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A MICRO COLORIMETRIC ASSAY USING CRYOPRESERVED MONOCYTES TO EVALUATE ANTIBODY-MEDIATED RED CELL-MONOCYTE INTERACTION

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ABSTRACT

Background and Objective. A number of *in vitro* assays based on the interaction of red cells with monocytes have been used to determine the clinical significance of red cell antibodies. When used in our laboratory, one of these assays (the monocyte-macrophage phagocytosis assay - MMPA), was very time consuming and showed great variability.

Methods. We set up a monocyte phagocytosis colorimetric assay (MPCA), using standard microtiter plate wells coated at 37°C for 1 hour with monocytes from healthy donors. After washing the wells to remove non-adherent monocytes, test red cells are added to the wells. Sensitized red cells bind to the monocytes, which are lysed after incubation to measure red cell phagocytosis. This is done by hemoglobin detection in the lysate through reaction with o-phenylenediamine and absorbance evaluation with a colorimeter. The results are expressed as the phagocytosis index (PI), which is calculated with the following formula: PI=[1-(A_{450} unsensitized red cells/A_{450} sensitized red cells)] $\times$ 100. In this study we determined: the source of MPCA variability; the precision of MPCA results; the correlation between MMPA and MPCA results; the MPCA reference values and the MPCA and MMPA execution times.

Results. MPCA variability depended largely on the monocyte source. The smallest variation coefficient of the results of replicate assays (19-21%) was found using pooled, cryopreserved monocytes. When performed with a pool of cryopreserved monocytes from 10 subjects, the SD of PI values obtained in replicate assays showed little variation (11-13) over the range of anti-D concentrations tested (from 18.75 to 300 ng/mL). A linear correlation coefficient *r* of 0.96 was obtained when MPCA and MMPA were performed in parallel, and the 95th centile of PI reference values determined with red cells of 40 non-transfused surgical patients free of irregular red cell antibodies was 7. MPCA execution time was 56% of that needed to perform MMPA.

Interpretation and Conclusions. These studies show that MPCA is an easy and reproducible assay which allows objective and automated evaluation of red cell phagocytosis.

Key words: monocytes, phagocytosis, red cell antibodies

It has been suggested that cellular immunoassays can provide information on the clinical significance of red cell antibodies.[1-5] These assays are based on the ability of mononuclear effector cells to bind, phagocyte and lyse opsonized red cells. The reaction between effector and target cells is identified in the antibody-dependent cellular cytotoxicity test (ADCC) by the detection of ^{51}Cr released from red cells.[6] It is identified in the chemiluminescence test (CLT) by the evaluation of the chemiluminescence produced when oxygen radicals formed by monocytes after red cell phagocytosis react with luminol,[7] and in the monocyte-macrophage phagocytosis assay (MMPA)[8] by microscopic determination of red cells rosetted to, and phagocytosed by monocytes plated onto a glass coverslip or a glass slide. At the onset of our studies we used the MMPA because, unlike CLT, it did not require expensive equipment, and, unlike ADCC, it did not use radioisotopes. Despite these advantages, in our opinion this method was time consuming and showed considerable variability.[9] Therefore, we modified a method developed by Jungi for other purposes,[10] and set up a monocyte phagocytosis colorimetric assay (MPCA) that uses standard microtiter plate wells instead of glass coverslips and an automated reader to collect the results.

In this paper we report: an evaluation of MPCA variability; the precision of MPCA results; the correlation of MMPA and MPCA results; MPCA reference values and a comparison of the times needed to perform MPCA and MMPA.

Materials and Methods

Monocyte purification

Five mL aliquots from buffy coats obtained from whole blood units within 2 hours of collection were

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each layered over 3 mL of lymphocyte separation medium (density 1.077 g/mL, ICN Pharmaceuticals Inc, Costa Mesa, CA, USA) in 10 mL polystirene tubes (LP, Milan, Italy) and centrifuged at 450 g for 20 minutes.

After centrifugation the interfaces containing mononuclear cells were harvested and the cells were washed three times with 5 mL phosphate buffer saline (PBS, Gibco, Grand Island, NY, USA). The cells were resuspended in 5 mL of RPMI medium (Gibco) containing 10% fetal calf serum (FCS) (Gibco) and layered over a 5 mL Percoll (Pharmacia, Uppsala, Sweden) gradient, prepared as follows: 2.3 mL of solution A (92.5% Percoll, density 1.13 g/mL and 7.5% of 10 x PBS) were added to 2.7 mL of solution B (RPMI/10% FCS, 275 mosm/L). The tubes were centrifuged at 550 g for 30 minutes. Then, the cells were harvested at the interfaces and washed twice by suspension in PBS and centrifugation at 440 g for 5 minutes. Lastly, the cells were resuspended in 2 mL RPMI/10% FCS.

Monocyte cryopreservation

Purified cells from 10 buffy coats were pooled and an equal volume of a cryoprotective medium containing 50% (v/v) RPMI, 30% (v/v) FCS and 20% (v/v) dimethylsulfoxide (DMSO, Carlo Erba, Milan, Italy) was slowly added to the cell suspension as the tube was gently stirred in crushed ice. Lastly, the cells were dispensed into vials in 1 mL aliquots and frozen at –80°C in a mechanical freezer.

Before use in the MPCA, the vials were gently shaken in a waterbath at 37°C. As the ice melted, the cells were transferred into a plastic tube, washed twice with 2 mL RPMI and finally resuspended in the same medium at the concentration of 2×10^6 cells/mL. The average freeze-thaw-wash yield was approximately 60%. In order to limit variability due to decreasing survival and function (adherence and phagocytosis) of thawed-washed monocytes, the plating phase of MPCA was started within 1 hour of immersion of the frozen monocyte vial in the water bath.

Preparation of positive and negative controls

We prepared positive controls by incubating one volume of 5% R1r red cells with one volume of anti-D antibodies (Rhesuman Berna, Berne, Switzerland) diluted with saline solution at a concentration of 150 ng/mL in a waterbath at 37°C for 1 hour. The cells were washed 3 times with saline solution and resuspended at 5% and 0.5% in RPMI/10% FCS for MMPA and MPCA respectively. Positive controls showed a 4+ agglutination score when tested with the antiglobulin test. Negative control samples consisted of a 5% or 0.5% suspension of the same unsensitized R1r red cells incubated with AB serum collected from male individuals that was serum free from red cell antibodies.

Monocyle-macrophage phagocytosis assay (MMPA)[8]

Fresh non-cryopreserved purified mononuclear cells obtained from one buffy coat were resuspended at 10^6 cells/mL in RPMI supplemented with 10% FCS. One mL of cell suspension was plated over a 22×22 mm coverslip supported by a 35 mm Petri dish. The Petri dishes were incubated for 1h at 37°C in 5% CO_2. After incubation, the coverslips were removed and rinsed vigorously in saline to remove non-adherent cells. The coverslips with the adherent cells were then placed into new Petri dishes and overlayed with the red cells to be tested (1 mL of 5% red cells in RPMI containing 10% FCS). The Petri dishes were incubated for an additional 2h at 37°C in 5% CO_2. After incubation, the coverslips were removed, rinsed gently to remove any unassociated red cells, fixed with methanol for 2 minutes, stained with May Grünwald-Giemsa and observed for adherent and phagocytosed red cells using a light microscope equipped with 10× oculars and a 100× objective. The total association index (TAI) was defined as the number of red cells adherent to, or phagocytosed by 100 monocytes.

Monocyte phagocytosis colorimetric assay (MPCA, modified from ref. #10)

One-hundred µL of monocyte suspension (2×10^6 cells/mL) were dispensed into the wells of a u-well polystirene microtiter plate (Sterilin, Staffordshire, UK). The plate was incubated for 1 h at 37°C in a humidified chamber at 5% CO_2. The wells were then carefully washed with 100 µL PBS to remove non-adherent cells and 100 µL of 0.5% sensitized red cell suspension were added. The plate was incubated for 90 min at 37°C and non-ingested red cells were removed by washing the wells three times, gently adding 100 µL of PBS to each well, tilting the plate and removing the fluid by vigorously inverting the plate. Lastly, 100 µL of a hypotonic solution (2 vol saline + 7 vol H_2O) were added to the wells for 5 minutes and then discharged. Then 100 µL of 0.3% sodium dodecyl sulphate (SDS, Bio-Rad Laboratories, Richmond, CA, USA) in PBS were dispensed into the wells to lyse cells and after 10 minutes 100 µL of o-phenylenediamine (OPD) solution were added as a chromogen.

OPD solution was prepared as recommended by the manufactuer by dissolving one OPD tablet in 6 mL of substrate buffer (Ortho Diagnostic Systems, Raritan, NJ, USA) and 100 µL of H_2O_2. After 20 min at room temperature in the dark, the absorbance (A) at 450 nm was evaluated with an automated reader (AutoReader III, Ortho Diagnostic Systems), and the phagocytosis index (PI) was calculated according to Rummage and Leu as follows:[11]

$$PI=[1-(A_{450}\text{ unsensitized red cells}/A_{450}\text{ sensitized red cells})]\times 100$$

Each assay was performed in triplicate and the PI was computed from the mean absorbance obtained. All solutions were stored at 4°C and used within one week of preparation, with the exception of OPD, which was prepared immediately before use.

Study 1: sources of MPCA variability

To evaluate the variability factors, we carried out 3 sets of experiments with cells from healthy donors. In each set a different variability factor was considered as explained below.

A) The first factor considered was variability due to the source of monocytes (10 non cryopreserved monocyte suspensions versus 1 red cell suspension). In 10 experiments performed in 10 days, we purified and tested non-cryopreserved monocytes of 10 donors in the MPCA. Each day a different donor was used. The different monocyte suspensions were tested against fresh R1r red cells collected from one subject who was not one of the monocyte donors, and sensitized v/v with 300 ng/mL anti-D antibodies on each of the 10 days.

B) The second factor was variability due to the source of red cells (1 cryopreserved monocyte pool versus 10 red cell suspensions). For this set of experiments we purified and cryopreserved a monocyte pool from 10 donors. On each of the 10 days we performed the MPCA with an aliquot of thawed monocytes from this pool, which were incubated with R1r red cells sensitized as described previously. Each day a different red cell donor was used.

C) Next, the technical variability was studied (1 cryopreserved monocyte pool versus 1 red cell suspension for 10 times). In this set we used aliquots of the monocyte pool described above. On each of the 10 days, monocytes were tested with R1r red cells collected from the same donor and sensitized as described in the first experiment.

We chose to use non-cryopreserved monocytes in the first study and cryopreserved monocytes in the last two studies because they seemed to be the most convenient conditions in case we would have ultimately chosen to use single-donor monocytes or pooled monocytes respectively. We evaluated the results of study 1 by using the F test.[12]

Study 2: evaluation of MPCA precision

In a series of 10 experiments (1 per day), we tested R1r red cells of different subjects sensitized with anti-D antibodies at concentrations of 18.75, 37.5, 75, 150, and 300 ng/mL, respectively, with the same pool of cryopreserved monocytes from 10 donors.

Study 3: correlation of MMPA and MPCA results

Correlation between the MMPA and MPCA results was determined using 31 red cell samples from autoimmune hemolytic anemia patients showing a positive direct antiglobulin test, with the MMPA and the MPCA assays being performed in parallel with the same pool of cryopreserved monocytes from 10 donors.

Study 4: determination of MPCA reference values

We performed the MPCA with 40 red cell samples from patients admitted to our hospital for surgical procedures who were found free of irregular red cell antibodies when examined by standard serological methods.

Study 5: evaluation of time required to perform MPCA and MMPA

We determined the times needed to perform the MMPA with fresh monocytes and MPCA with cryopreserved monocytes. The times needed for monocyte purification were determined experimentally on 5 occasions, and the average time was included in the computation. Because the preparation of the cryopreserved monocyte pool from 10 donors used in the MPCA provides sufficient monocytes for at least 25 assays, 1/25 of this time was used for the computation of time required for monocyte separation and freezing for the MPCA.

Results

Study 1. The results of study 1 are reported in Table 1. Variability was significantly greater in experiment A, in which the source of the monocytes was different in each replicate, than in experiments B and C ($0.01<p<0.05$). These results suggest that the source of monocytes is a major factor influencing the test variability.

Study 2. The precision of the results of MPCA is shown in Figure 1, which reports the mean±1SD of PI values of 10 replicates. Similar standard deviations of the results of replicate assays were found over the range of anti-D concentrations tested (from 18.75 to 300 ng/mL).

Study 3. Figure 2 shows the results of the study of 30 autoimmune hemolytic anemia patients with a positive direct antiglobulin test, performed in parellel with MMPA and MPCA. The Pearson correlation coefficient *r* of the MMPA and MPCA results[12] was 0.96.

Table 1. Results of study 1: variability of MPCA results evaluated in 3 sets of experiments performed under conditions A, B and C. The results are given as mean, standard deviation (SD) and coefficient of variation (CV) of the phagocytosis index (PI, see *Materials and Methods*).

	PI		
	Mean	SD	CV
A. 10 non-cryopreserved monocytes vs. 1 red cell (#10)	63.8	19.7	31%
B. 1 cryopreserved monocyte pool vs. 10 red cells (#10)	56.5	12	21%
C. 1 cryopreserved monocyte pool vs. 1 red cell (#10)	55.6	10.5	19%

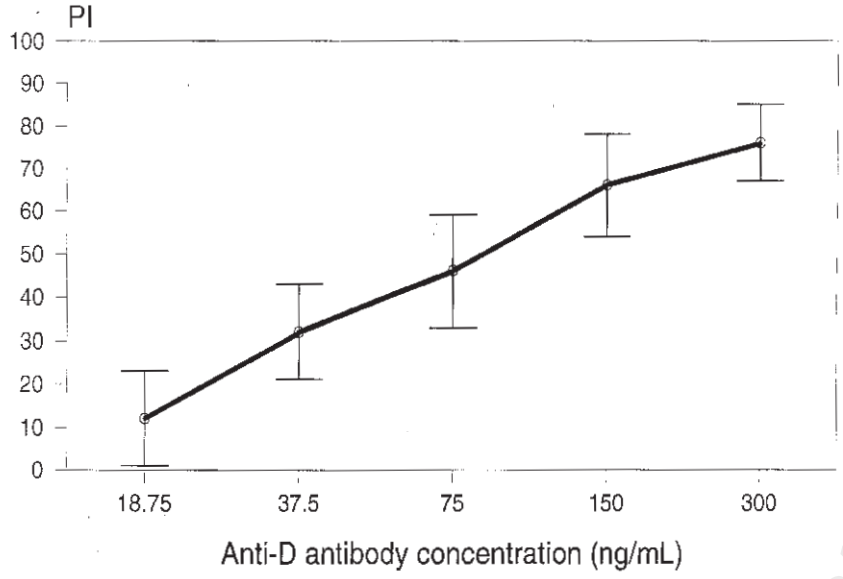

Figure 1. Results of study 2: phagocytosis index (PI, mean±1SD) obtained with MPCA by testing the same suspension of cryopreserved pooled monocytes from 10 donors with 1 suspension of red cells sensitized with different amounts of anti-D antibodies.

Study 4. The reference PI values ranged from 0 to 10. We set the upper limit of the reference values at 7, which corresponded to the 95th percentile.

Study 5. The times needed to perform the MMPA and MPCA are reported in Table 2, which shows that MPCA required 56% of the time needed for MMPA.

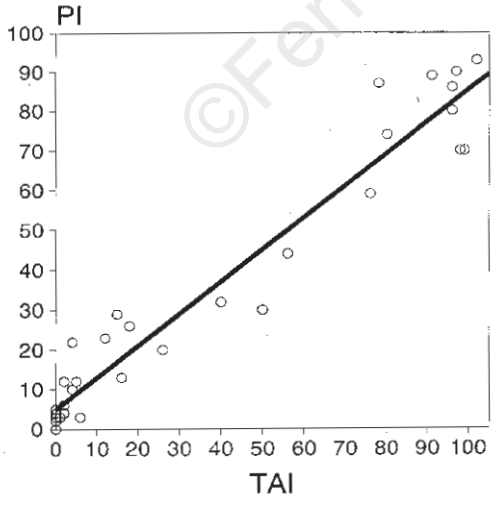

Figure 2. Results of study 3: correlation between MMPA and MPCA results, expressed as total association index (TAI) and phagocytosis index (PI), respectively.

Discussion

Traditional serological assays give limited information on the clinical significance of irregular red cell antibodies. In fact, the patient' sera can contain antibodies of different IgG subclasses which are able to affect the life span of red cells to different degrees.[13] To overcome these difficulties a number of assays based on the interaction of red cells with cells known to be involved in *in vivo* red cell destruction[14] have been developed.[2,6-8]

A number of studies reported findings which indicated a strong correlation between the above-mentioned functional assays and the antibody's clinical significance. In one of these studies, Schanfield *et al.* found much higher scores with clinically significant antibodies than with clinically insignificant ones, with no overlap between the two groups.[15] In addition, Branch and Gallagher found 100% correlation between their monocyte-phagocytosis assay results and *in vivo* clinical significance as well[16] although a preceeding investigation from the same group had shown that only 53% of all potentially clinically significant alloantibodies studied demonstrated significant *in vitro* interactions.[1] In 21 immunized patients, Gutgsel *et al.*[17] successfully selected red cell units that were serologically incompatible, based solely on the results of a red cell-monocyte assay. Similarly, Wren and Issitt[18] studied 11 cases of clinically significant antibodies and 4 cases of clinically insignificant antibodies and found 100% correlation of *in vitro* and *in vivo* antibody activity, with the exception of one case of anti-Lu^b antibodies that gave ambiguous results in the cellular assay. Additional investigations by Nance *et al.*[2] showed

Table 2. Results of study 5: steps and times needed to perform MMPA and MPCA.

MMPA steps	*average time (min)*
1. monocyte separation	150
2. monocyte incubation on coverslip	60
3. coverslip washing	5
4. red cell-monocyte incubation	120
5. coverslip washing	5
6. cell staining	30
7. results evaluation with microscope	40
Total time	410

MPCA steps	*average time (min)*
1. monocyte separation	6
2. monocyte freezing	2
3. monocyte thawing	15
4. monocyte incubation in plate	60
5. washing of wells	10
6. red cells-monocyte incubation	90
7. washing of wells	10
8. monocyte lysis	15
9. Hb reaction with chromogen	20
10. results evaluation with automated reader	2
Total time	230

that their red cell-monocyte assay was able to predict the clinical outcome of transfusion in 12 patients with antibodies against high-frequency antigens (*Lan, Ge, Yt^a, Yt^b*). Regarding the hemolytic disease of the newborn, Engelfriet *et al.* reported a strong correlation between the results of ADCC and clinical outcome.[14] Conversely, when the ADCC was used by other authors to investigate antibodies against high frequency antigens, the correlation was poor.[19]

At the onset of our studies with the MMPA, this method was found to show great variability and to be very time consuming, as well.[9] To support our findings, other investigators have reported that the microscopical estimation of phagocytosis is very subjective and variable from one laboratory to another.[20] To overcome these disadvantages, we developed the MPCA, which uses a microtiter plate and an automated reader to evaluate the results. In the studies reported in this article, we found that the use of a cryopreserved suspension of pooled monocytes from 10 donors showed less variability than the use of individual monocyte suspensions. This could be due to the well-known biological variability of cells collected by different subjects.[21] Moreover, MPCA results showed a strong correlation with the results obtained by skilled operators with MMPA, thus suggesting that the biological information provided by the two assays is similar. Additional advantages of MPCA include shorter execution times and the possibility of achieving objective and reproducible results automatically. In addition to the well-known potential applications of red cell monocyte assays, a new application was recently reported by Ducrot *et al.*,[20] who published an excellent study on the assessment of the functional properties of polyclonal and monoclonal Rh D antibodies. The main principle of this assay is similar to that of MPCA: it is based on the transformation of 2,7-diaminofluorene (DAF) into a colored molecule, fluorene bleu, by the pseudoperoxidase activity of free hemoglobin, released by red cells that are phagocytosed by monocytes. The data published by Ducrot *et al*. suggest that different anti-D monoclonal antibodies cause different levels of phagocytosis, which could be relevant in the selection of antibodies for therapeutic use. A direct comparison of the DAF assay and MPCA could be of interest in order to determine the practicality, the overall variability and the level of sensitivity of the two assays performed under similar conditions. In regard to sensitivity, our data from study 2 suggest that MPCA can reliably detect phagocytosis of red cells sensitized with more than 50 ng/mL of anti-D antibodies. This level could be compared to that reported by Ducrot *et al.*, who stated that "*...high OD values were obtained [with the DAF assay] even with weak concentrations of anti-D (0.5-0.1 µg/mL [500-100 ng/mL])*". Thus, it would be interesting to determine if the DAF assay and the MPCA, which show almost equally good sensitivity, allow the collection of data with similar biological significance.

In conclusion, our data suggest that the MPCA can be a useful tool in the technical repertoire of immunohematology. As a follow-up of the technical characterization of the MPCA reported in this article, further studies are encouraged in order to investigate its capability in predicting the clinical relevance of red cell antibodies.

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