Reduced Recruitment of Inflammatory Cells in a Contact Hypersensitivity Response in P-Selectin-deficient Mice

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Summary

The inflammatory response at sites of contact hypersensitivity induced by oxazolone was examined in the ears of P-selectin-deficient and wild-type mice. Accumulation of CD4$^+$ T lymphocytes, monocytes, and neutrophils was reduced significantly in the mutant mice, as well as mast cell degranulation. In contrast, there was no significant difference in vascular permeability or edema between the two genotypes. The results demonstrate a role for P-selectin in recruitment of CD4$^+$ T lymphocytes and show that P-selectin plays a role in long-term inflammation as well as in acute responses.

Leukocyte recruitment to sites of inflammation involves several steps: Leukocytes initially tether and roll on the activated endothelium; they subsequently become activated and bind firmly through leukocyte integrins; and they emigrate into surrounding tissues. Leukocyte rolling is a weak, reversible interaction mediated by the selectins, which appear to have overlapping functions (1–3). In the initial phase, P-selectin is the primary mediator of leukocyte rolling (4). L-selectin that is present on the leukocytes is also involved in rolling but most likely in a later time frame (5). E-selectin is expressed on activated endothelium hours after the onset of inflammation and may participate in rolling, as demonstrated recently in vitro (6).

The role of P-selectin in recruitment of neutrophils and monocytes has been studied in chemical peritonitis, acute lung injury, and ischemia reperfusion (3). However, little is known about its function in lymphocyte-mediated chronic inflammation in models such as contact hypersensitivity (CH), a form of delayed type hypersensitivity. In this cutaneous inflammatory model, the sensitizing agent penetrates the skin and binds Langerhans'/dendritic cells. These cells act as antigen-presenting cells and activate the T lymphocytes in the local lymph node to undergo proliferation (7). Upon subsequent challenge with the same antigen, the primed CD4$^+$ T lymphocytes, assisted by a subset of CD8$^+$ lymphocytes (7, 8), mount a CH response at the site of challenge by secreting several cytokines/lymphokines (7, 9). Some of these cytokines activate the endothelium to express adhesion molecules or their ligands (3), which help to recruit monocytes, more lymphocytes, and neutrophils to the site of challenge. The recruited macrophages further amplify the reaction by secreting TNF-α and IL-1 (10), which increase the expression of P-selectin, E-selectin, the ligand for L-selectin, intracellular adhesion molecule 1, and vascular cell adhesion molecule on the surface of endothelial cells (3). Histamine and serotonin released by mast cells and possibly other cells such as platelets (11, 12) may be important in the early phase of the reaction. Histamine and serotonin cause release of P-selectin from the Weibel-Palade bodies (13, 14), and this also induces leukocyte rolling in vivo (15, 16).

Antibodies directed against α_4 integrins and LFA-1 reduced the CH response (17, 18). Mice deficient in intracellular adhesion molecule 1 also have a reduced CH response (19). Similarly, antibodies against E-selectin and L-selectin diminished the recruitment of lymphocytes in delayed type hypersensitivity (20, 21). There are several reasons to think that P-selectin may also play a role in the CH response. P-selectin expression is regulated by various mediators present in the CH response, and it mediates adhesion of neutrophils, monocytes, and subsets of lymphocytes, all of which contribute to the inflammatory infiltrate in a CH reaction. P-selectin is partially responsible for rolling of CD4$^+$ T lymphocytes in vitro (22), and it is likely that lymphocytes, like other leukocytes, have to roll on the activated endothelium to infiltrate tissues. Since the rolling of leukocytes is practically absent in P-selectin-deficient mice (4), we decided to use the P-selectin-deficient mice to study the role of P-selectin in CH.

Materials and Methods

Mice. 2- to 5-mo-old 129Sv/C57BL wild-type or P-selectin-deficient females (4) were housed in the animal facilities at Massachusetts Institute of Technology (Cambridge, MA) or Tufts-New England Medical Center (Boston, MA).

Induction of CH to Oxazolone. On day 0, mice were painted on the inner surface of both hind legs with 25 μl oxazolone (Sigma Chemical Co., St. Louis, MO) (0.1 g/ml in 4:1 vol/vol acetone/olive oil); control animals received 25 μl of acetone/olive oil. The mice were challenged on day 5 with 5 μl of oxazolone on the inner side of the left pinna and vehicle on right pinna. 2 μCi [125I]iododeoxy-uridine (5.65 μCi/mg, Amersham Co., Arlington Heights, IL), in 0.1 ml PBS was injected into the tail vein 8 h after challenge (23), or 0.2 μCi 125I-albumin (125I-BSA, 1.59 μCi/mg, ICN Biomedicals, Inc., Costa Mesa, CA) (24) injection was given 16 h after challenge. 24 h after challenge, the mice were killed, and pinnae were cut off at the hairline and counted. This time point was chosen to reflect specific recruitment of inflammatory cells in response to the contact allergen (7). Ear thickness was measured in mice anesthetized with metafane (methoxyflurane; Pitman-Moore, Mundelein, IL), at 0 and 24 h after the challenge, using an engineer's micrometer.

Histology. Ears were cut in half longitudinally, and one-half was fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The other half was frozen with optimum cooling temperature compound (Miles, Inc., Diagnostic Division, Elkhart, IN), and sections were stained for CD4+ lymphocytes with rat anti–mouse antibody L3T4 (American Type Culture Collection, Rockville, MD) and for macrophages with F4/80 antibody (American Type Culture Collection) using the Biotin-Streptavidin biotin system (Zymed Laboratories Inc., South San Francisco, CA) and Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Serial sections from the same ears were stained with isotype-matched biotinylated rat IgG2b antibody as control (Phar-Mingen, San Diego, CA). Neutrophils and mast cells were stained in paraffin-embedded sections for a specific esterase, using the naphthol AS-D chloroacetate esterase kit (Sigma Chemical Co.). Cells were counted throughout the entire section. All of the sections were examined independently by two investigators. The focal infiltrates in the epidermis were ellipsoid in shape. The length and width of each focal infiltrate was measured using a linear grid on a light microscope (BX40F; Olympus Corp., Lake Success, NY) in the H&E sections. The area of each focal infiltrate was calculated (area of an ellipsoid = L/2 × W/2 × π).

Results

To elicit contact hypersensitivity, wild-type and P-selectin-deficient mice were sensitized with oxazolone, and 5 d later they were challenged with the same substance on their left ear. The right ear was painted with vehicle. 24 h after the challenge, the ears of these mice were examined for mononuclear cell and neutrophil infiltration and for vascular permeability.

Infiltration of Radiolabeled Mononuclear Cells. Dividing monocytes and lymphocytes were labeled with [125I]iododeoxyuridine 8 h after challenge (23), and infiltration of labeled cells at the site of the contact hypersensitivity reaction was determined 24 h after challenge by subtracting the counts in nonchallenged ears from those in challenged ears. The mutant animals had 53.8% lower infiltration (P <0.017) of radiolabeled mononuclear cells (Fig. 1). Animals challenged with
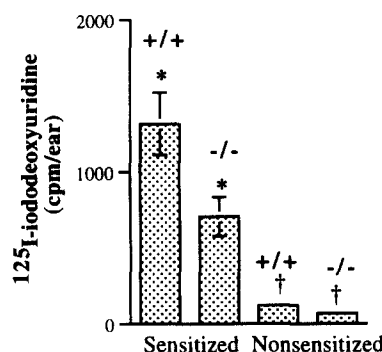

Figure 1. Infiltration of radiolabeled mononuclear cells. Oxazolone-sensitized wild-type (+/+) and P-selectin–deficient (−/−) mice were challenged 5 d later on their left ears. [125I]Iododeoxyuridine was injected to label dividing mononuclear cells. 24 h after the challenge, the infiltration of radiolabeled cells into the ears of the sensitized and nonsensitized mice was determined; counts of the nonchallenged ears were subtracted from those of the challenged ears. Bars, mean ± SEM for each animal group. *P <0.017, n = 11 for +/+ and n = 13 for −/−; †P = 0.252, n = 4 for +/+ and n = 5 for −/−.

oxazolone without prior sensitization showed much lower (<10%) infiltration of radiolabeled cells than did the sensitized animals (Fig. 1), and there was no statistical difference between wild-type and mutant animals.

Vascular Permeability. Vascular leak in delayed type hypersensitivity can be demonstrated by the local leakage of systemically injected radiolabeled albumin into the tissues (24). Since the vascular permeability in sensitized animals is reported to be maximum between 12 and 24 h (25), iodinated albumin was injected in the tail veins of mice 16 h after challenge, and both ears were counted 24 h after challenge. The leakage of radiolabeled albumin in mutant animals was 18.5% lower than in wild-type animals, but the difference was not statistically significant. Ear swelling was also measured using a micrometer before and 24 h after challenge. Change in thickness of ears before and after challenge was 2.55 ± 0.017 × 10⁻² mm (n = 17) for the wild-type mice and 2.33 ± 0.016 × 10⁻² mm (n = 18) for the mutants. The small difference in swelling (9%) was not statistically significant.

Histology of Ear Sections. The most notable feature of sections taken 24 h after challenge was the appearance of dense focal neutrophil infiltrates (confirmed by specific esterase staining) in the epidermis. These were less numerous and significantly smaller in the mutant than in wild-type mice (Fig. 2, *b, a*). Since the infiltrates were partially necrotic, it was not possible to count individual neutrophils. Therefore, the area of the focal collections was measured along the entire ear length and was 6.6 times smaller in the mutant mice (Fig. 3). The control nonchallenged ears had no focal infiltrates (Fig. 2, *c, d*). Nonsensitized mice had no or very few of these neutrophil clusters, ruling out the possibility that the neutrophil recruitment was an irritant response (Fig. 3). Necrosis of the epidermis was seen in the wild-type mice but was less prominent in the mutant mice. Neutrophil infiltration was also noted in the dermis (Fig. 2) but was more diffuse than in the epidermis. To determine neutrophil numbers in the
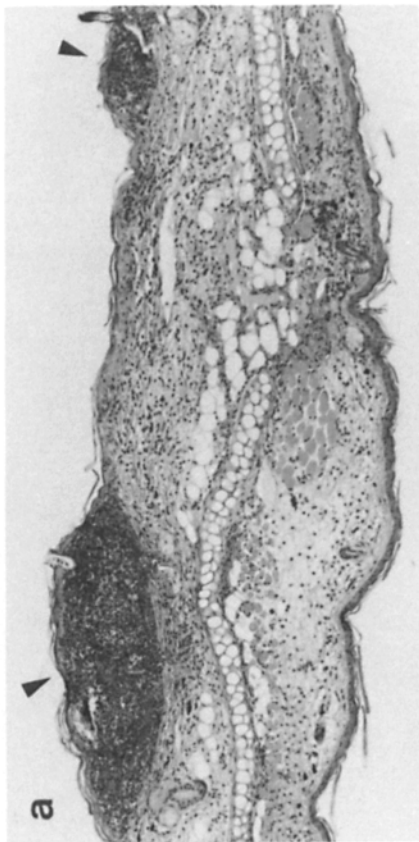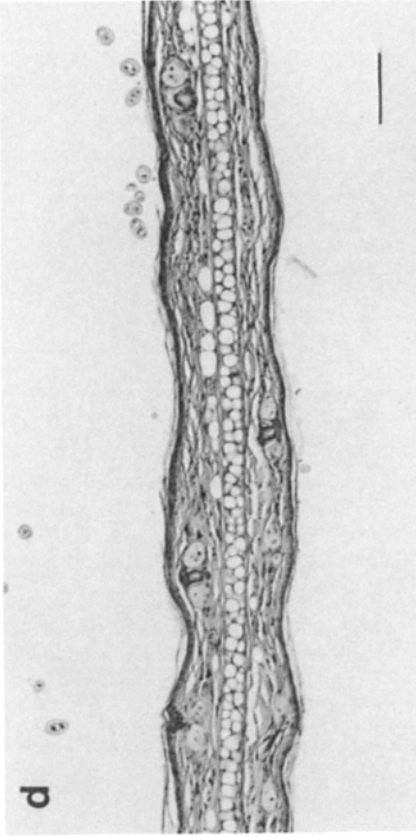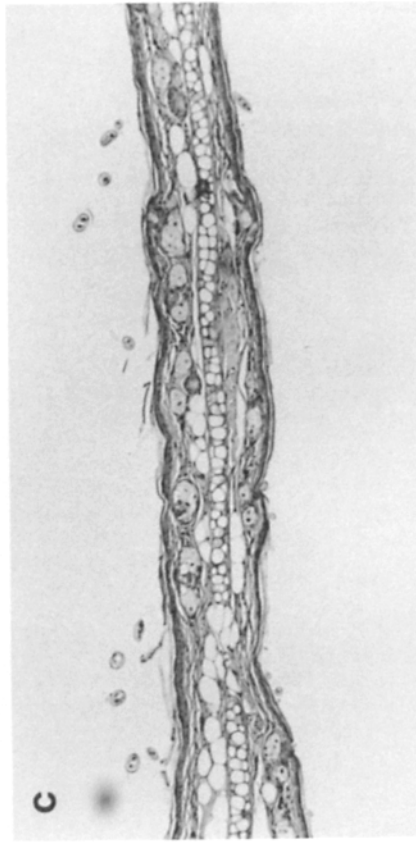
Figure 2. Histology of ear sections. Dense focal neutrophil infiltrates (*arrows*) were smaller in the challenged ears of the sensitized P-selectin–deficient mice (−/−) (*b*) as compared with the wild-type mice (+/+) (*a*). Necrosis of the epidermal lining over these infiltrates was seen in the wild-type mice and to a lesser extent in the mutants. The control, nonchallenged ears of the sensitized wild-type (*c*) as well as the mutant animals (*d*) had no focal infiltrates. Bar, 100 μm.
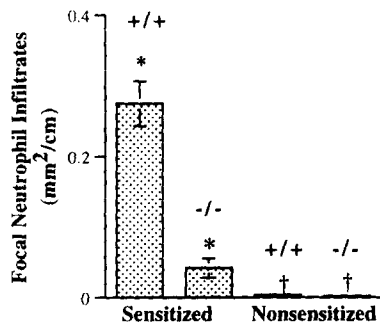
Figure 3. Focal neutrophil infiltrates in epidermis. The area of focal neutrophil infiltrates was measured along the entire ear length in H&E stained sections (see Fig. 2). Bars, mean ± SEM for each group of four animals. *P <0.001; †P = 0.696 (by Student's t test).

Figure 4. CD4+ T lymphocyte infiltration of the dermis. Frozen sections of challenged ears from sensitized animals were stained using biotinylated antibody to CD4+. Bars, mean ± SEM n = 10 for +/+, n = 9 for −/−). P <0.046 (by Student's t test).

dermis, paraffin sections were stained with a specific esterase stain that identifies neutrophils and mast cells; the mast cells are larger and have a central nucleus. The challenged ears of the mutant mice had 47.7% fewer neutrophils in the dermis than did wild-type mice (Table 1). We also examined whether mast cell degranulation differed in the two genotypes. The specific esterase stain identifies the granules of mast cells (26); therefore, it is likely that degranulated mast cells would not stain. Numbers of mast cells stained were lower in both genotypes in the challenged ears than in control nonchallenged ears (Table 1). However, the wild-type mice had significantly more degranulation, as their numbers of positive mast cells were 1.6 times lower than in the mutants. Some degranulation was also observed in the challenged ears of the nonsensitized animals but was less compared with sensitized animals. There did not appear to be any degranulation in nonchallenged ears of the sensitized and the nonsensitized animals (Table 1).

The mononuclear cells measured by the incorporation of [125I]iododeoxyuridine into dividing cells include both lymphocytes and monocytes (23). Monocytes/macrophages were
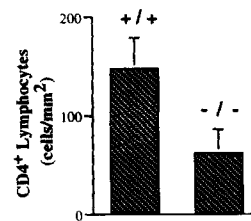
also stained with F4/80 antibody (27). Reduced numbers were seen in the mutant mice (data not shown), but the precise numbers could not be determined because of high nonspecific background. Since CD4+ T lymphocytes are the effector cells in the contact hypersensitivity reaction, their influx into the inflammatory site was determined with an mAb to CD4+. Parallel sections were stained with isotype-matched antibodies, and nonspecifically stained cells were counted and subtracted. The number of CD4+ lymphocytes infiltrating the dermis of the challenged ears of sensitized P-selectin-deficient mice was 2.3 times lower (P <0.046) than that in the sensitized wild-type mice (Fig. 4).

Discussion

Previously, our laboratory has demonstrated severe impairment in leukocyte/endothelial interaction in P-selectin-deficient mice and delayed neutrophil recruitment in acute peritonitis (4). These results imply that recruitment of leukocytes may be altered in other inflammatory models, such as delayed hypersensitivity reaction, a condition of clinical relevance. Therefore, we studied a well-established murine model of CH in P-selectin–deficient mice. We observed that both mononuclear cells and neutrophils were diminished in the challenged ears of the sensitized P-selectin–deficient mice as compared to wild-type mice. Most interestingly, numbers of CD4+ T lymphocytes in challenged ears of the P-selec-

Table 1. *Dermal Neutrophils and Mast Cells*

		Sensitized		Nonsensitized	
Genotype	Cell type	Challenged ear	Nonchallenged	Challenged ear	Nonchallenged
+/+	Neutrophils	622 ± 23*	12 ± 3	211 ± 125	8 ± 3
		(n = 4)	(n = 12)	(n = 4)	(n = 4)
−/−	Neutrophils	296 ± 54*	6 ± 0.7	162 ± 32	9 ± 6
		(n = 4)	(n = 13)	(n = 2)	(n = 3)
+/+	Mast cells	30 ± 3‡	64 ± 7	40 ± 8	64 ± 13
		(n = 12)	(n = 10)	(n = 5)	(n = 5)
−/−	Mast cells	49 ± 6‡	74 ± 7	69 ± 6	63 ± 8
		(n = 12)	(n = 12)	(n = 5)	(n = 4)

Paraffin-embedded sections of ears were stained with a specific esterase. The positive cells in all microscopic fields per tissue section were counted. Numbers represent cell count per square millimeter ± SEM. n indicates number of animals examined. *P <0.001 by Student's t test; ‡P <0.008 by Student's t test.

tin-deficient mice were half of that seen in the wild-type mice (Fig. 4). It is very likely that, like neutrophils, lymphocytes follow the multistep attachment process involving selectins and integrins (2). L- and E-selectin are used by lymphocytes for homing into lymph nodes and skin, respectively (28, 29). In another study, rolling of CD4$^+$ lymphocytes on TNF-α–stimulated endothelium in vitro could be partially blocked by anti–P-selectin antibodies but not by E- or L-selectin antibodies (22). Although in vitro studies have shown that subsets of T lymphocytes bind P-selectin (3), its role in the actual migration to the sites of inflamed tissues has not been studied. In this study, we have demonstrated for the first time the role of P-selectin in tissue migration of CD4$^+$ lymphocytes. Since the migration of CD4$^+$ lymphocytes into tissues was not completely blocked in the P-selectin–deficient mice, it is likely that other adhesion molecules are being used.

A marked difference in the infiltration of neutrophils between the two genotypes was also observed in the present CH model. The epidermal focal infiltrates were sixfold higher and the dermal infiltrates were twofold higher in the wild-type mice. The greater severity of the epidermal reaction, in contrast to the dermal reaction, was probably due to the epicutaneous application of the sensitizing agent. In fact, the inflammation of the epidermis was seen primarily on the ventral side, where oxazolone was applied. The murine CH reaction, unlike the human prototype, has greater neutrophil infiltration (30). It is possible that there is a species difference in section/up-regulation of distinct cytokines or chemokines in the CH reaction. It is known that cytokines and chemokines preferentially recruit different cell types (2).

We expected to see less edema in the P-selectin–deficient mice, since tissue infiltration of all inflammatory cells was reduced. However, only a marginal, nonsignificant decrease in edema was observed in the mutant animals. There are several possible reasons why the P-selectin–deficient mice did not show protection against edema. Although the mononuclear cells and neutrophils recruited in the mutant mice are fewer than in wild-type mice, it is possible that the numbers are sufficient to increase vascular permeability and that further recruitment in wild-type mice does not increase leakage any further. Alternatively, the two processes, leukocyte migration and leakage, could be independent of one another (31).

Since mast cell mediators, like histamine and serotonin, up-regulate P-selectin by releasing Weibel-Palade bodies (13, 14), we evaluated mast cell release in the CH reaction. In both genotypes, the nonchallenged ears had equivalent numbers of mast cells. The challenged ears of the wild-type mice had significantly fewer positive mast cells than did the nonchallenged ears. This was observed in the mutant mice as well, but to a significantly lesser extent, indicating that more mast cells degranulated in the wild-type mice. The more intense inflammatory response in the wild-type mice may be responsible for this observation. The actual role of mast cells in the development of contact hypersensitivity is not clear, because mast cell–deficient mice do not show in abnormal CH response (32). It is possible that the normal response in the mast cell–deficient mice may be due to an alternate source of serotonin from other cells like platelets (12).

P-selectin has been categorized as a molecule involved in acute inflammation because it is expressed on the surface within minutes after activation of endothelial cells (13). The results presented here clearly show an effect of the absence of P-selectin on a long-term inflammatory response. It is conceivable that this late effect reflects an earlier defect. For instance, P-selectin might play some role in the sensitization phase. Alternately, it is possible that the general reduction in cellular recruitment we observed 24 h after challenge was due, at least in part, to defective infiltration in the first hours of the response. Reduced numbers of recruited cells in the first hours, due to lack of P-selectin expression, might release lower amounts of cytokines and chemotactic proteins and thus result in lesser endothelial activation and lower additional cellular recruitment. It is also possible that, in the absence of P-selectin, the mononuclear cells are not fully activated, as P-selectin was recently shown to regulate cytokine secretion by human monocytes (33). On the other hand, since P-selectin is known to be transcriptionally regulated by cytokines (34), it is very likely to contribute directly to the late recruitment. Moreover, we have shown that after surface expression in endothelial cells, P-selectin is endocytosed, and a portion of the molecules travels into nascent storage granules and is therefore available for reuse (35). CH may represent a model that highlights P-selectin's role in long-term leukocyte recruitment. Here, for the first time, we have also demonstrated the crucial role that P-selectin plays in recruitment of CD4$^+$ T lymphocytes into inflammatory lesions.

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