Salicylates Inhibit T Cell Adhesion on Endothelium Under Nonstatic Conditions: Induction of L-Selectin Shedding by a Tyrosine Kinase-Dependent Mechanism

Roberto Gerli, Paolo Gresele, Onelia Bistoni, Cristina Paolucci, Luisa Lanfrancone, Stefano Fiorucci, Christopher Muscat and Vincenzo Costantini

*J Immunol* 2001; 166:832-840; doi: 10.4049/jimmunol.166.2.832
http://www.jimmunol.org/content/166/2/832

**References**

This article cites 76 articles, 33 of which you can access for free at:
http://www.jimmunol.org/content/166/2/832.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Salicylates Inhibit T Cell Adhesion on Endothelium Under Nonstatic Conditions: Induction of L-Selectin Shedding by a Tyrosine Kinase-Dependent Mechanism

Roberto Gerli,1* Paolo Gresele, ‡ Onelia Bistoni, † Cristina Paolucci, ‡ Luisa Lanfrancone, § Stefano Fiorucci, ‡ Christopher Muscat, † and Vincenzo Costantini‡

Salicylates inhibit T cell adhesion to and transmigration through endothelium by preventing integrin activation induced by contact with endothelial cells. In the present study the effects of aspirin and sodium salicylate on the first steps of T cell adhesion have been analyzed in a nonstatic in vitro system. Salicylates partially reduced adhesion to activated endothelium and, in parallel, L-selectin expression on resting T cells by inducing shedding of the molecule without affecting its mRNA transcript. The role of L-selectin down-regulation in reducing T cell adhesion in this system was supported by the fact that aspirin inhibited T cell adhesion also on plastic-immobilized L-selectin ligand or when α₄ integrin-mediated adhesion to endothelium was blocked by specific mAbs. In addition, preincubation of T cells with inhibitors of L-selectin shedding prevented both functional and phenotypic inhibitory effects of salicylates. The decrease in T cell adhesion and L-selectin expression seems to be dependent on intracellular calcium increase and tyrosine kinase activation, because these effects could be reversed by preincubating salicylate-treated T cells with EGTA, genistein, or tyrophostin. Finally, the infusion of aspirin into healthy volunteers induced down-regulation of L-selectin on circulating T cells. These results suggest that salicylates interfere not only with integrin activation, but also with the L-selectin-mediated first steps of T cell binding to endothelium. The Journal of Immunology, 2001, 166: 832–840.

Chronic inflammatory diseases are characterized by the presence of large numbers of leukocytes at the site of inflammation. Polymorphonuclear cells are a primary component of this inflammatory response, although it is thought that T lymphocytes play a key role in initiating as well as maintaining the inflammatory process (1, 2). The recruitment of leukocytes toward inflammatory sites follows a sequential multistep process that has been clarified in great detail in recent years (3–5). During the first step, the interaction between leukocytes and endothelium is mediated by selectins that cause circulating cells to slow their speed and roll along the vessel wall. This contact is not strong enough to stop leukocytes completely, but it is fundamental in allowing them to sample the local endothelium for the presence of stimulating factors, usually triggered by inflammation, that can activate integrins by enhancing the level of molecule expression and/or inducing conformational changes leading to increased binding avidity of the molecule. This allows the adhesion and migration of leukocytes to proceed. The different steps of leukocyte adhesion to and migration through endothelium have been reproduced by a number of in vitro systems employing different cell types bearing selectin and/or integrin receptors (3, 6). These experimental models have been employed under either static conditions, to simulate the integrin-mediated adhesion and transmigration, or flow conditions, to reproduce the initial rolling of leukocytes along endothelium (3, 6). The in vitro analysis of the different phases of leukocyte rolling, adhesion, and migration has also taken advantage of the different effects of various temperatures on the binding ability of integrins or selectins (7, 8).

Nonsteroidal anti-inflammatory drugs (NSAIDs)2 are potent inhibitors of inflammatory reactions and exert their effects mainly through the inhibition of the cyclooxygenase pathway of arachidonate metabolism (9). Several investigations, however, have shown that NSAIDs have also effects independent of cyclooxygenase inhibition, directly acting on the function of different inflammatory cells, including T lymphocytes (10–16). In this context we have demonstrated that salicylates reduce the ability of resting T cells to adhere to and transmigrate through endothelium in an in vitro static system via interference with integrin function (17). Moreover, an inhibitory activity of some NSAIDs on lymphocyte adhesion has been demonstrated in an in vitro nonstatic model at 37°C (18). Although it is well known that selectins play a pivotal role in the initial contact between leukocytes and endothelial wall (3–6), this study showed that the effect of NSAID appears to be exerted mainly by interference in integrin function similar to that observed in our above-mentioned study (17). This is in line with a number of data supporting an involvement of α₄ integrins in the rolling and tethering of lymphocytes (19–22). However, according to our preliminary observations (23), subsequently confirmed by others (18), salicylates and other NSAIDs are also able to reduce the expression of L-selectin on circulating leukocytes.

Received for publication February 4, 2000. Accepted for publication October 17, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Roberto Gerli, Dipartimento di Medicina Clinica e Sperimentale, Sezione di Medicina Interna e Scienze Oncologiche, Centro per lo Studio delle Malattie Reumatiche, Policlinico di Perugia, I-06122 Perugia, Italy. E-mail address: gerlir@unipg.it

2 Abbreviations used in this paper: NSAIDs, nonsteroidal anti-inflammatory drugs; ASA, acetylsalicylic acid; NaS, sodium salicylate; PTK, protein tyrosine kinase; PKC, protein kinase C; sLeX, sialyl-LewisX; sALac, 3′-sialyl-α2,3-LN-acetyl-lactosamine; sL-selectin, soluble L-selectin; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; VLA-4, very late Ag 4.
resting lymphocytes. The functional relevance of L-selectin down-regulation exerted by salicylates, however, and the mechanisms at the basis of it are still unclear. In the present paper we have investigated the effects of acetylsalicylic acid (aspirin; ASA) on the selectin-mediated initial phase of T/endothelial cell interaction studied with a rotating adhesion system at 4°C, which reduces the role of integrin-mediated adhesion (7). Parallel experiments were conducted with sodium salicylate (NaS), a drug that does not exert the inhibitory activity of ASA on cyclo-oxygenase (10, 11). Our results showed that these drugs partially block T cell adhesion via down-regulation of L-selectin on the surface of resting T lymphocytes. The effect does not appear to be due to interference with the mRNA transcript of L-selectin, but it is mediated by a protein tyrosine kinase (PTK)-dependent mechanism that leads to shedding of the molecule.

**Materials and Methods**

**Cell purification**

PBMC were isolated from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. T cells were purified from monocyte-depleted PBMC suspensions with SRBC and a nylon wool column technique, as previously reported (24), and resuspended in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (complete medium; Life Technologies, Gaithersburg, MD). On the basis of their reactivity with anti-CD3 mAb, >97% of these PBMC were T lymphocytes.

**Monoclonal Ab and reagents**

Anti-CD3 mAb was purified from supernatant of hybridoma cells obtained from the American Type Cell Culture Collection (Manassas, VA). For functional studies, it was immobilized on plastic in flat-bottom microtiter plates (no. 3596, Costar, Data Packaging Corp., Cambridge, MA), as previously described (17). Isotype-matched mAbs were used as negative controls in function-blocking experiments. For these experiments the anti-L-selectin DREG-200 (25), anti-ICAM-1 6.5B5 (26), and anti-VCAM 1G11 (27) mAbs were donated by Dr. D. O. Haskard (Hammersmith Hospital, London, U.K.), the anti-ICAM-1 4B9 (28) by Dr. J. M. Harlan (University of Washington, Seattle, WA), and E1/6 (29) by Dr. M. A. Gimbrone (Harvard Medical School, Boston, MA), while the αε integrin-blocking mAb HPIC (30) was purchased from Immunotech (Marseilleles, France). Anti-CD62L (TQI) and anti-CD29 (4B4) were purchased from Coulter Immunology (Hialeah, FL), anti-CD11a (IOT16) and anti-CD18 (IOT18) from Immunotech, anti-CD11b (OKM1) from Ortho Diagnostic Systems (Raritan, NJ), anti-CD11c (Leu M5) from Becton Dickinson (San Jose, CA), and anti-CD49d from Dakopatts (Copenhagen, Denmark). Human TNF-α (sp. act., 9.0 10^6 U/mg) was provided by Dr. T. F. Tedder (Duke University Medical Center, Durham, NC). Anti-CD18 (5E4, mAb plus rIL-2, or in the presence of ASA or NaS (300 μg/ml) at 37°C. After 20 and 50 min, cell suspensions were centrifuged, and supernatants were tested for sL-selectin using an ELISA kit (Bender MedSystem, Vienna, Austria).

**Northern blot analysis for the evaluation of L-selectin mRNA expression**

Total RNA was prepared according to established procedures (40). Samples (20 μg), treated as previously described (41), were analyzed by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Northern blot filters were hybridized at high stringency conditions. The human L-selectin (LAM-1) cDNA cloned from a human tonsil cDNA library was provided by Dr. T. F. Tedder (Duke University Medical Center, Durham, NC).

**Mitogen-activated protein kinase (MAPK) activation assay**

T lymphocytes were starved under serum- and growth factor-free conditions for 48 h and then treated with anti-CD3 mAb, PMA, or different amounts of ASA or NaS for 30 min. Cells were lysed, and 50 μg of total lysates were electrophoresed on a 11% polyacrylamide gel, blotted onto nitrocellulose, and incubated with anti-MAPK antisera. Polyclonal Abs to MAPK were purchased from Upstate Biotechnology (Lake Placid, NY) and used at 1 mg/ml for Western blot analysis (41).

In vivo experimental infusion of ASA

After obtained informed consent from the subjects and the approval of the local institutional ethics committee for human experiments, three healthy volunteers had a sample of venous blood taken. Then, 500 μg of ASA (lysine acetylsalicylate, Aspegic, Synthelabo, Milan, Italy) was injected i.v. over 1–2 min, and other blood samples were taken 30 and 120 min after the injection. The results were expressed in terms of the ratio of mean fluorescence intensities (MFI) for L-selectin or CD11b as described above. To minimize variations in fluorescence staining and FACS sensitivity in the different blood samples, the data were expressed as the ratio of mean fluorescence intensity (MFI) of L-selectin or CD11b...
expression to the MFI of CD11a, whose expression remained stable during NSAID treatment of T cells.

**Statistical analysis**

Due to the nonnormal distribution of the data, Kruskal-Wallis analysis of the variance was used for multiple comparisons. Then, the Mann-Whitney test was applied to evaluate differences between the various groups.

**Results**

**Adhesion of T lymphocytes to HUVEC in an in vitro nonstatic system**

The attachment of resting T lymphocytes was tested in vitro under nonstatic conditions, which reproduce the initial rolling of T lymphocytes along the vessel wall under a low shear flow rate (6, 42). T cell adhesion was first analyzed against rTNF-activated HUVEC, according to a previously described model (7). Because it is known that the very late Ag 4 (VLA-4)/VCAM-1 complex plays a role in the first dynamic interaction between lymphocytes and endothelium (19–22), we performed these experiments at 4°C, a condition that reduces the integrin adhesion capacity of lymphocytes (7). In an in vitro rotating system at low temperature, L-selectin plays a prominent role in the adhesion of T cells, as demonstrated by the observation that the addition of a blocking anti-L-selectin mAb diminished the binding of resting T cells to activated HUVEC by 68.2 ± 21% (p < 0.001). However, the addition of an anti-α4 integrin (HP2/1) or different anti-VCAM mAbs (1G11, 4B9, or E1/6) had minor, but still significant, effects. The addition of an anti-LFA-1 did not affect T cell adhesion (−9.5 ± 4% and −8.7 ± 3%, respectively; not significant).

Preincubation of HUVEC with different concentrations of ASA or NaS (30, 300, or 600 μg/ml) did not affect T cell attachment (data not shown). On the contrary, preincubation of resting T lymphocytes with the same doses of ASA or NaS significantly reduced, in a dose-dependent manner their adhesion to activated HUVEC (Fig. 1). To ascertain whether the salicylate inhibitory effect was linked to an interference with L-selectin and/or VLA-4 binding, we performed a set of experiments in which HUVEC were pretreated with either an mAb blocking L-selectin or the combination of mAbs blocking α4 integrin and three different epitopes of VCAM-1. In these experiments ASA was used at 300 μg/ml, the concentration that more strictly reproduces salicylate serum levels associated with optimal anti-inflammatory activity (43) and reduces T cell adhesion by 42.4 ± 11% (Fig. 1). The addition of ASA to anti-L-selectin mAb exerted inhibition of T cell adhesion (−67.6 ± 13%) similar to that produced by anti-L-selectin alone (−68.2 ± 21%; not significant). The four mAbs against the VLA-4/VCAM-1 complex induced a decrease in T cell adhesion to HUVEC (Fig. 2) similar to that obtained with each blocking mAb alone (see text above). However, when T cells were also pretreated with 300 μg/ml ASA, an additional decrease in T cell adhesion was demonstrated (Fig. 2).

Finally, to verify whether the ASA effect was reproducible under more physiological in vitro conditions, experiments were conducted with the above-described rotating system of adhesion, but at 37°C. The decrease in cell adhesion induced by blocking VLA-4/VCAM-1 complex in this condition was similar to that obtained at 4°C (mean ± SE of five different experiments, −36.8 ± 8%; p < 0.001), but the inhibition exerted by anti-L-selectin mAb at 37°C was clearly less than that seen at low temperature (−22.3 ± 5%; p < 0.01). However, it is noteworthy that 300 μg/ml ASA was also able to decrease T cell binding to HUVEC in the VLA-4/VCAM-1-blocked system at 37°C (−19.4 ± 4%; p < 0.01). This observation further supports a VLA-4/VCAM-1-independent inhibitory effect of ASA, probably exerted via L-selectin, because the addition of anti-L-selectin mAb to ASA in the VLA-4/VCAM-1-blocked system did not provide additional inhibition of T cell adhesion (−21.1 ± 4.6; p < 0.01 vs VLA-4/VCAM-1-blocked system; not significant vs ASA alone).

**Adhesion of T lymphocytes to plastic-immobilized sLeα in an in vitro nonstatic system**

To support the hypothesis that salicylates reduce the attachment of T lymphocytes to endothelium via interference with the binding of

**FIGURE 1.** Effects of ASA or NaS preincubation on adhesion of resting T cells to rTNF-activated HUVEC monolayers in a nonstatic adhesion system. Data (mean ± SEM of 21 separate experiments) are expressed as the percent variation compared with the T cell adhesion in medium alone (−). *, p < 0.05; **, p < 0.001.

**FIGURE 2.** Effect of 300 μg/ml ASA on T cell adhesion in a nonstatic system at 4°C with HUVEC pretreated with blocking mAbs against α4 integrin and three different VCAM-1 epitopes. Data are expressed as a percentage of the adherent cell number obtained in the same system without blocking mAbs (control). Paired observations refer to individual samples tested in the presence of blocking mAbs without (mAbs) or with (mAbs + ASA) ASA. Statistical comparison refers to the difference in adhesion in the mAb-blocked system in the presence or the absence of ASA. Horizontal and vertical bars indicate the mean ± SEM. Each sample was also tested in an anti-L-selectin-blocked system as a control (data not shown). The addition of ASA in this system (−67.6 ± 13%), however, did not enhance the inhibitory effect obtained with the anti-L-selectin alone (−68.2 ± 21%; not significant).
L-selectin to its counter-receptors, T cells were directly tested against neoglycolipids containing the oligosaccharide sLe^x, a ligand of human L-selectin (36–39), immobilized through a lipid carrier onto plastic wells (Table I). The results confirmed that preincubation of T cells with 300 μg/ml ASA significantly reduced cell adherence to the L-selectin counter-receptor sLe^x, but not to the oligosaccharide sALac, which was used as a negative control.

Expression of adhesion molecules on T cells

The expression of a number of adhesion molecules was evaluated on resting T cells after their incubation for 30 min with ASA or NaS. A significant down-regulation of L-selectin expression was induced by both drugs in a concentration-dependent manner (Fig. 3). In contrast, the expression of the CD11b molecule was slightly increased on T cells by preincubation with 300 or 600 μg/ml ASA, whereas there was no change in CD18, CD11a, CD11c, CD29, and CD49d molecule expression (data not shown), as previously shown (17). Similar results were obtained with 300 or 600 μg/ml NaS (data not shown).

L-selectin mRNA expression

The demonstration that the salicylate-induced impairment of T cell adhesion under nonstatic conditions is associated with down-regulation of L-selectin expression on the T cell surface prompted us to explore the cellular and molecular mechanisms of this effect. The difficulty in detecting mRNA signal in normal resting T lymphocytes, as previously reported (44) and confirmed in our laboratory (data not shown), forced us to employ the T lymphoblastic cell line Jurkat to evaluate whether ASA or NaS was able to influence the mRNA transcript of L-selectin. For this purpose, Jurkat T cells were incubated with medium alone, ASA, or NaS for 30 min, and then mRNA levels of the molecule were evaluated. L-selectin mRNA signal, well evident in the cells incubated with medium alone, was not modified by preincubation with 300 or 600 μg/ml ASA or NaS (data not shown).

L-selectin shedding

Considering the lack of effect of salicylates on L-selectin mRNA expression, we decided to ascertain whether salicylates could induce shedding of the molecule (25, 45, 46). Therefore, cell-free supernatant levels of the soluble form of L-selectin were measured after ASA or NaS incubation. A progressive increase in the levels of sL-selectin were found in the culture supernatants of T cells either triggered by immobilized anti-CD3 plus rIL-2 or incubated with ASA or NaS (Fig. 4). On the contrary, sL-selectin was undetectable in unstimulated T cultures over time.

Prevention by two different metalloprotease inhibitors of ASA-induced down-regulation of L-selectin expression and T cell adherence to HUVEC under nonstatic conditions

To analyze further the role of L-selectin in the decrease in T cell attachment to endothelium induced by ASA, we examined whether the inhibition of L-selectin shedding induced by the compounds KD-IX-73-4 and Ro 31-9790 was able to prevent the drug effect on L-selectin expression and/or adhesion of T cells to HUVEC. As shown in Table II, pretreatment of T cells with these metalloproteinase inhibitors before challenging them with ASA completely suppressed the drop in L-selectin expression and T cell binding to HUVEC, while they alone did not affect the expression of the molecule and the adhesion ability of T cells.

Effects of salicylates on MAPK activation

Because L-selectin shedding is a phenomenon consequent to cellular activation (46), MAPK activation was assayed after incubation of T lymphocytes with increasing concentrations of ASA or NaS. As shown in Fig. 5, anti-CD3 and PMA stimulation of resting T cells was capable of inducing MAPK activation, while different concentrations (30, 300, or 600 μg/ml) of both ASA or NaS were not.

Role of early intracellular signals in L-selectin expression and T cell adhesion decrease induced by salicylates

Despite the fact that salicylates do not elicit MAPK activation, it has been shown that they increase the level of intracellular calcium and activate PKC of T cells (17, 47, 48). Thus, to check whether early intracellular events were relevant for the observed down-regulation of L-selectin induced by salicylates, we tested the effect of preincubation for 30 min at 37°C of salicylate-treated T cells with EGTA, a calcium chelator, or with different PKC or PTK inhibitors before challenging them with ASA completely suppressed the drug effect on L-selectin expression and/or adhesion of T cells to HUVEC, while they alone did not affect the expression of the molecule and the adhesion ability of T cells.

![Supernatant levels of sL-selectin induced by anti-CD3 plus rIL-2 (see Materials and Methods) or 300 μg/ml ASA or NaS in T cell cultures. Data are the mean ± SEM of five separate experiments. Levels were <1 ng/ml in unstimulated T cell supernatants at both 20 and 50 min of culture. *, p < 0.05; †, p < 0.001.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Cell Pretreatment</th>
<th>Lipid Carrier</th>
<th>sALac</th>
<th>sLe^x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>19.8 ± 2*</td>
</tr>
<tr>
<td>ASA (300 μg/ml)</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>12.6 ± 2*</td>
</tr>
<tr>
<td>Anti-L-selectin</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± SEM of the number of adherent T lymphocytes (cell count/field) in eight separate experiments.

*, p < 0.001 vs lipid carrier or sALac; †, p < 0.04 vs sLe^x (Medium).
Table II. Effect of pretreatment with the KD-IX-73-4 and Ro 31-9790 compounds on L-selectin expression and adhesion capacity of ASA-treated T cells*

<table>
<thead>
<tr>
<th></th>
<th>L-Selectin Expression (MFI)</th>
<th>T Cell Adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA (300 μg/ml)</td>
<td>−46.5 ± 13*</td>
<td>−39.9 ± 15*</td>
</tr>
<tr>
<td>KD-IX-73-4 (25 μg/ml) + ASA</td>
<td>+2.7 ± 2†</td>
<td>+3.5 ± 4†</td>
</tr>
<tr>
<td>Ro 31-9790 (100 μM) + ASA</td>
<td>−1.4 ± 2</td>
<td>Not done</td>
</tr>
<tr>
<td>PMA (10 ng/ml)</td>
<td>−60.1 ± 16*</td>
<td>Not done</td>
</tr>
<tr>
<td>KD-IX-73-4 + PMA</td>
<td>−4.8 ± 4†</td>
<td>Not done</td>
</tr>
<tr>
<td>Ro 31-9790 + PMA</td>
<td>−6.1 ± 6†</td>
<td>Not done</td>
</tr>
</tbody>
</table>

Data (mean ± SEM) are expressed as the percentage of variations of L-selectin MFI or adherent T cells as compared with medium in 10 separate experiments. T cells were incubated with the metalloproteinase inhibitors (KD-IX-73-4 (10 min) or Ro 31-9790 (30 min), or its solvents alone at 37°C and, after washings, with ASA for 30 min or PMA for 1 h. The expression of L-selectin and the ability to adhere to activated HUVEC in the nonstatic system were then evaluated as described. The Ro 31-9790 alone did not affect L-selectin expression and T cell adhesion ability, whereas the compound KD-IX-73-4 slightly enhanced L-selectin expression (+15.9 ± 12, p < 0.05) but not T cell adhesion.

* p < 0.01 vs the basal values. † p < 0.01 vs ASA alone.

Discussion

The present study demonstrated that salicylates, such as ASA or NaS, reduce the ability of resting T cells to adhere to cytokine-activated HUVEC monolayers in an in vitro nonstatic model that simulates the first step of T cell adhesion to endothelial wall during the in vivo rolling under conditions of low flow (7, 20, 21). This effect appears to be mediated by a partial down-regulation, consequent to shedding, of L-selectin on the surface of resting T cells, as demonstrated by the finding of enhanced levels of the soluble form of the molecule in the supernatants of T cell cultures incubated with salicylates.

The role of L-selectin in leukocyte rolling along vascular wall has been well established (3, 4, 7, 25, 45). In addition, an L-selectin ligand, able to mediate lymphocyte binding in nonstatic adhesion systems, has been identified on the surface of activated HUVEC (49). A number of observations, however, have also shown an involvement of α4 integrin in the initial attachment of lymphocytes to endothelium (19–22). Because NSAIDs can interfere with lymphocyte integrin activity (17, 18), it might be concluded that the inhibitory effect of salicylates on T cell adhesion to HUVEC in nonstatic models is essentially exerted through an integrin-dependent mechanism. The data of the present investigation, however, suggest that these compounds also interfere with L-selectin-mediated adhesion of T cells.

A cause-effect relationship between reduction of L-selectin expression and decreased adhesion to endothelium in a nonstatic system is supported by a number of observations: 1) the inhibition can be attributed to an effect of salicylates on T lymphocytes rather than endothelial cells, because no significant reduction in T cell adhesion was noted after ASA or NaS preincubation of HUVEC; 2) our experiments were conducted in a rotating system at 4°C, simulating the first step of T cell adhesion to endothelial wall during in vivo rolling under conditions of low flow (7, 20, 21).

In vivo effect of salicylates on L-selectin expression of T cells

We finally examined whether the down-regulation of L-selectin on T cell surface induced by salicylates was reproducible in vivo. For this purpose, L-selectin was analyzed by flow cytometry on circulating T cells before and after the i.v. injection of 500 mg of ASA in three volunteers. Fig. 7 shows that L-selectin expression, relative to CD11a expression on T lymphocytes, significantly decreased 30 min after drug administration and reached a nadir at 2 h after the injection. On the contrary, a mild and later enhancement of CD11b expression was detectable 2 h after ASA administration (Fig. 7), while no significant changes in expression of other integrins, such as CD29, CD18, CD11a, CD11c, and CD49d, were observed (data not shown).

FIGURE 5. MAPK activation upon salicylate stimulation of T lymphocytes. Western blot analysis of MAPK expression in resting T lymphocytes stimulated with medium alone (lane 1), 30 μg/ml ASA (lane 2), 300 μg/ml ASA (lane 3), 600 μg/ml ASA (lane 4), 30 μg/ml NaS (lane 5), 300 μg/ml NaS (lane 6), 600 μg/ml NaS (lane 7), 10 ng/ml PMA (lane 8), and anti-CD3 mAb (lane 9). Arrows indicate the p42 and p44 isoforms of MAPK, as previously described (25).

![Image](http://www.jimmunol.org/)
FIGURE 6. Effect of pretreatment with PTK inhibitors on the capacity of ASA-treated T cells to adhere to HUVEC. Data are expressed as the percentage of variations in adherent T cells compared with medium (mean ± SEM of six separate experiments). T cells were incubated with 50 μg/ml genistein, 100 μM tyrphostin, and 300 μg/ml ASA as described in Table I and then evaluated for their ability to adhere to activated HUVEC in the rotating system. *p < 0.01 vs the basal values (medium); **p < 0.01 vs ASA alone.

where, as confirmed by the present data, T cell attachment is mediated more by L-selectin than integrins (7); 3) salicylates did not decrease T cell adhesion in this model when L-selectin-mediated binding was blocked by specific mAb, whereas a significant inhibitory effect of the drugs was still evident when the binding of VLA-4 to VCAM-1 was completely blocked by a mixture of four different mAbs against the two molecules; 4) in a rotating system at 37°C, where the role of the VLA-4/VCAM-1 complex in T cell adhesion prevails on that exerted by L-selectin, ASA significantly reduced cell adhesion also when the binding of VLA-4 to VCAM was blocked by specific mAbs, and this inhibition was not enhanced by the addition of anti-L-selectin mAb to ASA; 5) the down-regulation of L-selectin is associated with functional impairment, as demonstrated by the decrease in T cell adhesion to the L-selectin ligand sLe^a induced by ASA under rotating condition; 6) the rapid shedding of L-selectin correlated well with the rapid decrease in T cell adhesion; and 7) two different metalloprotease inhibitors that block L-selectin shedding (32, 33) prevented the salicylate-induced down-regulation of L-selectin expression and the inhibitory effect of ASA on T cell adhesion.

L-selectin is constitutively expressed on the T cell surface, and its expression is rapidly down-regulated upon Ag-driven cell activation (25, 45, 46). Although NSAIDs, including salicylates, induce both a rise in intracellular calcium and activation of PKC (17, 47, 48), the two main second messengers generated by the activation of the phosphatiidylinositol pathway that takes place after TCR stimulation (50), a full T cell activation induced by ASA or NaS is ruled out by the present demonstration that these drugs do not induce activation of the p42 and p44 MAPK, which have a key role in the signal transduction pathway leading to the activation of T lymphocytes (51) and by the fact that these drugs do not trigger significant T cell proliferation and/or IL-2 synthesis (17). We also analyzed whether the action of these drugs in reducing L-selectin expression was due to a decrease in mRNA expression. Indeed, it has been shown that the expression of L-selectin on T lymphocytes is regulated at least in part at the pretranslational level during T cell activation (44). In particular, an increase in intracellular calcium and activation of PKC appear to act in an opposite fashion in up- or down-regulating mRNA levels of L-selectin (44). According to the results of our experiments, however, ASA and NaS are not able to exert any modulatory effect on mRNA levels of the molecule in a Jurkat T lymphoblastic cell line, the responses of which to pharmacological activation usually parallel those of normal T cells in several respects (44, 52). Thus, the effect of salicylates appears to be essentially mediated via a proteolytic cleavage of the transmembrane domain of L-selectin.

L-selectin shedding not only occurs in response to cell activation, but also after treatment with nonactivating agents such as mAb against signal-transducing molecules promoting cell aggregation (53, 54) chemical cross-linkers (55), acute phase reactants (56), or drugs with anti-inflammatory properties, including colchicine (57–59). Although the signaling mechanisms regulating L-selectin shedding are largely undefined, it has been shown that the cleavage of this molecule from the T cell surface can be triggered through multiple independent intracellular signaling pathways that may depend on either PKC or PTK activity (25, 45, 53, 60). Anti-CD3 mAbs, for example, or PMA, a direct activator of PKC, induce L-selectin shedding via a PTK-independent, PKC-dependent pathway (61, 62). In contrast, mAbs against L-selectin or other surface molecules involved in the control of cell to cell interaction within lymphoid tissues, trigger L-selectin cleavage through a PTK-dependent, PKC-independent process (53, 60). In the present study we demonstrated that L-selectin down-regulation induced by salicylates is prevented by EGTA, suggesting a role for intracellular calcium in the activity of these drugs, although it cannot be excluded that the inhibitory action of EGTA on L-selectin shedding is the consequence of other mechanisms (44, 54, 63–65). In contrast, genistein or tyrphostin, two potent PTK inhibitors (66), were also able to prevent L-selectin down-regulation induced by salicylates. On the contrary, three different PKC inhibitors (67) were ineffective in reversing the inhibitory action of ASA or NaS on L-selectin expression. Taken together, these data support the concept that salicylates induce L-selectin shedding from the T cell membrane via a calcium/PTK-dependent, PKC-independent pathway. In line with this observation, we showed that PTK inhibitors are also able to prevent the inhibitory effect of salicylates on T cell adhesion, further supporting the link among PTK activation, L-selectin shedding, and decreased adherence of T cells.

It has been demonstrated that the shedding of L-selectin induced by PMA or anti-CD3 mAb is mediated by zinc-dependent metalloproteases (32, 33, 62). Although the biochemical and molecular nature of the proteolytic enzyme responsible for L-selectin cleavage has not been yet fully clarified, recent studies have suggested that the TNF-α-converting enzyme may have a central role in mediating L-selectin shedding on thymocytes (68). Moreover, a
PKC-dependent pathway has been proposed to cause a conformational change in L-selectin, which leads to enhanced susceptibility of the molecule to cleavage by constitutively active, membrane-associated proteases (61). Alternatively, intracellular signals may lead to downstream triggering of proteolytic activity, thereby increasing the rate of L-selectin shedding from the T cell surface. Despite the final mechanism implicated in the shedding of L-selectin elicited by salicylates remains to be determined, the results of our experiments, employing the inhibitors KD-IX-73–4 and Ro 31–9790, clearly show that the shedding of the molecule induced by these drugs is dependent on metalloproteinase activity. However, studies are ongoing to evaluate whether the PTK-dependent pathway triggered by ASA and the PKC-dependent pathway elicited by cellular activators, converge on a common mechanism, i.e., the same class of metalloprotease(s), to induce L-selectin shedding from the T cell membrane.

The lower degree of inhibition of T cell adhesion induced by ASA with respect to that obtained by anti-L-selectin mAb in the same experimental conditions at low temperature is explained by the only partial down-regulation of L-selectin expression induced by the drug. It has been suggested that under physiological conditions, the PTK-dependent, PKC-independent down-regulation of L-selectin may function to trigger a partial loss of the molecule during initial lymphocyte/endothelial cell interaction (53). This may be relevant in altering the velocity of rolling of T cells along blood vessels (29), as would occur in other nonpharmacologically induced conditions (55, 60).

In conclusion, these observations may have important implications in understanding the mechanisms of action of salicylates and, possibly, of other NSAIDs. As mentioned, it has been shown that these compounds are able to directly interfere with the integrin-mediated firm adhesion of lymphocytes to endothelium (17). We have here shown that salicylates also exert an inhibitory effect on the first phases of lymphocyte adhesion. We are aware that our results do not allow us to exactly establish the degree of interference exerted by salicylates on the different steps of T cell adhesion to endothelium and that more information may be obtained by using other in vitro systems, such as flow chamber assays (69). However, the present data, obtained in a system that permits distinguishing selectin- from integrin-mediated activity, clarify some important points at molecular level. According to our results, the inhibitory effect of ASA on the early step of T cell attachment is exerted through interference with L-selectin-mediated binding, although our finding that the degree of inhibition of T cell attachment in a nonstatic model was minor in the VLA-4/FVMCAM-1-blocked system compared with that in the nonblocked system argues for a possible bivalent effect of salicylates on L-selectin as well as integrins. Although this conclusion fits with previous data showing NSAID interference on VLA-4/FVMCAM-1-mediated attachment of T cells in similar nonstatic systems (18), our data suggest that the effect of the drugs is primarily exerted on L-selectin, because salicylate inhibition of L-selectin expression and T cell adhesion was completely prevented by inhibitors of L-selectin shedding. Therefore, on the basis of these findings, it is possible to postulate that the interference exerted by salicylates on integrin-mediated T cell adhesion in rotating models could be at least in part a consequence of the primary interference with L-selectin. This hypothesis appears to be supported by the demonstration that integrin activation and integrin-mediated adhesion can be induced by engagement of L-selectin on T cells via a PTK- and calcium-dependent, PKC-independent mechanism (70–72).

It is well known that L-selectin has a key role in the recirculation of naive T lymphocytes to the secondary lymphoid organs, as it mediates the binding of lymphocytes to high endothelial venules of peripheral lymph nodes (3, 6, 73), so that ASA may contribute to limit the traffic of these T cells, which are thought to be the precursors of effector and memory T cells. In this regard, experiments recently performed in our laboratories have confirmed that ASA reduces L-selectin expression on T cells circulating in the cord blood, a unique natural source of naive T cells highly expressing L-selectin (unpublished observations). However, the fact that the degree of reduction on cord T cells is similar to that observed in adults, in whom different T cell subsets circulate, suggests that salicylates exert their inhibitory effects not only on unprimed T cells, but also on memory or effector T cells, which display a great ability to migrate to inflammatory sites (74, 75). The assumption of an involvement of L-selectin in lymphocyte migration toward sites of inflammation is mainly supported by the demonstration of an impairment of lymphocyte recruitment into inflammatory sites in L-selectin-deficient mice (76, 77), but it is also sustained by the observations that memory T cells can recirculate in an L-selectin manner and that IL-12, a proinflammatory cytokine, is able to maintain L-selectin expression on Th1 effector cells (78, 79). Thus, the partial down-regulation of L-selectin expression induced by salicylates, confirmed in this study by in vivo administration of anti-inflammatory doses of ASA to healthy subjects, may contribute to limit the contact with endothelium of resting T cells and to reduce in different ways the immune response during inflammation.

Acknowledgments

We thank Dr. Thomas F. Tedder (Department of Immunology, Duke University Medical Center, Durham, NC) for providing the human LAM-1 cDNA; Dr. Dorian O. Haskard (Endothelial Cell Laboratory, Department of Medicine, Rheumatology Unit, Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K.) for the anti-VCAM-1 IgG1, the anti-L-selectin DREG-200, and the anti-ICAM-1 6.5B5; Dr. J. M. Harlan (Department of Medicine, University of Washington, Seattle, WA) for the anti-VCAM-1 4B9; Dr. Takashi Kei Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) for the compound KD-IX-73–4; and Dr. Bill Johnson (Roche Discovery, Welwyn, U.K.) for the Ro 31-9790. The anti-VCAM-1 E1/6 mAb, developed by Dr. M. A. Gimbrone (Department of Medicine, Harvard Medical School, Boston, MA), was obtained through Dr. Raffaele De Caterina (University of Pisa, Pisa, Italy).

References

phantom of sulphate in this interaction demonstrated by binding studies 
with a series of lipid-linked oligosaccharides. Biochem. Biophys. Res. Com- 
munications 188:244.
Springer-Verlag, New York, p. 577.
15. Kimmel, S. C., M. J. van de Stouwe, R. I. Levin, G. Weissman, and 
B. N. Cronstein. 1991. A final common pathway for anti-inflammatory agents: 
16. Milagres, A. M., R. R. E. M. Dollain, P. de Kuiper, C. L. Verweij, 
B. A. C. Dijkmaas, and F. C. Breedeweld. 1994. Tenidap inhibits T-cell prolifer- 
ation, cytokine-production, and the induction of mRNA encoding TNFα and 
17. Gerli, R., C. Paolucci, G. Grenele, S. Bistoni, S. Fiorucci, C. Muscat, S. Belia, 
A. Bertotto, and V. Costantini. 1998. Salicylates inhibit adhesion and transmi- 

gation of T lymphocytes by preventing integrin activation induced by contact 
with endothelial cells. Blood 92:2389.
18. Gonzalez-Alvaro, I., C. Munoz, R. Garcia-Vicuna, P. Sabando, C. Cabanas, 
F. Sanchez-Madrid, and F. Diaz-Gonzalez. 1998. Interference of nonsteroidal 
adhesion molecule L-selectin to 3-sulfated-Leα and Leβ oligosaccharides and 
the predominance of sulphate in this interaction demonstrated by binding studies
a hydroxamic acid-based protease inhibitor: identification with an L-selectin-
63. Laudanna, C., G. Constantin, P. Baron, E. Scarpini, G. Scarlato, G. Cabrini, 
increase of cytosolic free calcium and enhanced expression of tumor necrosis 
factor-α and interleukin-8 mRNA in human neutrophils: evidence for a role of 
ATP causes of loss of L-selectin from human lymphocytes via occupancy of P2Z 
shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by 
the same receptor but different metalloproteases. Blood 92:946.
66. Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Ishii, 
M. Shibuya, and Y. Fukami. 1987. Genistein, a specific inhibitor of tyrosine-
1986. Staurosporine, a potent inhibitor of phospholipid/Ca
2
1
-dependent protein 
69. Lawrence, M. B., and T. A. Springer. 1991. Leukocyte roll on a selectin at 
physiological flow rates: distinction from and prerequisite for adhesion through 
induces integrin-dependent adhesion: evidence for a signaling pathway involving 
71. Hwang, S. T., M. S. Singer, P. A. Giblin, T. A. Yednock, K. B. Bacon, 
S. I. Simon, and S. D. Rosen. 1996. GlyCAM-1, a physiologic ligand for L-
selectin, activates β1 integrins on naive peripheral lymphocytes. J. Exp. Med. 
184:1343.
L-selectin on T lymphocytes activates β1 integrins and promotes adhesion to 
73. Tangemann, K., M. D. Gunn, P. Giblin, and S. D. Rosen. 1998. A high endo-
theial cell-derived chemokine induces rapid, efficient, and subset-selective arrest 
of rolling T lymphocytes on a reconstituted endothelial substrate. J. Immunol. 
161:6330.
74. Gerli, R., A. Bertotto, P. Rambotti, P. Barbieri, M. L. Cionpi, and 
Rheum. 31:1073.
intrinsic migratory capacity of memory T cells contributes to their accumulation 
have impaired leukocyte recruitment into inflammatory sites. J. Exp. Med. 
181:2259.
involved in lymphocyte migration to sites of inflammation in the skin: delayed 
78. Tietz, W., and A. Hamann. 1997. The migratory behavior of murine CD4+
 cells 
Expression of L-selectin on Th1 cells is regulated by IL-12. J. Immunol. 
163:1214.