Characterization of a Computerized Assay for Rapid and Easy Determination of Leukocyte Adhesion to Endothelial Cells

Holger ULBRICH,^{*,a,b} Philip PRECH,^a Andreas LUXENBURGER,^a and Gerd DANNHARDT^a

^aJohannes Gutenberg-University of Mainz, Institute of Pharmacy; Staudingerweg 5, D-55099 Mainz, Germany; and

^bKarolinska Institutet, Department of Physiology and Pharmacology; Von Eulers Väg 8, SE-171 77 Stockholm, Sweden.

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We report on a facile and rapid computerized *in-vitro* assay for the quantification of leukocyte adhesion to endothelial cells under static conditions using bovine polymorphonuclear neutrophils (PMN) or human leukaemic Mono Mac 6 cells (MM6) and bovine aorta endothelial cells (BAEC). Images of leukocytes adherent to BAEC monolayers grown in microtiter plates were obtained by a digital camera attached to a conventional microscope and transferred to the public domain NIH ImageJ program for analysis. Using individually adapted program routines adherent leukocytes are easily discriminated and reproducibly quantified. The results obtained with our assay correspond to previous findings and demonstrate the suitability of the described procedure, which can easily be adapted to further standards as proven by the use of two different leukocyte species. This assay lends itself to the screening of pharmacological substances with different mechanism of action that might act on either leukocytes or endothelial cells.

Key words adhesion; leukocyte; computerized assay; antiadhesive compound; selectin

Adhesion of polymorphonuclear neutrophils (PMNs) to activated endothelial cells is a prerequisite for the recruitment of PMNs that may cause acute or chronic pathological reactions as observed in reperfusion injuries, stroke, psoriasis, rheumatoid arthritis, respiratory diseases and angiogenesis.¹⁾ Expression of a number of different cell adhesion molecules (CAMs) on PMNs and endothelial cells is required for the multistep adhesion process.²⁾

Because of the sequential characteristics of leukocyte accumulation in inflammation, blockade of any of the binding steps may have strong effects on inflammatory disease. Therefore, it is of great pharmaceutical interest to block interactions between leukocytes and endothelial cells by antagonizing the process of recognition. The function of inflammatory CAMs can be modulated *via* a number of different mechanisms including competitive blockade, altered expression on the cell surface or, for integrins, interference with integrin activation.^{3,4)}

In order to study the effect of CAM-inhibition on leukocyte adhesion to endothelial cells or to isolated CAMs, various assays under static or dynamic conditions have been developed. These assays often suffer from a lack of reproducibility because of heterogeneities in the components of the assays. Moreover the evaluation and data acquisition are often time consuming (reviewed by Simanek *et al.*).⁵⁾ Due to their simplicity and rapidity, static assays are the most widely used ones. In these assays the quantification of adherent leukocytes is often made using leukocytes pre-labeled with radioactive material, usually ⁵¹Cr,⁶⁾ or leukocytes stained with fluorescent dyes⁷⁾ like calcein-AM,⁸⁾ BCECF-AM⁶⁾ or TDA-AM.⁹⁾ Other techniques including those using ELISA,¹⁰⁾ horseradish peroxidase¹¹⁾ or biotinylated cells¹²⁾ require additional steps in the assay procedure (*e.g.* leukocyte manipulating) that may cause undesired activation or leakage of the labeling compounds. Assays based on non-labeled leukocytes and conventional microscopy to quantify cell adhesion have also been described (reviewed by Simanek *et al.*).⁵⁾ However, these techniques require time-consuming manual

counting procedures, which lack automated, observer-independent evaluation. The benefits of a computer assisted image analysis combined with an automated data evaluation system have been described using fluorescence-labeled leukocytes and confocal laser scan microscopy in a transmigration assay.¹³⁾ Here, we present a rapid method for quantification of leukocyte adhesion using unlabelled leukocytes, conventional microscopy and semi-automated computational image analysis that allows high-throughput to study interactions between leukocytes and endothelium and subsequent rapid testing of pharmaceutical adhesion inhibitors.

MATERIALS AND METHODS

Chemicals and Reagents The following cell culture media and reagents were purchased from Sigma (München, Germany): Medium 199, fetal bovine serum, penicillin-streptomycin solution, amphotericin B solution, L-glutamine solution, trypsin-EDTA solution, Dulbecco's buffered saline, recombinant human tumor necrosis factor alpha (rhTNF α), trypan blue solution 0.4%.

Bimosiamose (TBC-1269) was a generous gift from Revotar Biopharmaceuticals AG (Hennigsdorf, Germany).

Cell culture flasks were obtained from Becton Dickinson (Heidelberg, Germany). Microtiter plates and other cell culture ware were purchased from Greiner Bio-one (Frickhausen, Germany). Dispase was purchased from Roche (Mannheim, Germany), Accutase from PAA (Linz, Austria). Sialyl Lewis^x was acquired from Mobitec (Göttingen, Germany) and DMSO from Fluka (München, Germany).

Endothelial Cells Bovine aorta endothelial cells were isolated and cultured as described previously.¹⁴⁾ Segments of the thoracic aorta from freshly slaughtered 12 to 24 month old cattle were immersed in ice-cold PBS, supplemented with benzyl penicillin (100 U/ml), streptomycin (100 μ g/ml) and 5 ml amphotericin B solution (250 μ g/ml). The segments were opened longitudinally and clamped down to a frame with the intimal face upwards. The endothelium was

* To whom correspondence should be addressed. e-mail: ulbrich@uni-mainz.de

incubated with 6 ml dispase solution (480 mg/100 ml H₂O) for 40 min at 37 °C. Detached endothelial cells were collected in a sterile 50 ml tube containing 25 ml growth culture medium (medium 199 containing 20% heat-inactivated fetal bovine serum, benzyl penicillin (100 U/ml), streptomycin 100 µg/ml), and L-glutamine (2 mM). After centrifugation at 165×g for 10 min, the pellet was resuspended in culture medium. The cells were placed in 75 cm² cell culture flasks and maintained at 95% air and 5% CO₂ in a humidified incubator at 37 °C. The culture medium was replaced every three days. After reaching 70 to 85% confluence the cells were subcultured. The cell monolayer was rinsed with ice-cold PBS and incubated with 4–5 ml Trypsin-EDTA (0.025%) for 60 s at 37 °C/5% CO₂. The Trypsin-EDTA was then removed and the cells were left at 37 °C/5% CO₂ for 10 min before washing twice with 10 ml culture medium. The resulting endothelial cell suspension was centrifuged at 165×g for 10 min, the supernatant was discarded and the cell pellet was resuspended in culture medium. The cell suspension was distributed to a 24-well plate and maintained at 37 °C/5% CO₂ until at least 24 h after reaching confluence, usually for 36 to 72 h. The cells were identified as endothelial cells by their typical cobblestone-like appearance and their ability to take up fluorescently labeled acetylated low-density lipoprotein. Additionally, viability of the BAEC culture was verified using trypan blue exclusion.

Polymorphonuclear Leukocytes Bovine polymorphonuclear leukocytes (PMNs) were isolated using a technique adapted from procedures previously described.^{15,16}

Blood from the carotid of 12 to 24 month old cattle was collected immediately after the death of the animals from the sliced carotid in a vessel containing a solution of 0.077 M EDTA disodium in 0.21% saline (0.1 liter per liter blood). The anticoagulated blood was centrifuged at 200×g for 20 min, after which the platelet-rich plasma was carefully removed by aspiration. Lysis of erythrocytes was accomplished by adding 2 vol. of purified water and shaking gently. After 30 s 1 vol. of hypertonic PBS was added and the mixture was centrifuged at 475×g for 10 min. The pellet was resuspended in PBS and centrifuged at 475×g for 10 min. The cells were resuspended in PBS and placed on Histopaque-1077. After centrifugation at 675×g for 45 min the cells were washed again with PBS, centrifuged at 50×g for 10 min to remove remaining platelets and the cell concentration was adjusted to 1×10⁶ cells per ml (using a Sysmex F800 Microcellcounter (Sysmex, Hamburg, Germany)).

Viability of the PMNs was verified using trypan blue exclusion.

Mono Mac 6 Cells Mono Mac 6 (MM6) cells were kindly provided by Dr. O. Werz (Frankfurt, Germany) and maintained in RPMI 1640-Medium supplemented with 10% fetal calf serum, 100 µg/ml streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, 1×nonessential amino acids, 1 mM oxalacetic acid and 10 µg/ml human insulin.¹⁷ All cultures were seeded at a density of 2×10⁵ cells/ml.

Adhesion Assay The adhesion assay was performed on BAEC monolayer 7 d post seeding; passage 1–3, in 24 well plates. Prior to assay, the BAEC were examined microscopically, to confirm the integrity and uniformity of the monolayer. The culture medium was removed and the endothelial cell monolayer was washed twice with DPBS (1000 µl/well

at 37 °C). After the washing procedure the cells were treated with cell culture medium containing 20 ng/ml recombinant tumor necrosis factor alpha (TNFα) for 4 h and simultaneously the respective test substance dissolved in dimethylsulfoxide (DMSO) or vehicle for 4 h at 37 °C/5% CO₂. The stimulation times were optimised by kinetic measurements. We investigated the influence of a simultaneous treatment of TNFα and the compounds for 4 h and a treatment for 20 min with the compounds after 4 h stimulation with TNFα. Co-stimulation of TNFα and test substance was chosen for all further experiments since it gave good reproducible results as shown in the result section and additional washing steps can be avoided. Additionally, in initial experiments, BAEC were incubated with various concentrations of TNFα for 4, 6, 10 and 18 h. In all further experiments standard doses of TNFα (20 ng/ml) were used. The cells were washed twice with DPBS (1000 µl/well at 37 °C), before 900 µl of culture medium and 100 µl of PMNs (1×10⁶ cells per ml) per well suspension were added and the cells were incubated for 30 min at 37 °C/5% CO₂. Non-adherent cells were removed by washing three times with DPBS (1000 µl/well at 37 °C). Culture medium (1000 µl/well at 37 °C) was added prior to microscopic examination. (In inhibition experiments, various concentrations of the drugs were used (10⁻⁴–10⁻⁷ mol/l)). Cell viability of BAEC after incubation with cytokines or drugs was confirmed by trypan blue exclusion and microscopic examination.

Image Acquisition and Cell Counting The specimens were analyzed using the Leica DM IRB inverted microscope (Leica, Bensheim, Germany) at 100-fold magnification. All analysis was carried out at the centre area of each well where the endothelial cell layer with adherent PMNs can be brought in sharp focus.

Images from the specimens were taken with a Leica DC 200 digital camera connected to the microscope and saved on a personal computer in bitmap (bmp) or tagged image file format (tiff) using the Leica image archive software (Leica Microsystems, Bensheim, Germany) at a resolution of 1200×1000 pixels. The area covered by the digital image was smaller than the field of vision which reduces the effects of inhomogeneous illumination (shading). For manual counting of PMNs, several randomly selected fields totaling 0.25 mm² from the centre area of the well were used. Counting was accomplished by using the counter feature of the image archive software IM 1000 (Leica Microsystems, Bensheim, Germany). Briefly, every PMN in the chosen area was counted by a mouse click, leaving the counted cells with a mark. All manual counting was conducted blindfolded by two independent observers. This manual counting method is reported to have within-observer and between-observer variability of less than 10%.¹⁸

Image Analysis Image analysis was accomplished using the public domain software ImageJ (available at <http://rsb.info.nih.gov/ij>) running on a standard PC (Maxdata, Marl, Germany). All acquired images from one test run were grouped in a stack and subsequently analyzed in an automated procedure using a customized program (macro) written in Java language which integrates in the ImageJ software available from the authors. Operations during analysis consisted of converting the images to 8-bit greyscale (256 scales of grey, ranging from 0 (black) to 255 (white)), all images

show a typical distribution of grey values which is used for cell discrimination), plotting the distribution of grey values, thresholding and counting cells. For the latter purpose code from the “custom particle analyzer” macro (authors: Roos, G., Rasband, W.; <http://rsb.info.nih.gov/ij/plugins/particle-analyzer.html>) was used. Counted PMNs were displayed as filled black circles. For each particle the following parameters were recorded: area in pixels, minimum/maximum/mean grey value and circularity ($4\pi(\text{area}/\text{perimeter}^2)$). Limits for the area and circularity parameter were set to values corresponding to the size and shape of the PMNs used in the experiment (for more details see Results).

Statistics Data are presented as mean $\pm$ S.D. Statistical significances were calculated using Student's *t*-test for unpaired samples. Statistically significant difference was set at $p < 0.05$.

RESULTS

Detection of PMNs by Image Analysis For the validation of the computerized counting, PMN suspensions in DPBS at different concentrations ranging from 1×10^7 to 1.25×10^8 cells/ml were placed into 24-well microtiter plates without endothelial cells (150 μ l PMN suspension per well) and images from the centre area of the wells analyzed. Figure 1 indicates the correlation between cells counted by the image analysis program and the total number of cells added to each well. The number of counted cells shows a high linearity ($r = 0.9992\%$) to the original cell concentration in the wells.

Discrimination between BAEC, Partly Detached BAEC and PMNs After conversion of the original colored images (Fig. 2a) to 8-bit greyscale images (Fig. 2b), which consist of up to 256 scales of grey, ranging from 0 (black) to 255 (white), all images show a typical distribution of grey values which is used for cell discrimination (Fig. 3). The endothelial cells attached to the bottom of the well represent the largest area in each image, appearing at dark grey values, as shown by the maximum number of pixels in Fig. 3a (at a grey value of 51), which displays the distribution of grey values in an image (not shown) of a BAEC monolayer without PMNs. Junctions between BAECs appear at slightly brighter grey values, forming the second peak in the number of pixels (at a grey value of 63) in Fig. 3a. The same is found in Fig. 3b, which shows the distribution of grey values of Fig. 2b. PMNs adherent to the endothelial cells appear at brighter grey values (100–145, marked by an arrow in Fig. 3b). The small number of adherent PMNs in Fig. 2, representing less than 0.5% of the image area requires a logarithmic *y*-axis in the display of grey value distribution to make these grey values visible (grey graph in Fig. 3, black graph uses linear *y*-axis). These bright grey values are missing in control images of BAEC monolayers without PMNs. This information allows setting threshold values in a way that the particle analyzer counts only the bright grey values corresponding to PMNs. This protocol facilitates the discrimination between BAECs and PMNs.

However, in some cases few endothelial cells partially loose contact with the bottom of the well making the detached part of the cells appear brighter than fully attached cells (partly detached BAEC are shown by the arrows in

Fig. 1. Linearity of the PMN Counting by Image Analysis

Leukocytes were counted by image analysis software (ImageJ) in 24-well microtiter plates. 150 μ l of cell suspension (concentrations ranging from 1×10^7 to 1.25×10^8 PMNs/ml) were placed in each well. Images from the centre area were taken and the number of PMNs in 1 mm² was determined by image analysis. The number of counted cells shows a high linearity ($r = 0.9992\%$) to the original cell concentration in the wells. The values represent the mean of at least six independent experiments; $\pm$ S.D. is shown by the bar.

Figs. 2a–c), sometimes at grey values similar to those of the PMNs. Nearly completely detached BAECs appear brightest, but were excluded from the cell count due to their larger size. Partly detached endothelial cells appear dark at their still attached section while their detached section appears brighter. This section is excluded from the cell count due to its irregular shape, resulting in lower circularity values compared to PMNs. Use of these filters in the counting process facilitates an accurate determination of the number of adherent PMNs even in the presence of partly detached endothelial cells as proven by the size distribution histogram of counted cells which shows a Gaussian distribution (Fig. 4).

Image Analysis in Comparison with Manual Counting To evaluate the comparison of the results acquired by manual counting procedures and image analysis, the area used in the image analysis process was reduced from the usual 1 mm² to the 0.25 mm² used during manual counting. Within-observer and between-observer variability of the manual counting method was less than 5% (data not shown). Figure 5 reflects the comparison between assessing the number of MM6 cells adherent to BAEC by image analysis and manual counting procedure. Manual counting was performed in a blinded manner by two independent observers with the help of Leica IM 1000 image archive software using its “point-and-click” counting feature. The results obtained by image analysis equal the results achieved by manual counting for the three different concentrations of MM6 cell suspension ($1 \cdot 10^6$, $2 \cdot 10^6$, $8 \cdot 10^6$ cells/ml) used (36 vs. 38/61 vs. 63/220 vs. 223 MM6 cells).

Adhesion Assay TNF α is one of the immunomodulators commonly used to stimulate the expression of various adhesion molecules in endothelial cells. The kinetic of the expression in BAECs has been described in detail for E-selectin,¹⁹⁾ ICAM-1 and VCAM-1.²⁰⁾ From these data and own investigations (data not shown) we used a period of 4 h to stimulate the BAECs with TNF α (20 ng/ml) prior to addition of the PMNs. Thus we achieved a significant increase in the number of adherent cells after incubation for 30 min compared to unstimulated BAECs (Fig. 6a). In a different series of experiments we used the human leukaemic cell line Mono Mac 6 instead of PMNs while the other parameters of the assay procedure remained unchanged. Figure 6b shows the number of

Fig. 2. Images of Confluent BAEC Monolayer with Adherent PMNs and Partly Detached BAEC (Shown by the Arrows)

(a) A certain area of an image obtained directly from the Leica DM IRB microscope (100 fold magnification) with the Leica DC 200 digital camera. (b) The same area after conversion to 8-bit greyscale (256 scales of grey, ranging from 0 (black) to 255 (white), all images show a typical distribution of grey values which is used for cell discrimination (Fig. 3). (c) Result of the image analysis and conversion by ImageJ software: only PMNs are shown as black spots, partly detached BAEC are excluded, (shown by the arrows).

Fig. 3. Histogram of Grey Value Distribution from Two 8-Bit Images of 100000 Pixels Each; Black: Linear y-Axis, Grey: Logarithmic y-Axis

(a) Grey value distribution from an image showing only BAEC [grey value minimum: 45, max: 85, mean: 58, mode 51(6339 pixels)]. (b) Grey value distribution from an image showing BAEC with adherent PMNs (PMNs cover less than 0.5% of image area therefore only visible with logarithmic y-axis; marked by an arrow) [grey value minimum: 44, max: 145, mean: 61, mode: 50 (6573 pixels)].

adherent MM6 cells to $\text{TNF}\alpha$ -stimulated and unstimulated BAECs.

We also investigated the differences of a simultaneous treatment of $\text{TNF}\alpha$ (20 ng/ml) and the test compounds for 4 h or a treatment for 20 min with the test compounds after the

Fig. 4. Size Distribution Histogram of Counted Leukocytes (14956 Particles from 24 Stacked Images, Size from 10 to 55 Pixels; Mean 25 Pixels; Mode (Maximum) 26 Pixels (1032 Particles); S.D. 7.9)

Fig. 5. Comparison between Manual Counting of MM6 Cells and Counting by Image Analysis

Confluent BAEC were incubated with three different concentrations of MM6 cell suspension ($1 \cdot 10^6$, $2 \cdot 10^6$, $8 \cdot 10^6$ cells/ml) in PBS for 30 min. After removal of non-adherent MM6 cells by washing digital images of a 1 mm^2 area were taken and the number of adherent cells determined by manual counting (open circles) and image analysis "ImageJ" software (filled triangles). Each value represents the mean of 8 independent experiments; $\pm$ S.D. is shown by the bar.

Fig. 6a. Increase of Adhesion of PMNs to BAEC upon $\text{TNF}\alpha$ Stimulation

The Diagram shows unstimulated BAEC (377 adherent PMNs) (left bar) and BAEC stimulated by $\text{TNF}\alpha$ (20 ng/ml) (859 adherent PMNs) (right bar) (incubation time: 4 h). The number of adherent PMNs was determined in a 1 mm^2 area after addition of 1×10^6 PMNs/well. Non-adherent PMNs were removed by washing after 30 min. The values are the mean of 18 adhesion experiments; $\pm$ S.D. is shown by the bar. $**p < 0.001$, significantly different as compared to $\text{TNF}\alpha$ -stimulated cells by unpaired Student's *t*-test.

4 h $\text{TNF}\alpha$ (20 ng/ml) stimulation. The co-stimulation of $\text{TNF}\alpha$ (20 ng/ml) and test substance for 4 h was chosen for all further experiments since a longer time for the ligand receptor interplay was needed and additional washing steps can be avoided (Figs. 7, 8).

Effect of Sialyl Lewis^X and Bimosiamose Sialyl Lewis^X, a part of the larger natural selectin ligand PSGL-1, was the first E-, P- and L-selectin ligand identified in carbohydrate-mediated cell adhesion processes^{21,22}; and has been widely used as reference substance in adhesion studies. In our assay we determined the effect of Sialyl Lewis^X (100 μM) after 20 min treatment and 4 h co-incubation of sLex and

Fig. 6b. Increase of Adhesion of MM6 Cells to BAEC upon $\text{TNF}\alpha$ Stimulation

The Diagram shows unstimulated BAEC (299 adherent MM6 cells) (left bar) and BAEC stimulated by $\text{TNF}\alpha$ (20 ng/ml) (incubation time: 4 h) (598 adherent MM6 cells) (right bar). The number of adherent MM6 cells was determined in a 1 mm^2 area after addition of 1×10^6 MM6 cells/well. Non-adherent MM6 cells were removed by washing after 30 min. The values are the mean of 18 adhesion experiments; $\pm$ S.D. is shown by the bar. $**p < 0.001$, significantly different as compared to $\text{TNF}\alpha$ -stimulated cells by unpaired Student's *t*-test.

Fig. 7. Effect of Sialyl Lewis^X ($100\text{ }\mu\text{M}$) on PMN Cell Adhesion to BAEC Using Different Incubation Times

The number of adherent PMNs was determined in a 1 mm^2 area after addition of 1×10^6 PMN cells/well. Non-adherent PMNs were removed by washing after 30 min. Number of adherent PMNs at $\text{TNF}\alpha$ -stimulated endothelial cells is set to 100%. The values are the mean of 18 adhesion experiments; $\pm$ S.D. is shown by the bar. $*p < 0.05$, significantly different as compared to $\text{TNF}\alpha$ -stimulated cells by unpaired Student's *t*-test.

$\text{TNF}\alpha$ (20 ng/ml) on the adhesion of PMNs to BAEC. Figure 7 shows a significant decrease in the number of adherent PMNs to activated and Sialyl Lewis^X ($100\text{ }\mu\text{M}$) treated BAEC (20 min treatment or 4 h co-incubation) compared to $\text{TNF}\alpha$ (20 ng/ml) activated BAEC. The decrease of adhesive cells on Sialyl Lewis^X ($100\text{ }\mu\text{M}$) treated BAEC was independent from incubation time ($72 \pm 5\%$ for 20 min vs. $75 \pm 10\%$ for 4 h).

A representative of a new class of drugs, the synthetic Sialyl Lewis^X mimetic, Bimosiamose,²³ currently in phase-II trials for asthma and psoriasis, has been evaluated in our assay using PMN cells. Figure 8a shows that treatment of $\text{TNF}\alpha$ -stimulated (20 ng/ml) BAEC with Bimosiamose (100, 400, $600\text{ }\mu\text{M}$ for 4 h) reduces in a concentration dependent manner ($64 \pm 5\%$, $42 \pm 3\%$, $31 \pm 3\%$) the number of adherent PMN cells compared to the adhesion level found with stimulated BAEC. Interestingly, the decrease of adhesive cells was dependent from the incubation time and was stronger after 4 h co-incubation of BAEC with Bimosiamose ($100\text{ }\mu\text{M}$) compared to 20 min incubation time ($58 \pm 5\%$ for 4 h vs. $80 \pm 2\%$ for 20 min). After 16 h no significant change of ad-

Fig. 8a. Effect of Bimosiamose (100—400— $600\text{ }\mu\text{M}$) on PMN Cell Adhesion to BAEC

The number of adherent PMN cells was determined in a 1 mm^2 area after addition of 1×10^6 PMN cells/well. Non-adherent PMN cells were removed by washing after 30 min. Number of adherent PMN cells at $\text{TNF}\alpha$ -stimulated endothelial cells is set to 100%. The values are the mean of 18 adhesion experiments; $\pm$ S.D. is shown by the bar. $*p < 0.05$, $**p < 0.001$, significantly different as compared to $\text{TNF}\alpha$ -stimulated cells by unpaired Student's *t*-test.

Fig. 8b. Effect of Bimosiamose ($100\text{ }\mu\text{M}$) on PMN Cell Adhesion to BAEC Using Different Incubation Times

The number of adherent PMN cells was determined in a 1 mm^2 area after addition of 1×10^6 PMN cells/well. Non-adherent PMN cells were removed by washing after 30 min. Number of adherent PMN cells at $\text{TNF}\alpha$ -stimulated endothelial cells is set to 100%. The values are the mean of 6 adhesion experiments; $\pm$ S.D. is shown by the bar. $*p < 0.05$, significantly different as compared to $\text{TNF}\alpha$ -stimulated cells by unpaired Student's *t*-test.

herent PMNs to $\text{TNF}\alpha$ (20 ng/ml) activated and Bimosiamose ($100\text{ }\mu\text{M}$) treated BAEC compared to $\text{TNF}\alpha$ (20 ng/ml) activated BAEC was observed at this time-point (106 ± 2 for 16 h) (Fig. 8b).

DISCUSSION

In this report we present a rapid and facile computer-assisted method for quantification of *in vitro* neutrophil adhesion to bovine aortic endothelial cells. Our method aims at keeping the number of necessary steps during the assay procedure as low as possible while facilitating rapid acquisition of results. Most of the commonly used adhesion assays are based on pre-labeled PMNs⁸) while a relatively small number of assays forego pre-labeling by using visual methods for quantification.²⁴) These techniques are useful in studying adhesion processes but require time consuming analysis based on manual procedures which are unsuitable for large-scale use.^{25,26}) Pre-labeling is accomplished in various ways; iso-

topic methods have been widely used, while recently the use of different enzymatic and notably fluorimetric procedures have become more widespread.²⁷⁾ The use of these labels makes for rapid ways of analysis but is not without obstacles. For instance, it always adds steps to the assay procedure and there are conflicting reports about possible effects of label substances towards unwanted stimulation of cells or altered expression of adhesion molecules or adhesive capabilities and possible label leakage.^{7,28–30)} Furthermore, they are often susceptible to modifications in the assay procedure, for example the way non-adherent cells are removed.³¹⁾

We have demonstrated in our study that counting of neutrophil numbers by computer analysis of images acquired by contrast microscopy is possible and that this method is suitable to PMNs adherent to BAEC monolayers.

Moreover, the test with TNF α -stimulated BAEC shows that the results obtained with the described assay are in line with previous reports on increased adhesive capabilities of TNF α -stimulated endothelial cells.^{32,33)}

Furthermore, using the standard selectin-ligand Sialyl Lewis^X and the new synthetic pan-selectin ligand Bimosiamose with known influence on leukocyte adhesion we demonstrated the applicability of our assay for the evaluation of the influence of test substances on neutrophil adhesion to endothelial cells. Interestingly, we found that the antiadhesive effect of Bimosiamose but not of Sialyl Lewis^X was dependent from the duration time of the substance on the BAEC. It was more effective after 4 h co-incubation compared to only 20 min treatment.

After 4–6 h TNF- α pre-treatment of BAEC peak amounts of E-selectin¹⁹⁾ were expressed but no up-regulation of ICAM-1 mRNA or VCAM-1 mRNA was observed^{13,20)} in kinetic studies. ICAM-1 and VCAM-1 are the main counter receptors for β 2-integrin dependent firm adhesion and their expression peaked after 12–18 h vs. 6–12 h after TNF- α treatment on BAEC.^{19,20)}

Bimosiamose and Sialyl Lewis^X are designed as antagonists against E-, P-, and L-selectin. The time dependent inhibition of Bimosiamose suggests that it may need some changes in the conformation as a prerequisite for the receptor binding. According to their chemical structure and the kinetics of the expression of the adhesion molecules, mainly the selectins should be responsible for most of the adhesion in this assay after 4 h stimulation time. Other authors used for the screening for inhibitors of E-selectin expression 4 h incubation time and 16 h TNF- α treatment to investigate inhibitors of ICAM-1- or VCAM-1-expression (1,4-dihydroxy-xanthenes).³⁴⁾ Therefore it was not surprising that after 16 h no significant change of adherent PMNs to TNF α (20 ng/ml) activated and Bimosiamose (100 μ M) treated BAEC compared to TNF α (20 ng/ml) activated BAEC was observed, because the selectin expression was already downregulated on the endothelial surface at this time-point.

Thus our data suggest that by prolonging the incubation time up to 16 h it is possible to investigate integrin dependent adhesion in this model.

A lack of this assay is that it cannot separate effects on selectin-mediated capture and rolling, from effects on integrin-mediated immobilization and migration. This investigation would require models in which the full sequence can be observed and the steps evaluated separately. The major draw-

back of these models is that they are normally used to study the biophysics of rolling, and are not routinely used to evaluate large numbers of small antagonists to optimize a lead.⁵⁾

This monolayer adhesion assay is useful in defining adhesion-stimulatory or inhibitory substances with different mechanisms of action, that might act on either the leukocyte or endothelial cell, as well as characterizing the ligand-receptor pairs involved (e.g., through the use of specific antibodies or blocking reagents incorporated into the assay system as performed in other assays.¹³⁾ As shown for MM6 cells, this monolayer adhesion assay can be readily adapted to the study of other cell types (T-lymphocytes or e.g. tumor cells adhering to endothelial or nonendothelial monolayers).

In summary, we have developed a rapid assay which is easy to handle and allows a high throughput screening (HTS) of different test compounds (including small selectin blockers with low molecular weight, integrin antagonists and inhibitors of the expression of adhesion molecules).

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