Selectin Blockade Prevents Antigen-induced Late Bronchial Responses and Airway Hyperresponsiveness in Allergic Sheep

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Antigen challenge can elicit an allergic inflammatory response in the airways that involves eosinophils, basophils, and neutrophils and that is expressed physiologically as a late airway response (LAR) and airway hyperresponsiveness (AHR). Although previous studies have suggested that E-selectin participates in these allergic airway responses, there is little information concerning the role of L-selectin. To address this question, we examined the effects of administering an L-selectin-specific monoclonal antibody, DU1-29, as well as three small molecule selectin binding inhibitors, on the development of early airway responses (EAR), LAR and AHR in allergic sheep undergoing airway challenge with Ascaris suum antigen. Sheep treated with aerosol DU1-29 before antigen challenge had a significantly reduced LAR and did not develop postchallenge AHR. No protective effect was seen when sheep were treated with a nonspecific control monoclonal antibody. Treatment with DU1-29 also reduced the severity of the EAR to antigen. Similar results were obtained with each of the three small molecule selectin inhibitors at doses that depended on their L-, but not necessarily E-selectin inhibitory capacity. The inhibition of the EAR with one of the inhibitors, TBC-1269, was associated with a reduction in histamine release. Likewise, treatment with TBC-1269 reduced the number of neutrophils recovered in bronchoalveolar lavage (BAL) during the time of LAR and AHR. TBC-1269, given 90 min after antigen challenge also blocked the LAR and the AHR, but this protection was lost if the treatment was withheld until 4 h after challenge, a result consistent with the proposed time course of L-selectin involvement in leukocyte trafficking. These are the first data indicating that L-selectin may have a unique cellular function that modulates allergen-induced pulmonary responses. Abraham WM, Ahmed A, Sabater JR, Lauredo IT, Botvinnikova Y, Bjerkke RJ, Hu X, Revelle BM, Kogan TP, Scott IL, Dixon RAF, Yeh ETH, Beck PJ. Selectin blockade prevents antigen-induced late bronchial responses and airway hyperresponsiveness in allergic sheep. AM J RESPIR CRIT CARE MED 1999;159:1205–1214.
kocytes to areas of vascular trauma or inflammation. P-selectin (CD62P) is expressed upon the surface of activated platelets and endothelial cells, E-selectin (CD62E) upon the surface of activated endothelial cells, and L-selectin (CD62L) is expressed upon the surface of eosinophils, neutrophils, monocytes, and lymphocytes. The initial step in leukocyte migration from blood into extravascular tissue is thought to be adherence to the surface of activated endothelial cells that line vessel walls. This initial adherence is thought to be dependent on selectin-mediated processes (8–10). Based on these data, we hypothesized that treatment with selectin inhibitors should prevent the subsequent inflammatory events, i.e., the LAR and the AHR associated with antigen challenge. Partial support for this hypothesis comes from previous studies in primates that indicate that pretreatment with a monoclonal antibody to E-selectin blocks LAR (2). However, these studies did not assess the effect of E-selectin blockade on the post-antigen-induced AHR nor did they evaluate the effect of giving such drugs after antigen challenge. This timing may be critically important because selectins are involved early on in the inflammatory cascade. Thus, one would speculate that selectin inhibitors need to be given either before antigen challenge or soon after challenge (1-2 h) to show their maximal effect.

To test this hypothesis, confirm mechanism of action, and extend previous findings, we have utilized an anti-L-selectin blocking antibody and several small molecule selectin inhibitors and evaluated the effect of these compounds in the sheep model of allergic bronchoconstriction (11). In this model, airway challenge with specific antigen results in an early airway response (EAR), a LAR, and an AHR for up to 2 wk after challenge (4, 11). Our results indicate that the anti-L-selectin antibody and small molecule selectin binding inhibitors are able to block the LAR and AHR that follow antigen challenge in this animal model. These changes are associated with a reduction in the cellular inflammatory response as estimated by bronchoalveolar lavage (BAL).

**METHODS**

**Materials**

Tissue culture media, dialyzed fetal calf serum (FCS), phosphate-buffered saline (PBS), and antibiotics were obtained from Life Sciences Inc. (Gaithersburg, MD) and FCS from HyClone (Logan, UT). The anti-L-selectin antibody (SK11) was purchased from Becton-Dickinson (San Jose, CA), and antibody used in experiments was purified from ascites as described subsequently. The anti-L-selectin antibody (A12) was purchased from Becton-Dickinson.

Unless specifically stated, other immunocompounds purchased.

**TABLE 1**

**SUMMARY OF TREATMENT RESULTS**

<table>
<thead>
<tr>
<th>Treatment and Mode of Administration</th>
<th>n</th>
<th>Time of Treatment</th>
<th>EAR (%) + Drug</th>
<th>EAR (%) + Vehicle</th>
<th>LAR (%) + Drug</th>
<th>LAR (%) + Vehicle</th>
<th>PC Ratio + Drug</th>
<th>PC Ratio + Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg TBC-265 (aerosol)</td>
<td>2</td>
<td>30 min prior to challenge</td>
<td>58 ± 21†</td>
<td>173 ± 39</td>
<td>16 ± 6†</td>
<td>108 ± 14</td>
<td>0.45</td>
<td>0.31</td>
</tr>
<tr>
<td>15 mg TBC-265 (aerosol)</td>
<td>2</td>
<td>30 min prior to challenge</td>
<td>183 ± 32‡</td>
<td>199 ± 29</td>
<td>36 ± 4‡</td>
<td>145 ± 4</td>
<td>0.89</td>
<td>0.45</td>
</tr>
<tr>
<td>10 mg TBC-1269 (aerosol)</td>
<td>5</td>
<td>30 min prior to challenge</td>
<td>91 ± 22‡</td>
<td>97 ± 24‡</td>
<td>85 ± 9‡</td>
<td>136 ± 21</td>
<td>0.54</td>
<td>0.51</td>
</tr>
<tr>
<td>4 mg TBC-1269 (aerosol)</td>
<td>3</td>
<td>30 min prior to challenge</td>
<td>148 ± 12‡</td>
<td>247 ± 49</td>
<td>46 ± 15‡</td>
<td>214 ± 83</td>
<td>1.04</td>
<td>0.71</td>
</tr>
<tr>
<td>10 mg TBC-1269 (aerosol) IV</td>
<td>3</td>
<td>15 min prior to challenge</td>
<td>100 ± 13</td>
<td>172 ± 29</td>
<td>18 ± 6</td>
<td>69 ± 10</td>
<td>0.97</td>
<td>0.50</td>
</tr>
<tr>
<td>10 mg TBC-1269 (aerosol)</td>
<td>5</td>
<td>90 min after challenge</td>
<td>175 ± 28‡</td>
<td>222 ± 41</td>
<td>18 ± 6</td>
<td>201 ± 4</td>
<td>0.89</td>
<td>0.48</td>
</tr>
<tr>
<td>10 mg TBC-1269 (aerosol)</td>
<td>10</td>
<td>4 h after challenge</td>
<td>179 ± 20†</td>
<td>233 ± 33</td>
<td>88 ± 8†</td>
<td>169 ± 38</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>5 mg MBPA (aerosol)</td>
<td>2</td>
<td>30 min prior to challenge</td>
<td>91 ± 25‡</td>
<td>189 ± 58</td>
<td>12 ± 6†</td>
<td>156 ± 15</td>
<td>1.10</td>
<td>0.52</td>
</tr>
<tr>
<td>2.5 mg MBPA (aerosol)</td>
<td>2</td>
<td>30 min prior to challenge</td>
<td>123 ± 28‡</td>
<td>187 ± 44</td>
<td>23 ± 4‡</td>
<td>130 ± 13</td>
<td>1.01</td>
<td>0.42</td>
</tr>
<tr>
<td>1 mg MBPA (aerosol)</td>
<td>2</td>
<td>30 min prior to challenge</td>
<td>127 ± 29‡</td>
<td>195 ± 47</td>
<td>81 ± 7‡</td>
<td>105 ± 14</td>
<td>0.60</td>
<td>0.43</td>
</tr>
<tr>
<td>10 mg DU1-29 (aerosol)</td>
<td>4</td>
<td>30 min prior to challenge</td>
<td>71 ± 15</td>
<td>184 ± 20</td>
<td>14 ± 4†</td>
<td>112 ± 14</td>
<td>1.02</td>
<td>0.43</td>
</tr>
<tr>
<td>10 mg MD6 (aerosol)</td>
<td>4</td>
<td>30 min prior to challenge</td>
<td>146 ± 18</td>
<td>212 ± 33</td>
<td>118 ± 11</td>
<td>118 ± 24</td>
<td>0.52</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Definition of abbreviations: EAR = early airway response; LAR = late airway response; expressed as percent change from baseline; PC = the ratio of post/prechallenge value of PC400. A ratio of 1 indicates that there was no change in the airway responsiveness, whereas a ratio below 1 indicates the development of airway hyperresponsiveness. † p < 0.05 and ‡ p > 0.1 as determined by analysis using a two-tailed, unpaired, heteroscedastic Student’s t test with each indicated group compared with results obtained after antigen challenge of the same animals without drug treatment.

**TABLE 2**

**SELECTIN INHIBITORS**

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>IC50 Human E:Selectin (μM)</th>
<th>IC50 Human P:Selectin (μM)</th>
<th>IC50 Human L:Selectin (μM)</th>
<th>EL Inhibitory Capacity</th>
<th>M,</th>
<th>Description and/or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sLeα†</td>
<td>170 µM</td>
<td>160 µM</td>
<td>193 µM</td>
<td>0.88</td>
<td>821</td>
<td>(22)</td>
</tr>
<tr>
<td>TBC-265</td>
<td>150 µM</td>
<td>173 µM</td>
<td>177 µM</td>
<td>0.85</td>
<td>390</td>
<td>(23)</td>
</tr>
<tr>
<td>TBC-1269</td>
<td>105 µM</td>
<td>17 µM</td>
<td>87 µM</td>
<td>1.21</td>
<td>906</td>
<td>(12)</td>
</tr>
<tr>
<td>MBPA</td>
<td>382 µM</td>
<td>489 µM</td>
<td>115 µM</td>
<td>3.32</td>
<td>208</td>
<td>(24)</td>
</tr>
<tr>
<td>DU1-29</td>
<td>No effect @ 50 µg/ml</td>
<td>No effect @ 50 µg/ml</td>
<td>0.3 µg/ml – 2 nM</td>
<td>~150,000</td>
<td></td>
<td>Anti-sheep L-selectin</td>
</tr>
<tr>
<td>M6D</td>
<td>ND</td>
<td>ND</td>
<td>No effect @ 50 µg/ml</td>
<td>~150,000</td>
<td></td>
<td>Non-specific negative control antibody</td>
</tr>
</tbody>
</table>

* Selectin IgG fusion proteins were assayed for adherence to sLeα glycolipids prepared as described (27). Fusion protein binding in the absence of compound is defined as 100% and that for the mock control wells is designated 0%. The data represent the mean of two independent experiments run in duplicate. The inhibitory capacity was determined from respective IC50 values against human selectins. The higher the ratio, the more potent the compound is against L-selectin. Assays were performed as previously described (28).
from Calbiochem (San Diego, CA). Flexible 96-well assay plates and Probind 96-well ELISA plates were purchased from Falcon (Becton-Dickinson).

**Chemicals**

TBC-265 and TBC-1269 were synthesized as described elsewhere (12, 13). MBPA (3-(4-methoxybenzoyl) propionic acid) was purchased from Aldrich (Milwaukee, WI). Stock solutions used for animal experiments were freshly prepared in sterile, pyrogen-free 0.9% NaCl (VWR Scientific Products, West Chester, PA) and when necessary adjusted to pH 7.3 with NaOH.

**Antibody Purification**

Mice were primed by intraperitoneal injection of 0.1 ml pristane 7 to 10 d prior to intraperitoneal injection of $10^7$ hybridoma cells expressing DU1-29 antibody or MD6 antibody. Ten to 14 d after injection, peritoneal ascites fluid was evacuated by aspiration. After brief centrifugation at 600 x g, ascites was frozen and stored at -80°C until mouse IgG was fractionated from ascites by passing the diluted fluid (1:2 into PBS) over a 10-ml protein G (Amersham Pharmacia, Uppsala, Sweden) column and eluting as recommended by the manufacturer. Eluent was extensively dialyzed against 10 mM ammonium acetate (pH 7.5) and lyophilized. The amount of recovered antibody was determined by ELISA using the procedures of Harlow and Lane (14).

**Animal Preparation**

Sheep weighing between 27 and 36 kg that had previously been shown to develop both early and late bronchial responses to inhaled *Ascaris suum* antigen were conscious and were restrained in a modified shopping cart in the prone position with their heads immobilized as previously described (4, 11). After topical anesthesia of the nasal passages with 2% lidocaine, a balloon catheter was advanced through one nostril into the lower esophagus. The animals were intubated with a cuffed endotracheal tube through the other nostril with a flexible fiberoptic bronchoscope as a guide. All protocols used in this study

![Figure 1](image_url)

**Figure 1.** Effect of aerosol treatment with TBC-265 on antigen-induced airway responses. Values are mean ± SE for two sheep. *p < 0.05 versus control. (A) Effect of antigen challenge on the changes in SRL in sheep with and without treatment with TBC-265. TBC-265 was given as an aerosol 30 min before challenge. TBC-265 (30 mg) gave significant protection against the antigen-induced early and late increases in SRL. (B) Effect of TBC-265 on antigen-induced airway hyperresponsiveness in allergic sheep. Neither the high- nor low-dose treatment blocked the post-antigen-induced airway hyperresponsiveness (i.e., the fall in PC400).
were approved by the Mount Sinai Medical Center Animal Research Committee, which is responsible for assuring the humane care and use of experimental animals.

**Measurement of Airway Mechanics**

Breath by breath determination of mean pulmonary flow resistance ($R_L$) was measured with the esophageal balloon technique that has been described previously by us (4, 7). The mean of at least five breaths, free of swallowing artifact, was used to obtain $R_L$ in cm H$_2$O/L/s. Immediately after the measurement of $R_L$, thoracic gas volume ($V_{tg}$) was measured in a constant-volume body plethysmograph to obtain specific lung resistance ($SR_L = R_L \times V_{tg}$) in liter $\times$ cm H$_2$O/L/s (4, 7).

**Aerosol Delivery System**

Aerosols were generated using a disposable medical nebulizer that provided an aerosol with a mass median aerodynamic diameter of 3.2 $\mu$m as determined by a cascade impactor. The nebulizer was connected to a dosimeter system, consisting of a solenoid valve and a source of compressed air (20 psi). The output of the nebulizer was directed into a plastic T-piece, one end of which was connected to the inspiratory port of a respirator. The solenoid valve was activated for 1 s at the beginning of the inspiratory cycle of the respirator. Aerosols were delivered at a tidal volume of 500 ml and a rate of 20 breaths per minute (4, 7).

**Airway Responsiveness**

Airway responsiveness was determined from cumulative concentration–response curves to inhaled carbachol as previously described (4, 7). $SR_L$ was measured immediately after inhalation of buffer and after each consecutive administration of 10 breaths of increasing concentrations of carbachol (0.25, 0.5, 1.0, 2.0, and 4.0% wt/vol PBS). The cumulative carbachol concentration (in breath units [BU]) that increased $SR_L$ by 400% over the postsaline value ($PC_{400}$) was calculated from the dose–response curve. One BU was defined as one breath of a 1% wt/vol carbachol aerosol solution (4, 7).

**BAL**

The distal tip of a fiberoptic bronchoscope was wedged into three randomly selected subsegmental bronchi. Lung lavage was performed by infusion and aspiration of 30-ml aliquots of PBS (Sigma; pH 7.4) at 39°C. A different airway was used for each 30-ml aliquot (total 90 ml at each time point). The effluents were centrifuged and strained through gauze to remove mucus. The total number of cells was counted in a hemocytometer from a sample of unconcentrated lavage using phase microscopy. The effluent was then centrifuged at 420 g for 15 min and the cell pellet was resuspended in PBS. A cytocentrifuge separation was made and stained by Wright-Giemsa to identify cell populations. Five hundred cells per slide were enumerated to establish the differential cell count (100×; oil objective). Cell categories included macrophages, lymphocytes, neutrophils, and eosinophils (4).

**Protocol (In Vivo Studies)**

All studies were done in crossover fashion such that each sheep served as its own control. The same general protocol was used for all studies, except that the dosage and time of treatment with the different inhibitors were varied. Details of the doses and routes of administration of the compounds are given in Table 1. This basic protocol consisted of obtaining baseline dose–response curves to aerosol carbachol (i.e., $PC_{400}$) 1 to 3 d before antigen challenge. Then, on the antigen challenge day, baseline values of $SR_L$ were obtained after which the sheep were challenged with A. suum antigen. Measurements of $SR_L$ were obtained immediately after challenge, hourly from 1 to 6 h after challenge and on the half-hour from 6.5–8 h after challenge. Measurements of $SR_L$ were obtained 24 h after challenge followed by the 24-h postchallenge determination of $PC_{400}$. Drug and vehicle control trials were separated by at least 2 wk.

**Anti-inflammatory Studies**

To assess the anti-inflammatory capacity of TBC-1269, six sheep were challenged on two separate occasions, once without (PBS; placebo) and once after pretreatment (−0.5 h) with TBC-1269 aerosol in a randomized crossover fashion. In these studies, a baseline BAL was performed before treatment and then 6.5 h and 24 h after antigen challenge. Total cell and cell differential responses were expressed as cells/ml lavage return. In addition to the cell response, we measured tissue kallikrein activity in BAL, which has been previously shown by us to be a marker of inflammation (15). Tissue kallikrein was measured in aliquots (stored at −70°C until analysis) of the cell-free supernatant from each of the BAL samples using a modification of the procedure previously described by us (15). Briefly, tissue kallikrein was determined in unconcentrated samples from BAL using a microtiter assay. A volume of 150 μl BAL was incubated with 25 μl of trypsin (20 μg/ml in tris [hydroxymethyl] aminomethane [Trizma] buffer 0.05 M, pH 8.2) for 15 min at 37°C. Then, 25 μl of soybean trypsin inhibitor (4 mg/ml in 0.1 M Trizma buffer, pH 8.2) was added followed by 100 μl of substrate DL Val-Leu-Arg p-nitroanilide (pNA) dissolved in Trizma buffer 0.05 M, pH 8.2, with 0.05% albumin and
incubated for 24 h in a humidified CO₂ incubator. The values were reported as the change in optical density between zero and 24 h, measured at a wavelength of 405 nM. All assays were done in duplicate.

**Histamine Release**

To determine whether TBC-1269 had an effect on mast cell degranulation, six sheep were challenged with antigen on two separate occasions, once without (PBS; placebo) and once after pretreatment (−0.5 h) with 10 mg TBC-1269 aerosol in a randomized crossover fashion. In these studies, a baseline BAL (one 30-ml aliquot) was performed before treatment and then 30 min and 60 min after antigen challenge. The lavage return was centrifuged to remove the cells, and aliquots of the supernatants were analyzed for histamine using a commercially available enzyme immunoassay kit according to the manufacturer’s instructions. The sensitivity of the assay is 0.5 nM. Samples were done in duplicate (Immunotech, Marseille, France).

**Statistical Analyses**

For all airway mechanics studies, all probabilities were determined using two-tailed, unpaired, heteroscedastic Student’s t tests performed using Microsoft Excel version 5.0a. Nonparametric statistics were used to analyze the BAL cell results. For the cell responses, Friedman’s two-way analysis of variance was used to determine overall effects followed by Wilcoxon’s test to distinguish differences at individual time points (two-tailed). For the tissue kallikrein measurements, the data were log₁₀ transformed and then analyzed by a two-way analysis of variance to determine overall effects. Differences at individual time points were determined by paired t test (two-tailed) (Systat for Windows, Version 5; SYSTAT, Inc., Evanston, IL). The histamine results were also log₁₀ transformed and then analyzed by paired t test. Because the functional results suggested that mediator release was inhibited after treatment, we used a one-tailed test to determine significance (16).

**RESULTS**

The inhibitors discussed in the text together with the in vitro efficacies that were determined for inhibition of human selectin binding and the physical descriptions of the compounds or references detailing those descriptions are listed in Table 2. As illustrated, each of three low-molecular-weight inhibitors blocks all selectins, but each has a different ratio of E:L selec-

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**Figure 3.** Effect of TBC-1269 when administered after antigen challenge. Values are mean ± SE. *p < 0.05 versus control. (A) When TBC-1269 (10 mg) was given 90 min after challenge (n = 5), it provided significant protection against the late increases in SR₅, and (B) antigen-induced airway hyperresponsiveness. If treatment was withheld until 240 min after antigen challenge, (C) this protection against the late increases in SR₅, and (D) antigen-induced airway hyperresponsiveness was lost (n = 4).
tin inhibitory activity based on the values of the concentration that inhibits binding by 50% (IC\textsubscript{50}) for the respective selectins. As will be shown, these ratios are predictive of the doses of the different inhibitors necessary to show activity in the in vivo experiments, i.e., as the E:L selectin inhibitory ratio increases, the dose of compound required to give protection against the pathophysiological endpoints falls. Also shown are the studies that demonstrate that a commercially available anti-sheep L-selectin antibody, DU1-29, is able to recognize and inhibit human L-selectin binding to sheep neutrophils, whereas the control antibody, MD6, which does not recognize L-selectin, does not inhibit binding.

Initial in vivo studies indicated that pretreatment with 30 mg, but not 15 mg, aerosolized TBC-265 significantly reduced the EAR and blocked the LAR after antigen challenge (Figure 1A and Table 1). Although the 30-mg dose of TBC-265 was effective in blocking the LAR, there was no subsequent effect on the 24-h AHR as evidenced by the fall in the PC\textsubscript{400} (Figure 1B).

A similar series of experiments were then conducted with a
second, structurally related selectin inhibitor, TBC-1269, that displayed greater in vitro ability to inhibit E-, P-, and L-selectin binding. As predicted from the in vitro IC₅₀ values, a lower dose (10 mg) of TBC-1269 administered as an aerosol to the sheep 30 min before antigen challenge, significantly reduced the EAR and blocked the LAR (Figure 2A). However, in addition to inhibiting the LAR, treatment with TBC-1269 also blocked 24-h AHR (p < 0.02) (Figure 2B). The protective effects of TBC-1269 were lost if the dose was reduced (4 mg/sheep, Table 1). We found that the pretreatment time for nebulized TBC-1269 could be extended to 2 h, if the dose was increased appropriately (i.e., four times the 30-min pretreatment dose) and that TBC-1269 was also effective in blocking the three physiologic endpoints, EAR, LAR, and AHR when administered intravenously at 3 mg/kg (Table 1).

Because selectin expression and activation are part of the initial inflammatory event, one would expect that treatment with TBC-1269 should be effective if given after antigen challenge.

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**Figure 6.** Effect of aerosol treatment with TBC-1269 on antigen-induced leukocyte accumulation in BAL from allergic sheep. When sheep were given a single 10-mg aerosol dose of TBC-1269 (dark bars) 0.5 h before antigen challenge, there was a decreased cellular response as compared with saline-treated sheep (light bars). Values are mean ± SE for six sheep. *p < 0.05 versus placebo; #p < 0.10 versus placebo; +p < 0.05 versus baseline; **p < 0.05 versus 6.5 h postantigen.
lence provided that the time of treatment was in close proximity to the challenge. Figure 3 shows the effects of 10 mg of TBC-1269 given by aerosol either 90 min or 240 min after antigen challenge. Treatment at 90 min postchallenge effectively inhibited both LAR and the 24-h AHR, but the protective effect on the LAR and the 24-h AHR was lost if the compound was administered 240 min after challenge.

The collective data from the in vitro studies included in Table 2, the increased in vivo efficacy of TBC-1269 as compared with TBC-265, and the ability of both molecules to reduce the EAR make it likely that the primary target of these molecules is either P- or L-selectin, rather than E-selectin, which is maximally expressed 2 to 4 h after the initiation of the inflammatory response. To investigate this further, we next tested a small molecule selectin binding inhibitor that was 2- to 3-fold more effective at blocking L-selectin than either P- or E-selectin binding to sialyl-Lewis (sLex) glycolipids (Table 2). A secreted, based on the relative molecular mass (Mr) and in vitro efficacy data, both a 5-mg and a 2.5-mg dose of MBPA aerosol administered 30 min before challenge gave a profile similar to TBC-1269, significantly inhibiting all three allergen-induced responses (Figures 4A and 4B, and Table 1). The protection was lost if the dose of MBPA was reduced to 1 mg (Table 1).

Although the data with the small molecule inhibitors suggest that L-selectin is the primary target for these small molecules, more conclusive evidence is provided by the last series of studies where the sheep were treated with the anti-sheep L-selectin antibody, DU 1-29. A secreted from the data with the small molecule inhibitors, 10 mg DU 1-29 given 30 min before challenge as an aerosol significantly reduced the EAR and completely blocked the LAR and AHR after antigen challenge (Figures 5A and 5B). Treatment at 90 min postchallenge effectively blocked the LAR and the 24-h AHR that are induced by allergen challenge in allergic sheep. Specifically, this protective effect appears to be a function of inhibiting L-selectin because the nonoligosaccharide sLex mimetic, TBC-1269, and the smaller carbohydrate-free compound, MBPA, are more potent L- than E-selectin inhibitors and because this protection can be reproduced with an anti-L-selectin antibody. The additional evidence showing a reduction in histamine release during the EAR with TBC-1269 provides novel evidence that L-selectin binding may also influence mediator release.

Earlier studies have indicated that selectins are involved in allergic inflammation (2, 8, 17). Circulating E-selectin levels were significantly raised in patients with acute asthma when compared with concentrations in patients with stable asthma, atopic normal, or nonatopic normal volunteers (17). E-selectin was detected in biopsy specimens by immunolocalization in the bronchial submucosa of asthmatic subjects with airflow limitation (18), and was increased in skin biopsies from allergic subjects taken 3 to 6 h after intradermal injection of specific antigen. This expression correlated with the development of inflammatory cell infiltrates (8). Likewise, airway allergen challenge in primates resulted in increased expression of E-selectin exclusively on vascular endothelium 6 h after challenge (2), at which time the animals had an increase in BAL neutrophils and a LAR. The antigen-induced LAR and the neutrophil influx were blocked by pretreating the primates with an anti-E-selectin antibody. The effect of blocking E-selectin on post-antigen-induced AHR was not studied in these primates, but was examined in mice where P-selectin-deficient mice, sensitized to ovalbumin were found to exhibit less airway responsiveness and cell trafficking following ovalbumin challenge than did wild-type mice (19). The results of the present study trial, whereas in the treatment trial, the values remained at 1 nM (range 0; p = 0.0375).

**DISCUSSION**

The results of this study provide novel evidence that small molecule selectin binding inhibitors can significantly reduce the EAR, the LAR, and the 24-h AHR that are induced by antigen challenge in allergic sheep. Specifically, this protective effect appears to be a function of inhibiting L-selectin because the nonoligosaccharide sLex mimetic, TBC-1269, and the smaller carbohydrate-free compound, MBPA, are more potent L- than E-selectin inhibitors and because this protection can be reproduced with an anti-L-selectin antibody. The additional evidence showing a reduction in histamine release during the EAR with TBC-1269 provides novel evidence that L-selectin binding may also influence mediator release.
extend the previous findings by suggesting that L-selectin plays an important role in modulating antigen-induced responses. The protection against these physiologic endpoints obtained with lower doses of TBC-1269 and M BPA (better L- than E-selectin inhibitors) as compared with TBC-265 (better E- than L-selectin inhibitor) suggests that L-selectin is the primary target for these small molecules when given before antigen challenge. That similar results were obtained after treatment with the anti-sheep L-selectin antibody, DU 1-29, confirms the role of L-selectin in these events. These data, however, do not rule out the possible contributions of E- and/or P-selectin. For example, the experiments showing that TBC-1269 was effective in blocking the L A R and A H R when given 90 min after antigen challenge, but not 240 min after antigen challenge, are consistent with the molecule’s inhibitory profile (i.e., E-selectin blocker) and the time course of E-selectin protein expression. The experiments showing protective effects of TBC-1269, when given 90 min after challenge do, however, demonstrate that inhibition of the L A R and A H R with TBC-1269 is not dependent on a reduction in the early antigen-induced response (see Figures 3A and 3B).

The ability of DU 1-29 and TBC-1269, when given before antigen challenge, to modify the EAR, provides new data on the putative role of L-selectin in the modulation of allergic airway responses. That inhibition of L-selectin binding can reduce acute antigen-induced cell activation is new, but is consistent with previous findings in this animal model using anti-VLA-4 inhibitors (7). Based on the physiologic response to antigen in the presence of these L-selectin inhibitors, one could speculate that binding of L-selectin affects signal transduction in a way that reduces the initial cellular response to antigen. The finding that BAL histamine levels obtained during the EAR were reduced in treated animals supports this hypothesis.

The lavage data obtained with and without TBC-1269 pre-treatment confirm the inhibitory effect on recruited leukocytes with the treated animals showing a reduction in the numbers of inflammatory cells recovered in BAL L. Our result of decreased neutrophil numbers at 6.5 and 24 h after challenge confirms and extends the findings with primes using an E-selectin antibody. Our current results support our previous findings in sheep in which we demonstrated that the development of the L A R and A H R is dependent on the influx of activated granulocytes into the airways (20). Our previous work has also shown that increases in BAL tissue kallikrein activity are associated with airway inflammation during the L A R and the post-antigen-induced A H R (15). Here, we show for the first time that a selectin inhibitor blocks the antigen-induced increases in tissue kallikrein activity in BAL L, a finding consistent with the anti-inflammatory activity of these molecules.

A thorough present upon the surfaces of leukocytes rather than endothelial cells, the role of L-selectin in the development of allergen-induced response may be quite similar to that hypothesized for E-selectin. In fact, because SLE-mobilized L-selectin has been shown to be a ligand for E-selectin (21), it is possible that blockade of L-selectin might also result in blockade of E-selectin under some circumstances. However, since E-selectin has also been shown to bind to other glycoprotein ligands, this dual selectin blockade would probably be a disease- and tissue-specific phenomenon. Nevertheless, our results indicate that the physiologic abnormalities indicative of asthma can be modulated by inhibiting the binding of either L- or E-selectin to their natural cell-associated ligands. The results of this study provide the first evidence that aerosol administration of an L-selectin antibody or small molecule selectin inhibitors provides adequate protection against antigen-induced airway responses. Previous studies in this model have demonstrated similar efficacy using aerosol delivery for both monoclonal antibodies (4) and small molecule inhibitors to VLA-4 (7). The results of this study confirm and extend these previous observations and provide further evidence that local administration of such agents is a viable route for therapeutic administration of this class of compounds.

It is important to note that, while blocking effects of these agents are important to establish the role of selectins in allergic responses, the data in Table 1 showing dose-dependent effects of the different compounds on the physiologic responses, as well as the inability of the control antibody (M D 6) to block the EAR, L A R, and A H R, are important experiments as well. These negative studies indicate that appropriate drug levels of active compounds must be achieved for the desired effect and that inactive compounds do not give false-positive responses in the model.

In summary, we have presented evidence that blockade of selectin binding can prevent the pathophysiological responses to allergen inhalation. These findings suggest that blockade of L-selectin may provide the basis for a novel therapy to control the acute pulmonary inflammatory response in experimental asthma.

References
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