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E- and P-Selectins Are Essential for the Development of Cockroach Allergen-Induced Airway Responses

Nicholas W. Lukacs,1* Alison John,* Aaron Berlin,* Daniel C. Bullard,† Randall Knibbs,* and Lloyd M. Stoolman*

Peribronchial inflammation contributes to the pathophysiology of allergic asthma. In many vascular beds, adhesive interactions between leukocytes and the endothelial surface initiate the recruitment of circulating cells. Previous studies using OVA-induced airway hyperreactivity indicated that P-selectin, a member of the selectin family expressed by activated platelets and endothelium, contributed to both inflammation and bronchoconstriction. The current study used cockroach allergen (CRA), an allergen that induces asthmatic responses in both humans and mice, to further investigate the role of selectins in the development of peribronchial inflammation and airway hyperreactivity. P- and E-selectin mRNAs were detected in extracts of CRA-sensitized animals beginning shortly after intratracheal challenge with CRA. The P-selectin mRNA was transiently induced at early time points while up-regulation of the E-selectin mRNA was more prolonged. Mice with targeted deletions in E-selectin (E−/−), P-selectin (P−/−), and both genes (E−/−P−/−) showed 70–85% reductions in airway hyperreactivity, peribronchial inflammation, and eosinophil accumulation. The P−/− and E−/−P−/− groups showed the most profound reductions. The transfer of splenic lymphocytes from CRA-primed E−/−P−/− into naive wild-type (WT) mice produced the same level of airway hyperreactivity as transfers from CRA-primed WT into naive WT hosts, indicating that peripheral immunization was similar. The observed changes in the selectin-deficient animals were not related to inadequate sensitization, because CRA priming and challenge increased serum IgE levels. Furthermore, pulmonary Th2-type cytokines and chemokines in the E-selectin−/− and WT animals were similar. The findings indicate that both P- and E-selectin contribute to CRA-induced peribronchial inflammation and airway hyperreactivity. *The Journal of Immunology, 2002, 169: 2120–2125.

The severity of asthmatic pulmonary disease is dependent, in part, upon the intensity of the inflammatory response (1–3). Attenuation of leukocyte infiltration into the lung, especially eosinophils and lymphocytes, frequently correlates with clinical improvement and is a major therapeutic goal (4). In many vascular beds, adhesion molecules initiate leukocyte adhesion to the vascular endothelium. Chemokines produced locally then promote the arrest of leukocytes on the luminal surface and their migration into surrounding tissues. Both adhesion receptor-dependent and independent recruitment pathways have been identified in lung inflammation (5–8). In previous studies of allergic inflammation in the mouse, ICAM1/LFA-1, very late Ag-4/VCAM1, and P-selectin have been implicated in the development of both peribronchial inflammation and airway hyperreactivity (9–13). Additionally, early primate studies showed that blockade of E-selectin also reduced allergic pulmonary inflammation (8). Finally, increased expression of the E- and P-selectins has been observed in asthmatic patients (14–17), suggesting that these molecules are up-regulated in human disease and may contribute to the alteration of pathophysiology. Thus, the migration of leukocytes into an asthmatic lung is dependent upon multiple mechanisms that are initiated by the binding of leukocytes to the endothelial border to selectins. Blockade of the initial selectin-mediated adhesion event should inhibit subsequent migration into the lung during an allergen challenge.

The current study examined the contributions of the selectins to cockroach allergen (CRA)-induced airway inflammation and hyperreactivity. CRA is a potent allergen in both humans (18) and mice (19–22). The murine response to CRA resembles asthma in humans; thus, it compliments the classic murine OVA model of allergic inflammation. The findings herein indicate that both P- and E-selectin make highly significant, independent contributions to CRA-induced airway pathophysiology.

Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The E−/− and E/P-selectin−/− mice were bred and raised as previously described and backcrossed at least five generations onto the C57BL/6 strain and homozygotes were generated (23, 24).

Sensitization and induction of the airway response

C57BL/6 wild-type (WT) mice and selectin deletion mutants were sensitized and challenged with CRA as previously described (19, 20, 22). Briefly, mice were immunized with 10 μg of CRA (Bayer, Elkhart, IN) in IFA on day 0. On day 14, 10 μg of CRA, in 10 μl of sterile saline, was administered intranasally (IN) to enhance the immunologic response in the airway. Mice were then challenged 6 days later by intratracheal (IT) administration of 10 μg of CRA in 50 μl of sterile saline.

RT-PCR for E-selectin and P-selectin

Whole lung mRNA was isolated using a standardized TRIZol method of phenol extraction. Total RNA was determined by spectrometric analysis at 260 nm.
260 nm wavelength. Five micrograms of total RNA was reversed transcribed into cDNA using a reverse transcription kit (Life Technologies, Rockville, MD) and oligo(dT) 

12-18 primers. The primers used were 5'-tgctggcagcaggaaggaa-3' (sense) and 5'-actgagggttgctctggtg-3' (antisense) for β-actin, 5'-tgctggcagcaggaaggaa-3' (sense) and 5'-actgagggttgctctggtg-3' (antisense) for E-selectin, and 5'-agggacacttacagact-3' (sense) and 5'-gctgtctttcacttctg-3' (antisense) for P-selectin. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 mM MgCl₂. Specific oligonucleotide primer was added (200 ng per sample) to the buffer, along with 1 μl of the reverse transcribed cDNA samples. The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 5 min at 94°C then was cycled 30 times at 95°C for 30 s and 58°C for 45 s, and elongated at 72°C for 75 s. This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA. After amplification, the sample (20 μl) was separated on a 2% agarose gel containing 0.3 μg/ml (0.003%) ethidium bromide. The bands were visualized and photographed using UV transillumination.

**Morphometric analysis of airway and peribronchial eosinophil accumulation**

Eosinophil accumulation was assessed in fixed (4% paraformaldehyde) tissues at various time points after challenge. The fixed lungs were embedded in paraffin, sectioned at 5 μm, stained with Wright-Giemsa, and examined at a magnification of ×1000. Eosinophil counts were performed on 100 high-powered fields per lung at each time point. The fields covered the bronchial and peribronchial tissues because inflammation is restricted to these locations in the CRA model.

**Measurement of airway hyperreactivity**

Airway hyperreactivity was measured using a Buxco mouse plethysmograph (Buxco, Troy, NY) that is specifically designed for the low tidal volumes of mice (19, 20, 22, 25). Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was subsequently ventilated with a Harvard pump ventilator (tidal volume = −0.2 ml, frequency = 120 breaths/min, positive end-expiratory pressure = 2–2.5 cm H₂O) and the tail vein was cannulated with a 27-gauge needle for injection of the methacholine challenge. The plethysmograph was sealed and readings were monitored by computer. The trachea transducer was calibrated at a constant pressure of 20 cm H₂O. Resistance is calculated by the Buxco software by dividing the change in pressure (P) by the change in flow (F) (ΔP/ΔF, units = cm H₂O/ml/sec) at two time points. The mouse was attached to the box and ventilated for 5 min before acquiring readings. Once baseline levels were stabilized and initial readings were taken, a methacholine challenge (optimal dose, 100 μg/kg) was given via the cannulated tail vein. This dose induced little change in resistance in normal, nonsensitized mice. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyperreactivity.

**Quantitation of cytokines by specific ELISA**

The levels of cytokine and chemokine proteins in whole lung homogenate and from cell-free supernatants were measured by specific ELISA. The IL-5, IL-13, and C10 Abs were purchased and pretested by the company (R&D Systems, Rochester, MN). Briefly, lung tissue was homogenized on ice using a tissue-tearor (Biospec Products, Racine, WI) for 30 s in 1 ml of PBS containing 0.05% Triton X-100. The resulting supernatant was isolated following centrifugation (10,000 × g). Flat-bottom 96-well microtiter plates (Immunoplate 196-F; Nunc, Roskilde, Denmark) were coated with 50 μl/well rabbit polyclonal Abs, specific for the cytokine/chemokine in question, for 16 h at 4°C and then washed with PBS and 0.05% Tween 20. Nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer and cell-free supernatants were added ( neat and 1/10) followed by 100 μl/sample. The plates were washed four times and a secondary, biotinylated cytokine-specific Ab was added for 30 min, followed by four washes. In a final step, streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again and chromogen substrate (Bio-Rad) was added and incubated at room temperature to the desired extinction. The reaction was terminated with 50 μl/well 3 M H₂SO₄ solution and the plates were read at 490 nm in an ELISA reader. Standards were 0.5-log dilutions of recombinant protein from 1 pg/ml to 100 ng/ml. The ELISAs with purchased reagents were sensitive to 10 pg/ml.

**Cell transfer studies**

Splenic lymphocytes were teased into suspension, suspended in medium (RPMI 1640 with 10% FCS), and incubated in a T75 flask for 1 h at 37°C. The nonadherent (lymphocyte-enriched) cell populations from CRA-primed E/P and WT animals were infused (2 × 10⁷ cells per animal) into naive WT C57BL/6 mice. After 24 h, the recipients were challenged with CRA (IT) and airway hyperreactivity was assessed 24 h later.

**Statistics**

Statistical significance was determined using ANOVA with p values < 0.05.

**Results**

**E-selectin and P-selectin mRNA levels increase following CRA challenge**

The inflammatory and airway hyperreactivity responses of CRA-sensitized and IT-challenged mice have been reported (20, 21, 26, 27). In brief, an eosinophil-rich submucosal and peribronchial inflammatory infiltrate develops within 8–12 h, peaks at 24 h, and resolves 72 h after IT challenge of CRA-sensitized mice. The response to methacholine administration is increased for 8–48 h after IT challenges with CRA. In view of this time course, RT-PCR for P-selectin and E-selectin were performed on whole lung extracts beginning 4 h after IT challenge with CRA (Fig. 1). The P-selectin signal was detected at both the 4- and 8-h time points and fell below detectable levels by the 24-h time point. E-selectin expression peaked at the 4- and 8-h time points and remained detectable at the 24-h time point. Thus, both E- and P-selectin are up-regulated during CRA-induced airway responses.

**Airway hyperreactivity is attenuated in selectin-deficient animals**

Selectin-deficient animals, backcrossed five generations onto the C57BL/6 strain, were used to evaluate the contributions of the selectins to the development of airway hyperreactivity. In these experiments, WT C57BL/6 and selectin-deficient animals were “sensitized” to CRA with a single i.p. dose followed 14 days later by a single IN dose of the purified Ag in sterile saline. One week after the IN dose, animals received an IT dose of CRA followed by measurements of methacholine-induced bronchoconstriction at 24 h after challenge. The E-selectin-deficient (E-/-) and P-selectin-deficient (P-/-) mice showed 70 and >85% reductions in airway resistance when compared with WT mice 24 h after IT challenge (Fig. 2A). Double deficient mice (E-/-P-/-), examined in separate experiments, showed reductions of ~85% and ~75% in airway hyperreactivity 24 and 48 h, respectively, after IT challenge with CRA (Fig. 2B). There was no difference in airway resistance between unchallenged WT control and any of the selectin−/- mice (data not shown).

**Bronchial and peribronchial inflammation is attenuated in selectin-deficient animals**

The severity of asthmatic responses in humans and hyperreactivity responses in mice is directly related to the intensity of the inflammatory response, particularly the accumulation of eosiinophils. In

![FIGURE 1](http://www.jimmunol.org/)

Up-regulation of E- and P-selectin mRNA expression during allergen-induced airway inflammation. CRA-sensitized C57BL/6 mice were challenged IT with allergen and the whole lung mRNA was isolated at various time points. The isolated mRNA was reverse transcribed and subjected to primer-specific PCR for E- and P-selectin mRNA.
is the rise in serum levels of IgE following i.p. sensitization with the Ag. ELISA studies showed equal levels of IgE in WT and E−/P− animals, indicating that Th2 cytokine-mediated isotype switching was not altered by deletion of the selectins (Fig. 6A). We then further investigated whether the local immune response differed between WT and E-selectin mutant mice at 8 h postallergen challenge (peak of cytokine production). Whole lung homogenates demonstrated that E-selectin−/− mice displayed similar levels of Th2-associated cytokines, but interestingly the E-selectin−/− mice displayed higher levels of eosinophil-associated chemokines, eotaxin, and C10 (Fig. 6B). Thus, although the lungs of the selectin−/− mice had significant Th2 responses and local cytokine and chemokine levels, they demonstrated a decrease in eosinophil accumulation.

**Discussion**

CRA induces pulmonary asthmatic-type responses in both humans and mice. In humans, CRA may be particularly important in the growth of severe bronchial asthma among inner-city children (18, 28, 29). In susceptible humans and animal species, allergens induce Th2-driven production of allergen-specific IgE, airway hyperreactivity, peribronchial inflammation, and direct epithelial cell damage (2, 3, 30, 31). Airway hyperreactivity is a pathophysiological response to multiple stimuli released from peribronchial inflammatory cells. Eosinophil recruitment is prominent throughout the response to allergens, and mediators released by these cells foster the development of irreversible airway remodeling (32, 33). Lymphocytes and APCs contribute through the Th2-driven production of allergen-specific IgE Abs in secondary lymphoid organs. In addition, T cells, B cells, and monocyte-derived macrophages account for a significant portion of the peribronchial inflammatory cells in the CRA model and may contribute directly to airway pathophysiology (19, 20, 27).

The current study demonstrated that IT CRA in sensitized mice up-regulated the expression of both P-selectin and E-selectin mRNA during the period of maximal leukocyte influx. Furthermore, gene-targeted deletions in P-selectin, E-selectin, or both genes markedly reduced the development of airway hyperreactivity and peribronchial inflammation in response to CRA. Eosinophil counts fell significantly and histological evaluation indicated that lymphocyte- and monocyte-derived macrophage accumulation decreased as well. The successful transfer of the CRA-induced pulmonary responses by splenocytes from primed E−/P− mice indicated that splenic immunologic responses occurred normally in the selectin-deficient animals. In addition, the serum IgE production reached the same levels in the selectin-deficient and WT animals, indicating that the Th2-driven isotype switch was unaffected by the genetic alterations. The CRA-primed, E-selectin-deficient mice developed WT levels of pulmonary Th2 cytokines following IT CRA, implying that recruitment of Th2 cells (or their precursors) is not E-selectin dependent in this model. This finding is consistent with the low level of selectin ligands expressed on polarized Th2 cells (34, 35). The reduced inflammation in E-selectin-deficient mice is not due to a chemokine deficiency, because these animals expressed higher levels of eotaxin and C10, which may be a result of lower consumption locally because inflammation was reduced. Consequently, selectins are essential for the development of peribronchial eosinophilic inflammation and airway hyperreactivity induced by IT CRA in sensitized animals.

These results indicate that E-selectin contributes to allergen-induced inflammatory responses in the CRA model. The reduced accumulation of eosinophils in the E-selectin-deficient animals was unexpected because purified eosinophils interact primarily
with P-selectin and VCAM1 in vitro (36–39). However, the literature suggests that adhesion receptor use is broader in vivo. In ragweed-induced peritonitis, >90% of the eosinophil accumulation was P-selectin dependent (40). In contrast, 111In-labeled eosinophil trafficking during active cutaneous anaphylaxis induced by OVA used both P-selectin and E-selectin (41). Local availability may determine selectin use in these models because endogenous leukocyte rolling was P-selectin dependent in the former (40) and P-/E-selectin dependent in the latter (42). Thus, eosinophils may use both vascular selectins in vivo despite their preference for P-selectin in vitro.

In the CRA model, P-selectin mRNA was transiently expressed early in the allergen challenge. In contrast, E-selectin mRNA expression was prolonged and appeared to be at a significantly higher level than P-selectin. This pattern differs from the prolonged induction of P-selectin mRNA observed in Ag-sensitized mice following IT challenge with SRBCs (43), cutaneous challenge with oxalazone (44), or intrascrotal challenge with OVA (42). It more closely resembles the pattern observed in the OVA-induced active cutaneous anaphylaxis model in the skin (42). As a consequence, P-selectin may control leukocyte recruitment initially, with E-selectin predominating once P-selectin levels fall to baseline. Alternatively, both selectins may be necessary for optimal recruitment throughout the first 24 h of the response. In either case, the transient, low-level expression of P-selectin coupled with the prolonged, high-level expression of E-selectin following IT CRA

![FIGURE 3](image_url). Peribronchial inflammation is reduced in E-, P-, and E/P-selectin mice compared with WT mice. Lungs from allergen-sensitized and IT-rechallenged mice were inflated, fixed with 10% buffer formalin, and processed for histological analysis. The illustration represents inflammation in the various groups of mice as indicated. There appears to be an overall decrease in peribronchial inflammation.

![FIGURE 4](image_url). Enumeration of peribronchial eosinophil accumulation in allergic mice is attenuated in selectin-deficient allergic mice. Histological sections from allergen-sensitized and -rechallenged mice were subjected to morphometric analysis of peribronchial eosinophil accumulation. Data represent mean ± SE from six mice per group (p < 0.01).
challenge in WT animals supports the hypothesis that both adhesion receptors contribute to leukocyte recruitment in this model.

The deletion of one or both vascular selectins markedly reduced eosinophil accumulation 24 h after IT CRA, implying that VCAM1 was not a major factor during the initial period of recruitment. This result is consistent with previous studies showing that the contributions of the selectins and VCAM1 are time-dependent in allergen-induced inflammation. In the pulmonary response to aerosolized OVA, eosinophil accumulation was reduced by 70–90% in P-selectin-deficient animals when measured 3 h after the last in a series of aerosol challenges with OVA (45, 46).

At later time points, eosinophil accumulation approached the levels in WT control animals, implying that other adhesion receptors supplanted P-selectin over time. VCAM1 is clearly one of these receptors, because a 95% reduction in VCAM1 expression (45) or CD49d blockade of circulating leukocytes (47) completely inhibited eosinophil recruitment in the OVA model. Interestingly, Randolph et al. (48) found that Th1 recruitment preceded the development of Th2-driven VCAM1 expression and eosinophilic inflammation in the OVA model. Th1 cells generally express high levels of selectin ligands (35, 49), and selectin-mediated T cell trafficking into the airway occurs during the pulmonary immune response to IT Ag challenge (5, 43). Therefore, in the CRA model, selectins may contribute to eosinophil accumulation directly or indirectly through recruitment of Ag-primed effector T cell populations. In either case, the current findings imply that both E-selectin- and P-selectin-mediated leukocyte recruitment is necessary for the development of airway hyperreactivity following challenge with CRA. Nonetheless, the current findings show that selectin-mediated leukocyte recruitment into peribronchial tissues enhances airway hypersensitivity during the acute response to CRA challenge.

References

![FIGURE 5](http://www.jimmunol.org/DownloadedFrom)  
**FIGURE 5.** Allergen-induced airway hyperreactivity can be transferred by splenic lymphocytes from WT and E–/P-selectin mice. Nonadherent splenic mononuclear cells were isolated from allergen-sensitized mice and injected into naive C57BL/6 mice via the tail vein (2 x 107/mouse). Mice were IT challenged with CRA and examined for methacholine-induced airway hyperreactivity 24 h postallergen challenge. The data represent mean ± SE from five mice per group. Transfer of cells from spleens of naive mice does not induce a hyperreactive response to methacholine.

![FIGURE 6](http://www.jimmunol.org/DownloadedFrom)  
**FIGURE 6.** Serum IgE levels and local cytokine and chemokine levels are not attenuated in selectin–/– mice. Allergen-sensitized mice were given an IT CRA challenge. After 24 h serum was collected from five animals in each group of allergen-challenged animals and analyzed for serum IgE levels using a specific ELISA (A). After 8 h (the time of peak cytokine production) the lungs from five mice per group were removed, homogenized in buffer (see Materials and Methods), and assayed for IL-5, IL-13, eotaxin, and C10 (B). Results represent the mean ± SE.


