Determining the clinical significance of anti-M using monocyte monolayer assay

Brett C. Dunlap

LifeShare Blood Center

The MNS system is one of the 36 blood group systems currently recognized by the International Society of Blood Transfusion (ISBT). This blood group system (ISBT 002) was the second blood group described by Landsteiner and Levine in 1927. The MNS system consists of 48 antigens of which the M, N, S, s antigens are commonly encountered. These antigens are only expressed on red cells and are fully developed on fetal red cells. The MN determinants are carried on the transmembrane protein glycophorin A (Gagandeep, Sabita, Paramjit, & Ravneet, 2012).

Anti-M antibodies are usually not reactive at 37°C and hence are generally ignored in transfusion practice. However, when anti-M is active at 37°C, M antigen negative, crossmatch compatible red cell units should be selected for transfusion. The anti-M antibody is known to show dosage effect, reacting more strongly with homozygous cells (M+ N-) than heterozygous cells (M+ N+). This phenomenon is well demonstrated throughout this study. The prevalence of anti-M in donor sera is found to be 1 in 2500 units when tested with homozygous (M+N-) cells. This is reduced by half (i.e. 1 in 5000) when heterozygous (M+ N+) cells were used; indicating that some weaker examples of anti-M may be missed with heterozygous cells. Anti-M has also been described as a naturally occurring antibody in individuals whose red cells lack the M antigen and have no previous history of sensitization (Gagandeep, Sabita, Paramjit, & Ravneet, 2012).

Rarely has anti-M been implicated in immediate and delayed hemolytic transfusion reactions, which are supported by the results of 51Cr survival tests and monocyte phagocytosis assays. These examples demonstrate that anti-M can at times be of clinical importance and hence interpretation of test results should be done with caution (Gagandeep, Sabita, Paramjit, & Ravneet, 2012).

Anti-M antibodies have often been detected in antenatal mothers. Anti-M has been reported to cause hemolytic disease of the fetus and newborn (HDFN) of varying degrees of severity which may range from neonatal jaundice requiring exchange transfusion to intrauterine death. Reports in the literature describe this antibody as the second most common non-Rh antibody after anti-K (Kornstad, 1983). An unusual case in *Archives of disease in childhood: Fetal and neonatal* describes an anti-M of IgM and IgG nature responsible for neonatal red cell aplasia with substantial reduction in proliferation of erythroid cells in culture. It postulates that like an anti-K, anti-M may cause HDFN primarily by destroying erythroid progenitors rather than mature erythrocytes (Hinchliffe, Nolan, Vora, & Stamps, 2006).

This research outlines an experiment using monocyte monolayer assay (MMA) to discern whether the anti-M is clinically significant in 34 samples taken from thawed donor plasma known to contain anti-M.

Materials and Methods

RBC antibody testing technique

The presence of the anti-M antibodies in the sample plasma was tested and confirmed by using routine no enhancement media indirect antiglobulin (IAT) tube test. Two Medion (Grifols, Barcelona, Spain) screening cells, which were both homozygous for the M antigen, were selected to test the donor sera by IAT.

Treatment of plasma with 0.01M DTT (dithiothreitol)

The preparation of fresh 0.01M DTT was conducted by dissolving 0.154g of DTT powder in 100mL of a working solution of PBS at a pH of 7.3. To treat the plasma samples, a 1:1 ratio of 0.01M DTT and plasma were incubated at 37°C for 60 minutes. Using the previously stated antibody test method, the mixture was then retested. For each sample, a control tube was

prepared using a 1:1 ratio of plasma sample to 0.9% isotonic saline and tested in parallel with the test tubes. To ensure the removal of IgM antibodies, the control tube should remain positive, while the “test” tube should be negative.

Monocyte Monolayer Assay technique

This section describes the method for preparation of a monocyte monolayer, preparation of antibody-sensitized red cells, a combination of monolayer monocytes with sensitized red cells, and a calculation for the Monocyte Index (MI). The first step is to collect and separate the monocytes: two freshly drawn 7mL EDTA tubes are drawn from a healthy volunteer. The contents of the EDTA tubes are transferred to a 50mL sterile conical tube with equal parts phosphate buffer solution (PBS). In a separate 50mL sterile conical tube, 15mL of Sigma-Aldrich Histopaque® - 1077 is added and then tilted to a 30° angle. The diluted blood is slowly layered onto the Histopaque® - 1077 and then brought to a total volume of 45mL using PBS. After centrifugation for 60 minutes, the supernatant is removed and discarded. The white cell layer is carefully removed, transferred to another sterile 50mL tube and then diluted to a total volume of 45mL with PBS. After an additional 60-minute centrifugation step, the supernatant PBS is removed and discarded. 5mL of 5% Sigma-Aldrich® Fetal Bovine Serum (FBS) is then added to the remaining white blood cell pellet. This suspension is divided into equal amounts in each chamber of a tissue culture slide and incubated for one hour at 37°C in an atmosphere of 4-5% CO_2.

Aliquots from four units of donor blood, type O Rh negative, homozygous for M antigen were chosen for sensitization. The aliquots were manually washed three times with PBS and suspended to a 3% concentration. The test plasma was added to the 3% red cell solution to a ratio of 1:2 red cells/plasma and incubated at 37°C for 60 minutes. To allow for activation of

complement, fresh ABO compatible, non-immune serum was added to the red cells being sensitized with the test plasma and this mixture incubated for an additional 15 minutes at 37°C. After three more wash cycles with PBS, the mixture was suspended with 0.9% isotonic saline to a concentration of 3%. A drop from each tube was tested using Immucor Gamma anti-IgG and Immucor Gamma anti-C3b/C3d. The tubes were centrifuged once more, and the supernatant removed and discarded. The cells were then suspended in RPMI-1640 medium with 5% Sigma-Aldrich® Fetal Bovine Serum (FBS). The white cell monolayer was overlaid with the sensitized red cells and incubated at 37°C in an atmosphere of 4-5% CO_2 for 60 minutes. After incubation, the excess fluid was aspirated from each chamber on the tissue slide. The slide was removed from the media chambers and washed with 0.9% saline, then dried at 24°C. After drying, the slide was stained with a Wright stain and allowed to dry at 24°C. The monocytes were counted using a microscope with oil immersion lens. The slide was examined for at least 400 monocytes with a separate count of which cells were adhered (Adherence Index) and which were phagocytized (Phagocytized Index). Then, the Monocyte Index (MI) is calculated using the following formula:

$$MI = \frac{(\text{total \# of monocytes with adhered RBCs} + \text{total \# of monocytes with phagocytized RBCs}) \times 100}{\text{total number of monocytes counted}}$$

To calculate the Adherence Index (AI) use the following formula:

$$AI = \frac{(\text{total number of monocytes with adhered RBCs}) \times 100}{\text{total number of monocytes counted}}$$

To calculate the Phagocytized Index (PI) use the following formula:

$$PI = \frac{(\text{total number of monocytes with phagocytized RBCs}) \times 100}{\text{total number of monocytes counted}}$$

Table1. Significance of MI Values	
0	No adhered or phagocytized red cells
≤5%	Incompatible blood can be given without significant risk of hemolytic transfusion reaction (HTR) or hemolytic disease of the fetus and newborn (HDFN)
≥20%	Significant risk of HTR and HDFN

RBC antibody titration technique

Anti-M titration studies were conducted by the selection of a cell, which was homozygous for the M antigen, and then performing a serial dilution of the plasma samples. These samples reacted at the anti-human globulin (AHG) phase in the routine antibody screen. No enhancement media was added while performing the titrations. The titration endpoint was defined as the tube with the highest dilution demonstrating 1+ reactivity macroscopically. The titer was reported as the reciprocal value of this dilution.

Results

Antibody screens were performed on 34 donor plasma samples using no enhancement technique. These samples were previously known to contain anti-M. Twenty-four of the 34 samples had room temperature reactivity and no reactivity at AHG phase; therefore, no additional testing was performed on these samples. The remaining 10 samples, which did yield reactivity at the AHG phase, were treated with a freshly made working solution of 0.01M DTT, to remove any IgM component that might have been present in each sample. After the DTT treatment, an additional antibody screen was performed using no enhancement to determine which samples had IgG components. Five of the 10 samples treated yielded reactivity at AHG (see Table 2). These five samples are the group used for MMA testing (see Table 3 for MMA results).

Table 2. Serological Results of the Test Plasma

Sample ID	Neat Plasma		DTT-treated Plasma	
	Screen Cell I	Screen Cell II	Screen Cell I	Screen Cell II
W2801	3+	2+	0/0✓	0/0✓
W4716	2+	2+	0/0✓	0/0✓
W2397	2+	1+	2+	1+
W1643	Micro+	Micro+	0/0✓	0/0✓
W6840	2+	2+	W+	W+
W9437	2+	2+	1+	1+
W8793	3+	3+	0/0✓	0/0✓
W2370	1+	1+	1+	1+
W1552	2+	2+	3+	3+
W1202	1+	1+	0/0✓	0/0✓

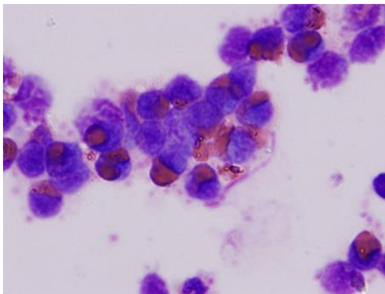

Image of MMA demonstrating RBC adherence and ingestion. Photograph credit: In house

Table 3. MMA Results				
RBC Source	**Antigen Profile**	**AHG IgG**	**AHG C3b/C3d**	**Monocyte Index**
Positive control	D+ M+N=	3+	Not done	60.25
Donor W9437	M+N=	1+	Micro+	4.5
Donor W2370	M+N=	2+	1+	5.5
Donor W2397	M+N=	2+	Micro+	2.5
Donor W6840	M+N=	1+	1+	0.75
Donor W1552	M+N=	2+	1+	15.5

Additionally, antibody titrations were performed on these five samples in order to correlate the clinical significance of the titer with the MMA results (see Table 4 for titer results).

Table 4. Antibody Titrations of anti-M present in MMA test samples	
Donor	**Titer**
Donor W9437	Titer = 8
Donor W2370	Titer = 16
Donor W2397	Titer = 16
Donor W6840	Titer = 4
Donor W1552	Titer = 32

Discussion

Prior to this study, using the MMA to determine the clinical significance of anti-M had yet to be performed. This study has the potential to uncover antibodies to the M antigen which are clinically significant and may not be detected by serology alone.

The low titers on samples from Donor W9437 and Donor W6840 are associated with the low MI percentages, which provides further proof that the antibodies present are not clinically significant. The higher titration result in the sample from Donor W1552 is in direct relation to the higher MI percentage, which indicates that this anti-M is more abundant and has more potential for clinical significance.

The MI percentage for sample from Donor W1552 is not >20%, which is considered predictive of hemolytic disease of fetus and newborn and/or cause for an adverse reaction to transfusion of M+ red cells (Rampersad, et al., 2005). However, the percentage is high enough that caution should be exercised if a patient and/or donor unit to be transfused has results similar to this in a clinical setting. More research is needed with a larger population of antibody positive samples in order to prove this could be common occurrence.

References

Gagandeep, K., Sabita, B., Paramjit, K., & Ravneet, K. (2012). Clinically significant anti M antibodies – A report of two cases. *Transfusion and Apheresis Science*(47), 259-261.

Hinchliffe, R. F., Nolan, B., Vora, A. J., & Stamps, R. (2006). Neonatal pure red cell aplasia due to anti-M. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, F467-F468. doi:doi:10.1136/adc.2006.102954

Kornstad, L. (1983). New Cases of Irregular Blood Group Antibodies Other Than Anti-D in Pregnancy. *Acta Obstet Gynecol Scand*(62), 431-436.

Rampersad, G., Suck, G., Sakac, D., Fahim, S., Foo, A., Denomme, G. L., & Branch, D. (2005). Chemical compounds that target thiol-disulfide groups on mononuclear phagocytes inhibit immune mediated phagocytosis of red blood cells. *Transfusion*, pp. 384-393.