Analysis of In Vitro Lymphocyte Adhesion and Transendothelial Migration by Fluorescent-Beads-Based Flow Cytometric Cell Counting

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In this report, we describe a new and simple method for flow cytometric quantitation of lymphocyte numbers in lymphocyte-endothelial adhesion/transendothelial migration assays. The method exploits fluorescent flow cytometer alignment beads as a counting reference. Known amounts of beads are added to samples with unknown lymphocyte numbers. Lymphocytes and a preset number of fluorescent beads are simultaneously analyzed. The total number of cells present in the sample can be subsequently calculated from the fixed ratio of added to analyzed fluorescent beads. Using this fluorescent-beads-based flow cytometric cell counting of lymphocyte numbers in adhesion/migration assays, labeling of cells and other time-consuming calibration procedures are not required and analysis time is short. Furthermore, we demonstrate that this cell counting method can be combined with concurrent single- or double-label fluorescence flow cytometric phenotyping of adherent and migrated lymphocytes. The method was applied to the in vitro study of the effects of lymphocyte activation status and binding of bispecific antibody (directed against CD3 x tumor cell-associated antigen) on lymphocyte adhesion and transendothelial migration. Cytometry 32:37–43, 1998. © 1998 Wiley-Liss, Inc.

Key terms: lymphocyte; adhesion; transendothelial migration; in vitro quantitation; fluorescent beads; flow cytometry

In recent years, new immunotherapeutical strategies for the treatment of solid tumors have been investigated. In our laboratory, we developed bispecific antibodies (BsMAb) capable of redirecting the cytolytic activity of cytotoxic T lymphocytes (CTLs) to tumor cells. These BsMAbs were able to kill tumor cells in vitro and showed significant antitumor activity in vivo in a rat tumor model (1) and after intratumoral administration in cancer patients (2). Upon intravenous administration of BsMAb BIS-1 in renal cell carcinoma patients, a rapidly occurring lymphopenia was observed (3). However, an antitumor effect could not be demonstrated and the mechanism(s) resulting in the lymphopenia and the fate of the lymphocytes are by now unknown.

To study the effects of BsMAb binding to lymphocytes on lymphocyte-endothelial cell interactions in vitro, we developed a simple and rapid method for quantitation of both lymphocyte adhesion to and migration through endothelial monolayers. The method applies the use of fluorescent beads, which have been shown to be adequate for quantitation of lymphocyte numbers per se (4,5). Known amounts of beads are added to adherent and migrated lymphocyte fractions containing unknown numbers of cells. Fluorescent beads and lymphocytes are simultaneously analyzed by flow cytometry and from the fixed ratio of added to analyzed beads, the total number of lymphocytes present in the sample can be calculated.

In the present study, we show that the results obtained by analysis of cell numbers by using this method are comparable to those obtained by microscopic analysis, a method commonly used in these types of studies (6–11). The presented method has, however, important advantages over the microscopic methods and assays using either radiolabeled or fluorescently labeled lymphocytes (11–14). Analysis time is short, a clear discrimination

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between adherent and migrated cells is obtained, and labeling of cells and other time-consuming calibration procedures are not required. Furthermore, we show that lymphocyte fractions can be subsequently phenotyped for markers of interest without the need for additional adhesion/transendothelial migration (tem) assays.

The fluorescent-beads-based cell counting was applied to study the effect of lymphocyte activation status and CD3 × tumor-associated antigen-directed BsMAB binding to lymphocytes on lymphocyte–endothelial cell interactions. These studies revealed that both anti-CD3/interleukin-2 (IL-2) and IL-2-activated lymphocytes were capable of adhering to endothelial cells and transendothelial migration. Coating of lymphocytes with BsMAB resulted in increased lymphocyte–endothelial cell interactions. Both BsMAB-coated helper T cells and CTLs displayed adhesion to endothelial monolayers and tem, albeit to a different extent. Migrated lymphocytes still carried the BsMAB bound to their surface, indicating that BsMAB-coated CTLs are in theory capable of reaching the tumor cells after intravenous administration of BsMAB in cancer patients.

MATERIALS AND METHODS

Antibodies

Anti-CD31 was purchased from Dako A/S (Glostrup, Denmark). FITC-labeled anti-mouse IgG was purchased from Dako A/S, and anti-CD4-FITC and anti-CD8-PE were purchased from Immuno Quality Products (Groningen, The Netherlands). Anti-mouse IgG3-FITC was purchased from Southern Biotechnology Associates (Birmingham, AL). Bispecific antibody BIS-1 F(ab')2 (a fusion between hybridoma RIV-9 directed against human CD3 (mouse IgG3) and MOC31 directed against tumor associated antigen EGP-2 (15) (mouse IgG1)) was enzymatically digested and purified as described elsewhere (3) and is hereafter referred to as BIS-1.

Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were routinely isolated and cultured as described by Mulder et al. (16). Occasionally, cells were phenotyped by flow cytometry for the presence of the endothelial cell marker CD31 and were found to be uniformly positive. HUVEC passages 1–4 were used for the adhesion/tem assays.

Lymphocytes

Human peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by density centrifugation on Lymphoprep (Nycomed, Oslo, Norway). After washing, the cells were resuspended in RPMI-1640 supplemented with 2 mM glutamine, 60 µg/mL gentamycin (all purchased from Gibco BRL/Life Technologies, Breda, The Netherlands) and 2% heat-inactivated pooled human serum and is hereafter referred to as RPMI complete medium. To activate the lymphocytes, PBMC were either cultured in the presence of 100 IU/mL recombinant human IL-2 (EuroCetus, Amsterdam, The Netherlands) or anti-CD3/IL-2 (3) for 5 or 6 d. The resulting lymphocyte fractions consisted of 80–98% T lymphocytes (CD3 positive): 45–60% of the cells were CD4 positive and 20–50% were CD8 positive.

Microscopic Determination of Lymphocyte–Endothelial Cell Interactions

Twelve to fourteen thousand HUVEC in 300 µl HUVEC culture medium were seeded on Labtek chambers (8 wells; 0.79 cm²/chamber; Nunc, Gibco BRL/Life Technologies) coated with 1% gelatin. After reaching confluency in 3–4 d, 300 µl lymphocyte suspension at 3–4 × 10⁵ cells/mL in RPMI complete medium and labeled with 5 µM HOECHST 33342 were added per chamber. Lymphocytes coated with BIS-1 BsMAB (PBMC_BIS) were obtained by incubating cells with 1 µg/mL BIS-1 in RPMI complete medium at room temperature for 45 min, followed by two washings. After incubation for 3 h at 37°C and 5% CO₂ in a humidified atmosphere, lymphocyte suspensions were removed and the chambers washed three times with HBSS⁺ (HBSS with Ca²⁺ and Mg²⁺; Gibco BRL/Life Technologies). Cells were fixed with 4% paraformaldehyde at 4°C, washed three times with phosphate buffered saline (PBS) at 4°C, embedded with Citifluor (UK Chemlab, Canterbury, UK) and analyzed at 200× magnification on a Leica Quantimet 600S microscope equipped with a UV filter. Per chamber, the number of lymphocytes in 10 separate microscopic fields were assessed and the total number of adherent/migrated lymphocytes per chamber was calculated.

Flow Cytometric Quantitation of Lymphocyte-Endothelial Cell Interactions

Adhesion/tem assay. Lymphocyte adhesion/tem assays were performed by using the method described by Kitayama et al. (9), with the following specifications. For lymphocyte adhesion/tem quantitation only, assays were performed in 24-well tissue culture plates. For adhesion/tem quantitation in combination with phenotyping, assays were performed in 12-well tissue culture plates (both from Costar, Cambridge, MA). A collagen gel was prepared from Vitrogen 100 solution (Collagen Corporation, Palo Alto, CA) according to the manufacturer’s instructions. After washing the collagen extensively with HUVEC culture medium, 35,000–40,000 HUVEC were seeded per square centimeter surface area and cultured for 2 d. Before starting the assay, HUVEC were washed with RPMI complete medium. To every square centimeter HUVEC monolayer, 250 µl lymphocyte suspension were applied at 5–6 × 10⁵ cells/mL, and the plates were incubated at 37°C, 5% CO₂ in a humidified atmosphere for 3 h. Coating of lymphocytes with BIS-1 BsMAB (PBMC_BIS) was performed as described above. Nonadherent lymphocytes were removed by washing three times with HBSS⁺. The wells were subsequently treated with 0.05% trypsin (ICN Biomedicals BV, Zoetermeer, The Netherlands) in 0.5 mM EDTA solution for 15–30 min at 37°C to release adherent lymphocytes. Optimal trypsinization time was determined by microscopically monitoring the release of the lymphocytes. Trypsin-
ized samples were collected in fetal calf serum (FCS; Biowhitaker, Brussels, Belgium) and put on ice. Migrated lymphocytes were released from the collagen gel by treatment for 15–20 min at 37°C with collagenase (2 mg/mL freshly prepared in PBS; collagenase was a kind gift from Knoll A/G, Ludwigshafen, Germany) and put on ice. Collagenase treatment was microscopically monitored to achieve release of all cells. Using trypan blue dye exclusion, lymphocyte viability in both the trypsin and collagenase released fractions was determined to be greater than 95%.

Flow cytometric sample analysis. Ten microliters of Immuno-Check EPICS® alignment fluorospheres (hereafter referred to as IC-beads; purchased from the Epics Division, Coulter Corporation, Hialeah, FL) solution were added to each sample tube immediately before analysis. Samples were analyzed on a Coulter Epics-Elite flow cytometer (Coulter Electronics, Hialeah, FL) with ELITE version 4.01 acquisition software. Forward scatter peak was used as a trigger signal. PMT voltage of the 675BP parameter was set at 1,000 V or lowered to such values that the very bright IC-beads fell in the higher channels, where autofluorescent cells did not interfere with the IC-beads signals. In a two-parameter dot plot of 575BP and 675BP (the latter being log recorded), the gate for the IC-beads was defined (gate R2 in Fig. 1B).

For each sample, forward scatter/side scatter (FSC/SSC; both in log scale; see Fig. 1A) and 575-nm/675-nm (675 nm in log scale; see Fig. 1B) data were acquired until 500 IC-beads were counted. During the analysis, samples were continuously mixed to prevent sedimentation of the IC-beads. Time of flight (TOF) was recorded to monitor the appearance of the IC-beads and the cells. Data analysis was done with Winlist (version 3.0) from Verity Software House (Topsham, ME).

Calculation of cell numbers. Based on the simultaneous analysis of a fixed number of IC-beads (i.e., 500) and an unknown number of lymphocytes, the following formula was used to calculate the total number of lymphocytes present in the sample:

\[
\text{total number of lymphocytes} = \frac{(\text{total number of IC beads added}/500)}{\text{number of lymphocytes in R3}}
\]

in which R3 is region 3, the region in the FSC/SSC dot plot demarcating the lymphocytes.

Accuracy of IC-Beads-Based Flow Cytometric Cell Counting

To determine the accuracy of IC-beads-based flow cytometric cell counting, lymphocytes suspensions with \(2.1 \times 10^3\), \(2.1 \times 10^4\), and \(2.1 \times 10^5\) cells were analyzed by flow cytometry, as described above. The numbers of cells calculated to be present in the suspensions were \(2.0 \pm 0.3 \times 10^3\) (96.4% \(\pm\) 12.1), \(2.1 \pm 0.1 \times 10^4\) (98.2% \(\pm\) 4.8), and \(2.1 \pm 0.1 \times 10^5\) (100.7% \(\pm\) 1.4; mean values \(\pm\) S.D. of three analyses), respectively.

Applicability of Flow Count® Coulter Fluorospheres in Quantitating Lymphocyte Adhesion/TEM

Shortly after validating the use of IC-beads for quantitating lymphocyte adhesion/TEM, the Coulter Company announced the withdrawal of the IC-beads from the commercial market due to instability of the fluorescence characteristics of the beads. For this reason, we determined whether the readily available Flow Count® Coulter Fluorospheres were as appropriate as IC-beads for use in the lymphocyte adhesion/TEM quantitation. Adhesion/TEM assays were performed as described above with the following modifications. Twenty-five microliters of Flow Count® Coulter Fluorospheres, hereafter referred to as FC-beads (catalog no. PN7547053, kindly provided by the Coulter Corporation), were added to each sample. Sample analysis was performed until 1,000 beads were recorded.
with the same flow cytometer set up as described for the analysis using the IC-beads.

**Combining Lymphocyte Adhesion/tem Quantitation and Lymphocyte Phenotyping**

BIS1-coated lymphocytes were subjected to the adhesion/tem assay as described. Adherent and migrated cells were recovered from the wells and divided into fractions. One fraction was used for the IC-beads-based quantitation of cell numbers, and the others were used for the phenotyping of lymphocytes.

Cells recovered with trypsin and collagenase from the adhesion/tem assay were centrifuged, and the pellet was resuspended in PBS/5% FCS containing anti-CD4-FITC plus anti-CD8-PE (both diluted 1:30) or anti-mouse IgG3-FITC (diluted 1:60), the latter staining the RIV-9 part of the BSMAb. After incubation for 30 min on ice, cells were washed twice and resuspended in 150 µl PBS/5% FCS. As a control for BIS1 staining, lymphocytes without bound BIS1 were stained with anti-mouse IgG3-FITC. Samples were analyzed on a Coulter Elite Cytometer (Coulter Electronics). Data were analyzed with Winlist.

**RESULTS**

**Flow Cytometric Characteristics of Lymphocytes, HUVEC, and IC-Beads**

Figure 1 shows flow cytometry dot plots from cell samples obtained by trypsin treatment (containing adherent lymphocytes) and collagenase treatment (containing migrated lymphocytes) from a regular adhesion/tem assay as described in Materials and Methods. In Figure 1A, a representative FSC/SSC dot plot of a sample recovered by trypsin treatment is shown. IC-beads are present in region 1 (R1). Based on their FSC/SSC characteristics, cells in region 3 (R3) and region 4 (R4) were identified as lymphocytes and HUVEC, respectively. Based on an absolute discrimination between IC-beads and cells in the 575-nm/675-nm dot plot (Fig. 1B), IC-beads in R2 were used as a counting reference for the quantitative analysis of lymphocytes in the samples. The FSC/SSC dot plot of cells recovered by collagenase treatment is shown in Fig. 1C. Although HUVEC rounded off with trypsin treatment, the majority did not detach from the collagen gel until collagenase treatment and were consequently recovered in the samples containing the migrated lymphocytes.

Figure 2 shows the flow cytometric analysis of samples, recovered with trypsin (Fig. 2A) and collagenase (Fig. 2B), of HUVEC grown on collagen gel without having participated in an adhesion/tem assay. In both samples, few events were recorded in R3, indicating that neither the endothelial cells nor the collagen gel were contributing to a significant extent to the events in this region of the dot plot.

The TOF recordings of the samples demonstrated that the IC-beads gated in R2 (Fig. 1B) consisted of a population of more than 95% single beads (TOF data not shown). Upon beads storage in time, no changes in TOF profile were seen, from which it was concluded that the beads did not form clusters in time. Furthermore, lymphocytes appeared as single cells, whereas a minority of HUVEC was present as doublets/triplets (data not shown).

**Comparison of Lymphocyte-Endothelial Cell Interactions Determined by Microscopy Versus IC-Beads-Based Flow Cytometric Cell Counting**

Because the number of lymphocytes applied per square centimeter of HUVEC were identical in the microscopic assay and the IC-beads-based cell counting assay, the results of both assays could be directly compared (Fig. 3). Using microscopy, the differentiation between adherent and migrated lymphocytes is difficult to make. Therefore, only the total number of cells present in the chambers could be determined. After incubation of lymphocytes and HUVEC for 3 h, 17% ± 5 (anti-CD3/IL-2 activated) and 6% ± 4 (IL-2 activated) of the cells applied firmly adhered to and/or had migrated into the endothelial cell layer. Using IC-beads-based flow cytometric analysis, these values were 18.4% ± 2.3 (consisting of 10.3% ± 1.7 adherent and 8.1% ± 0.6 migrated lymphocytes) and 9.7% ± 0.7 (4.3% ± 0.4 adherent and 5.4% ± 0.3 migrated cells), respectively.

**Reproducibility of IC-Beads-Based Lymphocyte Adhesion/tem Quantitation**

Lymphocyte adhesion/tem data obtained from four different assays performed in a period of several months are presented in Table 1. In the different experiments, the numbers of lymphocytes adhering to and migrating through the endothelial layers were highly variable. This most likely reflects biological variation in PBMC and HUVEC isolates and also has been observed by others (6).

The effect of BIS1 BsMab coating of PBMC on their adhesion/tem capacity was determined by comparing adhesion/tem of PBMC versus PBMC_BIS1 within one assay using the same PBMC and HUVEC isolates. The increased adhesion/tem capacity of PBMC_BIS1, expressed by the ratio of adherent or migrated PBMC_BIS1 to PBMC, ranged from 2.3 to 2.8 (mean ± S.D.: 2.5 ± 0.2) for adhesion and from 1.8 to 2.6 (2.1 ± 0.4) for migration. These data indicate that, whereas the absolute numbers of adherent and migrated cells differ significantly between experiments performed with different cell isolates, the effect of BIS1 binding to lymphocytes on adhesion/tem is reproducible in time.
Experiment-to-Experiment Variation of Beads-Based Flow Cytometric Analysis of Lymphocyte Adhesion/tem

<table>
<thead>
<tr>
<th>Adhesion/tem</th>
<th>Absolute cell numbers</th>
<th>Absolute cell numbers</th>
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<tbody>
<tr>
<td></td>
<td>with IC-beads</td>
<td>with FC-beads</td>
</tr>
<tr>
<td>PBMC, adherent cells</td>
<td>13.0 → 33.3</td>
<td>10.9 → 13.0</td>
</tr>
<tr>
<td>PBMC, migrated cells</td>
<td>23.3 → 61.1</td>
<td>21.2 → 23.3</td>
</tr>
<tr>
<td>PBMC&lt;sub&gt;BIS-1&lt;/sub&gt;, adherent cells</td>
<td>36.7 → 75.6</td>
<td>25.3 → 36.7</td>
</tr>
<tr>
<td>PBMC&lt;sub&gt;BIS-1&lt;/sub&gt;, migrated cells</td>
<td>44.9 → 119.9</td>
<td>44.9 → 590.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Adhesion</th>
<th>Migration</th>
</tr>
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<tbody>
<tr>
<td>PBMC&lt;sub&gt;BIS-1&lt;/sub&gt;:PBMC with IC-beads</td>
<td>2.5 ± 0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>PBMC&lt;sub&gt;BIS-1&lt;/sub&gt;:PBMC with FC-beads</td>
<td>2.6</td>
<td>2.3</td>
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</table>

Applicability of Flow Count<sup>®</sup> Coulter Fluorospheres in Quantitating Lymphocyte Adhesion/tem

Data on the use of FC-beads in determining lymphocyte adhesion/tem are presented in Table 1. FC-beads-based cell counting also showed significant differences in absolute values of lymphocyte adhesion and tem when determined on different days when using different PBMC and HUVEC cultures. However, analysis of the effects of BIS-1 binding to lymphocytes on lymphocyte adhesion/tem had outcomes similar to those measured by IC-beads. This result indicates that FC-beads are as suitable as IC-beads for flow cytometric cell counting purposes in lymphocyte adhesion/tem assays.

Combining Lymphocyte Adhesion/tem Quantitation and Phenotyping of Adherent and Migrated Lymphocytes

Adhesion/tem assays were performed with anti-CD3/IL-2-activated lymphocytes preincubated with BIS-1 BsMAb. With the assay set up for quantitation of adhesion/tem only, the percentages of adherent and migrated lymphocytes were 28.9 ± 0.6 and 13.7 ± 1.2, respectively (Fig. 4A). In the combination assay, these values were calculated to be 38.0% and 15.2%, respectively (Fig. 4B). The histograms of the subsequent phenotyping for CD4/CD8 expression by the adherent and migrated lymphocytes present in R3 of the FSC/SSC dot plot are shown in Figure 4C,D. In the adherent lymphocyte fraction, the ratio of CD4 to CD8 positive cells approximated 1, which is comparable to the control lymphocyte population not subjected to the adhesion/tem assay (Table 2). This ratio decreased to 0.7 in the migrated lymphocyte fraction. The levels of expression of CD4 and CD8 in the different lymphocyte fractions, represented by the mean fluorescence intensity (MFI) values, was somewhat variable but did not change considerably during the adhesion/migration process.

Phenotyping of surface bound BIS-1 demonstrated that all adherent and migrated cells were BsMAb positive, although lymphocytes reaching the subendothelial compartment contained less, approximately 40% of initial bound, BsMAb. Control lymphocytes without bound BIS-1 were negative (MFI < 1, data not shown) when stained with anti-IgG3-FITC. The observation that nonadherent cells stained similarly for BIS-1 presence as the adherent and migrated cells (data not shown) indicates that BIS-1 loss is likely due to internalization during the 3-h assay.

The presented histograms and the data in Table 2 are the results of one experiment and are representative of results of similar experiments. In all experiments performed, we observed a preference of CD8 positive cells to migrate under the given circumstances and a loss of surface-expressed BIS-1 in both the adherent and migrated cell fractions.

**DISCUSSION AND CONCLUSIONS**

In the present study, we present data on the application of fluorescent-beads-based cell counting to in vitro lymphocyte adhesion and tem analyses. The assay circumvents elaborate lymphocyte labeling or sample preparation prior to or after lymphocyte adhesion/tem studies. Furthermore, analysis time is short (30-40 samples can be analyzed per hour), and a clear discrimination between adherent and migrated cells is achieved. In addition, quantitative analysis can be combined with flow cytomet-
ric phenotyping of the cells involved without the need for additional adhesion/tem assays.

The results of the IC-beads-based cell counting of lymphocyte adhesion/tem were in agreement with the results of the microscopic evaluation (Fig. 3), thereby validating the use of IC-beads-based flow cytometric cell counting in lymphocyte adhesion/tem assays. From the small standard deviations for triplicate measurements, it was concluded that IC-beads-based sample analysis was highly reproducible. Variations in outcome of both assays are most likely the result of the use of different HUVEC and PBMC isolates in the experiments. These isolate differences can also explain the observed experiment-to-experiment variation (Table 1, Fig. 4A,B). Similar observations have been reported by Oppenheimer-Marks et al. (6). In seven different assays under identical assay conditions, they observed that the number of T cells bound to HUVEC ranged from $5.52 \times 10^4$ to $1.58 \times 10^5$. However, one may cope with the biological variation of the cell isolates used by studying the effects of lymphocyte manipulation (e.g. BIS-1 binding) through comparison with control lymphocyte populations that are not manipulated within the same assay.

Due to withdrawal of the IC-beads from the commercial market, we determined whether FC-beads were suitable for flow cytometric quantitation of lymphocyte adhesion/tem. By using 25 µl instead of 10 µl and by counting 1,000 instead of 500 beads, we obtained results similar to those with IC-beads. From these results, we concluded that FC-beads also can be used for the assay.

Assays using flow cytometry to analyze lymphocyte adhesion/tem have recently been described by others (17,18). Both analyses require an extra time-consuming step, either for measurement of total sample volumes (17) or for lymphocyte labeling with a fluorescent antibody (18). In the latter procedure, additional cell loss may occur, thereby introducing sample variations.

Studying the biology of chemokine and classical chemoattractant receptors, Campbell et al. also used polystyrene beads as a counting reference in flow cytometric determination of lymphocyte numbers (19). Whereas they discriminated between beads and cells in the FSC/SSC dot plot, we chose to discriminate between cells and beads by using the autofluorescence signal of the beads in the 575-nm/675-nm dot plot. Collagen debris and possibly some cell debris released during the adhesion/tem assay as we performed it might have interfered if the beads were to be gated in R1 of the FSC/SSC dot plot. Campbell et al. may not have encountered such interference because they performed migration assays using a two-compartment system with tissue culture inserts. This methodology has been described extensively for the characterization of chemotaxis of T lymphocytes (11). In our hands, however, we could not measure any fluorescently labeled lymphocyte migration toward the lower compartment of the system. In contrast, by using the present method, we were capable of measuring lymphocyte adhesion/tem. By combining the beads-based cell counting described in the present study with the chemotaxis assay described by Roth et al. (11), the detection limit of the assay may be improved, the lymphocyte labeling procedure may be circumvented, and sample preparation time and sample analysis time may be shortened.

We applied the method described in the present article to study the effects of lymphocyte activation status and BsMAb binding on their interaction with endothelial cells. A number of important observations was made. First of all, anti-CD3/IL-2-activated lymphocytes were as capable of adhering to and migrating through endothelial monolayers as IL-2-activated cells (20). Moreover, the activated and proliferating cells were even more prone to perform adhesive and migratory features. Second, the binding of the anti-CD3-directed BsMAb BIS-1 did not interfere with processes involved in lymphocyte movement. On the contrary, BsMAb binding to lymphocytes induced an increase in lymphocyte adhesion/tem. We recently observed that BsMabs directed against the CD5 epitope on T lymphocytes did not affect in vitro lymphocyte adhesion/tem (data not shown, manuscript in preparation), indicat-
ing a CD3-dependent effect. Especially for the intravenous infusions.

Acknowledged for isolating and culturing of HUVEC.

will be investigated.

migrated lymphocytes in inducing killing of tumor cells with part of the BsMAb still present on the surface.

positive cells, both CD4 and CD8 positive lymphocytes migrated lymphocyte population was enriched in CD8 positive cells. The contribution of vascular cell adhesion molecule-1-dependent and independent binding mechanisms. J Immunol 146:592–598, 1991.


Table 2

<table>
<thead>
<tr>
<th>CD4</th>
<th>CD8</th>
<th>BsMAb</th>
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<tbody>
<tr>
<td>% Positive cells</td>
<td>MFI</td>
<td>% Positive cells</td>
</tr>
<tr>
<td>Control lymphocyte population</td>
<td>47</td>
<td>501</td>
</tr>
<tr>
<td>Adherent lymphocytes</td>
<td>48</td>
<td>537</td>
</tr>
<tr>
<td>Migrated lymphocytes</td>
<td>38</td>
<td>481</td>
</tr>
</tbody>
</table>

aMFI, mean fluorescence intensity.

bLymphocytes coated with Bs1 BsMAb but not subjected to the adhesion/term assay.

ACKNOWLEDGMENTS

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LITERATURE CITED

