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Endothelial Cell E- and P-Selectin Up-Regulation in Murine Contact Sensitivity Is Prolonged by Distinct Mechanisms Occurring in Sequence[1]

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The selectins are adhesion molecules that mediate the tethering and rolling of leukocytes on vascular endothelium. Although E-selectin and P-selectin are known to be expressed by endothelial cells (EC) in response to proinflammatory stimuli, their pattern and mechanisms of expression in immune-mediated inflammation remain poorly understood. By quantifying luminal endothelial selectin expression via i.v. administration of radiolabeled mAb, we detected constitutive expression of P-selectin, but not E-selectin, in mouse skin. Both selectins were transiently up-regulated after intradermal TNF-α, IL-1α, or IL-1β. In contrast, during a contact sensitivity response to oxazolone, expression of both selectins was prolonged, with distinct peaks at 6 and 48 h. Experiments with P-selectin gene-targeted mice showed that the P-selectin measured was exclusively expressed by EC rather than platelets. The early and late phases of selectin expression in contact sensitivity were differentiated in terms of their requirement for prior sensitization, and the action of IL-1. Whereas the early phase was a nonspecific 'irritant' response to oxazolone, the late phase was Ag specific and was partially IL-1 dependent. Therefore, persistence of both E- and P-selectin expression in vivo can occur as a result of sequential and distinct EC activation processes that appear to be at least partially different from those previously reported as stimulating ICAM-1 and VCAM-1 expression. The further elucidation of mechanisms of EC activation in this model may help determine the relative roles of selectins and ligands for leukocyte integrins in the sequential recruitment of T cells and other leukocyte subsets during ongoing immune-mediated inflammatory responses. ***The Journal of Immunology*****, 1999, 163: 6860–6866.**

The selectins are a family of single-chain glycoprotein adhesion molecules that mediate a number of different adhesion events among leukocytes, platelets, and endothelial cells (EC)[4] during inflammatory responses (reviewed in Ref. 1). They are critically involved in the tethering and rolling of leukocytes on vascular endothelium during their migration from the circulation into tissues. L-selectin is constitutively present on granulocytes and most mononuclear cells. P-selectin is surface expressed by platelets, and E- and P-selectin are surface expressed by EC, in most instances only in response to an inflammatory stimulus. P-selectin is expressed within minutes in response to mediators such as histamine, through translocation to the plasma membrane of molecules stored in EC Weibel-Palade bodies and platelet α-granules. In contrast, TNF-α in vitro induces expression of both E- and P-selectin by EC, maximal at 2–4 h, that is dependent on gene transcription (2, 3). The kinetics and mechanisms of expression of E- and P-selectin in subacute and chronic inflammation are still unclear.

The fraction of circulating leukocytes that roll and adhere on EC depends in large part on the density of expression of the various adhesion molecules involved (4). Therefore, precise quantification of EC adhesion molecule expression in vivo could lead to a greater understanding of their pathophysiological role in leukocyte migration. Accordingly, we and others have administered radiolabeled mAb against EC adhesion molecules i.v. and quantified their localization in different vascular beds by gamma counting. With this approach, it has been possible to measure the expression of endothelial selectins during the course of inflammatory responses in the pig (5) and the mouse (6).

The murine contact sensitivity (CS) response is a model of focal, T cell immune-mediated inflammation, characterized by the migration of leukocytes into the skin over 48 h (reviewed in Ref. 7). We recently reported that the EC adhesion molecules ICAM-1 and VCAM-1 are up-regulated throughout the CS response elicited by 4-ethoxymethyl-2-phenyl-2-oxazolin-5-one (oxazolone (OX)) in the mouse. Even early in the response, this up-regulation was dependent on prior sensitization to Ag, and it was inhibited by treatment before challenge with a neutralizing anti-TNF-α antiserum (8). Recent studies have indicated that both E- and P-selectins are involved in generating the elicitation phase of CS (9, 10), whereas L-selectin may be necessary only for induction of sensitization to the Ag (11). In this study, we have compared the expression of E- and P-selectin over the time course of elicited CS responses and have related the results to previously demonstrated expression of ICAM-1 and VCAM-1.

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4 Abbreviations used in this paper: EC, endothelial cells; Pt, platelets; CS, contact sensitivity; ID, injected dose; i.d., intradermal; OX, 4-ethoxymethyl-2-phenyl-2-oxazolin-5-one (oxazolone).

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Materials and Methods

Abs and cytokines

MES1 is an IgG2a mAb against mouse E-selectin that was generated from splenocytes of a rat immunized with mouse E-selectin-transfected Chinese hamster ovary cells. RB40.3414 (RB40) is a rat IgG1 mAb against mouse P-selectin and was supplied by Professor Dietmar Vestweber (University of Münster, Münster, Germany). Rat hybridoma cell lines producing Abs against DNP were obtained from Dr. David Gray (Imperial College School of Medicine, London, U.K.) for the generation of rat IgG1, IgG2a, and IgG2b mAb for use as irrelevant negative control Abs. Hybridoma cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Paisley, U.K.). Hybridoma supernatants were purified by gel filtration over Sepharose 4B followed by affinity chromatography using protein G conjugated to Sepharose 4B (Pharmacia, Uppsala, Sweden). Abs were eluted from the column with the use of a glycine buffer (0.1 M, pH 3) and were dialyzed into HBSS (Life Technologies). Polyclonal sheep anti-murine TNF-α serum H92 and recombinant murine TNF-α were gifts from Dr. Tony Meager (National Institute for Biological Standards and Control, Potters Bar, Hertford, U.K.). Recombinant murine IL-1α and IL-1β were purchased from Peprotec (London, U.K.). The polyclonal sheep anti-murine IL-1α and IL-1β antisera were supplied by Dr. Roberto Solari (Glaxo Wellcome, Stevenage, U.K.).

Selectin localization in vivo by mAb targeting

Animals were maintained and studied according to U.K. Home Office regulations. Female mice were used for all experiments and were age-matched. BALB/c and C57BL/6 mice were obtained from Harlan Olac (Oxon, U.K.) at 6–10 wk of age and housed in standard cages. P-selectin deficient C57BL/6J-*selp* (*tm1 bay*) (12) were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs were housed in isolators, and progeny were housed in filter cages supplied with irradiated bedding, food, and acidified water. All mice were housed in proximity to sentinels, which were used for regular pathogen screening.

mAbs MES1 and RB40 and anti-DNP-negative control mAb, were differentially radiolabeled with ^{99m}Tc, ^{111}In, and ^{125}I to permit their detection in the skin after administration to mice, as previously described (8). They were then mixed together, and 2 μg of each were administered by tail vein injection. After 5 min, mice were placed under terminal anesthesia by i.p. injection of pentobarbitone sodium (Rhône Mérieux, Harlow, U.K.). The vasculature was then perfused with PBS containing heparin 10 U/ml (Leo Laboratories, Princes Risborough, U.K.). Tissues were harvested, weighed, placed in vials, and counted simultaneously in an automated gamma counter (Packard, Pangbourne, Berkshire, U.K.), with the use of separate channels for ^{99m}Tc, ^{111}In, and ^{125}I. Tissue cpm were corrected for background, spill between channels, and ^{99m}Tc decay over the counting period and for tissue sample weight in grams. They were then divided by the total cpm injected into that animal, which was calculated with the volume of injected material and the cpm in a standard dilution of the injection mixture. This value was either made into a percentage (referred to as the percent injected dose (ID) per gram; %ID/g) or, when experiments involved comparisons between knockout and wild-type mice, normalized for plasma volume by multiplying by the weight of the animal in grams (referred to as normalized %ID/g). Specific uptake of anti-selectin mAb was calculated by subtracting the %ID/g for the control mAb from that of the test mAb.

Alternatively, localization of i.v.-administered mAb was detected by immunohistochemistry. Mice received 50 μg of anti-selectin or control mAb; 5 min later they were placed under terminal anesthesia, and the vasculature was perfused as above. Harvested tissues were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) and snap frozen over liquid nitrogen. Then 5-μm sections were mounted on glass slides, air-dried, and fixed for 10 min in acetone. After blocking with 20% normal rabbit serum (Dakopatts, Glostrup, Denmark), rabbit anti-rat Ig Ab and alkaline phosphatase + anti-alkaline phosphatase complex (Dakopatts) were applied in succession. The sections then were developed with Fast Red (Sigma, Poole, U.K.), counterstained with Harris' hematoxylin, and coverslipped with Aquamount (BDH, Poole, U.K.).

Models of skin inflammation

Acute dermal inflammation was induced by the intradermal (i.d.) administration of TNF-α or IL-1 as follows. Mice were anesthetized i.m. with a 20-μl mixture of ketamine (Parke Davis, Gwent, U.K.), xylazine (Bayer, Leverkusen, Germany), and PBS (4:1:5 v/v/v). The flanks were then shaved, after which each flank received a single i.d. injection of TNF-α (5 ng), IL-1α (5 ng), or IL-1β (5 ng) in HBSS containing 0.01% Evans blue (Sigma). These concentrations of cytokines were found to be optimal in preliminary experiments. Control mice received HBSS, 0.01% Evans blue alone. After varying incubation times, and 5 min after injection of radiolabeled mAb mixture, mice were killed by cervical dislocation, and the flank skin was removed. Any trace of blood was removed from the skin by blotting with gentle pressure, and then injection sites were excised with a 5-mm punch, weighed, and counted.

Mice were sensitized for CS responses by topical application of OX (Sigma) (50 μl) in acetone-olive oil vehicle (4:1 v/v) to the shaved right flank. Unless specified, the sensitizing and challenging concentrations of OX for BALB/c mice were 1%, whereas C57BL/6 were sensitized and challenged with 5% OX, because our preliminary studies had shown that the CS response to OX is more difficult to elicit in this strain (data not shown). Control mice received vehicle alone. From 5 to 7 days later, mice received a 10-μl challenge on one or both ears of varying concentrations of OX in acetone-olive oil or of vehicle alone. After various time periods, selectin expression in the ears was ascertained as above. The ears were transected at a line measuring 9 mm from the apex, and their thickness was measured with a micrometer (Cadar, Sheffield, U.K.), before weighing and gamma counting excised ears.

Our preliminary data suggested that small differences in the radiolabeled mAb preparations for each experiment resulted in some variation between experiments in the absolute level of the outcome measure, %ID/g. Therefore, no attempt was made to pool data from different experiments, and each experiment was designed to include appropriate internal controls. Individual comparisons between groups were made by Student's *t* test; multiple comparisons were made using a one-tailed ANOVA followed by Bonferroni's multiple comparison of means test.

Transfer of bone marrow cells

Mice selectively deficient in either platelet or endothelial P-selectin were created by the transfer of bone marrow cells between wild-type and P-selectin-deficient mice (13, 14) to determine the cell type responsible for uptake of i.v.-injected anti-P selectin mAb. Bone marrow cell recipients received a lethal dose of irradiation (9.5 Gy) from a cesium source (IBL 637; Cis Bio International, High Wycombe, Buckinghamshire, U.K.) 18 h before cell transfer. Bone marrow donor mice were killed by cervical dislocation, and then bone marrow cells were harvested from both femora and resuspended in sterile PBS. Each recipient was injected with 10^7 cells by i.v. tail vein injection and was left to generate bone marrow-derived cells for at least 8 wk before use. There was no mortality or morbidity among recipients. The blood cell phenotype of recipients was ascertained by purifying platelets from whole blood and determining their level of P-selectin expression with a solid phase ELISA and with RB40 as the primary Ab, as described (15).

Results

Quantification of dermal vascular selectin expression in vivo

We first used immunohistochemistry to detect the luminal expression of E- and P-selectin on vascular endothelium in mouse skin of mice previously sensitized to 1% OX both under basal conditions and 48 h after local topical challenge with 1% OX (Fig. 1). Control IgG, anti-E-selectin mAb MES1, or anti-P-selectin mAb RB40 that had been administered i.v. were localized in tissue sections using anti-rat Ig. Staining with hematoxylin and eosin (Fig. 1, *A* and *B*) showed typical changes of a CS response, with dermal edema, a mixed leukocyte infiltrate, and epidermal microabscesses. E-selectin was not detectable under resting conditions (Fig. 1*E*) but was expressed on the lumina of small venules 48 h after OX challenge (Fig. 1*F*). In contrast, P-selectin was detected on venular EC in noninflamed (Fig. 1*G*) as well as inflamed skin (Fig. 1*H*). Control IgG did not localize to vascular endothelium (Fig. 1, *C* and *D*).

EC expression of E-selectin after administration of i.d. TNF-α

To achieve more quantitative data on E- and P-selectin expression, we administered radiolabeled mAb and determined their uptake in skin tissue by gamma counting. Initially, we performed experiments to validate radiolabeled mAb targeting as a means of quantifying E-selectin expression in vivo. We chose to study the effect over time of i.d. TNF-α, in that we previously had shown a marked, transient up-regulation in E-selectin expression after this stimulus in pigs (16). Fig. 2 shows that after i.d. injection of 5 ng

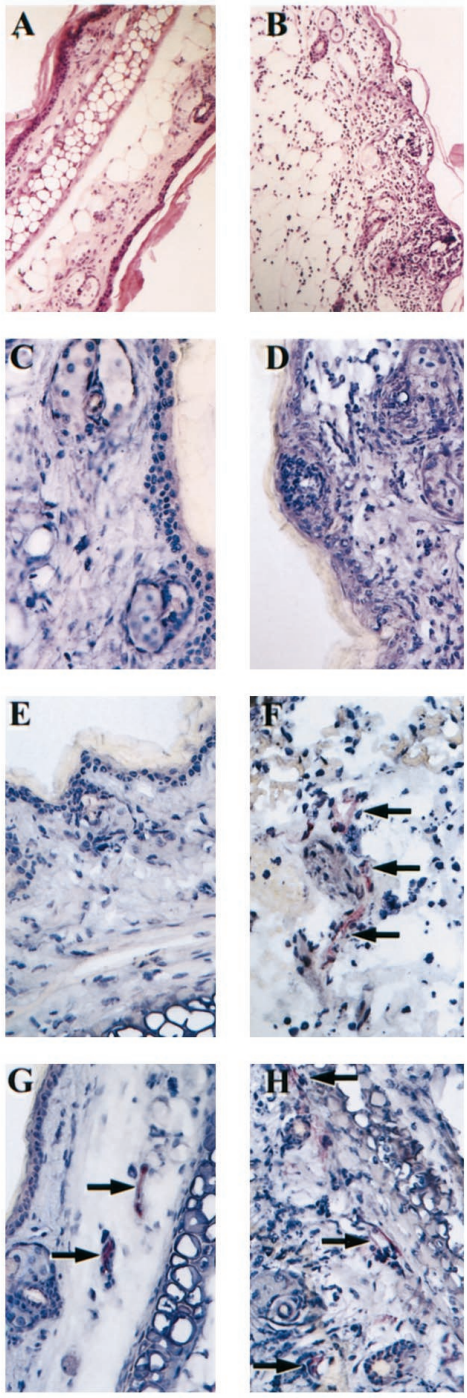

FIGURE 1. Immunohistochemical localization of i.v.-injected anti-E- and anti-P-selectin mAb in mouse ear skin during a CS response to OX. Ear tissue was taken from 1% OX-sensitized BALB/c mice that were challenged topically with 1% OX (*B, D, F, H*) or vehicle (*A, C, E, G*) for 48 h. *A* and *B,* Sections stained with hematoxylin and eosin (×20). Skin painted with vehicle showed no obvious inflammation (*A*). *B,* Typical changes of a CS response, with an edematous dermis, a mixed inflammatory dermal cell infiltrate, and epidermal microabscesses. *C–H,* sections immunostained with anti-rat Ig, developed with Fast Red and counterstained with hematoxylin (×40). Mice had received i.v. control IgG (*C, D*), anti-E-selectin MES1 (*E, F*), or anti-P-selectin RB40 (*G, H*) mAb 5 min before killing. No staining is seen with control IgG (*C, D*) or in noninflamed skin with anti-E-selectin (*E*). Arrows indicate vessel representative staining of E-selectin in inflamed skin (*F*) and of P-selectin in skin painted either with vehicle (*G*) or with OX (*H*).

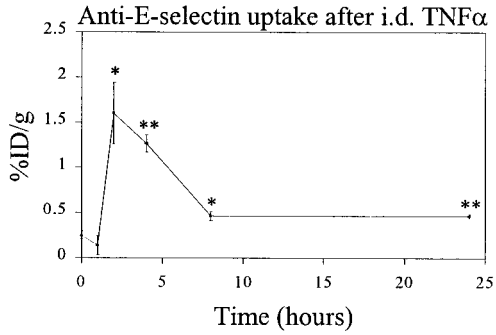

FIGURE 2. Determination of E-selectin expression in vivo by radiolabeled anti-E-selectin mAb (MES1). Localization at TNF-α-injected sites. Uptake of i.v.-administered ^{99m}Tc-labeled anti-E-selectin mAb (MES1) after 5 min circulation, in skin of BALB/c mice previously treated with 5 ng TNF-α for varying times ($n = 3$ per time point). E-selectin expression is presented as % ID/g of MES1 localizing to the skin site (corrected for weight) after subtraction of that of a ^{111}In-labeled isotype-matched control mAb. Values represent mean ± SD. E-selectin expression peaked at 2–4 h. *, $p < 0.05$, **, $p < 0.005$ vs 0 h, Student's unpaired *t* test.

TNF-α, the specific uptake of anti-E-selectin mAb (MES1) increased to a peak at 2–4 h, after which it declined to a low but significant level that was maintained until at least 24 h. A further experiment (data not shown) showed that radiolabeled mAb MES1 localization was also increased 24 h into the elicitation phase of a CS response to OX and that this increase could be completely inhibited by coadministration of excess unlabeled MES1.

EC expression of P-selectin after i.d. administration of TNF-α or IL-1

We then sought to validate similarly the technique for P-selectin by quantifying skin expression of P-selectin at various times after i.d. TNF-α administration using ^{125}I-labeled anti-P-selectin (mAb RB40). Fig. 3*A* shows that TNF-α led to transient up-regulation of P-selectin expression, with kinetics broadly similar to those of E-selectin, peaking at 4 h, and returning to baseline at 24 h. We then determined P-selectin expression 4 h after i.d. IL-1α and IL-1β, using TNF-α as a positive control. Fig. 3*B* shows that P-selectin was up-regulated in response to IL-1-α and IL-1-β to a comparable degree to that seen after i.d. TNF-α.

P-selectin-deficient (−/−) mice were used to establish the specificity for P-selectin of anti-P-selectin targeting in the skin. By reconstituting the bone marrow of lethally irradiated wild-type (+/+) or −/− mice with that of P-selectin −/− or +/+ mice, we were able to generate mice selectively deficient in expression of platelet or endothelial P-selectin and thereby determine the relative involvement of platelets and EC in anti-P-selectin uptake after i.v. administration. Wild-type (+/+) mice reconstituted with −/− bone marrow (Pt^-, EC^+) had no platelet P-selectin detectable by ELISA, whereas the platelets of P-selectin −/− mice reconstituted with +/+ marrow (Pt^+, EC^-) expressed P-selectin to the same degree as +/+ animals (data not shown). As noted for BALB/c mice, P-selectin expression in the skin of C57BL/6-+/+ mice was increased significantly by i.d. TNF-α compared with vehicle alone (Fig. 3*C*). In contrast, there was no detectable specific anti-P-selectin mAb uptake in the skin of P-selectin −/− or Pt^+, EC^- mice (Fig. 3*C*). However, in Pt^-, EC^+ mice, the anti-P-selectin uptake was not significantly different from that seen in +/+ mice. This result indicated that mAb RB40 was specifically targeting P-selectin expressed by EC rather than platelets. The finding that +/+ skin injected with HBSS vehicle showed significantly more P-selectin expression than the TNF-α-treated skin of P-selectin

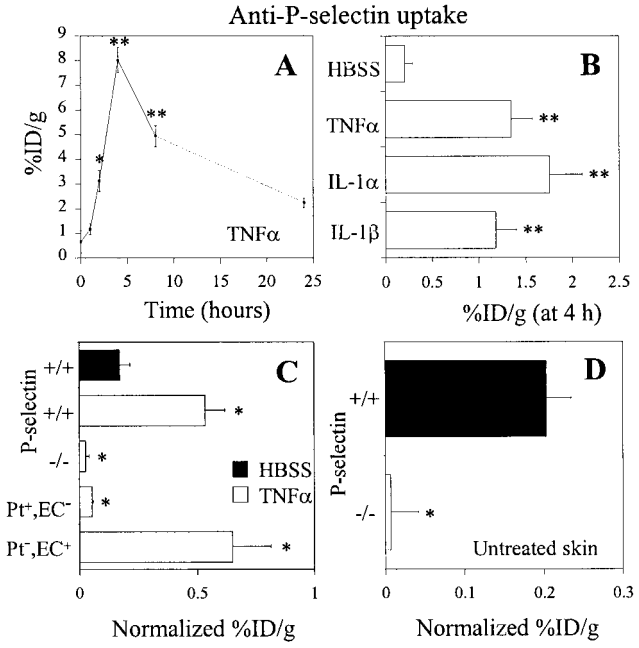

FIGURE 3. Determination of P-selectin expression in vivo by radiolabeled anti-P-selectin mAb RB40. P-selectin expression in the skin of BALB/c mice was quantified by measuring the skin uptake of ^{125}I-labeled anti-P-selectin mAb (RB40), after subtraction of the uptake of a ^{111}In-labeled isotype-matched control mAb. The two mAb were administered i.v. 5 min before the end of the experiment. *A*, P-selectin expression was measured at varying times after i.d. injection of 5 ng TNF-α ($n = 3$ per time point) and is expressed as %ID/g. Values represent mean ± SD. Peak up-regulation was seen at 4 h. *B*, P-selectin expression was determined in the skin of BALB/c mice 4 h after i.d. injection of 5 ng TNF-α, IL-1α, IL-1β, or HBSS alone ($n = 3$ per group). Both IL-1α and IL-1β caused an up-regulation of P-selectin similar to that seen after TNF-α. *C*, C57BL/6 mice, selectively deficient in either EC (Pt^+, EC^-) or platelet (Pt^-, EC^+) P-selectin, obtained by reconstituting the bone marrow of wild-type (+/+) or P-selectin-deficient (−/−) mice with P-selectin −/− or +/+ bone marrow cell transfer, respectively. P-selectin expression was determined 4 h after i.d. injection of 5 ng TNF-α (□) or vehicle (■) ($n = 5$ per group). The normalized %ID/g represents the %ID/g corrected for mouse weight (see *Materials and Methods*). Bars represent mean + SD. P-selectin expression was not detectable in TNF-α-stimulated skin of the two groups of mice lacking P-selectin on EC (i.e., −/− and Pt^+, EC^-). Uptake of anti-P-selectin in the Pt^-, EC^+ mice was no different from that seen in P-selectin +/+ wild-type mice. There was also a significant uptake of anti-P-selectin in P-selectin +/+ wild-type skin treated with vehicle (*C*) and untreated (*D*) ($n = 6$ per group), compared with the skin of P-selectin −/− mice. Comparisons were made with 0 h in *A*, with HBSS-treated mice in *B* and *C*, and with untreated P-selectin +/+ mice in *D*. *, $p < 0.05$; **, $p < 0.005$; Students' unpaired *t* test.

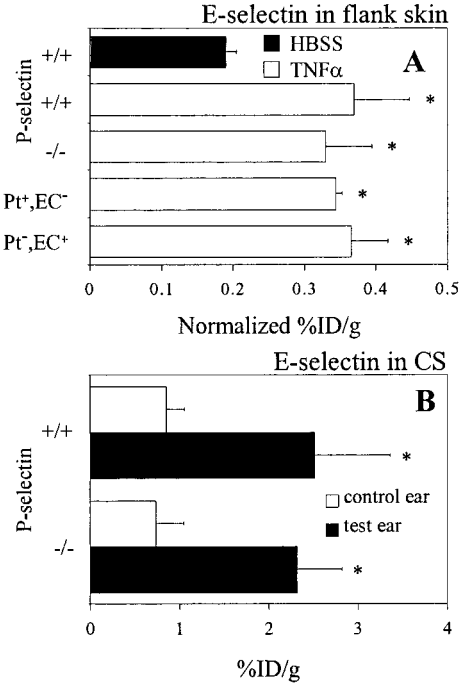

FIGURE 4. Targeted disruption of the P-selectin gene does not alter E-selectin up-regulation in vivo in response to TNF-α or in the CS response to OX. *A*, E-selectin expression was quantified 4 h after the i.d. injection of 5 ng TNF-α (□) or vehicle (HBSS; ■), in P-selectin +/+ C57BL/6 mice and in those deficient in endothelial (Pt^+, EC^-) or platelet (Pt^-, EC^+) P-selectin, or both (−/−) ($n = 3$ per group). The up-regulation of E-selectin expression seen in all groups of P-selectin-deficient mice in response to TNF-α was the same as that seen in P-selectin +/+ mice. *, $p < 0.05$ compared with HBSS-injected +/+ mice using Student's unpaired *t* test. *B*, Increased expression of E-selectin was quantified in the ears of 5% OX-sensitized mice 24 h after challenge with 5% OX (test ear) or vehicle alone (control ear) ($n = 5$ per group). The up-regulation seen in the test ears of P-selectin +/+ and −/− mice was not significantly different. *, $p < 0.05$ compared with the control ear; Students' unpaired *t* test ($n = 5$ per group). Bars represent mean + SD.

−/− mice (Fig. 3*C*) suggested that P-selectin is normally basally expressed in the skin (cf. Fig. 1). However, it was possible that i.d. application of vehicle alone was sufficient to induce P-selectin expression. We therefore proceeded to evaluate P-selectin expression in entirely untreated ear skin, of +/+ and −/− mice. Fig. 3*D* clearly shows a significant low level constitutive luminal expression of P-selectin in the skin of untreated C57BL/6 mouse ears.

E-selectin expression in CS responses of P-selectin-deficient mice

Previous work with E and P-selectin gene-targeted mice has raised the possibility that disruption of the gene encoding one selectin molecule might cause compensatory changes during ontogeny in the control of expression of other selectin molecules (17–19). We were able to directly address this question by quantifying E-selectin expression in wild-type and P-selectin −/− mice in both the TNF-α skin spot model (Fig. 4*A*) and in CS responses to OX (Fig. 4*B*). We found no differences in TNF-α- or OX-induced E-selectin expression between wild-type and P-selectin −/− mice, suggesting that a compensatory up-regulation of E-selectin in response to P-selectin gene-targeting had not occurred.

Selectin up-regulation has different mechanisms early and late in the CS response

We delineated the precise kinetics of E- and P-selectin expression during the CS response to OX in BALB/c mice. Fig. 5*A* shows that E-selectin expression had two overlapping phases of expression, with peaks around 6 and 48 h, after which expression returned toward baseline. The kinetics of P-selectin expression closely paralleled that of E-selectin (Fig. 5*B*). In contrast to the biphasic kinetics of selectin expression, ear thickness increase measured during this period (i.e., 6–72 h) was monophasic (Fig. 5*C*). We then determined the earliest time point at which increased P-selectin expression was detectable during the onset of the CS response. As shown in Fig. 5*D*, significant P-selectin up-regulation was first noted at 2 h after application of OX. No difference was detected between the time of onset of P-selectin and that of E-selectin expression (data not shown).

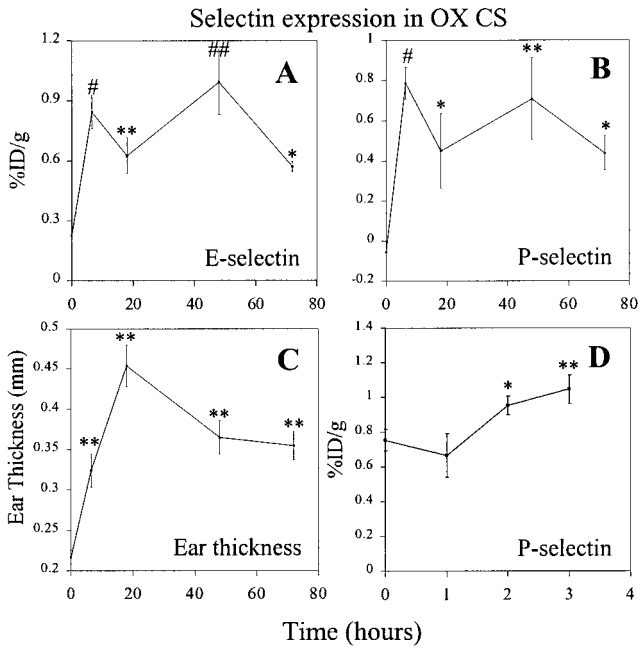

FIGURE 5. Endothelial E- and P-selectin expression in CS is prolonged and biphasic. Expression of E-selectin (*A*) and P-selectin (*B*) and ear thickness (*C*) were determined at various times during 72 h in 1% OX-sensitized BALB/c mice (n = 3–7) challenged on both ears with 1% OX. Values represent mean ± SD. Both selectins were up-regulated during 72 h, with two distinct peaks in expression at 6 and 48 h. Ear thickness increase also persisted over 72 h and was monophasic when beginning at 4 h, with a peak at 24 h. Results shown are representative of three experiments. *D*, P-selectin expression was determined during the first 3 h after application of the 1% challenge dose of OX (n = 5 per time point). Specific uptake of ^{111}In-labeled anti-P-selectin (mAb RB40) did not differ from baseline at 1 h but was significantly increased from 2 h. Results are representative of two experiments. Groups were compared using the one-tailed ANOVA with Bonferroni correction. ∗, $p < 0.05$ and ∗∗, $p < 0.001$ vs 0 h; #, $p < 0.05$ and ##, $p < 0.001$ vs 18 h.

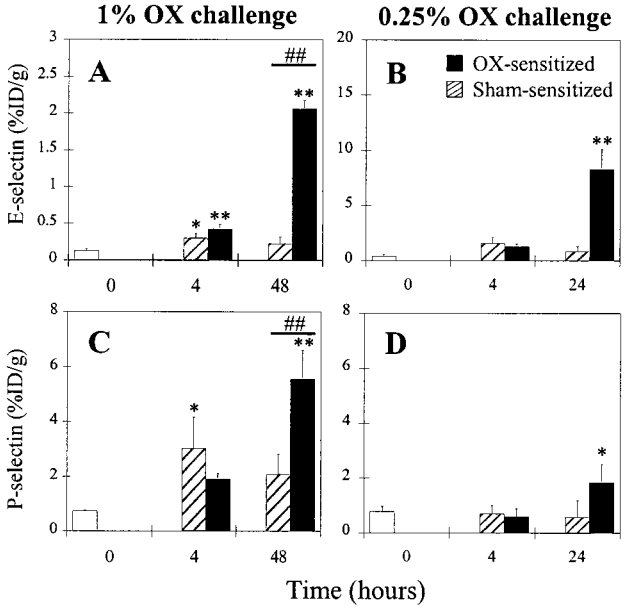

FIGURE 6. Early and late phases of E- and P-selectin expression in CS differ with respect to dependency on OX concentration used for challenge and on previous sensitization. Expression of E-selectin (*A*, *B*) and P-selectin (*C*, *D*) was determined at various times after challenge with 1% (*A*, *C*) or 0.25% OX (*B*, *D*) in BALB/c mice (n = 4 per group) previously sensitized with 1% OX (■), or with vehicle alone (▨). Columns represent mean + SD. From 24 h after 0.25% OX and 48 h after 1% OX challenge, selectin up-regulation was detected only in sensitized mice. At 4 h after 1% OX challenge, both E- (*A*) and P-selectin (*C*) up-regulation was detected at similar levels in sensitized and sham-sensitized mice; 0.25% OX was not sufficient to induce a detectable early selectin up-regulation. Groups were compared by one-tailed ANOVA with Bonferroni correction. ∗, $p < 0.05$, ∗∗, $p < 0.001$ in comparison with the 0-h group; ##, $p < 0.001$ comparing OX- vs sham-sensitized groups at 48 h.

The experiment illustrated in Fig. 6 was designed to establish the contribution of Ag-specific immunity to the two phases of expression of E- and P-selectin. OX-sensitized mice were compared with those sham-sensitized with vehicle alone. Responses were then elicited with 1% OX and with a lower OX challenge dose of 0.25%. The late phase of expression of both E-selectin (Fig. 6, *A* and *B*) and P-selectin (Fig. 6, *C* and *D*) observed in sensitized mice was not detected in sham-sensitized mice. In contrast, at 4 h, selectin expression did not differ significantly with respect to previous exposure to OX. Furthermore, this early expression was highly dose dependent, given that it was not detected with the 0.25% OX challenge (Fig. 6, *B* and *D*). Thus, selectin expression at 4 h after the elicitation of CS was Ag nonspecific, whereas by 24 h expression of the two selectins was largely dependent on prior immunization.

We next determined the effect of inhibiting TNF-α, both IL-1α and IL-1β, or all three cytokines on selectin expression during CS, using specific sheep polyclonal neutralizing antisera. These had been shown to inhibit the expression of ICAM-1 and VCAM-1 (8) and E- and P-selectin (data not shown) in response to i.d. injection of the appropriate recombinant cytokines. Cytokine blockade during the CS response was achieved by i.p. administration of antisera 24 h before OX challenge and thereafter every 24 h until termination of the experiment. As expected, the antisera significantly reduced the increase in ear thickness seen in control mice at 48 h after challenge (from 0.11 ± 0.012 mm to 0.060 ± 0.019 mm for TNF-α and to 0.074 ± 0.028 mm for IL-1; $p < 0.005$ and $p < 0.05$ by Student's unpaired t test, respectively). As shown in Fig. 7, *A* and *C*, expression of both E- and P-selectins at 4 h after challenge was not detectably affected by inhibition with these anti-cytokine antisera. In contrast, at 48 h there was a modest but significant inhibition of P-selectin expression with anti-IL-1α and -β and with a combination of anti-IL-1 and anti-TNF-α. In contrast, we observed no statistically significant effect of IL-1 and TNF-α blockade on E-selectin expression (Fig. 7*B*).

Discussion

Measurement of EC surface determinants with i.v.-injected radiolabeled mAb is a relatively new technique that allows a detailed analysis of changes in the EC surface over the course of inflammatory responses. In the case of P-selectin, the technique offers the advantage over quantitative immunohistochemistry in distinguishing luminal Ag from that contained in intracellular Weibel-Palade bodies. By comparing anti-P-selectin mAb uptake in wild-type and P-selectin −/− mice and by immunohistochemical localization of mAb RB40, we obtained direct evidence supporting suggestions from functional experiments that P-selectin is expressed constitutively on the luminal surface of EC in murine dermal vasculature (20–22). In parallel, we found no evidence of basal E-selectin expression in mouse skin, in contrast with our findings in pig skin

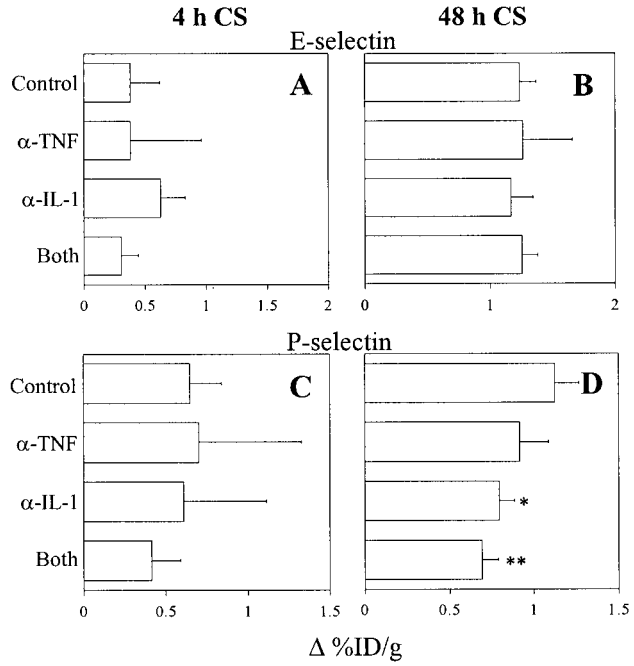

FIGURE 7. Effects of TNFα and IL-1 inhibition on early and late phases of E- and P-selectin up-regulation in CS. BALB/c mice previously sensitized with 1% OX ($n = 5$ per group) were challenged with 1% OX on the right ear and with vehicle on the left ear 4 h (*A, C*), or 48 h (*B, D*) before E-selectin (*A, B*) and P-selectin (*C, D*) quantification. For each mouse, %ID/g in the control ear was subtracted from that in the test ear to give an increase (Δ) in %ID/g due to the CS response. Mice were pretreated by i.p. injection of neutralizing sheep antisera to TNF-α (100 μl), to the combination of anti-IL-1α (100 μl) and anti-IL-1β (100 μl) (i.e., anti-IL-1), or to both. Normal sheep serum was used to make up the volume of all anti-cytokine injections to 300 μl, and control mice received this volume of normal sheep serum. E- and P-selectin were significantly up-regulated in the control groups (comparing %ID/g in the test ear with that in the control ear by Student's paired *t* test). At 48 h, the Δ %ID/g of anti-P-selectin (*D*), but not that of anti-E-selectin (*C*), was significantly reduced by the anti-IL-1 antisera, and addition of anti-TNF-α serum provided no further inhibition (*, $p < 0.01$, **, $p < 0.001$, ANOVA with Bonferroni correction).

(23). These observations, together with species differences in cytokine responsiveness of EC P-selectin (24, 25),[5] highlight the importance of establishing differences in expression patterns of selectins and other adhesion molecules in individual models. The functional significance of species differences in the relative quantitative expression of P- and E-selectins is at present still unclear.

Besides providing data on the expression of P-selectin, experiments with the P-selectin −/− mice also allowed us to clarify whether the relatively small effect of targeted disruption of the P-selectin gene on leukocyte trafficking into inflamed skin is caused by a developmental compensatory increase in E-selectin expression. We found no difference in E-selectin expression between wild-type and P-selectin −/− mice, supporting the view that in the mouse the two EC selectins are to some extent redundant (9, 10, 18).

In CS, we found prolonged up-regulation of both selectins throughout the time course of the elicited response. Up-regulation was detected in two phases: an early phase peaking at 4–6 h; and a later phase at 24–48 h. Our data support previous observations on E-selectin expression made using the radiolabeled mAb technique in pig skin (5) and by immunohistochemistry in the mouse (26). It now appears that the kinetics of P-selectin expression is similar to that of E-selectin. We have distinguished mechanisms underlying the early and the late phase. The early phase was independent of immunity to Ag and highly OX dose dependent, consistent with an "irritant" effect of the hapten (27), whereas the late phase was immune dependent. Unlike the early phase, the late phase was inhibited by mAb to α_4 and β_2 integrins (data not shown), suggesting a dependence on the migration of leukocytes into the lesion for its expression. Local elaboration of both TNF-α and IL-1 have been shown to be important in mediating inflammation in CS (28, 29). Our studies attempting to establish roles for these cytokines in the stimulation of E- and P-selectin showed evidence for IL-1 mediating P-selectin expression in the late phase, but otherwise were negative. It is possible that the inhibitory effects of anti-cytokine antisera lay below the level of detection in this experimental system. However, the failure of anti-TNF-α to inhibit E-selectin expression is consistent with our experience in the porcine delayed-type hypersensitivity response to tuberculin purified protein derivative (30). The exact mediators responsible for up-regulation of selectins in the two phases therefore remain to be determined but, in the case of the early phase, could include a direct effect on EC of hapten itself (31).

Comparison with the results of our earlier study (8) shows that expression of the EC selectins differs from that of ICAM-1 and VCAM-1 in at least three ways: 1) expression of the selectins is more obviously biphasic; 2) expression of the integrin ligands was immune dependent in both the early and later phases of the CS response, whereas the early expression of E- and P-selectins was not Ag-specific; 3) expression of ICAM-1 and VCAM-1 at 4 h after challenge was inhibited by anti-TNF-α, which had no detectable inhibitory effect on expression of the selectins at this or later time points. These differences were also observed in our study comparing E-selectin and VCAM-1 expression in the porcine delayed-type hypersensitivity response to purified protein derivative (30), suggesting that they are not unique to the murine model.

Taken together, our studies suggest that the two EC selectins perform a different function to the integrin ligands in the control of leukocyte recruitment in CS responses. The ability of EC to express selectins transiently in response to irritant stimuli may allow leukocytes to be exposed to factors in the local vascular microenvironment without committing them to firm adhesion. In sensitized mice, however, the additional up-regulated expression of ICAM-1 and VCAM-1 may facilitate the firm adhesion and transendothelial migration of activated CS-effector T cells critical to the development of the response.

In conclusion, this study has demonstrated that the persistence of both E- and P-selectin expression in vivo can occur in cutaneous immune responses as a result of sequential EC activation, a process likely to be relevant in chronic, immune-mediated inflammatory diseases. We postulate that selectin expression in response to different stimuli enables leukocyte rolling in a number of pathophysiological states, whereas the more tightly controlled expression of ICAM-1 and VCAM-1 may be a rate-limiting step for in the leukocyte adhesion cascade leading ultimately to recruitment across the endothelium and into the tissues.

[5] C. J. Stocker, K. L. Sugars, O. A. Harari, R. C. Landis, B. J. Morley, and D. O. Haskard. TNF-α, IL-4 and IFN-γ regulate differential expression of P- and E-selectin expression by porcine aortic endothelial cells. *Submitted for publication.*

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