Journal of Immunological Methods, 24 (1978) 363—370

BRIEF COMMUNICATION: TECHNICAL MODIFICATIONS OF THE HUMAN AGAROSE MICRODROPLET LEUKOCYTE MIGRATION INHIBITION ASSAY

JAMES L. WEESE [1,2], JAMES L. McCOY [1], JACK H. DEAN [3], JOHN R. ORTALDO [1], KATHLEEN R. BURK [1] and RONALD B. HERBERMAN [1]

[1] *Laboratory of Immunodiagnosis, National Cancer Institute, Bethesda, MD 20014,* [2] *Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48104 and* [3] *Department of Immunology, Litton Bionetics Research Laboratories, Kensington, MD 20975, U.S.A.*

(Received 6 January 1978, accepted 14 June 1978)

Direct leukocyte migration inhibition assays using the capillary tube technique can be used to demonstrate cell-mediated immunity in vitro. Unfortunately, the cumbersome nature of this technique makes it time consuming and difficult to perform. Similar results have been obtained using the direct agarose microdroplet leukocyte migration inhibition assay. In this paper, modifications of the agarose technique are outlined which insure standardization of droplets and ease of performance of the assay. Additionally a technique is described to reduce the time required for calculation of results.

INTRODUCTION

Direct capillary tube leukocyte migration inhibition (LMI) assays have been used to demonstrate in vitro cell-mediated immunity (CMI) to purified protein derivative (PPD) and tumor antigens (Bendixen and Soberg, 1969; Andersen et al., 1970; Kjaer, 1974; McCoy et al., 1974, 1975, 1976b, c, 1977a, b; Boddie et al., 1975a, b; Elias and Elias, 1975; Brandes and Goldenberg, 1976; Kadish et al., 1976). Several major problems have been associated with this technique: The assay is technically very time consuming and difficult to perform, with variation in results among different technicians; large volumes of blood are required (approximately 40—50 ml) for even small tests; reading and calculating the data require sophisticated equipment and are very time consuming. Harrington and Stastny (1973) developed an agarose microdroplet technique using guinea pig and mouse (Harrington, 1974) peritoneal exudate cells for LMI. We have found a similar technique to give reproducible results with PPD (McCoy et al., 1976a, 1977c), and more recently with tumor antigens using human leukocytes (McCoy et al., 1976d) and animal peritoneal exudate cells (Landolfo et al., 1977; McCoy et al.,

Supported in part by NCI Contract No. NO1-CB-63975.

1977d). The agarose microdroplet technique allows testing of many antigens with minimal amounts of blood (2—10 ml). Because of some remaining technical problems with the assay, including delivery of droplets of consistent size, maintenance of the liquid state of the agarose-cell suspensions, difficulties in drawing and measuring migration areas and calculating migration index, we performed the present study in an attempt to further simplify the assay and to reduce technical variation. We have found that with several technical changes and the recognition of several critical time intervals, the agarose microdroplet assay can be set up in less time than previously described (McCoy et al., 1976a), the replicates have less variation, and migration indices can be calculated more quickly.

METHODS

The technique for the agarose microdroplet human leukocyte migration inhibition assay has been described in detail (McCoy et al., 1976a). Briefly, 2—10 ml of whole blood is drawn into a plastic syringe containing 100 units/ml of preservative-free heparin and is mixed with Plasmagel (PG) (1 ml PG/6 ml whole blood) in a 15 ml conical tube and is allowed to settle at $1 \times g$ for 1 h in a 37°C incubator. The leukocyte-rich plasma is carefully aspirated to within 1 mm of the erythrocyte pellet and is placed in a 15 ml conical tube. The cells are centrifuged at $200 \times g$ for 10 min at room temperature. The plasma is discarded, and the cells are washed twice in McCoy's 5A medium containing heat-inactivated 10% fetal bovine serum (FBS) and 100 μg/ml of gentamicin. The cells are counted before the final wash and are resuspended at 2×10^7 leukocytes/ml in complete medium. During this period, 1 ml of 0.4% Seakem agarose is brought to a boil and placed in a 37°C water bath for several minutes. One ml of 2 × medium 199 (warmed to 37°C), containing 20% heat-inactivated FBS and 200 μg/ml gentamicin, is added to the liquefied 0.4% agarose. The final mixture, containing 0.2% agarose, is placed in a 37°C water bath until the cells are ready.

At this time, 1 ml of the leukocytes (2×10^7 cells) is dispensed into a plastic tube and centrifuged at $200 \times g$ for 10 min. Afterward all medium is carefully removed by a Pasteur pipet connected to suction. 0.1 ml of the 0.2% agarose mixture is added to the leukocytes and the tube is vortexed until an even suspension is obtained. Two microliter droplets of this suspension (containing 4×10^5 leukocytes per droplet) are placed into each well of the top half of a flat bottom Microtest II plate with a Drummond microdispenser. After droplets dry for 2—5 min, 0.1 ml aliquots of McCoy's 5A medium containing 10% FBS, Hepes buffer (25 mmoles/500 ml), gentamicin (100 μg/ml) are added to at least 4 control wells using a Biotip pipet. Similarly, antigen-containing medium is added to appropriate wells. The plates are covered and are kept in a 37°C, humidified 5% CO_2 atmosphere for 18—24 h.

After incubation, light mineral oil is added to each of the wells to improve

projection. Each well is projected onto paper using an inverted microscope fitted with a projection lens, and the inner area (the agarose droplet) and the outer area of migration are drawn for each well. These areas are carefully measured by a manual planimeter, and the area of migration determined by subtracting the smaller area from the larger area. The areas of 4 replicate droplets are averaged. A migration index (MI) was calculated using the formula:

$$MI = \frac{\text{Mean migration of 4 or more replicate droplets in the presence of antigen}}{\text{Mean migration of 4 or more replicate droplets in the absence of antigen}}.$$

METHOD MODIFICATIONS

It has been our experience that several technical adaptations can reduce the time needed to set up the test, and reduce the variability of the agarose microdroplet assay. These changes are related to several phases of the assay: (1) preparation and delivery of the droplets; (2) standardization of the time intervals for various steps in the assay; (3) the projection and recording devices used to measure migration; and (4) the technique by which migration index is calculated.

Initially, we used the Drummond 'Dialamatic microdispenser' for delivery of droplets. This instrument can only dispense a preset quantity, 2 μl, and requires refilling between droplets. Because of this limited capacity, the necessity for frequent refills, and the rapid settling of the cells, plating of 48 droplets (as are needed for a standard 12 antigen, 4 replicate/antigen test) requires repeated mixing (8—12 times on a vortex mixer) of the agarose-cell suspension during the plating procedure. Each mixing step requires removing the agarose mixture from the 37°C water bath, can cause loss of some cells on the sides of the tube, and can result in premature cooling and solidification of the agarose. For these reasons we have found that the Hamilton 0.1 ml gas-tight Luer lock syringe (No. 1710 TefLL) with the Hamilton repeater (No. PB-600-1) is more effective for droplet delivery. A Teflon adapter (No. 31330) allows connection to a 0.1 ml plastic tip (Volumetrics V-1 tips, Volumetrics Inc., Woburn, MA 01801) which will hold the entire 0.1 ml mixture and allow dispersing of 50—2 μl droplets in rapid succession. The advantages of this instrument include: (1) need for only one mixing step for every 50 droplets: (2) less variation among droplets (6.3% coefficient of variation among droplet areas compared to 8.0% coefficient of variation with the Drummond); (3) speed of delivery (48 droplets in approximately 50 sec compared to 210 sec for the Drummond); (4) no apparent change in consistency of agarose between the 1st and 48th droplet; (5) the disposable tips allow delivery of different patient cells in rapid sequence. An example of assays set up using both the Drummond and Hamilton syringes with the same PPD positive and negative donors is shown in Table 1. The results obtained with the Hamilton syringe were quite comparable to those obtained with the Drummond pipet.

TABLE 1

COMPARISON OF MIGRATION INDICES FOR A PPD-POSITIVE AND A PPD-NEGATIVE DONOR USING THE DRUMMOND PIPET OR HAMILTON SYRINGE

PPD conc. (μg/ml)	Migration indices			
	Drummond pipet		Hamilton syringe	
	PPD—	PPD+	PPD—	PPD+
50	1.17	0.75 [a]	1.00	0.68 [a]
5.0	1.18	0.80	1.02	0.70
5×10^{-1}	1.29	0.79	0.98	0.71
5×10^{-2}	1.13	0.89	1.00	0.72
5×10^{-3}	1.10	0.96	1.05	0.87

[a] Migration indices arbitrarily considered positive if MI value ≤ 0.80.

Timing and proper temperature equilibration were found to be critical factors in the agarose microdroplet technique. It was important for the agarose-medium solution to equilibrate at 37°C before addition to the leukocytes. Additionally, to maintain the desired temperature, we have found that before dispensing the droplets, an incubation of at least 4 min at 37°C is useful after the agarose-medium mixture is added to the cell pellet. When the cell suspension was dispensed before equilibration to 37°C, the agarose often began to solidify before all the droplets were placed, substantially reducing the migration areas of the later droplets.

After dispensing the droplets in the flat bottom Microtest II plate wells (Falcon No. 3040), the time for addition of media and antigen was found to be critical. When working at room temperature (approximately 24°C), a minimum delay period of 2.5 min between placing the droplets and addition of the medium or antigen was necessary to prevent slippage of the droplets. Additionally, a delay of greater than 8 min at room temperature after the droplets were dispensed resulted in drying, with substantially decreased areas of migration (Table 2). We have found that adding 0.1 ml of medium or

TABLE 2

EFFECT ON MIGRATION OF TIME INTERVAL BETWEEN DROPLET PLACEMENT AND ADDITION OF MEDIUM

Time intervals [a]	2.0	2.5	3.5	5	6.5	8	9.5	11	12.5
Migration index [b]	1.00	1.00	1.01	1.05	0.98	1.01	0.64	0.75	0.78

[a] Minutes between placement of droplets and addition of medium.

[b] Calculated as: $\frac{\text{Mean of 4 replicates for particular time interval}}{\text{Mean of first set of 4 replicates}}$.

medium containing antigen to each well from a graduated 1 ml pipet allowed completion of the technical aspects of a 48-droplet assay within the 'safe' time period.

The techniques previously described for reading this assay, projecting the circles onto paper, tracing their image, and then measuring this by planimetry are quite time consuming and tedious. Because the migration index represents the ratio of two average areas, and because the circular or elliptical migration areas can be calculated using geometric formulae, we expected that the ratio of calculated areas could be substituted for those determined by planimetry.

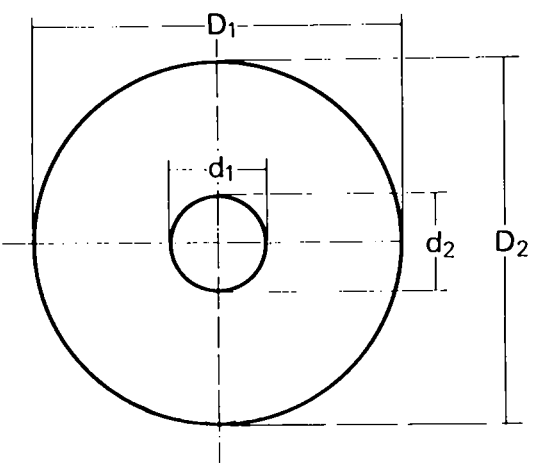
The area of the large outer ellipse in Fig. 1 can be determined by the formula:

$$A_1 = \pi \left[\left(\frac{D_1}{2}\right)\left(\frac{D_2}{2}\right)\right] = \frac{\pi}{4}(D_1 D_2)$$

The area of the inner ellipse can be represented by the formula:

$$a_1 = \pi \left[\left(\frac{d_1}{2}\right)\left(\frac{d_2}{2}\right)\right] = \frac{\pi}{4}(d_1 d_2)$$

The area of cell migration, the differences between the outer and inner ellipses, can be determined by subtracting a_1 from A_1. Using this formula a



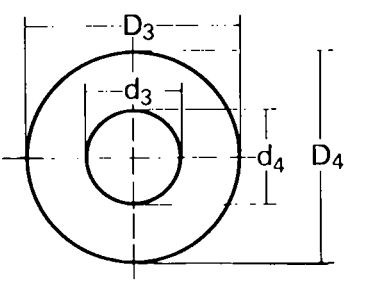


Fig. 1. Representative area of migration in the absence of antigen. D_1 and D_2 represent the diameters of the large ellipse, d_1 and d_2 represent the diameters of the small ellipse.

Fig. 2. Representative area of migration in the presence of antigen. D_3 and D_4 represent the diameters of the large ellipse, d_3 and d_4 represent the diameters of the small ellipse.

mean can be calculated for any number of replicates when the diameters of the ellipses are measured.

$$A_1 - a_1 = \frac{\pi}{4}(D_1D_2 - d_1d_2)$$

A representative area of migration in the presence of antigen would be (Fig. 2):

$$A_2 - a_2 = \frac{\pi}{4}(D_3D_4 - d_3d_4)$$

Using the above formulae, a migration index (MI) can be calculated by the

TABLE 3

COMPARISON OF MIGRATION INDICES CALCULATED USING EITHER DIAMETER MEASUREMENTS OR PLANIMETRY OF AREAS

Circle number	Diameter calculation	Planimetry
1	1.06	1.08
2	1.06	1.09
3	0.93	0.96
4	0.96	0.95
5	0.94	1.00
6	0.99	0.94
7	1.00	1.02
8	0.96	0.99
9	0.95	0.97
10	0.87	0.87
11	0.95	0.93
12	0.93	0.96
13	0.99	1.07
14	0.92	1.06
15	1.05	1.06
16	1.07	1.11
17	1.09	1.12
18	1.05	1.14
19	1.03	1.06
20	0.93	0.93
21	1.03	1.07
22	0.99	1.03
Range	0.87–1.09	0.87–1.14
Mean MI	0.98	1.01
S.D.	0.05	0.07
S.E.	0.01	0.01

[a] Migration indices listed calculated as: $\frac{\text{Area of individual droplet migration}}{\text{Mean area of 22 droplet migrations}}$.

following ratio:

$$MI = \frac{A_2 - a_2}{A_1 - a_1} = \frac{\frac{\pi}{4}(D_3D_4 - d_3d_4)}{\frac{\pi}{4}(D_1D_2 - d_1d_2)} = \frac{(D_3D_4 - d_3d_4)}{(D_1D_2 - d_1d_2)}$$

Projecting the migration plate onto a screen (Dokumator, DL2, Microfilm Reader, International Micro-optics, Fairfield, NJ) defines the margins of the migration area very clearly without the addition of mineral oil, and perpendicular diameters for each ellipse/circle can be easily read directly off the screen. A migration index can be calculated directly from these measured diameters with the use of a programmable calculator (Wang 700 or Texas Instruments SR-52). Measurement of MIs for the same migration areas, by planimetry and this formula (Table 3), demonstrated the comparability of results. Additionally, reading and calculating the data by this method took less than 30 min per 48 droplets compared to 2.5—3 h using the original technique.

CONCLUSIONS

The direct capillary tube leukocyte migration inhibition assay has been shown to give reproducible results demonstrating CMI against PPD and tumor-associated antigens. More recently, the direct agarose microdroplet assay has been shown to give similar results (McCoy et al., 1977c). The agarose microdroplet technique allows: (1) testing many antigens on a small blood sample; (2) ease and speed of performance; and (3) minimal technician to technician variation. Some of the earlier disadvantages of this assay included difficulties in delivering droplets of consistent size; maintaining the liquid state of the agarose-cell suspensions; and difficulties in drawing and measuring migration areas. We have found that the use of a Hamilton repeating syringe with a 0.1 ml disposable tip allows quick delivery of a large number of nearly identical droplets with minimal waste of cells. With experience in performing the assay, essentially all of the migration areas are circular, or elliptical, and based on this, a technique has been demonstrated which significantly increases the speed at which this assay can be read and a migration index calculated. Using this revised technique a 48-droplet assay could be set up, and after overnight incubation, migration areas could be read and migration indices calculated in approximately 30 min, compared to 2.5—3 h using the original technique.

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