The migration of neutrophils from blood vessels to peripheral tissues is a key step of inflammation (Harlan, 1985). This requires the formation of transient gaps between endothelial cells with concomitant leucocyte squeezing through these narrow apertures and immediate restoration of endothelium continuity. It is currently considered that the main role of selectins is to mediate the initial contact between flowing leucocytes and endothelial cells. We show here that the binding of E- or P-selectins by specific antibodies induces a marked ‘rounding up’ of interleukin-1- or thrombin-activated human endothelial cells, respectively. Also, anti-E-selectin antibodies trigger a transient increase in cytosolic calcium involving intracellular calcium stores. No such effect is observed when von Willebrand factor or intercellular adhesion molecule 1 are similarly bound. Thus, in addition to promoting the initial interaction between activated endothelium and moving leucocytes, selectins might play a role in the induction of subsequent endothelial deformation, which would facilitate leucocyte arrest and transmigration towards peripheral tissues, and enhance the diffusion of soluble molecules between intravascular and peripheral compartments. Our results are consistent with this hypothesis and demonstrate a new property of endothelial selectins.

Key words: inflammation, endothelial cell, calcium, confocal microscopy, scanning electron microscopy
induction. This may play a role in the coordinated set of events leading to transmigration of the endothelium by neutrophils.

**MATERIALS AND METHODS**

**Endothelial cells**

Human umbilical vein endothelial cells (HUVEC) were obtained following standard methods (Jaffe et al., 1973). Umbilical veins were perfused with Hanks’ solution containing 0.3 units/ml of collagenase A (Boehringer Mannheim, Germany). They were incubated for 15 minutes at 37°C, then gently massaged and washed with Hanks’ solution supplemented with 20% fetal calf serum (Gibco, Glasgow, Scotland). Cells were collected and washed. Incubation was done in 25 ml Falcon culture vials (Becton and Dickinson France, Grenoble) and described by Wellcome et al. (1990). Anti-ICAM-1 (clone 84H10; Makgoba et al., 1988), anti-von Willebrand factor (Genzyme, supplied by Tebu, le Perray en Yveline, France) and described by Wellcome et al. (1990). Anti-ICAM-1 where stimulation was performed for 24 hours. Thrombin incubations were done for 4 hours, excepted for experiments with anti-ICAM-1 where stimulation was performed for 24 hours. Thrombin treatment was performed by 10 minutes of incubation at 37°C with 2 units/ml of thrombin (from human plasma, Sigma) in the absence of fetal calf serum.

**Antibodies**

All antibodies were murine IgG1 monoclonal antibodies (mAbs). Anti-E-selectin mAb (clone BBIG-E4) was supplied by British Biotechnology (Oxon, UK) It was specific for the terminal (lectin/EGF domain) region of the molecule (Pigott et al., 1991) and it was found to impair granulocyte adhesion to IL-1-stimulated HUVEC in a flow chamber assay (Kaplanski et al., 1993). In some experiments, we also used 1.2B6 supplied by Immunotech (Mar-seilles, France) and described by Wellcome et al. (1990). Anti-ICAM-1 (clone 84H10; Makgoba et al., 1988), anti-von Willebrand factor and anti-P-selectin mAbs were provided by Immunotech.

Standard incubations were performed at room temperature for 30 minutes with or without 50 µg/ml antibodies immediately before fluorescent staining.

**Fluorescence microscopic study of endothelial cells**

Cells were labeled with 5 µg/ml octadecyl rhodamine (Molecular Probes, Eugene, OR) for 10 minutes at room temperature. This procedure resulted in rapid labeling of the plasma membrane (within 5-10 minutes) with subsequent diffusion to intracellular membranes. It had been previously checked that this procedure did not affect the adhesive behavior and mobility of membrane molecules in conjugates made between cytotoxic lymphocytes and target cells (André et al., 1990). After washing, coverslips were inverted and deposited on a glass slide with a fixed volume of 30 µl phosphate buffered saline solution. They were then examined with a confocal laser scanning microscope (Leica, Heidelberg) operated under 2× mode to observe sections perpendicular to the monolayers. A ×40 dry objective (0.70 NA) was used to avoid any mechanical action on the coverslip.

Images were transferred to an IBM-compatible desk computer by real-time digitization of the video output of the confocal microscope with a PCVision + card (Imaging Technology, Bedford, MA) allowing 8-bit accuracy (i.e. 256 gray levels). The linearity of the acquisition was checked by digitizing artificial images constructed with the confocal microscope computer and spanning the whole range of intensities. Images were then processed with a previously described analysis system developed in the laboratory (André et al., 1990). The background fluorescence always comprised between about 20 and 30% of the labeled endothelial monolayer was then constructed with a standard boundary-follow algorithm, using a threshold higher than the background by 16 units. The mean monolayer thickness was then determined on standard fields of about 88 µm width. Mean values of about 40 such determinations obtained in four separate experiments were then calculated (see Results).

**Determination of the height of isolated HUVEC by conventional microscopy**

We made use of a previously described method developed in our laboratory (Mège et al., 1985). Briefly, saline containing 2.5% by weight latex beads of 0.8 µm diameter (Sigma) was added to nonconfluent HUVEC monolayers. Since beads were readily deposited on cells and intercellular substrata, cell height was determined by examining samples with a ×40 lens on the stage of an Olympus IMT2 inverted microscope that was sequentially focussed on the beads located on the beads located on the highest point of each cell, then on the glass near the cell. The height was read on the microscope micrometer with about 1 µm accuracy. About 50 cells were studied in a typical experiment.

**Observation of the shape of isolated HUVEC by scanning electron microscopy**

Confuent HUVEC monolayers were incubated with or without mAbs as described above. They were then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and postfixed with 1% OsO4 (Prolabo, Paris) dissolved in water. The preparation was dehydrated in a series of ethanol and dried in a Balzer critical-point CO2 apparatus. The samples were placed in a Nanotech SEMREP 2 sputter coater for gold deposition. They were examined in a Jeol 35CS scanning electron microscope, operated at 60 kV.

**Monitoring of cytosolic calcium concentration**

Nonconfluent HUVEC monolayers were labeled with fluo-3/AM as previously described (Zaffran et al., 1993) by a 20 minute incubation at room temperature in a solution of 20 nM HEPES, 1 mM CaCl2, 1 mM MgCl2, 5 mM KCl, 10 mM glucose, 135 mM NaCl, 1% bovine albumin, pH 7.4, containing 3 µM fluo-3/AM (Molecular Probes). They were then washed and examined at 37°C with an Olympus IMT2 inverted microscope equipped with a Reichert heating stage. They were immediately stimulated with mAbs. Fluorescence measurements were performed with a Lehsa 4036 ST video camera (Lhesa, Cergy Pontoise, France) mounted on the microscope and connected to a
A novel role for E- and P-selectins

 цифр 1. Маркировка эндотелиальных клеток октадецилродамином. Клетки HUVEC, маркированные октадецилродамином, были исследованы с помощью сканирующего лазерного микроскопа. Зона, похожая на яркое свечение, выявлена в клетках. Масштабный столб, 25 мкм.

 цифр 2. Анти-E-селектины вызывают существенные изменения в эндотелиальных клетках. Конфлюэнтные клетки HUVEC были активированы интерлейкином-1, затем инкубированы с или без моноклональных антител. Использование конфокального лазерного микроскопа для прямого визуализации трехмерной структуры. Как показано на рис. 2, основным эффектом моноклональных антител было сильное утолщение. Антибоды вызывали яркое свечение в клетках (а и с). Антибоды вызывали явные отклонения в структурной организации (стрелка). Масштабный столб, 12.5 мкм.

 цифр 3. Количественная оценка среднего толщины конфлюэнтных клеток HUVEC. Конфокальные изображения поперечного сечения конфлюэнтных клеток HUVEC были исследованы. Обычные контроли (а) и анти-E-селектины (b) показаны. В каждом случае, исходное изображение (вверху) обрабатывалось с помощью алгоритма поиска границы, чтобы получить контур клетки. Выделение яркости экстраклеточных пикселей использовалось для непосредственной визуализации интраклеточных областей (снизу). Толщина каждого монослоя рассчитывалась как отношение общего клеточного объема к ширине клетки. Масштабный столб, 10 мкм.

 RESULTS

 Anti-E-selectin and anti-P-selectin mAbs, not anti-ICAM-1 or anti-von Willebrand mAb, induce a marked rounding up of confluent HUVEC monolayers

 Эндотелиальные клетки активировались интерлейкином-1, чтобы вызвать экспрессию E-селектина. Клетки были инкубированы с или без моноклональных антител, маркированных октадецилродамином и исследованных с помощью конфокального лазерного микроскопа. Как показано на рис. 1, октадецилродамин был концентрирован в клеточных мембранах, хотя область, которую можно было назвать ярким свечением, была видна в цитоплазме клеток (стрелка). Масштабный столб, 10 мкм.

 The effect of mAbs on endothelial cell thickness was then studied by observing optical sections perpendicular to monolayers. As shown in Fig. 2A, controls exhibited fairly smooth surfaces with constant thickness. However, a 30 minute incubation with monoclonal antibodies specific for E-selectin triggered dramatic morphological changes with increased height and images suggesting intercellular gaps (Fig. 2B).

 Since ICAM-1 is thought to play an important role in leucocyte attachment to and transmigration through endothelial cells, it was important to know whether anti-ICAM-1 mAb...
induced a rounding up similar to that obtained with anti-E-selectin antibody. Experiments were done after 4 hours and 24 hours of incubation with interleukin-1, since the latter treatment induced maximal expression of ICAM-1 (Zimmerman et al., 1992). However, no change in shape of

stimulated cells was found (Fig. 2C). Also, cells extensively bound anti-von Willebrand factor mAb without any deformation (not shown). Finally, endothelial cells were stimulated with thrombin to induce P-selectin expression, and then exposed to anti-P-selectin mAb: similar morphological changes were found to those with anti-E-selectin mAb.

Confocal images were studied quantitatively by measuring the height of endothelial cells. The principle of the analysis is exemplified in Fig. 3. Preliminary experiments were done with different threshold values for boundary determination: in contrast to absolute thickness values, the relative variations in endothelial thickness were not heavily dependent on the threshold level. As shown in Table 1, our qualitative conclusions were supported, since anti-E-selectin and anti-P-selectin mAbs repeatedly induced a substantial (about 50%) and significant (P<0.0001) increase in the height of endothelial cells.

Additional experiments (Table 2) revealed that after stimulation with anti-E-selectin mAb, deformations were not apparent at 5 minutes, were detectable at 10 minutes and maximal at 30 minutes.

It was important to ascertain that the rounding that we observed was not triggered by some activating factor contaminating anti-selectin antibodies. In order to rule out this possibility, experiments were repeated with another anti-E-selectin mAb (1.2B6). Indeed, in the same series of experiments, the mean endothelial thickness was 4.6 µm (±0.32 µm s.e.m., 9 determinations) for the control, it was 6.9 µm (±0.60 µm, n=8) after exposure to 1.2B6 mAb antibody, and 7.1 µm (±0.6 µm, n=13) after treatment with the first anti-E-selectin that we used. The latter antibody was used throughout all subsequent experiments. Finally, HUVEC were treated with anti-E-selectin

### Table 1. Antibody-mediated height increase of endothelial cell monolayers

<table>
<thead>
<tr>
<th>Endothelial cell pretreatment</th>
<th>Control</th>
<th>E-selectin</th>
<th>ICAM-1</th>
<th>P-selectin</th>
<th>von Willebrand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-1 (4 hours)</td>
<td>5.4±0.22 (n=37)</td>
<td>8.5±0.36 (n=57)</td>
<td>5.4±0.19 (n=53)</td>
<td>−</td>
<td>5.8±0.21 (n=11)</td>
</tr>
<tr>
<td>Interleukin-1 (24 hours)</td>
<td>6.0±0.42 (n=28)</td>
<td>6.9±0.42 (n=34)</td>
<td>6.7±0.35 (n=30)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Thrombin (10 minutes)</td>
<td>5.1±0.26 (n=38)</td>
<td>−</td>
<td>−</td>
<td>8.10±0.32 (n=58)</td>
<td>−</td>
</tr>
</tbody>
</table>

Endothelial cell monolayers were pretreated with thrombin or recombinant interleukin-1 and exposed for 30 minutes to 50 µg/ml of mAbs of different specificities. They were then labeled with a fluorescent probe and examined with a confocal microscope. Images were quantitatively processed for determination of the mean HUVEC height on a field of 125 µm width. The results of four separate experiments were pooled and mean values are shown ± standard error of the mean. Number of averaged fields n in brackets.

### Table 2. Kinetics of anti-E-selectin mediated height increase of HUVEC monolayers

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Height increase</td>
<td>0</td>
<td>2±8</td>
<td>25±7</td>
<td>54±6</td>
</tr>
<tr>
<td>(n=13)</td>
<td>(n=22)</td>
<td>(n=33)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Series of interleukin-1-activated-endothelial cell monolayers were treated with anti-E-selectin mAb and labeled with octadecylrhodamine. The height of different monolayers was measured at regular intervals with confocal microscopy and results were expressed as percent of controls. Mean values are shown ± standard error. Number of averaged fields n in brackets.

### Table 3. Use of conventional microscopy to determine the height of control and antibody-treated HUVEC monolayers

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Control</th>
<th>IL-1</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean height (µm)</td>
<td>6.96</td>
<td>7.50</td>
<td>10.18</td>
<td>11.24</td>
</tr>
<tr>
<td>Standard error (µm)</td>
<td>0.18</td>
<td>0.30</td>
<td>0.34</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Endothelial cell monolayers were incubated with or without anti-E-selectin antibodies after IL-1 pretreatment and coated with small diameter latex beads for determination of their average height. Mean values determined on about 25 cells are shown with standard error (mean). Number of averaged fields n in brackets.

**Fig. 4.** Interaction between human granulocytes and confluent endothelial cell monolayers. Human granulocytes were labeled with fluo-3 and deposited on octadecyl rhodamine-labeled HUVEC. Cells were examined by confocal microscopy using dual color display to allow easy discrimination between granulocytes (green) and endothelial cells (red). Bar, 12.5 µm.
Fig. 5. Observation of anti-E-selectin-induced HUVEC deformation by scanning electron microscopy. Control HUVEC (A) or cells treated with anti-ICAM-1 (B) or anti-E-selectin (C) mABs were observed by scanning electron microscopy. Only the latter treatment induced marked cell thickening with formation of conspicuous intercellular gaps.
antibody without any previous stimulation with interleukin 1: cell height was 5.3 \(\mu\)m (±0.39 \(\mu\)m) and 6.5 \(\mu\)m (±0.57 \(\mu\)m), respectively. However, these results were difficult to interpret, since unstimulated HUVEC expressed low but detectable levels of membrane selectins (not shown).

**Interaction between granulocytes and endothelial cells may induce a significant rounding up**

In order to assess the physiological relevance of the described results, fluo-3-labeled granulocytes were deposited on rhodamine-labeled endothelial cells and examined by confocal microscopy. As shown in Fig. 4, images suggesting contact-induced endothelial rounding up were obtained; however, they were too sparse to allow quantitative analysis.

**Anti-E-selectin-mediated endothelial cell deformation can be measured by conventional microscopy**

It was considered useful to confirm our finding by a previously described method allowing cell observations with visible light. Monolayers were covered with a suspension of latex beads and examined by conventional microscopy. Cell height could thus be determined by sequentially focussing the microscope on beads deposited on the cell surface and the slide. As shown in Table 3, our conclusion that anti-E-selectin mAb induced a significant (about 50%) increase in cell height was confirmed by this method.

**Anti-E-selectin-mediated rounding up of confluent HUVEC is clearly visible on scanning electron micrographs**

Control HUVEC and cells treated with anti-ICAM-1 or anti-E-selectin were fixed and examined by scanning electron microscopy. As shown in Fig. 5, controls (Fig. 5A) and anti-ICAM1-treated (Fig. 5B) cells exhibited full spreading, whereas anti-E-selectin treatment (Fig. 5C) resulted in marked thickening of the cell body with cell retraction and formation of conspicuous intercellular gaps.

**Anti-E-selectin mAb induced a transient increase in free cytosolic calcium in confluent and nonconfluent endothelial cell monolayers**

If selectin-mediated endothelial cell deformation were an active process, it should be preceded by a generation of second messengers involved in cell shape-control. Since calcium changes were recently suggested to play a role in endothelium deformations induced by pro-inflammatory mediators (Northover, 1992), endothelial cells were labeled with a fluorescent calcium probe, then stimulated with anti-E-selectin or anti-P-selectin mAb under continuous fluorescence monitoring. Qualitative observation of confluent fluo-3-labeled HUVEC monolayers revealed transient fluorescence increases after addition of anti-E-selectin, not anti-P-selectin antibodies. In order to achieve a quantitative estimate of changes in calcium concentration, nonconfluent monolayers were used, which allowed easy determination of total cell fluorescence after boundary determination. As shown in Fig. 6, transient and asynchronous increases in calcium were observed during the first minutes following the stimulation of interleukin-1-activated endothelial cells with anti-E-selectin mAb. In a representative series of 11 cells, cytosolic calcium rose from an initial value of 100 nM (13 nM standard deviation) to 836±164 nM within ten seconds, and initial values were restored a few minutes later. Results of sequential calcium determinations performed on individual cells are shown on Fig. 7: marked differences in the kinetics of the calcium response were found, with heterogeneity of peak duration and lag before the increase in calcium. Also, a few cells did not display any measurable increase in calcium during the period of observation.

However, this increase in calcium was not found when thrombin-activated cells were stimulated with anti-P-selectin antibodies. This lack of response was not due to a depletion of intracellular calcium stores following thrombin treatment (thrombin indeed triggered an increase in cytosolic calcium - not shown), since thrombin-treated HUVEC displayed a marked increase in cytosolic calcium after being treated with histamine (not shown).

The effect of anti-E-selectin mAb on intracytosolic calcium might be due to a release of intracellular calcium, opening of plasma-membrane calcium channels, or both. We tried to get some information on the involved mechanism by treating fluo-3-labeled HUVEC with anti-E-selectin mAb in the presence of a calcium chelator: cells still displayed calcium increases in the presence of 1 nM EGTA, but the lag between antibody stimulation and calcium response was longer than 2 minutes.
whereas most cells displayed an increase in calcium within 60 seconds in calcium-containing medium.

To assess the role of the increase in calcium in anti-E-selectin mAb-induced cell deformations, adherent cell monolayers were treated with BAPTA-AM to buffer intracellular calcium. However, under these conditions, a significant rounding up was observed in the absence of antibody treatment, making results uninterpretable.

Finally, cells were treated with 0.1 µM ionomycin and examined by confocal microscopy. The mean heights of controls and ionophore-treated monolayers were, respectively, 4.70 µm (±0.20 µm s.e.; 35 fields examined) and 5.15 µm (±0.24 µm s.e., n=33). In separate experiments, the ionophore was found to increase cell calcium concentration from 53±3.4 nM to 365±30 nM. In contrast to antibody-induced calcium increases, which were transient, ionophore-triggered increases in calcium persisted for at least several minutes.

DISCUSSION

We demonstrated that antibody-mediated stimulation of E- and P-selectins borne by endothelial cells induced a marked rounding up of these cells. No such effect was observed when von Willebrand factor or ICAM-1 molecules were similarly stimulated. Also, stimulation of E-selectin molecules induced a transient increase in cytosolic calcium in endothelial cells. These results raise several important points.

First, we may speculate that granulocytes adhering to endothelial walls might induce a similar deformation by stimulating the same E- and P-selectins through carbohydrates borne by their membrane molecules (Picker et al., 1991). This endothelial response might enhance the local diffusion of extravascular chemotactic substances towards blood cells, and facilitate the passage of endothelium-bound leucocytes towards peripheral tissues by opening intercellular apertures. This hypothesis is supported by the preliminary observations exemplified in Fig. 4. This might seem at variance with previous reports suggesting that selectins were not involved in leucocyte transmigration through endothelial monolayers in vitro, in contrast to other molecular species such as β2-integrins, ICAMs or PECAM-1 (platelet endothelial cell adhesion molecule-1)/CD31 (Hakkert et al., 1991; Furie et al., 1992; Muller et al., 1993; Vaporciyan et al., 1993). However, this discrepancy is only apparent, since the absence of transmigration blocking by anti-E-selectin antibodies is difficult to interpret for the following reasons: it is possible that different molecular pathways are able to promote the transmigration process, thus allowing leucocyte passage to proceed when only one of these pathways is inhibited by a specific antibody. Further, even if selectin-induced endothelial rounding up was a prerequisite to leucocyte transmigration, this process could not be prevented by anti-selectin antibodies, since we have shown that they are able to induce the very same rounding that might no longer be triggered by leucocytes in their presence.

Secondly, we formally demonstrated a transduction capacity...
of selectin molecules. This is in accordance with a recent report suggesting a signalling role for L-selectin: it was found that the binding of E-selectins to ligands borne by leucocytes, particularly L-selectin (Picker et al., 1991), was able to activate these leucocytes as evidenced by a functional enhancement of β2-integrins (Lo et al., 1991).

Thirdly, an important question is whether there was a causal link between the observed increase in calcium and endothelial cell deformation. The finding that anti-E- and P-selectin mAbs induced similar morphological changes in endothelial cells, whereas only the former antibody triggered an increase in cytosolic calcium, strongly suggests that the calcium response was not the main mediator of shape changes. This conclusion is supported by the absence of significant cell deformation after iomycin treatment. This is consistent with our recent finding that calcium did not play a major role in the control of neutrophil deformation after chemical or mechanical stimulation (Zaffran et al., 1993). It must be emphasized that various different results were reported by different authors who studied the metabolic sensitivity of endothelial cell retraction induced by pro-inflammatory mediators. Thus, thrombin-induced shape changes were inhibited by inhibitors of calcium fluxes such as verapamil and nifedipine, according to some authors (Galdal et al., 1983) but not others (Northover, 1992). However, it is difficult to compare these results, since quite different methods were used to measure the endothelial deformations induced by various mediators of inflammation or coagulation, including conventional microscopy (Northover, 1992; Galdal et al., 1983; Laposata et al., 1983), electrical resistance determinations (Trippathi et al., 1992) or assay of permeability to different macromolecules (Lynch et al., 1990). This might explain why under our experimental conditions thrombin did not induce a marked cell rounding up (Table 1), although it triggered an immediate increase in calcium (not shown). It must be emphasized that our experimental approach is probably the most immediate way of studying cell morphology.

Fourthly, it might seem surprising that E- and P-selectin exhibited different signalling capacities, since only the former molecules induced an increase in calcium in endothelial cells after antibody stimulation. However, this finding is consistent with the hypothesis that P-selectin might be less extensively associated with cell membrane and submembrane components than E-selectin. Indeed, P-selectin is transiently expressed by rapid externalization of the intracellular granule content (Sugama et al., 1992), whereas E-selectin is synthesized after mRNA and protein induction by interleukin-1 (Zimmerman et al., 1992). This point is significant in view of the recent finding that gp150/95 β2-integrins, appearing on the surface of freshly stimulated leucocytes, were functionally different from apparently similar molecules already present on the cell membrane before stimulation (Buyon et al., 1988). The possibility that E- and P-selectins might exhibit different functional properties is further supported by the finding that there is no significant homology between the sequences of their intracytoplasmic domains. This was recently pointed out by Kansas et al. (1993) and we checked it with the Fastscan program of Intelligenetics PCgene package (not shown).

In conclusion, we demonstrated that selectin molecules are not only involved in the first binding events between endothelial cells and flowing leucocytes, but are also endowed with a capacity for signalling and a role in the regulation of endothelial shape. This might be useful for endothelial gap formation and leucocyte transmigration in vivo.

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