Inhibition of Delayed-Type Contact Hypersensitivity in Mice Deficient in Both E-Selectin and P-Selectin

By Nigel D. Staite, James M. Justen, Laurel M. Sly, Arthur L. Beaudet, and Daniel C. Bullard

Leukocyte rolling and emigration in response to inflammatory stimuli appears to involve both E-selectin- and P-selectin-dependent adhesion, which suggests that these molecules have overlapping functions. To clarify their relative contributions in chronic inflammation, we examined delayed-type contact hypersensitivity (DTH) responses in P-selectin, E-selectin, and E-/P-selectin-deficient mice. Oxazolone-induced increases in ear thickness and ear weight were equivalent in wild-type mice and in P-selectin and E-selectin mutants, but were significantly reduced in E-/P-selectin mutants. The number and area of microabscesses on the ears of E-/P-deficient mice were decreased by 72% and 93%, and the number of leukocytes invading the subdermal ear tissue was reduced. T cells from E-/P-deficient mice transferred oxazolone reactivity into naïve wild-type mice. However, when donor T cells from wild-type mice were transferred into E-/P-selectin-deficient mice, the DTH response was significantly impaired. These results show that leukocyte recruitment into a subacute inflammatory reaction can occur when either P-selectin or E-selectin is present, but is significantly reduced when both selectins are absent. Both P- and E-selectin are likely to play important roles in the development and maintenance of inflammatory diseases.

Studies using neutralizing antibodies to LFA-1/ICAM-1, ICAM-1-deficient mice, or antibodies to VLA-4 have suggested roles for both β1- and β2-integrins in the development of DTH reactions. In L-selectin-deficient mice, ear swelling was decreased by 69% compared with wild-type controls, indicating that in this model, L-selectin-dependent adhesion is necessary for antigen sensitization, leukocyte recruitment, or both. However, there are conflicting reports concerning the relative importance of P-selectin- and E-selectin-dependent adhesion in the development of a CHS response. In P-selectin-deficient mice, a normal ear swelling response but a reduction in leukocyte infiltration has been reported. In contrast, no effect of an anti-P-selectin antibody was observed on ear swelling or on the number of infiltrating leukocytes in wild-type mice and a normal response to oxazolone was seen in E-selectin-deficient mice. However, the combination of anti-P-selectin antibody administration to E-selectin-deficient mice resulted in suppression of both edema and leukocyte recruitment. Blocking the expression of adhesion molecules in mice via gene targeting offers an alternative strategy to neutralizing antibodies, the use of which can be complicated by the need to maintain saturating levels of antibody and the effect of antibody- ligand binding on the activation status and function of the target cell. In addition, neutralizing antibodies are typically administered only during the efferent phase of the model, whereas gene targeting results in absence of a molecule during both the sensitization and inflammatory phases of a response. In view of this, we used mice unable to express P-
selectin, E-selectin, or both E- and P-selectin to investigate the role of these molecules in the development of oxazolone-induced DTH reactions.

MATERIALS AND METHODS

Generation of E-selectin/P-selectin double-mutant mice. Mice that contained null mutations in E-selectin, P-selectin, or both genes were generated by gene targeting in embryonic stem cells, as described.\(^6,8\) All mice used in these experiments were 8 to 12 weeks old and were from a mixed 129/Sv × C57BL/6 background. Only mutant mice that had no loss of facial hair and no symptoms of opportunistic infection or lymphadenopathy were used in these studies. Routine screening showed no evidence of infection with 10 common murine viruses, *Mycoplasma pulmonis*, or pin worms. All procedures in this study were in compliance with Animal Welfare Act Regulations (9CFR Parts 1, 2, and 3) and with the Guide for the Care and Use of Laboratory Animals (DHEW Publications [NIH] 85-23, 1985).

Induction and measurement of DTH responses to oxazolone. DTH reactions were induced with oxazolone (Sigma, St Louis, MO). Mice were sensitized by applying 50 \(\mu\)L of 3\% oxazolone in acetone/olive oil (4:1) to shaved abdominal skin and 5 \(\mu\)L to each paw. Mice were resensitized 24 hours later. After an additional 4 days, a DTH response was elicited by challenging the dorsal surface of the right ear with 20 \(\mu\)L of 2\% oxazolone in acetone/olive oil. The left ear (control) received only acetone/olive oil. Reactions were quantitated 24 hours postchallenge by two methods. Left and right ear thicknesses were measured using a MAX Series Electronic Caliper (KBC Tools, Sterling Heights, MI). In addition, biopsies of the left and right ears were performed using a 5-mm punch (G. Tiemann, Plainview, NY) and the individual biopsy weights recorded. Left ear thickness measurements and punch biopsy weights were subtracted from right ear measurements to obtain response values. Peripheral blood was collected by cardiac or retro-orbital puncture under Metofane anesthesia (Pitman-Moore, Mundelein, IL) anticoagulated with sodium edathamil (EDTA) and anesthetized mice killed by cervical dislocation. Total leukocyte counts were obtained using a Coulter Counter ZM (model SSII; Hialeah, FL).

Adaptive transfer of oxazolone-induced hypersensitivity reactions. Mice were sensitized to oxazolone as described earlier. A single-cell suspension was made from the spleens and both popliteal and inguinal lymph nodes of mice 6 days after oxazolone sensitization. Erythrocytes were lysed with tris-ammonium chloride buffer and T cells purified by passage over nylon wool columns. A total of 3 \(×\) 10\(^8\) cells in 4.0 mL of RPMI that contained 10\% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD) was incubated for 45 minutes at 37°C in prewarmed 30-cc plastic syringes packed with 1.5 g of sterilized nylon wool (NEN Products, Boston, MA). Columns were washed with 25 mL of medium and the cell recovery was 4\% for 24 hours, rinsed with distilled water, and placed in 70\% ethanol. Each biopsy was then cut in half, placed in formalin, and dehydrated with alcohols using an automatic tissue processor (Fisher Histomac, Pittsburgh, PA). Specimens were cleared with xylene and embedded in paraffin before sectioning and staining with hematoxylin, eosin, and phloxine. Histomorphometry was performed on coded specimens, in a blinded manner, using a Leitz Laborlux microscope (Leitz Instrument Division, Calumet City, IL) fitted with a Sony 3CCD color video camera (Mager Scientific Inc, Dexter, MI), connected to a personal computer. The digital image was captured with a Color Frame Grabber, 24-bit image processor (VISIONplus-AT; Imaging Technology, Bedford, MA) and analyzed with OPTIMAS software (Bioscan, Edmonds, WA). The areas of microabscesses were calculated from user-defined outlines using software and calibrations provided within OPTIMAS. Cell counts were performed after spectral scans and size limits of cell nuclei were calibrated to ensure that all individual cells were counted. Cell counts were performed inside user-defined areas located in the subdermal tissue, above the lateral cartilage layer of the ear. Microabscess areas, expressed in square microns, were totaled for each ear and then averaged to obtain a mean total area per ear. The average frequency of microabscesses per ear was also calculated. Microabscesses were excluded from the analysis of infiltrating cells in the subdermal tissue. The number of cells that infiltrated the subdermal tissue was averaged for three separate regions per ear and the cell density expressed as cells per square millimeter.

RESULTS

Reduced oxazolone-induced contact hypersensitivity in E-selectin/P-selectin double mutants. Groups of eight to 10 mice were sensitized to oxazolone and challenged 5 days later on the dorsal surface of the right ear. For comparison, left ears were painted with acetone–olive oil vehicle. Twenty-four hours after oxazolone challenge, the DTH reaction was measured by comparing left and right ear thickness and 5-mm punch biopsy tissue weights. Increases in ear thickness in wild-type mice averaged 6.67 \(±\) 0.11 \(\times\) 10\(^{3}\) in (SEM, \(n = 29\)) and comparable responses were seen in E-selectin and P-selectin mutant mice. In E-selectin/P-selectin double mutants, oxazolone-induced ear swelling was reduced by 53\% and 50\% compared with wild-type mice and single-selectin mutants, respectively (Fig 1). The reduced response observed in E-/P-selectin double mutants was confirmed by ear biopsy weights, taken immediately after ear thickness measurements. The mean difference in left and right biopsy weights was 7.47 \(±\) 0.20 mg (SEM, \(n = 29\)) in wild-type mice. A significantly smaller increase in biopsy weight was seen in E-/P-selectin double mutants. This response was reduced by 53\% compared with wild-type mice and 54\% and 48\% compared with E-selectin or P-selectin single mutants (Fig 1; \(P < .001\)).

Histologic assessment of DTH responses from E-selectin/P-selectin mutant mice. Examination of ear biopsies from sensitized, nonchallenged, and challenged ears showed that in wild-type mice, oxazolone induced a pronounced inflammatory reaction compared with vehicle-treated ears. The reaction was characterized by interstitial edema, an accumulation of scattered, extravascular leukocytes in the subdermal tissue, and microabscesses on the ear surface containing focal accumulations of polymorphonuclear leukocytes (Fig 2A and B). Histomorphometric analysis was performed to determine the number of microabscesses per ear and their average
IMPAIRED DTH IN E/P-SELECTIN-DEFICIENT MICE

The emigration of leukocytes from the vasculature into the subdermal tissue. As shown in Table 1, increases in both the number and average area of microabscesses were noted on the surface of oxazolone-challenged ears from E-selectin or P-selectin single mutant mice, compared with wild-type mice. In contrast, the frequency of microabscesses was reduced by 72% in E/P-double mutants, and the average area of these lesions was decreased by 93%. The increase in number and area of microabscesses in the single mutants was not associated with any notable change in the type of infiltrating cells. The majority of the cells were polymorphonuclear leukocytes, as was seen in wild-type mice.

The emigration of leukocytes from the vasculature into the subdermal ear tissue of E/P-double mutants was also attenuated. While the number of leukocytes that had infiltrated the tissue was approximately the same in wild-type mice and in E-selectin and P-selectin single mutants, cell accumulation in the E/P-double mutants was significantly reduced, even when the numbers were corrected for differences in ear thickness between the groups (Table 1). No consistent differences were observed in the relative proportions of mononuclear and polymorphonuclear leukocytes in the subdermal infiltrate of P-selectin, E-selectin, or E/P-double mutants. Representative ear sections from the non-challenged and challenged ears of a representative sensitized E/P-double mutant mouse are shown in Fig 2C and D.

Circulating numbers of peripheral blood leukocytes. A reduction in severity of the DTH response in the ears of E/P-double mutant mice would be consistent with impaired adhesion of cells to vascular endothelium and inhibition of emigration into tissue. Consequently, we examined circulating numbers of leukocytes in the peripheral blood of sensitized mice before and after oxazolone challenge to look for evidence of cell accumulation in the vasculature. Leukocyte numbers in the blood of sensitized and sensitized/challenged wild-type mice were essentially the same, whereas cell numbers in E-selectin or P-selectin mutant mice decreased after challenge (Table 2). The number of blood leukocytes circulating in E- or P-selectin mutants after challenge was similar to wild-type mice. In contrast, the number of leukocytes in the peripheral blood of E/P-double mutants was significantly higher than the other groups before challenge and increased slightly after challenge, consistent with impaired recruitment of cells into the DTH reaction.

Adoptive transfer of contact hypersensitivity in E-selectin/P-selectin mutant mice. The effect of selectin deficiency on the sensitization of mice to oxazolone, the ability of oxazolone-reactive T cells to migrate to the challenged ear, and the subsequent recruitment of leukocytes were examined in adoptive transfer experiments. T cells from the lymph nodes and spleen of wild-type mice or E/P-double mutants sensitized to oxazolone were injected intravenously into naive wild-type mice. Recipient mice were then immediately challenged on the left ear with vehicle and on the right ear with oxazolone. Increases in ear thickness and ear biopsy weight were essentially the same in wild-type mice, regardless of whether the transferred cells were from wild-type mice or E/P-double mutants (Table 3). However, when cells from oxazolone-sensitized wild-type mice were transferred into naive E/P-double mutants, the DTH reaction was significantly attenuated compared with control mice (P < .0001).

DISCUSSION

In these studies, we have examined the roles of P- and E-selectin in the development of a DTH response to oxazolone. Our results show that in mice unable to express P-selectin or E-selectin alone, ear swelling and accumulation of leukocytes are not significantly different from wild-type mice. These findings are consistent with the observation that oxazolone-induced inflammation was unaffected by administration of anti-P-selectin antibody or lack of expression of E-selectin. Studies in P-selectin−deficient mice, in which a substantially weaker DTH response to oxazolone was induced, have shown that while leukocyte infiltration was reduced, ear swelling and vascular permeability were not significantly inhibited. Interestingly, our histomorphometric analysis showed that both the number and size of oxazolone-induced microabscesses on the ears of P-selectin or E-selectin−deficient mice was significantly higher than wild-type mice, which parallels the observation that at 24 hours, thio-
Fig 2. Histologic features of oxazolone-induced DTH responses in the ears of wild-type mice and E-/P-selectin-deficient mice. Representative ear sections from a wild-type mouse and an E-/P-selectin-deficient mouse are shown. (A) Ear biopsy from a sensitized wild-type mouse challenged with vehicle; normal appearance (size bar = 180 μm). The cartilage layer in this and subsequent panels is indicated by a large arrow. (B) Part of an ear biopsy from a sensitized wild-type mouse 24 hours after oxazolone challenge at the same magnification. Multiple focal accumulations of neutrophils can be seen at the epidermal surface forming microabscesses (smaller arrows). The subdermal tissue contains large numbers of infiltrating leukocytes and there is marked edema distending the connective tissue and muscle fibers. (C) Ear section from a sensitized E-/P-selectin-deficient mouse challenged with vehicle; normal appearance. (D) Ear section from a sensitized E-/P-double mutant 24 hours after oxazolone challenge is shown at the same magnification. Both the number and size of microabscesses, together with the number of leukocytes invading the connective tissue, were significantly reduced in this group. A reduction in the degree of tissue swelling can also be seen.

Table 1. Histomorphometric Analysis of Oxazolone-Induced DTH Reactions

<table>
<thead>
<tr>
<th>Histology</th>
<th>Wild-Type Mice</th>
<th>E-Selectin Mutants</th>
<th>P-Selectin Mutants</th>
<th>E/P-Double Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microabscess (no./ear)</td>
<td>10.90 ± 0.55</td>
<td>16.50 ± 0.76†</td>
<td>16.20 ± 1.11†</td>
<td>3.10 ± 0.48†</td>
</tr>
<tr>
<td>Microabscess (area/ear, μm² × 10³)</td>
<td>74.33 ± 5.83</td>
<td>104.57 ± 10.58*</td>
<td>135.90 ± 12.05†</td>
<td>4.90 ± 1.36†</td>
</tr>
<tr>
<td>Subdermal infiltrate (cells/mm²)</td>
<td>4,232 ± 806</td>
<td>4,164 ± 345</td>
<td>4,972 ± 376</td>
<td>1,724 ± 98†</td>
</tr>
</tbody>
</table>

Oxazolone-induced ear DTH reactions were examined microscopically (n = 10 per group). The mean number of epidermal microabscesses that contained focal accumulations of neutrophils was determined for each challenged ear ± SEM. Lesion areas and cell infiltration into the subdermal tissue above the central cartilage layer were measured using image analysis software. Data are expressed as lesion area per ear or the mean number of infiltrating cells per mm² of tissue ± SEM. Microabscesses were excluded from the infiltration analysis.

Significant differences by ANOVA are shown by † P < .05, * P < .001 v wild-type; ‡ P < .001 v wild-type, E-selectin, and P-selectin; †† P < .01 v wild-type and E-selectin; ‡‡ P < .001 v P-selectin.
glycolate-induced neutrophil accumulation was increased in P-selectin–deficient mice. Our results show that in sensitized E- or P-selectin mutant mice, the number of peripheral blood leukocytes is approximately 68% to 100% higher than in wild-type mice, but decreases to wild-type values after challenge. This suggests that leukocyte recruitment in these mutants is essentially normal and that the increased severity of the DTH reaction in these mice may be due to the availability of a larger than normal reservoir of leukocytes. Alternatively, lack of P- or E-selectin may impair the emigration of a regulatory cell population into the tissue, which normally exerts a suppressive effect on the inflammatory response. However, in E-/P-selectin–deficient mice, both the number and area of microabscesses was significantly decreased, as well as the number of leukocytes infiltrating the subdermal tissue. Inhibition of the DTH reaction occurred despite the large number of blood leukocytes available for recruitment, which was two to three times the leukocyte pool available in E- or P-selectin mutants and fivefold higher than in wild-type mice. Suppression of the inflammatory response was paralleled by a slight increase in the number of peripheral blood leukocytes following challenge, which would be consistent with impaired recruitment of cells from the vasculature. The ability of leukocytes from E-/P-double mutant mice to successfully transfer oxazolone reactivity into naive wild-type mice indicates that antigen-specific T cells from these mice are functional and can transmigrate across endothelial cells normally. In contrast, the inability of T cells from wild-type mice to transfer oxazolone reactivity into E-/P-double mutants shows that the endothelial cells in these mice are unable to support the adhesive interactions necessary for leukocyte migration and recruitment. Since P- and E-selectin have been implicated in T-cell, as well as neutrophil rolling on activated endothelium, inhibition of the DTH reaction could be due to impaired T-cell migration, neutrophil recruitment, or both.

Our data support the contention that inhibition or lack of expression of P-selectin or E-selectin alone is insufficient to prevent leukocyte migration into a DTH reaction. This would suggest that L-selectin or other molecules, either alone or in combination with P- and E-selectin, can maintain the adhesive interactions required for rolling. Intravital microscopy studies have shown that early (0 to 60 minutes) rolling is dominated by P-selectin interactions, while later rolling (60 to 120 minutes) involves both P- and L-selectin. In the presence of activating cytokines, L-selectin–mediated adhesion appears to dominate, and leukocyte rolling can be completely blocked when both L- and P-selectin are inhibited. This paradigm is also supported by results from peritonitis models in vivo using antiselectin antibodies or selectin-deficient mice. Inhibition or lack of expression of P- or L-selectin resulted in a 50% to 60% decrease in neutrophil accumulation in the peritoneal cavity 1 to 4 hours following thioglycolate administration. Interestingly, at 24 hours, the number of neutrophils in the peritoneal cavity of P-selectin–deficient mice was not reduced compared with controls, whereas the response was inhibited by 50% to 60% in L-selectin–deficient mice. These observations suggest that L-selectin may play an increasingly important role in the recruitment of neutrophils as the peritonitis reaction becomes more chronic. The contribution of E-selectin to neutrophil and lymphocyte adhesion has been unclear. E-selectin alone appears to be unable to maintain leukocyte rolling in mice in the absence of P- and L-selectin. However, while anti-E-selectin antibody partially inhibits early (1 to 4 hours) thioglycolate-induced neutrophil influx, the response in E-selectin–deficient mice appears to be normal. Blocking both E- and P-selectin has also given different results. While the effect of a combination of antibodies to E- and P-selectin was not significantly different compared with either antibody alone, the addition of an anti-P-selectin antibody to E-selectin–deficient mice produced an additive effect on thioglycolate-induced neutrophil accumulation. These apparently conflicting results appear to be due to differences between anti-

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Sensitized</th>
<th>Sensitized + Challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>7.33 ± 0.56 (n = 13)</td>
<td>1.31 ± 0.47 (n = 29)</td>
</tr>
<tr>
<td>E-selectin mutants</td>
<td>12.29 ± 0.60 (n = 11)</td>
<td>8.88 ± 0.51 (n = 34)*</td>
</tr>
<tr>
<td>P-selectin mutants</td>
<td>15.00 ± 0.93 (n = 13)</td>
<td>7.59 ± 0.42 (n = 19)**</td>
</tr>
<tr>
<td>E-/P-double mutants</td>
<td>36.86 ± 2.73 (n = 13)</td>
<td>43.14 ± 2.47 (n = 29)</td>
</tr>
</tbody>
</table>

Peripheral blood was obtained from the retroorbital sinus of mice 5 days after oxazolone sensitization and again 24 hours after challenge. Blood leukocyte counts are expressed as the mean number of cells × 10⁶/mL ± SEM.

A significant difference between prechallenge and postchallenge counts is shown by * P < .005, ** P < .001 (two-tailed Student's t-test).

<table>
<thead>
<tr>
<th>Donor = wild-type</th>
<th>Recipient = wild-type (n = 9)</th>
<th>4.17 ± 0.14</th>
<th>3.72 ± 0.34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor = E-/P-mutant</td>
<td>Recipient = wild-type (n = 9)</td>
<td>3.94 ± 0.16</td>
<td>4.91 ± 0.48</td>
</tr>
<tr>
<td>Donor = wild-type</td>
<td>Recipient = wild-type (n = 9)</td>
<td>3.61 ± 0.26</td>
<td>2.94 ± 0.27</td>
</tr>
<tr>
<td>Donor = wild-type</td>
<td>Recipient = E-/P-mutant (n = 9)</td>
<td>1.11 ± 0.07*</td>
<td>0.84 ± 0.11*</td>
</tr>
</tbody>
</table>

Enriched T cells were obtained from pooled spleen and lymph nodes of oxazolone-sensitized wild-type or E-/P-selectin–deficient mice and injected intravenously into the corresponding naive recipient mice, as shown, in 2 separate experiments. At the time of injection, recipient mice were immediately challenged on the right ear with oxazolone and on the left ear with vehicle. The results shown represent the increases in ear thickness and ear punch biopsy weight obtained 24 hours after challenge of right ears after subtraction of left ear values. Significance versus the response obtained when wild-type cells were transferred into wild-type recipients is shown by * P < .0001 (two-tailed Student's t-test).
E-selectin antibodies. The antibody 9A9E3 blocks E-selectin-mediated rolling in vivo, while 10E9.6 has no effect.8 Our results show that loss of P-selectin alone did not influence antigen-specific leukocyte recruitment and that lack of expression of both P- and E-selectin was required to see significant effects. The residual inflammation seen in the ears of E-/P-double mutants may well be due to L-selectin-mediated cell recruitment, since oxazolone reactivity is impaired in L-selectin-deficient mice.15 Alternatively, other adhesion pathways may also be involved, such as the recently described ability of VLA-4 to support tethering and rolling by binding to VCAM-1.27

E-/P-selectin mutant mice appear to have the most severe deficiency in selectin function reported to date and recent studies show that both early and delayed leukocyte rolling are absent.6 As these mice age, they frequently develop oral and ocular infections; consequently, we were careful in our experiments to use mice that had no signs of opportunistic infections. While the surface density of L-selectin on bone marrow cells from these mice is approximately 80% to 85% that of wild-type mice, the surface density on blood lymphocytes and granulocytes is approximately 50% and 12% to 17% of normal levels, respectively,6 probably due to cell activation and L-selectin shedding.25 However, despite the reduction in surface density, the number of L-selectin-positive leukocytes in E-/P-selectin mutants is still twofold to threefold higher than in wild-type mice. Decreased expression of L-selectin is unlikely to account for all of the suppression of oxazolone DTH reported here, since our findings agree with those of Labow et al, who used E-selectin-deficient mice treated with an anti–P-selectin antibody.17 Furthermore, if these mice were deficient in L-selectin, the ability of oxazolone sensitized T cells from E-/P-mutants to transfer DTH reaction should have been inhibited, since L-selectin deficient mice have an impaired DTH response 4 to 5 days after contact sensitization26 and migration of antigen-specific lymphocytes to a site of antigen challenge involves L-selectin-mediated adhesion.26

In conclusion, our data demonstrate that mice unable to express both E-selectin and P-selectin have an impaired DTH response, whereas no such impairment was seen in E- or P-selectin-deficient mice. This defect appears to result from impaired leukocyte emigration and recruitment. These data support the concept that E-selectin and P-selectin have some overlapping functions in the recruitment of cells to a site of inflammation.

ACKNOWLEDGMENT

The authors are grateful to J.G. Chosay for assistance with histomorphometric image analysis and to L.A. Hurley and I. Lorenzo for technical assistance.

REFERENCES

8. Asherson GP, Puk W: Contact and delayed hypersensitivity in the mouse I. Active sensitization and passive transfer. Immunology 15:405, 1968
Inhibition of delayed-type contact hypersensitivity in mice deficient in both E-selectin and P-selectin

ND Staite, JM Justen, LM Sly, AL Beaudet and DC Bullard