Sensitive and quantitative determination of monocyte adherence

Gerrit D. Keizer, Carl G. Figdor and Jan E. De Vries *

Division of Immunology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

(Received 7 July 1986, accepted 18 August 1986)

A new sensitive and highly reproducible one-step ELISA is described to quantitatively determine the adherent capacity of monocytes and related cell lines. Cells were labelled with a monoclonal antibody/peroxidase conjugate which did not affect the adhesive properties of these cells. The labelled cells were allowed to adhere for 1 h and subsequently stained by the addition of substrate. The results demonstrate that there is a good correlation between the number of peroxidase-labelled adherent cells and the absorbance measured at 450 nm. Furthermore the assay permits the use of very low cell numbers since adherent cells could be measured efficiently at a level of only 100–500 cells/well.

The method may be very useful in the selection of hybridomas that secrete antibodies which inhibit adherence of cells. In addition it can be applied to study the adhesive properties of any cell type, provided that appropriate monoclonal antibodies are available.

Key words: Monocyte; Adherence; ELISA

Introduction

Monocytes originate from committed bone marrow stem cells and differentiate via monoblasts to promonocytes (Begemann and Rastetter, 1979). The latter already have the adherent and phagocytic characteristics of mature peripheral blood monocytes (Kaplan and Gaudernack, 1982). The promonocytes divide and differentiate into monocytes which leave the bone marrow compartment to circulate in the peripheral blood. After a short period of time these peripheral blood monocytes are thought to migrate into the various tissues where they develop into tissue macrophages (Kaplan and Gaudernack, 1982).

Human myeloid cell lines such as KG-1, HL-60 and U937 can be induced to differentiate in vitro along the monocytic pathway by chemical means (e.g., TPA) or biological response modifiers (e.g., γ-interferon) (Perussia et al., 1983). After such treatment the cells acquire morphological and functional characteristics of mature monocytes/macrophages. One of the properties gained by these cells is that they become adherent (Koeffler et al., 1981). Recently, various laboratories have developed monoclonal antibodies (moabs) that react with surface molecules which are associated with adherence. These molecules have been shown to play an important role in T cell-mediated cytotoxicity, being involved in the adhesion of effec-
tor cells to targets (Sanchez-Madrid et al., 1982; Spits et al., 1983) and in the interaction of T lymphocytes specifically interacting with soluble antigens presented by monocytes (Keizer et al., 1985).

Our aim has been to define antigens on human monocytes which are associated with cell adhesion. However, to date no sensitive, quantitative method to conveniently measure adhesion of monocytes or monocyte-like cell lines has been reported (for instance for screening of hybridoma supernatants). In the present communication we describe an ELISA which we believe will permit such determinations of cell adherence to plastic surfaces in a quantitative manner.

Materials and methods

Media and reagents

12-O-tetradecanoylphorbol 13-acetate (TPA), dimethylsulphoxide (DMSO) and 3,3',5,5'-tetramethylbenzidine (crystalline T2885; TMB) were obtained from Consolidated Midland Corporation (Brewster, NY), Merck (Darmstadt, F.R.G.) and Sigma (St. Louis, MO), respectively. Horseradish peroxidase (HRPO; type VI RZ = 3.0) and RPMI were purchased from Sigma and from Gibco Europe (Paisley, Scotland), respectively. Fetal calf serum (no. 401050, FCS-1; no. 401015, FCS-2), newborn calf serum (no. 103050; NBCS-1) and donor horse serum (no. 404057; HS) were all obtained from Sera Lab (Sussex, U.K.). Newborn calf serum (NBSC-2; batch no. U9700301) from Gibco Europe (Glasgow, Scotland) was also used. Human serum was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (HS(AB)).

Monoclonal antibodies

The monoclonal antibodies (moabs) CEM, Bear-1, Bear-2, Bear-3, Rupi-1, Rupi-2, SPV-L7 (anti-LFA-1) and Gepi-1 were raised in our laboratory. The moab CEM (anti-KLH) does not react with cells and served as a negative control. Bear-1, Bear-2 and Bear-3 react with the antigens Mo 1 (Todd et al., 1981), Mo 2 (Todd et al., 1982) and MAC 120 (Raff et al., 1980), respectively. Rupi-1 reacts with determinants on monocytes and granulocytes with $M_1, 35000$; Rupi-2 moabs detect a 12 kDa antigen on both monocytes and granulocytes. The antigen detected by Gepi-1 is present on bone marrow cells, monocytes, myeloid cell lines and granulocytes and as yet has no defined function. Preliminary experiments indicate that Gepi-1 is reactive with a sugar moiety on these cells. The moabs CLB-54 (CLB-LFA 1/1), and MHM-23 both reacting with the beta chain of the LFA-1 family (Keizer et al., 1985) were kindly provided by Dr. F. Miedema (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) and by Dr. A. McMichael (Nuffield Department, John Radcliffe Hospital, Oxford, U.K.), respectively. Hybridoma supernatants of SHCL-3, reacting with the alpha chain of p150,95 (Schwarting et al., 1985) were a gift from Dr. Roland Schwarting (Institute for Pathology, Berlin University, West-Berlin, Germany).

Cells

Human monocytes were isolated from a buffy coat of 500 ml of blood from healthy volunteers by centrifugal elutriation as described previously (Figdor et al., 1982). Monocytes obtained in this way were > 95% pure as judged by staining for non-specific esterase. The myeloblastic cell line KG-1 (Koeffler et al., 1978) was cultured in RPMI 1640 supplemented with glutamine ($2 \times 10^{-3}$ M), pyruvate (50 mg/ml) and 5% FCS. KG-1 cells were induced to differentiate to adherent monocyte/macrophage-like cells (Koeffler et al., 1981) by stimulation of $10^7$ cells with 10 ng TPA/ml in Falcon (3024) tissue culture flasks in a total volume of 20 ml. Cells were restimulated every third day with TPA (2 ng/ml) until use in the assay. Adherent cells were rinsed from the bottom of the culture flask, collected and washed with serum-free medium (Yssel et al., 1984) before use. In this study we used only TPA-stimulated KG-1 cells which were termed KG-1 cells.

Coupling of peroxidase to moabs

Gepi-1 moabs were labelled with horseradish peroxidase as described (Hudson and Hay, 1976). Briefly, 5 mg of moab (IgM) were dialysed against 0.01 M sodium carbonate buffer (pH 9.5). 4 mg HRPO were dissolved in 1 ml distilled water to which 200 µl freshly made 0.1 M NAIO₄ was
added. This solution was incubated for 20 min at room temperature (RT) followed by dialysis against 1 mM sodium acetate buffer (pH 4.0). After 16 h (4°C), 20 μl sodium carbonate (pH 9.5, 0.2 M) were added to the HRPO. Immediately afterwards the immunoglobulin preparation was added and the mixture incubated at RT for 2 h. Finally 100 μl NaBH₄ (4 ng/ml) were added to stop the reaction and the solution incubated at 4°C for an additional 2 h. Subsequently the preparation was chromatographed on a Sephadex G-100 column (85 × 1.5 cm), equilibrated in PBS in order to purify the HRPO mab conjugates. Absorbance was measured at 280 and 403 nm to distinguish HRPO conjugates from unbound HRPO. Between 8–12 ml peroxidase-labelled antibody (0.5 mg/ml) were obtained from 1 ml of ascites fluid.

Adherence assay

To test the capacity of human monocytes and KG-1 cells to adhere to a plastic surface, 5 × 10⁶ cells were incubated for 45 min with 100 μl of Gepi-1-peroxidase conjugates. Subsequently the cells were washed twice with PBS/BSA (0.2%) and resuspended in serum-free medium. 5 × 10⁴ cells were seeded in flat-bottom microtitre wells (Costar no. 3596) in the presence or absence of moabs (ascites dilution 1 : 250). After an incubation period of 60 min at 37°C the plates were washed twice by gently shaking the microtitre plates after the addition of 200 μl warm (37°C) PBS/BSA to each well. Subsequently the PBS was removed. The number of cells which remained attached to the substrate after the washing procedure were referred to as adherent cells. 100 μl TMB (100 mg TMB/10 ml DMSO, 1 : 100 diluted in 0.1 M NaAc, pH 6.0) were added. The reaction was stopped after 5–60 min depending on the intensity of the colour of the substrate, by the addition of 100 μl 0.8 M H₂SO₄. The staining intensity reflected the number of adherent cells according to a calibration curve which was determined with a multiscan photospectrometer (Titertek) at a wavelength of 450 nm.

A calibration curve made for each test served as a control. Known numbers of cells (Coulter counter analysis or determined by microscopy) were seeded in wells in 25 μl PBS and TMB substrate was added. By employing this method it was possible to quantitate the total number of HRPO-labelled cells instead of only the number of adherent cells. A linear relation between cell number and E₄₅₀ was reached within the range of 10²–2.10⁶ cells/well.

Gepi-1-peroxidase did not affect the adherent properties of the monocytoïd cells since equal numbers of labelled and unlabelled cells were adherent.

Results

Determination of the sensitivity of an ELISA to measure adherence

To investigate whether the adhesion of monocytes and KG-1 cells could be measured quantitatively, 5 × 10⁶ KG-1 cells were suspended in 100 μl PBS and incubated with 35, 175, or 350 μg of peroxidase-labelled Gepi-1 moabs. The calibration curves shown in Fig. 1 demonstrate that there is a linear correlation between the number of cells and the E₄₅₀. The sensitivity of the test could be increased by adding larger quantities of Gepi-1-peroxidase conjugates to the same number of cells (Fig. 1), probably due to the large number of binding sites for Gepi-1 on these cells (results not shown). KG-1 cells could be detected up to a level

![Fig. 1. Correlation between cell number and E₄₅₀](nap.lk/assets/images/143.png)
of 100 cells/well, when incubated with 350 μg Gepi-1-peroxidase conjugates/5 × 10⁶ cells. Monocytes could be detected up to a level of 500 cells/well. This difference is probably due to the fact that the antigen recognized by Gepi-1 mAbs is less abundantly present on monocytes than on KG-1 cells. A linear correlation between E₄₅₀ and the number of monocytes or KG-1 cells was obtained up to the maximum cell concentration tested, i.e., 2 × 10⁶ cells/well (results not shown).

To determine whether lymphocyte contamination in monocyte preparations could disturb the calibration curve and/or the adherence of the cells, we carried out mixing experiments. As shown in Table I lymphocytes did not affect the ELISA for determining the number of adherent cells nor did it affect the calibration curve. In addition, monocyte adherence was not diminished in the presence of lymphocytes. Furthermore it was shown both by ELISA and by Coulter counter analysis that 98–100% of the monocytes were adherent. The number of non-adherent cells corre-

TABLE I

EFFECTS OF LYMPHOCYTE CONTAMINATION IN THE MONOCYTE PREPARATIONS ON THE ELISA E₄₅₀ MEASUREMENT

5 × 10⁶ monocytes and lymphocytes were incubated separately with 175 μg Gepi-1 peroxidase, washed and mixed as indicated (total cell number, 5 × 10⁴/well). After an incubation period of 1 h, adherent cells were determined by ELISA or Coulter counter analysis.

<table>
<thead>
<tr>
<th>Monocytes %</th>
<th>Lymphocytes %</th>
<th>ELISA (E₄₅₀)</th>
<th>Counted number of adherent cells (×10⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calibration</td>
<td>Adherence</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>342 ± 16</td>
<td>337 ± 12</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>279 ± 17</td>
<td>269 ± 11</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>210 ± 7</td>
<td>198 ± 7</td>
</tr>
<tr>
<td>25</td>
<td>75</td>
<td>143 ± 5</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>65 ± 2</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>63 ± 3</td>
<td>63 ± 3</td>
</tr>
</tbody>
</table>

*a* Measurement of all seeded cells (without washing).

*b* Measurement of all adherent cells (after washing).

*c* The number of the adherent cells as calculated by subtraction of the counted number of non-adherent cells from the total cell number.

The correlation between the % of monocytes and \( a^b \) and \( c \) was 0.999, 0.999 and 0.996, respectively.

Inhibition of adherence of monocytes and KG-1 cells by monoclonal antibodies

To investigate whether the ELISA could be used to measure inhibition of adherence, we tested a panel of ten mAbs (ascites dilution 1:250) that reacted with several differentiation antigens on human monocytes. Two out of ten mAbs, MHN-23 and CLB54, both reacting with the beta chain of LFA-1, Mo 1 and p150,95, effectively blocked the adherence of both KG-1 and monocytes to plastic surfaces (Table II). The results shown in Fig. 2 demonstrate that the inhibition directly correlated with the amount of mAb present and that dilution of ascites up to 1/2000 could block the adherence of KG-1 cells. Not only ascites fluid of CLB-54 inhibited monocyte and KG-1 cell adherence, but also hybridoma supernatants containing this antibody were able to inhibit adhesion. In addition, culture supernatants of another hybridoma, producing antibodies called SHC1-3 (Schwarting et al., 1985), blocked the ad-

TABLE II

EFFECTS OF MONOClonal Antibodies ON THE ADHERENCE OF MONOCYTES AND KG-1 CELLS TO PLASTIC SURFACES

The cell adherence was detected as described in the materials and methods section. The monoclonal antibodies were added as ascites to a final dilution of 1:250. Monocytes were obtained by centrifugal elutriation (Figgler et al., 1982).

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>% Inhibition</th>
<th>KG-1 (TPA)</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 1</td>
</tr>
<tr>
<td>CEM</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bear-1</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Bear-2</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Bear-3</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>CLB54</td>
<td>95</td>
<td>60</td>
<td>55</td>
</tr>
<tr>
<td>Gepi-1</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>MHN-23</td>
<td>85</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>Rupi-1</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Rupi-2</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>SPV-L7</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>
Fig. 2. Inhibition of adherence obtained using several types of monoclonal antibody peroxidase-labelled KG-1 cells were incubated with different concentrations of moabs.

Inhibition of adherence of monocytes and KG-1 cells even more strongly than CLB-54 moabs (Keizer et al., submitted for publication). Furthermore it was observed that the adherent capacity of human monocytes was donor dependent, since inhibition of adherence was highly variable (results not shown).

To exclude that inhibition of adherence by moabs was due to the inhibition of enzyme activity, we compared the results obtained using the ELISA with those obtained by Coulter counter analysis (Table III). The calculated number of adherent cells showed a linear correlation with the extinction as found in ELISA \( r = 0.99 \). Therefore, the percentage inhibition calculated from the \( E_{450} \) does not differ from the percentage inhibition found by Coulter counter analysis, indicating that a decrease in \( E_{450} \) is due to a lower number of cells instead of an inhibition of enzyme activity caused by binding of moabs.

**TABLE III**

<table>
<thead>
<tr>
<th>moab dilution</th>
<th>ELISA extinction</th>
<th>Counted number of adherent cells ((\times 10^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>212 ± 16</td>
<td>185 ± 22</td>
</tr>
<tr>
<td>250</td>
<td>380 ± 23</td>
<td>318 ± 27</td>
</tr>
<tr>
<td>500</td>
<td>531 ± 9</td>
<td>408 ± 38</td>
</tr>
<tr>
<td>1000</td>
<td>575 ± 24</td>
<td>452 ± 11</td>
</tr>
<tr>
<td>2000</td>
<td>656 ± 29</td>
<td>496 ± 36</td>
</tr>
<tr>
<td>4000</td>
<td>649 ± 29</td>
<td>500 ± 24</td>
</tr>
<tr>
<td>Medium</td>
<td>640 ± 9</td>
<td>492 ± 33</td>
</tr>
</tbody>
</table>

\( ^a \) moab CLB54 was used to inhibit adherence.

\( ^b \) Extinction measured at 450 nm.

\( ^c \) The number of adherent cells was calculated by subtraction of the counted number of non-adherent cells from the total cell number.

The correlation between \( ^b \) and \( ^c \) was 0.998.

Fig. 3. Dose-response curve of several sera in relation to the adherence of KG-1 cells (A) and monocytes (B). HS = horse serum, HS(ab) = pooled human AB serum, FCS = fetal calf serum (two batches), NBCS = newborn calf serum (two batches). The moab CLB54 was added as ascites at a 1 : 250 dilution.
Finally, the Gepi-1 moabs which were used to prepare the HRPO conjugates did not affect adherence of monocytes and KG-1 cells, since equal numbers of labelled and unlabelled cells were adherent as judged microscopically. In addition increased amounts of Gepi-1 did not reduce the number of adherent cells (Fig. 2).

**Influence of pH and sera on monocyte and KG-1 cell adherence**

Hybridoma cells can be cultured in RPMI 1640 medium complemented with NBCS, FCS or HS or even in serum-free medium (results not shown). This prompted us to investigate the influence of different sera on the adherence of monocytes and KG-1 cells. As depicted in Fig. 3A the adherence of KG-1 cells varies depending on the concentration of NBCS and FCS. The presence of NBCS or FCS could even mask the capacity of CLB54 to inhibit adherence of KG-1 cells. In contrast, the adherence of KG-1 cells was minimally affected by pooled HS(AB) and HS. The adherence of monocytes was not or only marginally serum dependent (Fig. 3B).

The adherence of both cell types was also influenced by the pH of the culture medium. Adherence of monocytes and KG-1 cells was maximal under physiological conditions. The adherent capacity of KG-1 cells decreased very rapidly below pH 6.4 and above pH 7.4. Similar results were obtained with monocytes at pH < 6.1 and > 7.3 (Fig. 4).

**Discussion**

In this paper we present a quantitative method for measuring adherence of KG-1 cells and monocytes. The test is a one-step ELISA: cells are labelled with a moab conjugated with horseradish peroxidase, allowed to adhere for 1 h and after the addition of a substrate, the staining intensity correlated directly with the number of adherent cells.

The moab Gepi-1 was selected for conjugation with peroxidase because it possessed two unique characteristics. In the first place, this moab recognizes a determinant present predominantly on cells of the myeloid lineage, whereas it does not affect the adhesive properties of the cells. Hence, lymphocytes that may be present in monocyte preparations do not influence the calibration curve nor the adherent capacity of the monocytes (Table I). Secondly, Gepi-1 moabs are extremely useful since the antigen recognized by this antibody cannot, apparently, be saturated as detected by indirect immunofluorescence (FACS IV analysis, results not shown). Therefore, monocytes and related cell lines can be labelled with large amounts of Gepi-1-HRPO conjugates which increases the sensitivity of this assay (Fig. 1).

We observed that the culture conditions may alter the adherent capacity of monocytes and KG-1 cells. This may affect the results of a screening procedure in which hybridomas are selected, producing antibodies which inhibit the adherence of cells. In contrast to monocytes, KG-1 cells were particularly sensitive to the serum used to supplement the culture medium (Fig. 3). However, if the hybridomas are grown in serum-free medium or medium that contains horse serum, KG-1 cells are advantageous compared to monocytes for various reasons. KG-1 cells can be grown to large numbers whereas monocytes do not divide and have to be isolated from peripheral blood which is rather time consuming. Moreover, we observed that the capacity of antibodies to inhibit the adherence of monocytes is donor dependent (not shown). The results presented in Figs. 2 and 3 show that KG-1
cells are much more sensitive with respect to inhibition of adherence than monocytes. Furthermore both cell types were sensitive to low pH (Fig. 4). Measurement of the pH of individual wells with fast growing hybridomas demonstrated large fluctuations in pH (not shown). Therefore, hybridomas should be cultured in Hepes-containing media in which variations in pH were less extreme.

The data in Table II demonstrate that two out of nine moabs were capable of inhibiting the adhesion of monocytes. The reduced monocyte adherence, as measured by the ELISA was not caused by inhibition of enzyme activity, since the same results were obtained with Coulter counter analysis (Table III).

Cell adherence has been determined in several ways, including DNA detection (Cookson and Adams, 1978), by counting adherent cells microscopically (Rovera et al., 1979; Wallis et al., 1985) or by means of a Coulter counter (Zimmerman et al., 1985). These methods, together with the method of Nakagawara and Nathan (1983) which is based on the counting of cell nuclei, and that of Kelly and Thong (1984) who used a nylon fibre microcolumn technique, are not suitable for large screening procedures, since they are very laborious and time consuming, whereas the sensitivity is in general too low to permit successful screening of hybridomas. In addition large numbers of cells are required in the assays. More sensitive methods are described by DiCorletto and De la Motte (1985) and Charo et al. (1985), who used radioactive markers for the determination of adherent cells. The serious drawbacks of these methods when used as a screening assay are that they are also laborious and require the use of radioactive markers. The method presented here is fast, requires low cell numbers, short incubation times and is, therefore, extremely suitable for large-scale screening procedures to detect moabs that recognize structures associated with adherence. Furthermore recent studies have demonstrated that the method is also applicable to the investigation of monocyte adherence to other substrates such as monolayers of endothelial cells and melanoma cells. In addition, the method described in the present study can easily be modified to measure the adherent capacity of other cells by using other moab/peroxidase conjugates (A.A. Te Velde, manuscript in preparation).

Acknowledgements

This work was supported by a grant from the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds), Grant no. NKI 83-19.

We gratefully acknowledge Wietze Visser for technical assistance, and thank Marie Anne Van Halem for excellent secretarial help.

References

Koeffler, H.P., M. Bar-Eli and M.C. Territo, 1981, Cancer Res. 41, 919.