Correlation Between Warm Reactive Autoantibodies

And The Monocyte Monolayer Assay

ABSTRACT

BACKGROUND: The monocyte monolayer assay (MMA) measures the adherence and phagocytosis of sensitized red cells with peripheral monocytes. The result of MMA therefore may reflect to some extent the *in vivo* destruction of sensitized red blood cells (RBCs). However, serological and clinical features of patients with warm reactive autoantibodies do not always show strict correlation with *in vivo* activity. To evaluate the correlation of warm reactive autoantibody between the MMA result and the clinical data may add value in distinguishing whether or not the autoantibodies are harmful.

STUDY DESIGN AND METHODS: Blood samples from 20 patients with warm reactive autoantibodies were investigated using the MMA. The Monocyte Index (MI) values > 5% were considered positive while MI values ≤ 5% were considered negative.

RESULTS: Of the 20 patients, only one patient had a positive MMA result with an MI of 30%. All other 19 patients demonstrated a negative MI value (<5%). There was no correlation between serologically detectable warm reactive autoantibodies and the MMA or DAT.

CONCLUSION: Our findings were only a preliminary study; the final conclusion demands more comparative studies between the clinical and serologic features of patients with warm autoantibodies and the MMA tests. The mechanism of red cell destruction depends on many factors. Furthermore, there are many variables that exist within the MMA which may have an effect on the outcome. These variables should be taken into account when interpreting MMA results.

INTRODUCTION

Warm reactive autoantibodies are red blood cell (RBC) directed immune responses that are maximally reactive at 37°C. These are often associated with the destruction of red blood cells *in vivo*, causing hemolytic anemia in patients.[1] These autoantibodies present a complex serological problem with a positive direct antiglobulin test (DAT) and crossmatch incompatibility with all RBC units tested. *In vitro* serological tests are routinely used to detect and characterize autoantibodies. However, serological studies performed in patients with warm reactive autoantibodies do not always show strict correlation with *in vivo* occurring phenomena.[2] The clinical significance of autoantibodies may vary between patients. Further investigation of warm autoantibody interaction *in vivo* in relation to *in vitro* activity may add value in distinguishing whether or not the autoantibodies are harmful.

It is well known a positive DAT indicates antibodies are present on circulating RBCs *in vivo*. Nevertheless, individuals with a positive DAT do not always demonstrate obvious signs of RBC destruction while some patients demonstrate overt hemolysis with a weak or negative DAT.[3] This is unexpected since damage *in vivo* is not only dependent on direct contact between the erythrocyte and autoantibody but on several other factors. These factors include: antibody subclasses, interaction between Fc receptors and cells of the mononuclear phagocytic system, ability to activate complement, concentration of antibody with corresponding antigen molecules on the RBC surface, and chemical and physical characteristics of the antigens on the red cell membrane[4]. Among all of these the most important factor influencing the destruction of red cells is the interaction between the sensitized RBCs and cells of the mononuclear phagocytic system. As a result, many attempts have been made by investigators to evaluate *in vivo* red blood cell destruction using cellular assays.[4-8]

The premise of all *in vitro* cellular assays is to mimic in the laboratory what occurs *in vivo*. The monocyte monolayer assay (MMA) acts as an *in vitro* cellular assay that represents the mechanism of red cell destruction via macrophages in the spleen[9]. It is performed by incubating sensitized erythrocytes with peripheral monocytes bearing Fc-receptors and assessing different stages of the interaction, such as phagocytosis and adherence.[9] Many variables are involved in the MMA procedure. Those include: the source from which the monocytes are collected,[2,9-10] the RBC sensitization procedure (including whether or not fresh normal serum as a source of complement is added),[9,11] the culture conditions (a CO_2 atmosphere versus ambient air),[9,12] and how the results are expressed. Although such variables exist, the MMA is as close to conditions *in vivo* as is possible to establish *in vitro* and provides insight into the potential clinical significance of autoantibodies.[9]

The MMA has been used successfully in many studies to determine the clinical significance of red cell alloantibodies directed towards high frequency antigens.[10,13-17] The procedure has also been successful in investigating the survival of transfused RBCs.[14,17] However, assessing the clinical importance of warm autoantibodies using the MMA has not been evaluated thoroughly. When patients possess warm reactive autoantibodies, presumed serological and clinical laboratory data may not always correlate with *in vivo* activity of autoantibody.[16] Evaluation of the functional activity of warm reactive autoantibodies using the MMA will contribute additional value in confirming the significance of warm autoantibodies. The aim of this study was to evaluate the correlation of warm reactive autoantibodies between the MMA result and the clinical data. This study also compared the DAT reaction strength and Hemoglobin (Hb) level of each patient with the MMA results to see if a correlation exists.

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MATERIALS AND METHODS

Patients

Study samples were selected from blood samples referred to an immunohematology laboratory due to difficulties in resolving serological problems caused by autoantibodies when grouping or crossmatching compatible RBC donor units. The Hb and Hematocrit (Hct) level with each patient's transfusion history was obtained upon admission.

RBCs Tested

RBCs used in the *in vitro* sensitization phase of the MMA were selected based on the patient's RBC phenotype and the specificity of the antibody(ies) each patient possessed in addition to the autoantibody. Phenotypically matched antigen negative RBCs were from two different sources; screening cells from commercial reagents (Panocells, Immucor Gamma; Data-Cyte Plus, Medion Diagnostics AG) and segments from donor RBC units.

The MMA

The MMA was performed using the two-stage sensitization method, as previously described by Garratty et al.[18] The procedure isolated mononuclear cells from a healthy volunteer donor using a Ficoll-sodium diatrizoate density gradient (Histopaque-1077, Sigma-Aldrich Inc.). The separated mononuclear cells were then washed with phosphate-buffered saline (PBS, 0.8% NaCl) and suspended in culture media (RPMI Medium-1640, Sigma-Aldrich) containing 5% fetal bovine serum (Sigma-Aldrich). The mononuclear cell suspension was added to an 8-well tissue culture chamber slide (Lab-Tek chamber slide with cover, Nalge Nunc Int.) and incubated for one hour at 37°C in atmosphere of 4-5% CO_2.

Meanwhile, the sensitization of RBCs was prepared using phenotypically matched RBCs and patient plasma containing warm autoantibodies and alloantibodies. After one hour of incubation

at 37C in saline, RBCs were washed and the fresh normal serum was added as a source of complement. RBCs were incubated for another 15 minutes at 37C, washed and re-suspended in culture media containing 5% fetal bovine serum. The small aliquot of sensitized RBCs was tested by indirect antiglobulin test (IAT) with anti-IgG and anti-C3 to determine the degree of sensitization.

After incubation of the mononuclear cell suspension in a tissue culture chamber slide, the supernatant containing non-adherent lymphocytes was removed via pipette and a sensitized RBC suspension was added to the monocyte monolayer. After one hour incubation at 37°C in atmosphere of 4-5% CO_2, the non-adherent RBCs in supernatant were aspirated via pipettes. The gasket from the slide was removed then rinsed in phosphate-buffered saline to remove RBCs that did not react with monocytes.

The slides were stained with a Wright-Giemsa stain and observed microscopically using oil immersion of 100x lens. A total of 500 monocytes were counted for reactivity which includes the number of RBCs adhered and phagocytized. For each RBC tested, the percentage of reactive monocytes with RBCs adhered (AI), phagocytized (PI), and the Monocyte Index (MI) was determined using the following formula:[17]

$$\textbf{Adherence Index (AI)} = \frac{\text{Total number of monocytes with adhered RBCs}}{\text{Total number of monocytes counted}} \times 100$$

$$\textbf{Phagocytosis Index (PI)} = \frac{\text{Total number of monocytes with phagocytized RBCs}}{\text{Total number of monocytes counted}} \times 100$$

$$\textbf{Monocyte Index (MI)} = AI + PI$$

Positive and negative controls were performed along with patients' MMAs. The positive

control consisted of a 1:40 dilution of Rh immune globulin (Rh_oGam) anti-D+C with 6% Bovine

Serum Albumin (BSA) in PBS that reacted 3+ by antiglobulin test. The negative control was

prepared and tested in parallel with the sensitized RBCs, including RBCs sensitized with fresh

volunteer donor plasma that previously did not react in the assay. The same positive and

negative controls were used throughout the study and RBCs were incubated with PBS under the

same conditions as the test RBCs' sensitization. Each run of MMA was considered valid only

when the positive control MI was $\geq 70\%$ and when the negative control had not exhibited

reactivity.

RESULTS

Blood samples from 20 patients with a positive DAT were investigated. Of the 20 patients, 10

patients were female (age range 20 – 90 years old) and 10 patients were male (age range 42 – 88

years old). Fifteen of our 20 patients had a history of previous transfusions and 5 patients had

never been transfused before.

The MMA

The MMA was performed using allogeneic monocytes with phenotypically matched red cells to

each patient respectively. The red cells were then sensitized with autoantibodies obtained from

patients. A total of 500 monocytes were counted for reactivity which include the number of

monocytes that had RBCs either adhered or phagocytized. The percentage of adhered RBC

index (AI), phagocytized index (PI), and the monocytes index (MI) were calculated for each

patient. A cut off of 5% MI value was used to determine positive or negative MMA result. The

interpretation of MMA was based on Garratty's proposal: $\leq 5\%$ (0-5%) MI indicates that the

antibody is not clinically significant. The MI values in the range of 5.1-20% range may or may not cause increased RBC destruction while those with an MI value over 20% are clinically significant when incompatible RBCs are transfused. Therefore, the transfusing of selected incompatible blood is not recommended.[18]

Table 1 shows the summary of MMA results of the 20 patients. Only one patient showed a positive MMA result with an MI value of 30%. Both AI and PI values were also significant in this patient compared to other patients. All other 19 patients demonstrated a negative MI value (<5%). The mean MI value of 10 patients with warm autoantibodies only was 1.34% and the mean MI value for 9 patients with concurrent warm autoantibodies and alloantibodies was 0.91%. There was no significant difference between the MMA results obtained from the autoantibody only group and the autoantibodies with concurrent alloantibodies group.

The DAT reaction strength and MMA result

The DAT was reactive for IgG in all patients with warm autoantibodies but none displayed C3 reactivity. **Figure 1** has the number of patients with DAT reaction strength. The DAT strength ranged from microscopically reactive to a strong 3+. The DAT was microscopically positive in 5 patients, 1-2+ in 9 patients, and 3+ in 6 patients. **Table 2** and **Figure 2** compare the DAT strength of each patient to their corresponding AI, PI, and MMA result. Of all, only one patient (patient #11) demonstrated a positive MMA result while the other 19 patients showed a negative MMA result despite their DAT reaction strength. Of 19 patients, no correlation ($r = 0.21$) was seen between MMA results and the DAT reaction strength in this study.

The MMA results in relation to Hemoglobin (Hb)

The 20 patients with warm reactive autoantibodies exhibited Hb level between 4.0 and 14.0 g/dl. The Hb level was divided into 4-6, 6.1-8.0, 8.1-10.0, and 10.1–14.0 g/gL. **Table 3** displays the

comparison between the MMA result patients Hb level. There were 10 patients who had a Hb level between 6.1 and 8.0 but only one patient demonstrated a clinical significant MI value. This is Patient #11 who may have an alloantibody to a high frequency antigen. All of the other 19 patients demonstrated MI values less than 5 % regardless of their Hb level. No correlation (r = -0.12) was seen between the MMA results and patient's Hb level.

Patient with Positive MMA Result

Patient #11 was a 43 year old female who was admitted to the hospital with GI bleeding. At the time of admission, the patient's hemoglobin level was 6.3g/dl. An accurate transfusion history was not available but the patient stated she had been transfused. No other clinical data was accessible. Her antibody screen was 3+ at LISS-37°C/AHG and the DAT strength was 3+. A very strong AHG reactive autoantibody was confirmed using the patient's plasma and an acid elution from the red cells. Reacting autoantibody was not completely removed by adsorption (x3) onto ficin-treated allogeneic red cells. Only a partial phenotype of the patient was performed due to the DAT being positive after EGA treatment (x2); hence the genotype was requested. Therefore, the MMA was performed using partially genotype-similar RBCs: negative for C, E, K, S, Fy^a, Fy^b and Jk^b.

The outcome of Patient #11's MMA result demonstrated considerable phagocytic activity of monocytes with sensitized phenotype-similar tested red cells. There was a preponderance of phagocytosis (PI=21.6%) in this patient compared to the adherence index (AI=8.4%). Given the above history, an alternative explanation for these results is this patient may have an antibody to a very rare high frequency antigen such as anti-En^{a}FR. Anti-En^{a}FR is often not recognized because it looks just like an idiopathic IgG warm autoantibody. Perhaps immunoblotting studies

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may give a better clue of autoantibody specificity however, there is no compatible blood available for someone with anti-En[a]FR.[19]

DISCUSSION

The MMA measures the adherence and phagocytosis of sensitized red cells with peripheral monocytes. The monocyte index value is calculated using the adherence and phagocytosis index and is then expressed as either positive or negative using a 5% cutoff point.[18] A number of variations of the MMA have been performed by different investigators over the past 30 years,[10,14-20] and variables associated with the MMA also have been reviewed.[9] It is important to note that all of those variables may affect the outcome of MMA.

Van der Meulen, et al. were the first group to correlate an *in vitro* monocyte assay (measuring RBC adherence only) with in vivo hemolysis in patients with positive DATs.[20] In their study, RBCs from 42 patients with a positive DAT were added to monocytes. *In vitro* adherence occurred with RBCs from all 22 patients who had evidence of hemolytic anemia, and no adherence was noted with RBCs from 20 patients without increased RBC destruction. Thus, the correlations of the MMA result and the in vivo hemolysis with positive DAT patients were reported. A good correlation was also found in Gallagher et a1study.[21] However, our study did not demonstrate these findings. Nineteen of our 20 patients yielded a negative MMA result regardless of their DAT reaction strength. The DAT strength of each patient was also compared with their corresponding MMA result. Wheeler et al.[1] reported the DAT reaction strength correlates well with the presence or absence of hemolysis *in vivo*, however no correlation was found between the MMA result and the DAT reaction strength in our study.

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The MMA was performed using free unbound autoantibodies from patient's plasma instead of eluates in our study. In the patient, RBC-bound autoantibody and free plasma autoantibody exists in a reversible dynamic equilibrium.[22-23] The amount of autoantibody in plasma depends upon the amount of antibody present and the affinity of the antibody for RBC antigens. In the study of Wikman et al., the MMA index was significantly higher when RBCs were sensitized with autoantibodies from eluates than when RBCs were sensitized with free unbound autoantibodies from plasma.[24] They indicated this occurred because the concentration of autoantibodies in eluates was higher than free unbound autoantibodies in plasma or serum. A report by Packman stated that the warm reactive autoantibodies are predominantly IgG globulins which possess relatively high affinity for human RBCs at body temperature (37°C).[25] As a result, most of the autoantibody is bound to the patient's circulating RBCs with only a small amount circulating free in plasma. Further, the low affinity autoantibodies can be easily detached during sensitization of red cells.[2,9] Thus, the concentration of autoantibodies in plasma and the binding affinity of autoantibodies for RBC antigens may also play a role in red cell destruction. The use of an eluate in the MMA may yield a different outcome than using free unbound autoantibodies from plasma.

Most destruction of the autoantibody-coated RBCs is mediated primarily by sequestration and phagocytosis by macrophages in the spleen.[26-27] The macrophages express surface receptors for the Fc region of IgG, particularly for IgG1 and IgG3 and for opsonic fragments of complement proteins.[28] When present together on the RBC surface, IgG and complement fragments act synergistically to enhance phagocytosis.[28-29] Brojer and colleagues demonstrated that the number of RBC-bound IgG molecules correlate with the presence of phagocytosis and the degree of *in vivo* hemolysis in positive DAT patients.[30,31] In their study, phagocytosis was

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always observed when there were greater than 2,000 IgG molecules per RBC. In patients having less than 700 IgG molecules per RBC, the interaction with monocytes was observed only if IgG3 was present on the RBCs. The minimum number of IgG3 molecules per RBC initiating phagocytosis was 150 to 640 as opposed to IgGl where 1,250 to 4,020 were needed. Thus, the quantity and type of IgG on the red cell surface may influence the degree of hemolysis. Quantification of IgG and its subtype in relation to MMA values may assist in determining the degree of hemolysis.

Gallagher et al studied the MMA using both autologous and allogeneic monocytes.[21] The MMA result was significantly elevated with autologous monocytes in their study and a similar finding was also observed from the study of Branch et al.[2] Therefore, the sources of monocytes may have an effect on the MMA outcome. The use of autologous monocytes to perform the MMA may have demonstrated a better *in vivo* phenomenon. However, it is not always possible to obtain autologous monocytes in a condition suitable for the MMA.

Our study indicated there was no correlation between serologically detectable warm reactive autoantibodies and the MMA or DAT. There was also no correlation between the MMA results and Hb level. These findings were only a preliminary study; the final conclusion demands more comparative studies between the clinical and serologic features of patients with warm autoantibodies and the MMA tests. The mechanism of red cell destruction depends on many factors. Furthermore, there are many variables that exist within the MMA which may have an effect on the outcome. These variables should be taken into account when interpreting MMA results.

REFERENCES

1. Wheeler CA, Calhoun L, Blackall DP. Warm reactive autoantibodies; clinical and serologic correlations. *Am J Clin Pathol* 2004;122:680-685.

2. Branch DR, Gallagher MT, Mison AP, Sy Siok Hian AL, Petz LD. In vitro determination of red cell alloantibody significance using an assay of monocyte-macrophage interaction with sensitized erythrocytes. *Br J Haematol* 1984;56:19-29.

3. Gorst DW, Rawlinson VI, Merry AH, Stratton F. Positive direct antiglobulin test in normal individuals. *Vox Sang* 1980;38:99-105.

4. Garratty G. Factors affecting the pathogenicity of red cell auto- and alloantibodies, in Nance SJ (ed) Immune Destruction of Red Blood Cells. Arlington, VA., American Association of Blood Banks 1989, pp.109-169.

5. von dem Borne AEGKr, Beckers D, Engelfriet CP. Mechanisms of red cell destruction mediated by non-complement binding IgG antibodies: the essential role in vivo of the Fc part of IgG. *British Journal of Haematology* 1977;36:485-493.

6. Engelfriet CP, von dem Borne AEGKr, Beckers U, van der Meulen FW, Fleer A, Roos D, Ouwehand WH. Immune destruction of red cells. American Association of Blood Banks. Washington. D.C. 1981 pp.93-130.

7. Engelfreit CP, Overbeeke MAM, von dem Borne AEGKr. Autoimmune hemolytic anemia. *Seminars in Hemotalogy* 1992;29:3-12.

8. Zupanska B, Brojer E, McIntosh J, Seyfried H, Howell P. Correlation of monocyte-monolayer assay results, number of erythrocyte-bound IgG molecules, and IgG subclass composition in the study of red cell alloantibodies other than D. *Vox Sang* 1990;58:276-280.

9. Garratty G. Predicting the clinical significance of red cell antibodies with in vitro cellular assays. *Transfus Med Rev* 1990;IV:297-312.

10. Zupanska B, Gronkowska A, Ziemski M. Comparison of activity of peripheral monocytes and splenic macrophages in the monocyte monolayer assay. *Vox Sang* 1995;68:241-242.

11. Nance SJ, Arndt PA, Garratty G. The effect of fresh normal serum on monocyte monolayer assay reactivity. *Transfusion* 1988;28:398-399.

12. Branch DR, Gallagher MT. The importance of CO2 in short-term monocyte-macrophage assays (letter). *Transfusion* 1985;25:399.

13. Nance SJ, Amdt P, Garratty G. Predicting the clinical significance of red cell alloantibodies using a monocyte monolayer assay. *Transfusion* 1987;27:449-452.

14. Garratty G, Nance S, O'Neill P. Correlation of monocyte monolayer assays and RBC survival in patients with alloantibodies of questionable clinical significance. *Transfusion* 1985;25:474 (Abstr).

15. Schanfield MS, Stevens JO, Bauman D. The detection of clinically significant erythrocyte aIloantibodies using a human mononuelear phagocyte assay. *Transfusion* 1981;21:571-576.

16. Zupanska, B. Assays to predict the clinical significance of blood group antibodies. *Curr Opin Hematol* 1998;5(6):412-416.

17. Garratty G. Predicting the clinical significance of alloantibodies and determining the in vivo survival of transfused red cells in Judd WJ, Bames A. eds. Clinical and Serological Aspects of Transfusion Reactions: A Technical Workshop. Washington DC: American Association of Blood Banks, 1982:91-119.

18. Arndt PA, Garratty G. A retrospective analysis of the value of monocyte monolayer assay results for prediction the clinical significance of blood group alloantibodies. *Transfusion* 2004;44(9):1273-1281.

19. Storry JR, Castilho L, et al. International Society of Blood Transfusion Working Party on red cell immunogenetics and blood group terminology: Berlin Report. *Vox Sang* 2011.

20. van der Meulen FW, van der Hart M, Fleer A, et al. The role of adherence to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies. *Br J Haematol* 1980;38:541-549.

21. Gallagher MT, Branch DR, Mison A, Petz LD. Evaluation of reticuloendothelial function in autoimmune hemolytic anemia using an in vitro assay of monocyte-macrophage interaction with erythrocytes. *Exp Hematol* 1983;11:82-89.

22. Evans RS, Bingham M, Turner E. Autoimmune hemolytic disease: Observations of serological reactions and disease activity. *Ann NY Acad Sci* 1965;124:422–440.

23. Gilliland BC, Leddy JP, Vaughan JH. The detection of cell bound antibody on complement-coated human red cells. *J Clin Invest* 1970;49:898–906.

24. Wikman A, Axdorph U, Gryfelt G, et al. Characterization of red cell autoantibodies in consecutive DAT-positive patients with relation to in vivo haemolysis. *Ann Hematol* 2005;84:150-158.

25. Packman CH. Hemolytic anemia due to warm autoantibodies. *Blood Rev* 2008;22:17-31.

26. Mollison PL. Measurement of survival and destruction of red cells in haemolytic syndromes. *Br Med Bull* 1959;15:59–67.

27. Mollison PL, Crome P, Hughes-Jones NC, Rochna E. Rate of removal from the circulation of red cells sensitized with different amounts of antibody. *Br J Haematol* 1965;11:461–470.
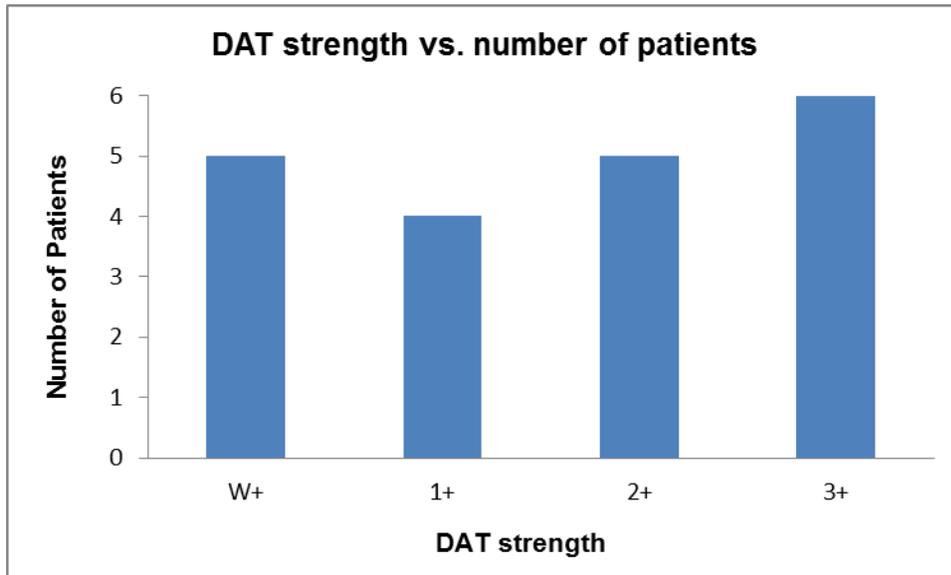
28. Engelfriet CP, Overbeeke MA, Dooren C, Ouwehand WH, von dem Borne A. Bioassays to determine the clinical significance of red cell alloantibodies based on Fc receptor-induced destruction of red cells sensitized by IgG. *Transfusion* 1994;34: 617–626.

29. Schreiber AD, Frank MM. Role of antibody and complement in the immune clearance and destruction of erythrocytes: I. In vivo effects of IgG and IgM complement-fixing sites. *J Clin Invest* 1972;51:575–582.

30. Brojer E, Zupanska B, Michalewska B. Adherence to human monocytes of red cells from autoimmune haemolytic anaemia and red cells sensitized with alloantibodies. *Haematologia* 1982;15:135-145.

31. Zupanska B, Brojer E, Thomson EE, et al. Monocyte-erythrocyte interaction in autoimmune haemolytic anaemia in relation to the number of erythrocyte-bound IgG molecules and subclass specificity of autoantibodies. *Vox Sang* 1987;52:212-218.

Table 1. MMA results of studied patients warm reactive autoantibodies.

	Patient #	AI (%)	PI (%)	MI (%) <5	5.1 - 20	>20
Warm autoantibodies only	1	1.8	0.2	2.0		
	2	0.8	0.0	0.8		
	3	0.0	0.0	0.0		
	4	1.4	0.0	1.4		
	5	1.2	0.0	1.2		
	6	1.8	0.0	1.8		
	7	0.0	0.0	0.0		
	8	2.2	0.4	2.6		
	9	3.4	0.2	3.6		
	10	0.0	0.0	0.0		
	11	8.4	21.6			30.0
Warm autoantibodies with concurrent alloantibodies	12	1.8	0.0	1.8		
	13	3.0	0.8	3.8		
	14	0.0	0.0	0.0		
	15	0.0	0.0	0.0		
	16	2.2	0.0	2.2		
	17	0.0	0.0	0.0		
	18	0.0	0.0	0.0		
	19	0.0	0.0	0.0		
	20	0.4	0.0	0.4		

MMA = monocyte monolayer assay; AI = adherence index; PI = phagocytosis index;
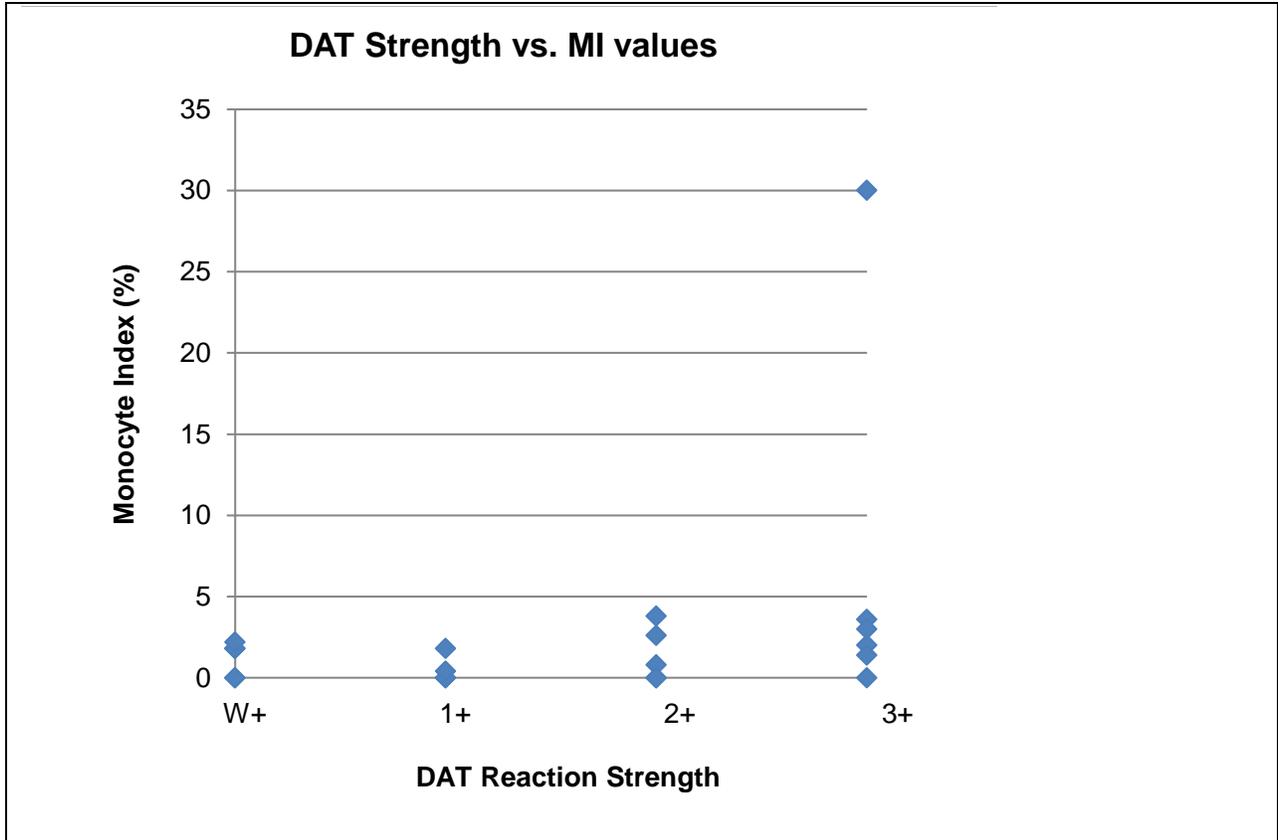MI = monocyte index

Figure 1. DAT strength of patient samples.

DAT (IgG) = direct antiglobulin test

Table 2. DAT strength in relation to MMA results.

DAT strength (anti-IgG) (range)	# of Patients evaluated	Patient showing positive AI	Patient showing positive PI	Patient showing positive MI
Microscopic	5	0	0	0
1+	4	0	0	0
2+	5	0	0	0
3+	6	1	1	1
Totals	20	1	1	1

MMA = monocyte monolayer assay; DAT = direct antiglobulin test; AI = adherence index; PI = phagocytosis index; MI = monocyte index

Figure 2. DAT strength with corresponding MI values.

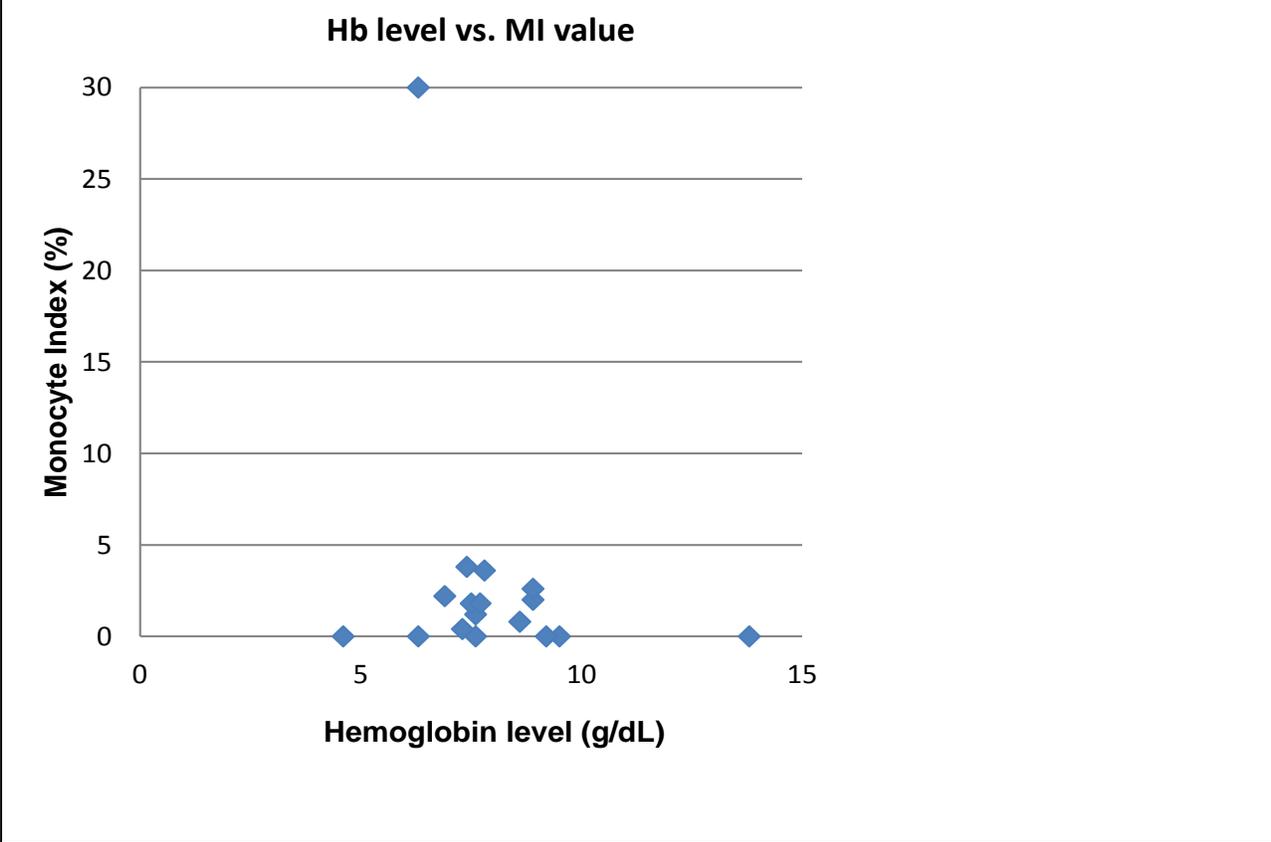DAT = Direct Antiglobulin Test; MI = Monocyte Index

Table 3. MMA results and the patient Hemoglobin levels.

Hb level (g/dL)	Number of Patients evaluated	Number of showing significant AI	Number showing significant PI	Number showing significant MI
4.0 - 6.0	1	0.0	0.0	0.0
6.1 - 8.0	10	1	1	1
8.1 - 10.0	5	0	0	0
10.1 - 14.0	1	0	0	0
total	17	1	1	1

*Hemoglobin level of 3 patients was not accessible.

MMA = monocyte monolayer assay; Hb = hemoglobin; AI = adherence index; PI = phagocytosis index; MI = monocyte index

Figure 3. Hemoglobin levels with corresponding MI values.

MI = monocyte index; Hb = hemoglobin