron terapia transfusional se realizaron las Pruebas de Compatibilidad Transfusional y el EE incubando monocitos del paciente con Glóbulos Rojos de las unidades seleccionadas, sensibilizados previamente con suero del enfermo. En 35 pacientes con PCD positiva el % de CFA fue de 32.1 ± 1.7 y en los 11 pacientes con PCD negativa fue de 17.8 ± 1.3. Este último valor fue significativamente mayor que el obtenido con controles negativos (3.7 ± 0.3) (p ≤ 0.01). Con respecto a la evaluación de la terapéutica aplicada, los pacientes que respondieron favorablemente (n=6) presentaron una disminución significativa del porcentaje inicial de CFA (31.8 ± 1.6 a 15.3 ± 2.4; p ≤ 0.05) mientras que la PCD y PCI permanecieron positivas. En aquellos pacientes que requirieron terapia transfusional las pruebas de compatibilidad fueron positivas con todas las unidades ensayadas, mientras que el porcentaje de CFA presentó valores diferentes con cada una de ellas. Las unidades fueron seleccionadas de acuerdo al menor porcentaje de CFA.

Palabras clave: eritrofagocitosis, células fagocíticas activas, anemia hemolítica autoinmune

Serological studies performed in patients with presumed AIHA do not always show strict correlation with the *in vivo* occurring phenomena. The binding of an antibody to an erythrocyte antigen does not affect the lifespan of this cell. The immune destruction of red blood cells (RBC) is produced by two mechanisms that may be activated secondarily to the antibody-antigen interaction. These mechanisms involve the adherence to Fc receptors of the monocyte macrophage system and the activation of complement[1-3].

Abbreviations:
AIHA: autoimmune hemolytic anemia
APC: active phagocytic cells
DAT: direct antiglobulin test
EA: erythrophagocytosis assay
IAT: indirect antiglobulin test
RBC: red blood cells

The functional cellular assays developed so far, are performed in a relatively simple way by incubating sensitized erythrocytes with peripheral blood cells bearing Fc-receptors, usually monocytes and assessing different stages of the interaction such as phagocytosis and adherence by the monocytes monolayer assay[4, 5].

In recent years, several groups have used some tests for assessing the clinical significance of red cell antibodies through different methods. Hemolysis is often difficult to diagnose and attempts have been made to evaluate *in vivo* red blood cell destruction using cellular immunoassays: it reflects RBCs adherence to and phagocytosis by peripheral blood monocytes[6, 7].

The aim of this study is to evaluate the practical value of EA in the diagnosis, therapeutic assessment and selection of blood for transfusion in patients with AIHA.

Materials and Methods

A*) Diagnosis:* Blood samples from 46 patients were investigated. The patients were referred to the Immunohematology Laboratory because of suspected autoimmune hemolysis. All procedures using human samples were performed in accordance with the ethical standards established by the University of Rosario.

DAT with polyspecific and monospecific (anti-IgG and anti-$C_{3b/d}$) antibodies was determined in these patients. DAT in low - ionic strength saline and bromeline techniques were also performed. Titres and specificities of free serum and eluate antibodies were determined by classic techniques[8].

The patients were categorized on the basis of routine serological tests into two groups: 1) patients with positive DAT (warm autoantibodies) and 2) patients with negative DAT.

Peripheral blood monocytes were obtained through their glass-adhering property. The adhered cells (90% monocytes according to the morphological criteria with May Grünwald Giemsa method and the presence of peroxidase and esterase) were overlayered with 0.5% suspensions of patients' erythrocytes in Hank's solution supplemented with 20% serum AB. The mixture of cells was then incubated for 3 hours at 37°C and thereafter the unbound erythrocytes were washed out and the cells on the glass were fixed with methanol, stained by the May Grünwald Giemsa method and observed under the light microscope. Two hundred cells taken from different glass spots were analyzed to determine the percentage of monocytes with phagocytosed and adherent red cells (active phagocytic cells, APC). Non sensitized RBCs were used in negative control *in vitro* assays. The positive control was performed with *in vitro* sensitized RBCs. Values greater than 4% were regarded as positive[9].

The hemolytic state was evaluated using the clinical and laboratory findings, including the presence of anemia, the reticulocyte count and the level of bilirrubin and haptoglobin.

B) *Treatment:* Nine patients with positive DAT were selected in order to evaluate the therapeutic assessment with the techniques previously described: EA, DAT and IAT[8]. The applied treatments consisted in different corticoid doses, chemotherapy and immunomodulators.

C) *Transfusion therapy:* We studied 14 patients with AIHA diagnosis, who required transfusional therapy. Their sera had antibodies against antigens of very high frequency.

The transfusional compatibility proofs were performed in different media: saline, macromolecular (albumine), enzymatic (bromeline) and IAT with 20 units of red cell concentrates for each patient[10].

The EA for the evaluation of the clinical significance of the antibodies was performed using monocytes from the patient and erythrocytes from selected units, previously sensitized with patients' sera.

Statistical analysis

Differences in the % of APC among patient groups and positive and negative controls were assesed by the Kruskall-Wallis non parametric analysis of variance and multiple comparison. The Wilcoxon test was employed to evaluate the therapeutic assessment on the applied treatments.

Results

A) *Diagnosis*:

Positive results were obtained in all the classical globular immunohematologic assays of 35 samples, and in 5 patients the determinations with DAT in low - ionic strength saline and bromeline techniques. Seric antibodies were positive in 50% of the cases and in most of the eluates. The immunohematologic tests performed in the rest of the patients were negative. There was no presence of crioagglutinins in the cases studied (Table 1).

The percentages of APC of the first 35 patients were 32.1±1.7 and those of the other 11 patients were 17.8±1.3, lower than those cases in which the erythrocytes were sensitized *in vitro*: 32.5±1.9 (positive control) (p≤0.01), but higher than in those in which they were not: 3.7±0.3 (negative control) (p≤0.01) (Figure 1).

TABLE 1.– *Immunohematologic Classic Tests*

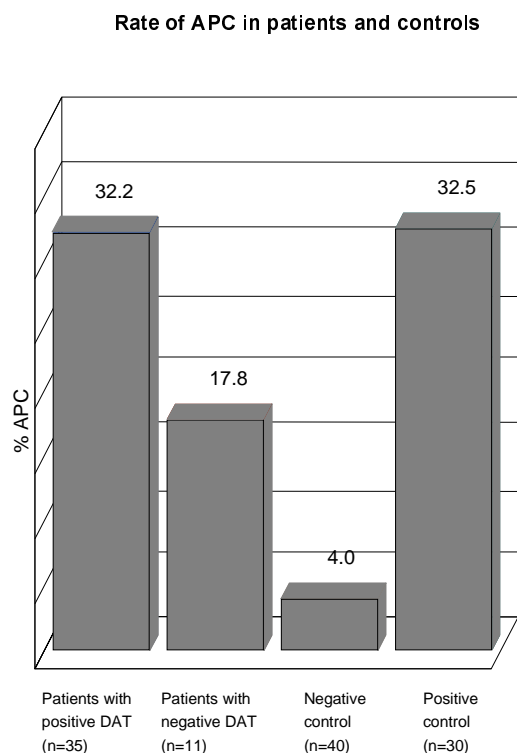| Patients	DAT	DAT in low-ionic strength saline	Bromeline	IAT	Eluates	Crioaglutinines
n = 35	+	+	+	+ (n = 18)	+	-
				- (n = 17)		
n = 11	-	+ (n = 5)	+ (n = 5)	-	-	-
		- (n = 6)	- (n = 6)			

Rate of APC in patients and controls

Fig. 1.– Rate of APC in patients with positive and negative DAT and controls.

Rate of APC in patients before and after treatment

Fig. 2.– Rate of APC in patients before and after treatment.
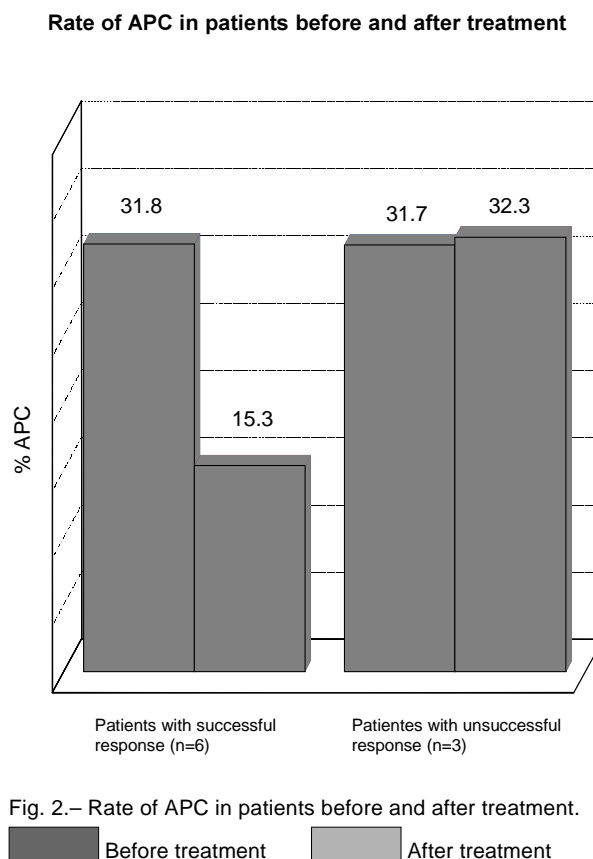
◼ Before treatment   ◻ After treatment

B) *Treatment*

In the 9 patients selected for therapeutic assessment, DAT and IAT were always positive, but EA was modified by the applied treatment. Patients with a successful response (n=6) showed a decrease in the initial % APC (31.8 ± 1.6 to 15.3 ± 2.4; $p\leq0.05$) while in the three patients with no response to the applied treatment, % APC was not modified (Figure 2).

C) *Transfusion therapy*

Compatibility transfusional tests were positive with all the media applied to the units to be transfused, whereas APC % varied for each unit.

Blood units were selected according to the lower APC % (9-12). Clinical improvement was obtained in most patients. There was an increase in hematocrit and in hemoglobin concentration.

Discussion

In patients with AIHA, erythrocyte autoantibodies are detected and characterized by serological methods[3, 10]. However, in some patients with hematological and clinical characteristics of this pathology, the search for antibodies is systematically negative. These results are attributed to the presence of antibodies, mainly IgG_3 subclass[4] in levels which are inferior to the sensitivity of classical techniques (200 - 400 molecules of IgG for each red cell), which are capable of producing *in vivo* hemolysis[5, 11, 12].

Taking into account that one of the main objectives of all laboratory tests is to correlate the determinations performed *in vitro* with what happens *in vivo*, we have used a functional simple test (EA) in the study of AIHA.

The results obtained with classical immunohematologic assays allowed the diagnosis of AIHA in 35 of the cases studied. In these patients, the results obtained with their own red cells were similar to those found with sensitized red cells *in vitro* with IgG anti-D. This might be attributed to the high number of molecules of IgG bound *in vivo* to the erythrocyte membranes. These molecules contributed to the positive results in routine determinations[8, 13] (Table 1).

With the remaining patients, the functional tests allowed AIHA diagnosis. The values of active phagocytic cells with own red cells were significantly higher than those observed with normal erythrocytes, but lower than those obtained with sensitized red cells (Figure 1). These results

could be explained by the presence of a reduced number of autoantibodies bound to the red cell which are not detected by classic techniques[5, 11, 12]. Similar behaviour was observed in 5 samples of patients with positive DAT in low-ionic strength saline and bromeline techniques. The erythrophagocytosis functional assay might contribute to the AIHA diagnosis, allowing a better correlation with clinical findings. This study would be particularly adequate when the number of molecules of autoantibodies is not enough for its determination by classical techniques.

The 9 patients selected to evaluate the treatment had positive DAT (semiquantified in 4 +). As it happens with the indirect test, the hematological response appears later owing to the high autoantibody concentration. These results did not allow us to perform the follow up of studied patients because of the maximum positivity found in the DAT and the presence of seric antibodies[6, 14]. The APC percentage obtained varied according to the treatment response. This decrease coincides with clinical improvement (Figure 2). This response might be attributed to a modification in the IgG levels present in red cells. The EA is more adequate than classic immunohematologic tests to obtain a better evaluation of the patients´ response to the treatment.

It is important to find out whether antibodies against antigens of a very high frequency are clinically significant since compatible blood is usually not available[1, 15]. Transfusions are often avoided because the donor's red cells constitute an additional antigenic charge which might stimulate the production of antibodies, which is an effect opposite to that of corticoids, although sometimes it is necessary to correct the symptomatic anemia[16, 17].

Pre-transfusional tests made in patients systematically studied had positive results in all assayed media, while the APC percentage obtained with EA varied with different blood units. The patients received the blood units with the lower APC percentage. They had clinical improvement, indicating that the transfusion was effective. The EA results correlate with the clinical significance of the antibodies found. The lower percent of APC suggests that incompatible blood may be transfused, without much risk, in a patient with antibodies of questionable significance. However, it is not certain whether such blood will survive as long as compatible red blood cells. An antibody may change its activity in a given patient, hence, it is important to repeat the assay before each transfusion.

The results of EA reflect to some extent the *in vivo* destruction of sensitized red blood cells. We can use this test in order to predict the clinical significance of antibodies.

The results obtained would indicate that it is necessary to perform, not only the classical laboratory tests but also to confirm the studies with functional assays. These should be used for diagnosis, following of treatment and selection of blood to be transfused in cases of autoimmune hemolytic anemia.

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