Infectious Susceptibility and Severe Deficiency of Leukocyte Rolling and Recruitment in E-Selectin and P-Selectin Double Mutant Mice

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Summary

During the initial phase of the inflammatory response, leukocytes marginate and roll along the endothelial surface, a process mediated largely by the selectins and their ligands. Mice with mutations in individual selectins show no spontaneous disease and have mild or negligible deficiencies of inflammatory responses. In contrast, we find that mice with null mutations in both endothelial selectins (P and E) develop a phenotype of leukocyte adhesion deficiency characterized by mucocutaneous infections, plasma cell proliferation, hypergammaglobulinemia, severe deficiencies of leukocyte rolling in cremaster venules with or without addition of TNF-α, and an absence of neutrophil emigration at 4 h in response to intraperitoneal *Streptococcus pneumoniae* peritonitis. These mice provide strong evidence for the functional importance of selectins in vivo.

Leukocyte and endothelial cell adhesion molecules play an important role in inflammatory and immune responses (1, 2). The initial steps in leukocyte emigration in response to inflammatory stimuli involve leukocyte rolling that is mediated primarily by interactions between selectins and selectin ligand molecules (3). P-selectin is expressed on endothelium and platelets, E-selectin on endothelium, and L-selectin on the majority of leukocytes. Selectins bind to carbohydrate portions of glycoproteins that serve as selectin ligands, many of which are mucin-like proteins (4–7). Leukocyte rolling is followed by firm attachment and emigration, processes largely dependent on the interaction of the β_2 leukocyte integrins and immunoglobulin family members including ICAM-1 and related molecules (1, 8). The leukocyte integrins are heterodimeric proteins and include LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), p150,95 ($\alpha_X\beta_2$), and $\alpha_d\beta_2$.

The functional importance of these adhesion molecules is demonstrated by the occurrence of two human genetic disorders, leukocyte adhesion deficiency types I and II (LAD I/II)[1] (9). LAD I is caused by genetic deficiency of the common β-subunit of the β_2 leukocyte integrins (CD18) and is characterized by increased granulocyte counts, impaired leukocyte emigration, and susceptibility to life-threatening bacterial infections (10, 11). LAD II is caused by an unspecified defect in fucose metabolism and is also characterized by increased granulocyte counts, and recurrent bacterial infections (12, 13). The defect in fucose metabolism is thought to affect the carbohydrate portion of selectin ligand molecules. The known LAD II patients also suffer from a variety of disorders outside the immune system leading to severe mental retardation and short stature (12). The functional abnormality in LAD I is primarily impairment of firm adhesion and emigration, whereas impaired leukocyte rolling is characteristic of LAD II (9, 14, 15).

We are studying the functional importance and the effects of genetic deficiency for the selectin molecules. The three known selectins (L, P, and E) occur as a gene cluster in mice and humans (16), and null mutations for each of the individual genes have been reported using gene targeting in mice (17–20). Modest reductions of leukocyte rolling and emigration occur in mice lacking L-selectin or P-selectin, but not in mice lacking E-selectin, and all three mutant strains remain healthy under normal laboratory conditions (17–21). To further define the role of selectins in inflam-

[1] *Abbreviations used in this paper:* ES, embryonic stem; LAD, leukocyte adhesion deficiency.

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matory responses, we have prepared a line of mice with null mutations in both endothelial selectins (P and E); and find that these mice demonstrate spontaneous susceptibility to infections with a phenotype of leukocyte adhesion deficiency.

Materials and Methods

Gene Targeting for E-Selectin. A cDNA for mouse E-selectin (gift of Christie Ballantyne, Baylor College of Medicine) was used to screen a 129/Sv genomic library (Stratagene, La Jolla, CA); genomic clones were partially characterized using restriction digests and PCR and compared to the published genomic structure (22). An E-selectin replacement construct was generated by replacing ~1.4 kb of DNA between the PstI site in exon 4 and the HindIII site in exon 6 with an HPRT minigene (23); (gift of Allan Bradley, Baylor College of Medicine). This construct was electroporated into P-selectin mutant AB2.1 ES cells (24) carrying the previously described P-selectin mutation (20). Digestion with EcoRI and hybridization with the probe indicated were used to identify homologous recombinants on Southern blots. Several clones were isolated that correctly targeted the E-selectin gene, and injection of one of these clones into C57BL/6 embryos resulted in germline transmission. This construct was also used to generate mice deficient in E-selectin alone following electroporation into unmanipulated AB2.1 embryonic stem (ES) cells. All mice used in these experiments were from a mixed 129/Sv and C57BL/6 background and were kept in a specific pathogen-free barrier facility. Only mutant mice that had no hair loss and no symptoms of opportunistic infection or lymphadenopathy were used in the experiments described.

Pathology. Necropsies were performed on 12 affected E-/P-selectin-deficient mice and compared with four age-matched control mice. Tissue was obtained from major organ systems as well as cervical and perioral skin, gingiva, and oral mucosa. The tissue was fixed in formalin, embedded in paraffin and examined following hematoxylin and eosin staining. Additional tissue was cryopreserved at −80°C for immunocytochemical studies. Bacterial and fungal cultures were performed from lymph node, lung, liver, spleen, and blood. Methenamine silver and gram stains, for the detection of fungi and bacteria, were performed on tissue sections. Immunocytochemical studies were performed on deparaffinized sections of the enlarged cervical lymph nodes using kappa and lambda light chain antibodies (Dakopatt, Carpintera, CA) to determine if the plasmacytoid lymphocytic proliferation was monoclonal. Portions of lung were fixed in glutaraldehyde and processed for electron microscopic examination.

Intravital Microscopy Experiments. Mice were anesthetized exactly as described (21). Some animals were pretreated with an intrascrotal injection of 0.5 μg mouse TNF-α (Genzyme, Cambridge, MA) in 0.3 ml saline for 2 to 2.5 h. The cremaster muscle was prepared for intravital microscopy as described (21, 25), and superfused with thermocontrolled (37°C) bicarbonate-buffered saline as described (26). Microscopic observations were made on a Zeiss Axioscope (salt water immersion objective 40/0.75 numerical aperture) as described previously (27). Microvascular centerline red blood cell velocity was measured using a dual phototransistor and an automatic tracking correlator implemented on a personal computer based on software described previously (28). Throughout the experiment, small blood samples (20 μl each) were withdrawn from the carotid catheter at ~45-min intervals and analyzed for leukocyte concentration (hemocytometer) and differential leukocyte counts (Leukostat, Fisher-Scientific, Pittsburgh, PA). Microvessel diameter was measured interactively using a digital image processing system (28), and rolling leukocyte flux was determined by counting the number of leukocytes passing each venule as described (21). Total leukocyte flux was estimated as the product of measured systemic leukocyte concentration and blood volume flow calculated from the venule cross-sectional area multiplied with mean blood flow velocity. Leukocyte rolling flux fraction is defined as the flux of rolling leukocytes as a percentage of total leukocyte flux, which is independent of variations in systemic leukocyte counts. Leukocyte rolling flux fraction in E-selectin/P-selectin-deficient mice was compared with age-matched wild-type controls using Student's *t*-test.

Peritonitis Studies. Anesthetized mice received an intravenous injection of ^{125}I-albumin into the tail vein, and edema formation was calculated as described (20). After 15 min, *Streptococcus pneumoniae* ($1–5 \times 10^9$ organisms/mouse) were injected in the peritoneal space. After either 4 or 24 h ($n = 5$ in each group), the mice inhaled an overdose of halothane. The peritoneal space was lavaged using 5 ml of phosphate-buffered saline three times for a total of 15 ml. The circulating neutrophil counts and neutrophil counts in the peritoneal lavage fluid were quantitated using a hemocytometer and Wright-stained smears on cytospin preparations. The clearance of bacteria from the peritoneal space was determined by measuring the number of colony-forming units (CFU) in the peritoneal instillate and in the peritoneal lavage fluid after 4 or 24 h.

Results

Gene Targeting of E- and P-Selectin. Since E-selectin and P-selectin are tightly linked members of a gene cluster (16), the double mutation could not be obtained by breeding the single mutations, and it was necessary to introduce both mutations onto a single chromosome in ES cells. An ES cell line carrying the previously reported P-selectin mutation (20) was electroporated with a plasmid construct designed to produce a null mutation in E-selectin (Fig. 1 *a*). Only 50% of the targeted cell clones would be expected to carry both mutations on the same chromosome as is required to produce double homozygous mice. Chimeric males obtained from one clone, P2D6, transmitted both mutations on the same chromosome to their progeny, and double homozygous mice were obtained by intercrossing as documented by Southern blotting (Fig. 1 *b*). The same plasmid construct was used to obtain mice carrying the null mutation for E-selectin alone.

Phenotype of E-/P-Selectin Deficient Mice. There was no evidence for prenatal or early postnatal lethality in the double homozygous mice. The evidence that the P-selectin mutation represents a null allele was published previously (20). The E-selectin mutation is predicted to be a null mutation since a portion of exon 4, all of exon 5, and a portion of exon 6 are deleted, leaving no mechanism for synthesis of the normal protein (Fig. 1 *a*). Immunohistochemical staining of lung tissue from the E-/P-selectin double mutant mice, and from the single mutant mice using monoclonal antibodies against E- or P-selectin, failed to show any reactivity for the mutated gene(s) after intratracheal administration of *Escherichia coli* endotoxin (data not shown).

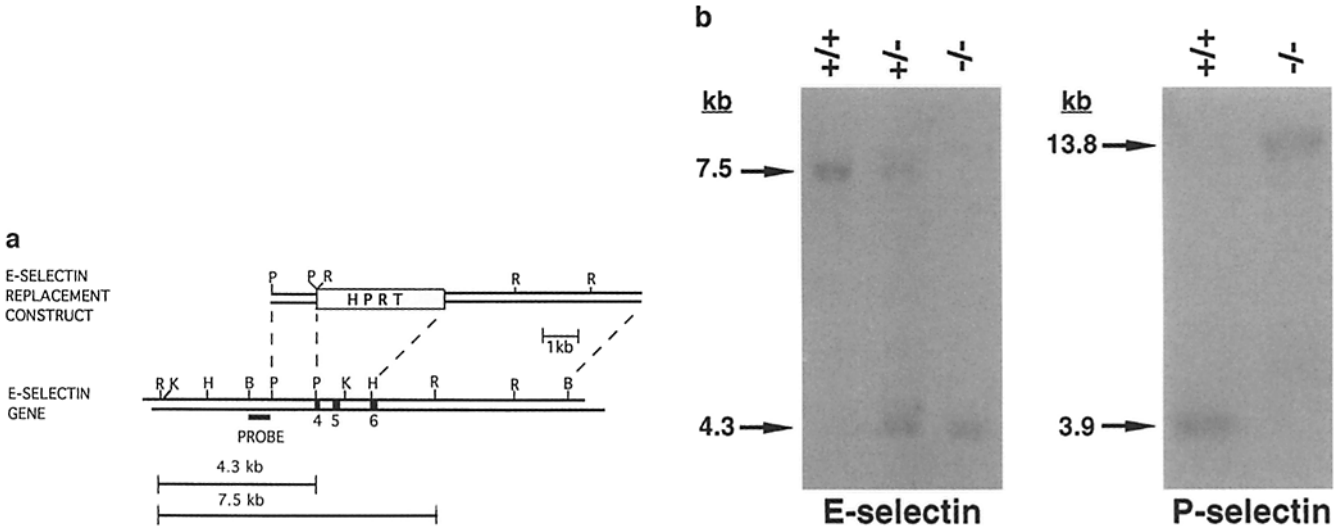


Figure 1. Gene targeted mutations for E-selectin and P-selectin. (*a*) E-selectin replacement construct and partial restriction map of the endogenous E-selectin locus. Exons 4, 5, and 6 are indicated as solid boxes; the positions of the other exons are not shown. *P*, PstI; *R*, EcoRI; *B*, BamHI; *H*, HindIII; not all PstI sites are shown. The HindIII site present in exon 6 and the BamHI site present at the 3′ end of the construct were destroyed in the construct. The E-selectin-specific probe indicated in *a* and a probe specific for the P-selectin gene (20), were hybridized separately to tail DNA from nonmutant, double heterozygote, and double homozygote mutant mice. The E-selectin probe identifies two EcoRI fragments of 4.3 and 7.5 kb corresponding to the mutated and endogenous E-selectin locus, respectively. The P-selectin probe identifies a 3.9-kb endogenous EcoRV fragment and a 13.8-kb EcoRV mutant fragment. (*b*) blot confirmation of E- and P-selectin mutations.

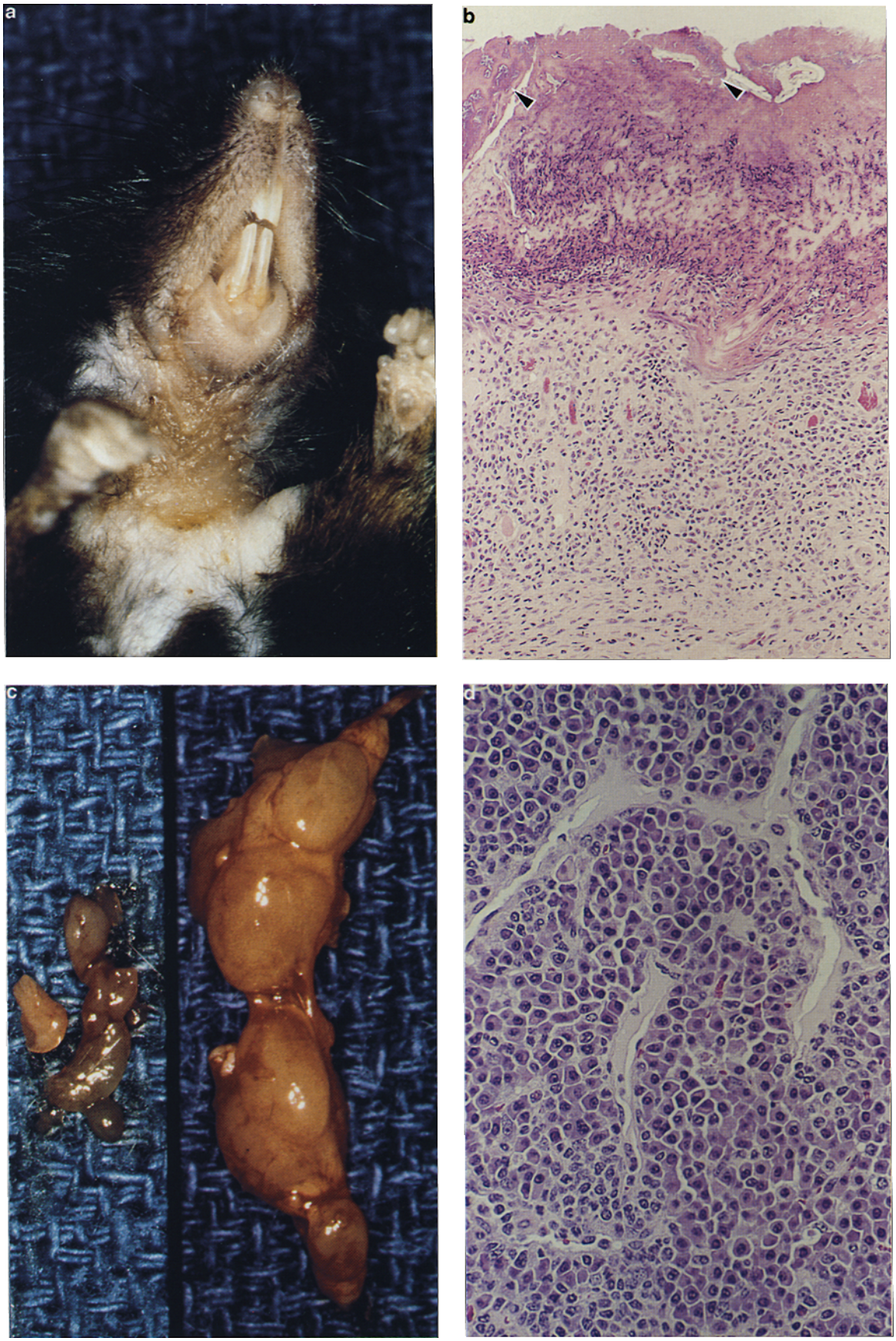
In contrast to the healthy status of mice carrying either the E- or P-selectin mutation alone, double homozygous mice began to show abnormalities at variable ages after weaning. Findings included nonulcerative excoriative skin lesions with hair loss in the head and neck regions, generalized redness and swelling of the oral mucosa, and overgrowth of the mandibular and maxillary incisors (Fig. 2 *a*). Clipping of the incisors regularly was necessary for the mice to feed. These manifestations were present in a few mice by the time of weaning, and the proportion of affected mice increased continuously so that >90% had obvious signs of the disease by 6–8 mo of age.

Leukocyte counts in E-/P-selectin double mutant mice without obvious signs of disease were extremely elevated for neutrophils, lymphocytes, and monocytes; mean/μl ± SEM were as follows: 12,020 ± 200 neutrophils for mutants versus 1690 ± 690 for controls; 10,930 ± 1224 lymphocytes for mutants versus 4930 ± 249 for controls; and 873 ± 188 monocytes for mutants versus 148 ± 13 for controls (all $P < 0.001$). Blood smears revealed a marked increase in immature as well as mature neutrophils.

Histologic sections from affected skin and oral mucosa showed loss of the overlying squamous epithelium and superficial colonization of these tissue surfaces by bacteria (Fig. 2 *b*); cultures were positive for *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*, and other common bacterial flora. These organisms did not invade the underlying dermis or submucosa, and these regions contained a mixed granulocytic and mononuclear cell infiltrate. Histologic sections of the oral cavity also demonstrated periodontitis with a chronic leukocytic infiltrate of the periodontal tissue and evidence of gingival recession. A few mice spontaneously developed conjunctivitis with bacterial flora similar to that recovered from infected skin. Repetitive cultures for bacterial and fungal organisms taken from blood, spleen, liver, lymph node, and lung were negative.

Necropsy examination also revealed massive cervical lymphadenopathy (Fig. 2 *c*) due to replacement of the nodal architecture with an infiltrate consisting predominantly of plasmacytoid lymphocytes and plasma cells on histologic examination (Fig. 2 *d*). Increased numbers of plasma cells were also observed in sections from spleen and bone marrow in affected mice. Immunohistochemical staining of plasma cells with kappa or lambda chain antibodies was positive for both, indicating that the proliferation was not monoclonal (data not shown). The IgG levels in serum of double mutant mice were increased ~10-fold; mean ± SEM were as follows: 34,760 ± 4312 μg/ml for mutants versus 3339 ± 708 μg/ml for controls ($P < 0.0001$). IgM levels were not significantly increased. The accumulation of plasma cells in the cervical lymph nodes is unusual compared to what is observed in chronic bacterial infections due to various forms of leukocyte dysfunction and may imply a defect in plasma cell traffic in addition to the chronic infection (9).

Paraffin-embedded histologic sections of lung tissue revealed an increased cellularity in the alveolocapillary walls and no emigration of leukocytes into the alveolar space or the airways. The most striking observation on ultrastructural studies was that the increased cellularity was due to a large increase in the number of neutrophils and other leukocytes marginated within the capillary lumina. Many of


a
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these marginated neutrophils (24%) showed ultrastructural features of apoptosis. Almost no neutrophils had spontaneously migrated into either the interstitium or the alveolar spaces.

Leukocyte Rolling. In order to investigate the effects of the combined deficiency of E- and P-selectin on leukocyte rolling, we used intravital microscopy to determine the leukocyte rolling flux fraction in venules of the cremaster muscle (21, 27). In this model, the muscle was exteriorized (which provides a mild inflammatory stimulus), and rolling was assessed for 2 h. In P-selectin-deficient mice, rolling is initially absent, and the rolling leukocyte flux fraction increases over time (21). In contrast, L-selectin mutant mice display normal initial rolling with the fraction of rolling leukocytes declining significantly at later times (21). We have not observed a significant reduction in rolling flux fraction in E-selectin mutant mice (data not shown). However, leukocyte rolling is induced in P-selectin mutant or P-selectin/ICAM-1 double mutant mice by TNF-α, a cytokine that stimulates expression of both E- and P-selectin, and this rolling is blocked by antibodies to either E- or L-selectin (21, 27). In the E-/P-selectin double mutant mice studied before onset of obvious disease symptoms, leukocyte rolling was completely absent in venules of the cremaster muscle at all time points up to 2 h after exteriorization and when mice were pretreated with TNF-α (Fig. 3). The leukocyte rolling flux was <0.5% of leukocytes passing through the vessels in all cases. Isolated leukocytes were seen to firmly attach to the vessel wall in TNF-α treated mice.

Streptococcus Pneumoniae-induced Peritonitis. We also examined neutrophil emigration during *Streptococcus pneumoniae*-induced peritonitis to further assess leukocyte function in double mutant mice. Previous studies in mice with single selectin deficiencies have shown a positive correlation between impairment of leukocyte rolling in P-selectin- and L-selectin-deficient mice with reductions in neutrophil emigration during peritonitis (17, 19, 20). In response to *S. pneumoniae*, the E-/P-selectin double mutant mice showed complete absence of neutrophil emigration at 4 h after instillation compared to a moderate reduction in P-selectin-deficient mice and normal emigration in E-selectin-deficient mice (Table 1). Neutrophil emigration in double mutant mice was increased between 4 and 24 h, resulting in accumulation of similar numbers of intraperitoneal neutrophils compared to wild-type mice at 24 h. Edema formation in the peritonitis model correlated well with neutrophil emigration with significant reductions in double mutant mice and P-selectin-deficient mice at 4 h. Edema was significantly increased in E-/P-selectin double mutants at 24 h. Clearance of *S. pneumoniae* from the peritoneal space was not different for any of the mutant mice at 4 h, but was significantly reduced at 24 h in the double mutant mice.

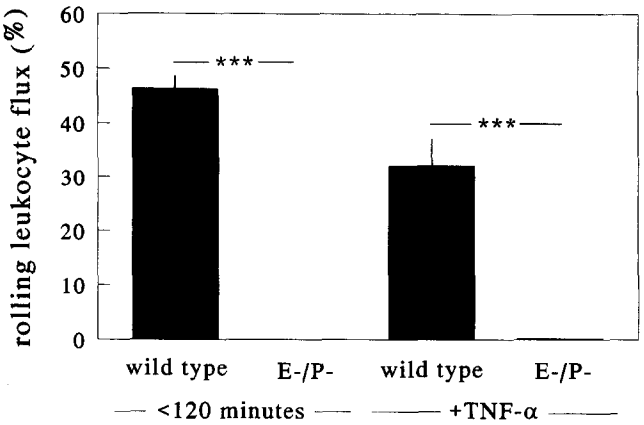

Figure 3. Leukocyte rolling flux fraction in untreated and TNF-α-treated venules in wild-type and E-selectin/P-selectin-deficient mice. Average leukocyte rolling flux fraction in untreated E-/P-selectin-deficient mice (82 venules) was significantly lower ($P < 0.001$) than in untreated wild-type mice (130 venules) 10–120 min after cremaster exteriorization. TNF-α-treated mice were pretreated with an intrascrotal injection 2–2.5 h before the initiation of surgery. Consistent with previous findings (21) TNF-α treatment does not increase the flux of rolling leukocytes in wild-type mice, primarily because firm adhesion is greatly increased in the TNF-α-treated venules. Rolling in TNF-α-treated E-/P-selectin-deficient mice (42 venules) is also significantly lower ($P < 0.01$) than that seen in similarly treated wild-type mice (17 venules). Data shown as mean ± SEM.

L-Selectin Expression. The defect observed in the E-/P-selectin double mutant mice is the most severe deficiency of leukocyte rolling described in any selectin-deficient mouse to date, suggesting that L-selectin and possible selectin-independent mechanisms are insufficient to provide the adhesive forces necessary for rolling. This observation is of particular interest in view of the deficiency of leukocyte rolling observed in L-selectin-deficient mice (19, 21), suggesting that L-selectin is required for effective rolling but by itself is not sufficient. To further clarify the role of L-selectin in E-/P-selectin mutant mice, its expression on the surface of peripheral blood leukocytes was examined by flow cytometry. Both older (8–10 wk) and younger (3 wk) mice displayed a substantial reduction (1/6 to 1/8 of normal mean channel fluorescence) of L-selectin expression on Gr-1-positive granulocytes (data not shown). Expression of L-selectin on Thy 1.2-positive lymphocytes was also reduced to about one half of normal. However, when CD45-positive bone marrow cells from E-/P-selectin double mutant mice were analyzed, the expression of L-selectin was 80–85% of normal. These observations suggest that the reduction in L-selectin on the surface of peripheral leukocytes may be due to increased shedding and activation in association with chronic infection (29, 30). Decreased levels of L-selectin expression on neutrophils from LAD I pa-

Figure 2. Gross morphology and histologic analysis of E-/P-selectin-deficient mice. (*a*) Ventral view of the neck of an affected E-/P-selectin mutant with excoriative skin lesions and gingival recession. (*b*) Histopathologic appearance of affected skin with bacterial colonization (*arrows*) of the epidermis and a mixed inflammatory dermal infiltrate. (*c*) Markedly enlarged cervical lymph nodes from an affected double mutant (*right*) compared with non-mutant (*left*). (*d*) Histopathologic appearance of a cervical lymph node from an affected animal.

Table 1. *S. pneumoniae-induced Peritonitis*

Mutation	4 h			24 h		
	Neutrophil accumulation	Edema formation	Bacteria recovered	Neutrophil accumulation	Edema formation	Bacteria recovered
Wild type	0.57 ± 0.15	8.0 ± 0.8	40 ± 7	4.55 ± 2.04	3.2 ± 0.5	0.3 ± 0.3
P-selectin	0.19 ± 0.003*	3.8 ± 1.1*	44 ± 10	2.84 ± 0.39	3.9 ± 0.6	16 ± 14
E-selectin	0.37 ± 0.07	5.7 ± 1.5	32 ± 14	2.56 ± 0.18	2.9 ± 0.5	28 ± 28
E-/P-selectin	0.06 ± 0.01*	2.1 ± 0.7*	39 ± 13	6.51 ± 1.76	6.9 ± 1.1‡	102 ± 53‡
Wild type, no organisms	0.01 ± 0.01	0.5 ± 0.1	N.D.	0.01 ± 0.00	0.9 ± 0.1	N.D.
E-/P-selectin, no organisms	N.D.	N.D.	N.D.	0.04 ± 0.01	1.3 ± 0.1	N.D.

Neutrophil emigration is expressed as the number of neutrophils × 10^{-5}/ml peritoneal lavage fluid. The formation of edema is expressed as the percent of ^{125}I-albumin recovered in the peritoneal fluid. Clearance of *S. pneumoniae* from the peritoneal space is expressed as percent recovery of organisms instilled in the peritoneal lavage fluid. Data are expressed as mean ± SEM. N.D. = not determined.
*Significantly less than value observed in wild-type mice that received organisms, $P < 0.05$.
‡Significantly greater than value observed in wild-type mice that received organisms, $P < 0.05$.

tients, but normal expression on neutrophils from LAD II patients have been reported (15, 31). The absence of leukocyte rolling in the venules and of neutrophil emigration into the peritoneum of E/P-selectin double mutant mice may therefore result from complete loss of E- and P-selectin on endothelial cells and perhaps a partial loss of L-selectin on leukocytes.

Discussion

The role of selectins has been evaluated using one or more blocking monoclonal antibodies in wild-type mice and using single selectin mutations with or without additional monoclonal antibodies. The results indicate that inactivation of multiple selectins leads to severe defects in early leukocyte rolling and/or peritoneal emigration of neutrophils (18, 21, 32). Monoclonal antibodies are not suitable for chronic administration, but long term observations of single mutant mice reveals no evidence of spontaneous infection. Here we demonstrate genetic deficiency of E- and P-selectin in mice results in increased susceptibility to spontaneous infections over time. In the human syndrome LAD type II, a disease that affects the expression of sialyl Lewis X and thus selectin ligands, the two patients described suffer from recurrent bacterial infections, neutrophilia, and a decrease in neutrophil rolling (9, 12–15). Given the hypothesis that LAD type II is primarily a defect of selectin ligand expression on leukocytes, it is instructive to compare this with the double deficiency of endothelial selectins in mice. Generally the phenotyes are quite similar, although expression of L-selectin is normal on LAD II granulocytes and is reduced in the selectin-deficient mice. We have observed no evidence for neurological damage or abnormal growth patterns in the double mutant mice.

In the peritonitis studies reported here, the most remarkable finding was that the E-/P-selectin double mutant mice showed a complete defect in neutrophil emigration at 4 h, but no defect compared to wild-type mice at 24 h. This is in contrast to either single mutation, since the P-selectin mutant mice showed only a partial reduction at 4 h and no defect at 24 h, while E-selectin mutants were similar to wild-type mice at both time points. Thus, the initial deficiency of rolling in E-/P-selectin double mutant mice is not associated with absence of *S. pneumoniae*–induced neutrophil emigration at later times points. Leukocyte emigration is also not completely deficient in other tissues as illustrated by the observation of a mixed inflammatory infiltrate in the dermis and epidermis of infected skin from E-/P-selectin double mutant animals. This result suggests that other E- and P-selectin-independent mechanisms can mediate adhesion and emigration. It is possible that the poorly controlled bacterial proliferation provides a greater stimulus which now recruits additional adhesion pathways. There is other evidence for differences in adhesion molecules involved in acute inflammatory responses within the first 6 h compared to 24–48 h (33, 34). These double mutant mice will be extremely valuable for delineation of the mechanisms and molecules that allow neutrophil emigration at later times.

It is interesting that L-selectin mutant mice show partial reductions in neutrophil emigration into the peritoneal cavity at 4, 24, and 48 h during thioglycollate-induced peritonitis (19, 34). The reduction observed at later timepoints in L-selectin-deficient mice compared to normal emigration for the other mutants may indicate a separate role for L-selectin that is not shared by E- and P-selectin. However, the differences in the two models (a single chemical stimulus vs. proliferating bacteria) may affect the nature and the extent of the stimulation of adhesive pathways and may contribute to the differences observed at 24 h.

These data raise questions regarding the extent to which the functions of selectins (particularly E and P) are distinct

versus overlapping. The mutant mice described here demonstrate that the combined deficiency of E- and P-selectin causes a disease phenotype that is not seen with either deficiency alone. Given the evolutionary conservation of each of the selectins and the considerable differences in patterns of expression, we believe that the roles of E- and P-selectin are only partially overlapping. Preparation of mice with other combinations of mutations in selectins and selectin ligands and more detailed analysis of each mutant should further clarify the distinct roles of individual molecules.

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