Influence of Verapamil and Diclofenac on Leukocyte Migration in Rats

Luis Lopez Martinez, Maria Aparecida de Oliveira, Zuleica Bruno Fortes

Abstract—Nonsteroidal anti-inflammatory drugs and calcium channel blockers can reduce inflammatory responses. Leukocytes play an important role in these responses. An increased expression of adhesion molecules may increase leukocyte migration. Verapamil and diclofenac are known to reduce leukocyte-endothelium interaction. To investigate a possible synergism between these drugs that could be beneficial in cardiovascular diseases, we studied leukocyte behavior by using intravital microscopy. Venules of the spermatic fascia of anesthetized Wistar rats were observed with a closed-circuit TV coupled to an optical microscope. The number of leukocytes rolling along the venular endothelium ("rollers"), sticking after application of a stimulus such as leukotriene B₄ or zymozan-activated plasma ("stickers"), or migrating after a carrageenan stimulus was reduced by verapamil at the dose of 10 mg/kg IP and by diclofenac at the dose of 2.5 mg/kg IP. The combination of both did not augment the effect of each agent alone. Verapamil, diclofenac, or their combination did not interfere with vessel diameter, number of circulating leukocytes, blood pressure levels, or heart rate. Verapamil alone or together with diclofenac reduced venular blood flow velocity and in consequence, the venular shear rate. Our data allow us to suggest that these drugs might interfere with the expression of adhesion cell molecules to reduce cell migration in inflammation. The lack of synergism between the drugs might be explained by the reduction in venular shear rate induced by verapamil, which might not be sufficient to hinder the effect of verapamil alone but hindered the summation effects of both. (Hypertension. 1999;34[part 2]:997-1001.)

Key Words: anti-inflammatory agents, nonsteroidal \textbullet{} verapamil \textbullet{} cell adhesion molecules \textbullet{} endothelium \textbullet{} cell movement \textbullet{} leukocytes \textbullet{} microcirculation

Leukocyte extravasation is essential in the inflammatory response and can be divided into 3 steps: initial interaction of leukocytes with the activated endothelium (rolling), leukocyte activation with firm adhesion to endothelial cells (sticking), and leukocyte extravasation into the surrounding tissues.\textsuperscript{1,2} Several adhesion molecules are involved in the interaction between leukocytes and vascular endothelial cells, which include E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1).\textsuperscript{3} An increased expression of adhesion molecules may increase leukocyte migration and contribute to end-organ damage in cardiovascular diseases.\textsuperscript{3} Calcium channel blockers\textsuperscript{4} and nonsteroidal anti-inflammatory drugs\textsuperscript{5} are known to reduce the expression of several adhesion molecules and reduce leukocyte-endothelium interaction.

Diclofenac, a nonsteroidal anti-inflammatory drug, reduces inflammation, swelling, and arthritic pain by inhibiting the production of prostaglandins.\textsuperscript{6}\textsuperscript{–}\textsuperscript{8} Diclofenac affects polymorphonuclear leukocyte function in vitro, reducing chemotaxis, superoxide radical generation, and neutral protease production.\textsuperscript{9} Diclofenac also reduces the expression of several adhesion molecules such as L-selectin (ELAM),\textsuperscript{5} E-selectin, ICAM-1, and VCAM-1.\textsuperscript{10}

Verapamil, a calcium channel blocker, acts on the cardiovascular system and has antianginal, antiarrhythmic, antihypertensive, and cardiovascular-protective effects.\textsuperscript{11} Verapamil has been reported to inhibit superoxide production in human neutrophils\textsuperscript{12}\textsuperscript{–}\textsuperscript{14} and to reduce the expression of adhesion molecules such VCAM-1\textsuperscript{4} and ICAM-1\textsuperscript{15} in endothelial cells.

An interaction between diclofenac and verapamil has been demonstrated. Verapamil enhanced the anti-inflammatory effect of diclofenac in vivo (paw edema) and potentiated the diclofenac inhibitory effect on the chemiluminescence response of isolated human polymorphonuclear leukocytes in vitro.\textsuperscript{16}

To investigate a possible synergism between diclofenac and verapamil on leukocyte migration that could be beneficial in cardiovascular diseases, we studied leukocyte behavior with intravital microscopy.

Methods

Animals

Male Wistar rats that weighed 180 to 200 g were used. All rats were derived from breeding stock maintained at our institute and were randomized into 4 groups that were matched for age and weight, with
Leukocyte Counts

Leukocyte counts were performed on blood samples collected at the time of the animals were killed. Total leukocyte counts were obtained by counting wave forms generated on the physiograph (model 908, PTM, San Diego, Calif) incorporated into the system. Vessels selected for study were postcapillary venules, and their diameters ranged from 12 to 16 μm. In another series of experiments, the number of leukocytes that moved in the periphery of the axial stream in a Neuhaus chamber. Stained blood films were used for differential leukocyte counts. Blood samples for these measurements were collected from the abdominal aorta while the rats were under anesthesia.

Direct Vital Microscopy of the Microcirculation:
Surgical Preparation

The animals were anesthetized with an injection of 40 mg/kg IP sodium pentobarbital, and the internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination in situ. This was done through a longitudinal incision of the skin and dartos muscle in the midline over the ventral aspect of the scrotum and opening of the cremaster muscle to expose the internal fascia. This procedure does not require extensive surgical manipulation for observation of the vascular network and provides a valuable means for transilluminating a tissue for quantitative studies of the microcirculation. In addition, the preparation is not affected by respiratory movements of the animal, and its microcirculatory characteristics remain basically invariant throughout the course of the experiment. The animals were maintained on a special board thermostatically controlled at 37°C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed (37°C) Locke-Ringer’s solution, pH 7.20 to 7.40, containing 1% gelatin. The composition of the solution (in mmol/L) was as follows: 154 NaCl, 5.6 KCl, 2 CaCl₂, 2 H₂O, 6 NaHCO₃, and 5 glucose. The rate of outflow of the solution onto the exposed tissue was controlled to maintain the preparation in continuous contact with a thin film of physiological saline and topically added to the preparation. Adhesion of leukocytes was assessed after 10 minutes of addition of zymosan. Plasma treated identically, except for the addition of zymosan, was used as a control. Leukocyte adhesion was also quantitated with the same protocol in animals given a local application of LTB₄ (1 ng/mL, 0.1 mL). Each section of the vascular bed was tested only once, and no more than 2 determinations were performed on a single animal. The 2 measurements were averaged for each animal.

Carrageenan-Induced Leukocyte Transmigration

In another series of experiments, the number of leukocytes that accumulated in a 2500-μm² standard area of connective tissue adjacent to a postcapillary venule was determined after the induction of a local inflammatory response. Cells were counted on the recorded image. Five different fields were evaluated for each animal to avoid variability on the basis of sampling. Data were then averaged for each animal. The inflammatory reaction was evoked by injecting 100 μg of carrageenan in 0.1 mL of saline into the scrotum of the animals, and the number of migrated cells was counted after 2 hours of carrageenan injection. At the end of the experiments, the preparations were stained with toluidine blue for 15 minutes to check mast cell degranulation.

Drugs and Reagents

The following reagents were used: LTB₄, zymosan, carrageenan (all from Sigma Chemical Co, St. Louis, Mo); diclofenac, potassium salt (Cataflan–Geigy); verapamil (Dilacoron–Knoll); sodium pentobarbital (Hypnol–Cristalá); heparin (Liquemine–Roche S/A, RJ, Brazil); toluidine blue (ECIBRA Brazil S/A); and NaCl, KCl, CaCl₂ · 2H₂O, NaHCO₃, and glucose (all from Merck S/A, RJ, Brazil).

Statistical Analysis

Data are given as mean±SEM. One-way ANOVA followed by the Tukey-Kramer multiple comparisons test and Student’s t test were used, when pertinent. The minimum acceptable level of significance was P at a value ≤0.05.

Results

Leukocyte Counts

Animals treated with verapamil, diclofenac, or a combination of both showed similar total and differential leukocyte counts relative to control animals (Table 1).

Direct Vital Microscopy of the Microcirculation

The number of leukocytes rolling along the venular endothelium (rollers), sticking after stimulus with LTB₄ or ZAP (stickers), and migrating after carrageenan stimulation was reduced by verapamil at the dose of 10 mg/kg IP, diclofenac

Experimental Protocols

Leukocyte Rolling

In a series of experiments, interaction of leukocytes with the luminal surface of the venular endothelium was studied in a segment of the vessel. Leukocytes moving in the periphery of the axial stream in contact with the endothelium were considered to be “rollers.”19 and their number was determined in 10-minute periods. These leukocytes moved slow enough to be individually visible and were counted as they rolled past a 100-μm-length venule.20

Chemoattractant-Induced Leukocyte Adhesion

Leukocytes adhering to the endothelium were quantified after the application of an irritant stimulus such as leukotriene B₄ (LTB₄) or zymozan-activated plasma (ZAP). A leukocyte was considered to be adherent to the venular endothelium if it remained stationary for >30 seconds.21 Adherent cells (“stickers”) were expressed as the number per 100-μm-length of venule. Adhesion was investigated under 2 conditions. In 1, the internal spiermatic fascia, after a suitable control period of normal circulation, was exposed to 0.1 mL of a solution containing 10% homologous ZAP in physiological saline. To obtain activated plasma, zymosan, an enzyme from Saccharomyces cerevisiae, was incubated (1 mg/mL) with plasma from normal animals for 1 hour at 37°C. After centrifugation at 1600 g for 10 minutes, the supernatant fraction ZAP was collected and diluted 1:10 with physiological saline and topically added to the preparation. Adhesion of leukocytes was assessed after 10 minutes of addition of zymosan. Plasma treated identically, except for the addition of zymosan, was used as a control. Leukocyte adhesion was also quantitated with the same protocol in animals given a local application of LTB₄ (1 ng/mL, 0.1 mL). Each section of the vascular bed was tested only once, and no more than 2 determinations were performed on a single animal. The 2 measurements were averaged for each animal.
at the dose of 2.5 mg/kg IP, or the combination of verapamil (10 mg/kg IP) and diclofenac (2.5 mg/kg IP) in comparison with control rats (Table 2). The drug combination did not augment the effect of each agent alone (Table 2). Neither treatment tested interfered with vessel diameters (Table 2).

**Mean Arterial Blood Pressure, Heart Rate, Blood Flow Velocity, and Wall Shear Rate**

Under baseline conditions, untreated and treated animals had similar arterial blood pressure levels and heart rates (Table 3). Arterioles of treated animals had a similar centerline red blood cell velocity (Table 4) and wall shear rate relative to untreated controls (data not shown). However, in venules, verapamil alone or in combination with diclofenac reduced blood flow velocity and consequently the venular wall shear rate when compared with control animals (Table 4). On the other hand, in venules no differences were observed between diclofenac-treated and untreated control animals (Table 4).

**Discussion**

Postcapillary venules in particular were chosen for observation of leukocyte-endothelial interactions because they are considered to be the major site for leukocyte adhesion to the vascular wall in response to noxious stimuli. A reduced number of leukocytes rolling along the venular endothelium, sticking, and migrating was observed in rats treated with verapamil, diclofenac, and the combination of both. The findings were not paralleled by any significant decrease in the number of circulating leukocytes, thereby suggesting that adhesion changes were induced by the treatments affecting the leukocyte-endothelial interaction.

Adhesion of leukocytes induced by both LTB4 and ZAP was reduced by the different treatments tested. No differences were observed between the effects of the drugs on the magnitude of the reduction. During an acute inflammatory reaction induced locally by the injection of carrageenan, animals treated with verapamil, diclofenac, or the combination of both exhibited a similar pattern: only a few cells accumulated in the perivascular tissue. In controls, however, cells emerged massively into the connective tissue around the vessel under the influence of the inflammatory stimulus.

It is well known that during the inflammatory process, leukocytes are rapidly transported via the circulatory system to areas of tissue injury, in which they adhere to the endothelium and emigrate to the perivascular space. These events depend on the interaction between hemodynamic parameters (flow and resistance) that affect the transport of leukocytes to an injury site as well as a balance of forces to sweep them away. To evaluate the possible interference of hemodynamic changes on leukocyte behavior (rolling, sticking, and migration) studied, we measured arterial blood pressure (to estimate vascular resistance), blood flow velocity (to estimate blood volume), and venular diameter. We also calculated the wall shear rate, because the dependence of leukocyte adhesion on shear rate has been demonstrated in vivo and in vitro. Low shear rates promote leukocyte adherence to the microvascular endothelium in postcapillary venules. Treatment of the animals with verapamil, diclofenac-treated and untreated control animals (Table 4).

**Table 1. Blood Leukocyte Counts (10⁹/L) in Control Rats (C) or in Rats Treated With Verapamil (V), Diclofenac (D), or Verapamil+Diclofenac (V+D)**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control (C)</th>
<th>Vapamil (V)</th>
<th>Diclofenac (D)</th>
<th>Vapamil+Diclofenac (V+D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>9.40±0.67</td>
<td>10.67±1.52</td>
<td>10.92±1.37</td>
<td>12.18±0.90</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.32±0.16</td>
<td>0.21±0.07</td>
<td>0.25±0.05</td>
<td>0.22±0.07</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>7.40±0.52</td>
<td>8.32±1.26</td>
<td>8.59±1.16</td>
<td>9.23±0.73</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.68±0.34</td>
<td>2.14±0.34</td>
<td>2.03±0.24</td>
<td>2.72±0.30</td>
</tr>
</tbody>
</table>

Values are mean±SEM for 8 animals.

**Table 2. Venular Diameters and the Numbers of Rollers, Stickers, and Migrated Cells 2 Hours After Carrageenan in the Venules of Untreated Control Rats (C) and in Verapamil (V)-, Diclofenac (D)-, or Verapamil+Diclofenac (V+D)-Treated Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diameter, μm</th>
<th>Rollers, No./10 min</th>
<th>Stickers, No./10 min</th>
<th>Migrated, No./2500 μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ZAP</td>
<td>LTB4</td>
</tr>
<tr>
<td>C</td>
<td>16.5±0.5</td>
<td>90.3±6.1</td>
<td>22.6±3.0</td>
<td>13.5±1.0</td>
</tr>
<tr>
<td></td>
<td>n=12</td>
<td>n=12</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>V</td>
<td>15.0±0.6</td>
<td>34.4±4.2*†</td>
<td>7.6±0.9*</td>
<td>3.5±0.3*</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>D</td>
<td>16.1±0.6</td>
<td>60.3±6.3*</td>
<td>9.5±0.9*</td>
<td>6.0±0.4*</td>
</tr>
<tr>
<td></td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>V+D</td>
<td>16.8±0.3</td>
<td>42.0±4.3*</td>
<td>7.7±0.4*</td>
<td>5.7±0.5*</td>
</tr>
<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
<td>n=8</td>
<td>n=8</td>
</tr>
</tbody>
</table>

n indicates number of animals in each case. Values are mean±SEM.

*P<0.05 vs control; †P<0.05 vs diclofenac.
on the leukocyte surface\(^3\) and ICAM-1, VCAM-1, and E-selectin on endothelial cells.\(^{10}\) Similarly, verapamil inhibited VCAM-1 expression on endothelial cells.\(^4\) However, Hailer et al.\(^{50}\) could not find any inhibition of ICAM-1, VCAM-1, and ELAM-1 on these cells. On the contrary, in higher concentrations, increased expression of ICAM-1 and ELAM-1 was found.\(^{50}\) Therefore, the lack of synergism between verapamil and diclofenac might be explained by an increased expression of these adhesion molecules that compensates for the reduction in expression induced by diclofenac. Studies are in progress to identify the adhesion molecules involved and altered by the treatments tested.

In conclusion, our data allow us to suggest that verapamil and diclofenac, by interfering with adhesion molecule expression, reduce leukocyte migration in vivo. The lack of synergism between the drugs might be explained by the reduction of venular shear rate induced by verapamil that might not be sufficient to hinder the effect of verapamil alone but hindered the summation of effects of verapamil and diclofenac when together.

The mechanisms underlying leukocyte accumulation in a tissue depend on the interaction between the cells and the vascular endothelium. During the development of inflammatory responses, leukocytes roll along the lining endothelium of postcapillary venules and eventually become firmly attached to the vascular wall before migrating into tissues. Specific adhesion glycoproteins expressed on the surface of leukocytes and endothelial cells play a relevant role in the adhesion phenomenon.\(^{25}\) Members of the selectin family of cell adhesion molecules are thought to mediate leukocyte rolling along the walls of the microvasculature.\(^{26}\) Glycoproteins of the CD11/CD18 complex (\(\beta_2\) integrins) expressed on leukocytes interact with ligands such as ICAM-1 on endothelial cells to mediate leukocyte adhesion and emigration.\(^{27}\) Blockade of adhesion molecules on leukocytes, endothelial cells, or both can effectively inhibit leukocyte adhesion. Therefore, another explanation for the abnormal leukocyte function observed after treatment with verapamil, diclofenac, and the combination of both might be an interference of the drugs used on the adhesion molecule expression in vivo. In fact, in vitro studies, diclofenac reduced ELAM expression

### TABLE 3. Mean Arterial Blood Pressure (MAP) and Heart Rate (HR) in Untreated Control Rats (C) and Verapamil (V)—, Diclofenac (D)—, or Verapamil+Diclofenac (V+D)—Treated Rats

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>V</th>
<th>D</th>
<th>V+D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>94.8±3.3</td>
<td>92.8±4.6</td>
<td>100.0±3.8</td>
<td>84.4±3.4</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>352.5±9.4</td>
<td>354.0±7.8</td>
<td>352.5±8.9</td>
<td>330.0±11.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM for 8 animals.

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### TABLE 4. Blood Flow Velocity and Venular Shear Rate in Untreated Control Rats (C), and Verapamil (V)—, Diclofenac (D)—, or Verapamil+Diclofenac (V+D)—Treated Rats Determined in the Microcirculation of the Internal Spermatic Fascia

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Flow Velocity, mm/s</th>
<th>Shear Rate, Venule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arteriole (14–18 μm)</td>
<td>Arteriole (&gt;25 μm)</td>
</tr>
<tr>
<td>C</td>
<td>2.5±0.2</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>V</td>
<td>2.6±0.2</td>
<td>3.0±0.5</td>
</tr>
<tr>
<td>D</td>
<td>2.4±0.2</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td>V+D</td>
<td>2.8±0.1</td>
<td>3.0±0.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM for 8 animals. *\(P<0.05\) vs control.

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