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Role of selectins on IgE-mediated skin reaction

*,[1]Yukihisa Wada, [1]Akemi Kuzuhara, [1]Mikiko Hanamura, [1]Rie Kida, [1]Tsuyoshi Yoshinaka & [1]Tadayuki Saito

[1]R&D Laboratories, Nippon Organon K.K., 5-90. Tomobuti-cho 1-chome Miyakojima-ku, Osaka 534-0016, Japan

1 Selectins play an important role on leukocytes infiltration into inflammatory tissues. To understand the role of selectins, we investigated the effects of selectin-IgG chimeras and anti selectin antibodies on the murine IgE-mediated skin inflammation model.

2 Biphasic skin reactions were induced by intradermal challenge with ovalbumin (OA) to ears of actively sensitized mice. This reaction was characterized by immediate and late phase responses observed as which were induced *via* a rapid increase in capillary permeability and leukocyte infiltration, respectively. The expression of E-selectin mRNA was significantly increased to reach its highest level at 2 h after OA challenge.

3 E-, P-, and L-selectin-IgG chimeras inhibited the late phase responses, i.e. ear swelling, neutrophil infiltration and eosinophil infiltration at 24 h after OA challenge in a dose-dependent manner at dose range of 0.1–10 mg kg^{-1}, i.v. Antiselectin antibodies did not inhibit the increase of ear swelling. But anti E- and P-selectin antibodies significantly inhibited neutrophil infiltration and eosinophil infiltration.

4 These results indicate that selectins play an important role on the late phase response of the murine IgE-mediated skin inflammation model by mediating inflammatory cell adhesion to endothelium.

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Abbreviations: EPO, eosinophil peroxidase; IPR, immediate-phase response; LPR, late-phase response; MPO, myeloperoxidase; OA, ovalbumin; PBS, phosphate-buffered saline; sLe^x, sialyl Lewis x

Introduction

Selectins are a family of three Ca^{2+}-dependent membrane-bound lectins that interact with not only small sialylated oligosaccharides such as sialyl Lewis x (sLe^x) (Foxall *et al*., 1992; Varki, 1994), but also glycans displayed on a limited number of glycoproteins with higher affinity. E-selectin, expressed by cytokine-activated endothelial cells, and P-selectin, expressed by thrombin activated platelets and endothelial cells, bind to ligands on myeloid cells and subsets of lymphocytes. L-selectin, expressed on leukocytes, binds to constitutive or inducible ligands on endothelial cells. These selectins participate in the initial phase of adhesion between inflammatory cells and endothelial cells, and play an important role on leukocytes infiltration into inflammatory tissues (Berg *et al*., 1991; Picker *et al*., 1991; Lorant *et al*., 1993; Symon *et al*., 1994; Akbar *et al*., 1991).

Atopic dermatitis is a common inflammatory skin condition characterized by severe pruritis, chronic relapses with frequent periods of exacerbation, and distinctive clinical morphology and distribution of skin lesions. After the challenge with relevant antigen, sensitized animals or atopic individuals exhibit immediate responses such as the appearance of weals and flares on the skin. When a high concentration of allergen is used, oedema and erythema usually persist at the challenged site for a 6–24 h period. These dual responses are of interest because of the similarity to the clinical manifestations of chronic allergic diseases (Patterson, 1969; 1983; Ray *et al*., 1983; Butler *et al*., 1983; Katayama *et al*., 1990; Nagai *et al*., 1995). Pathogenic examination of the cutaneous late-phase response (LPR) has shown infiltration of mononuclear cells, neutrophils, basophils and eosinophils (Solley *et al*., 1976; Dolovich *et al*., 1973). Cell infiltration into inflammatory sites is one of the causes of chronic allergic inflammation (Charlesworth *et al*., 1989).

In the present study, we have measured cell infiltration into skin tissue in the LPR in a murine IgE-mediated skin inflammation model, and investigated the role of selectin molecules in this model.

Methods

Animals

Balb/c mice (female, 7 weeks-old) were purchased from Japan SLC (Hamamatsu, Japan). They were housed in an air-conditioned room (temperature 23±2°C, humidity 55±10%) with a controlled light-dark cycle (light on 07:30–19:30 h) and freely available food and water. All the experiments were performed according to the Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Mouse selectin-immunoglobulin fusion proteins

Mouse E-selectin-IgG chimera, mouse P-selectin-IgG chimera and mouse L-selectin-IgG chimera, were produced as described previously (Watson *et al*., 1991; Erbe *et al*., 1993; Ohmoto *et al*., 1996; Wada *et al*., 1996). Briefly the proteins were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS (−)). Mouse IgG2a (Zymed, San Francisco, CA, U.S.A.), dissolved in PBS (−), was used as control. Mouse E-selectin-IgG chimera was given intravenously 2 h after the

*Author for correspondence.

OA challenge. Other test agents were administered intravenously 1 min prior to OA challenge.

Monoclonal anti-E-, P- or L-selectin antibodies

The monoclonal antibodies were purchased from PharMingen (San Diego, CA, U.S.A.). Anti-E-selectin antibody (10E9.6), anti-P-selectin antibody (RB40, 34), anti-L-selectin antibody (MEL-14) were dissolved in PBS (−). Mouse IgG2a, dissolved in PBS (−), was used as control. All anti-selectin antibodies used, have been reported to have inhibitory activity on cells to sLe^x binding *in vitro* (Bosse & Vestweber, 1994; Fink *et al*., 1985; Weller *et al*., 1992). Anti-E-selectin antibody was given intravenously 2 h after the OA challenge. The others were given intravenously 1 min prior to OA challenge.

IgE-mediated murine skin inflammatory model

IgE-mediated murine skin inflammatory model were carried out as described previously (Nagai *et al*., 1995). Eight-week-old female Balb/c mice were sensitized by intraperitoneal injection with 3 μg of OA and 4 mg of aluminium hydroxide gel (alum). Reactions to OA were elicited by intracutaneous injection of 10 μg of OA to each ear of mice 2 weeks after the sensitization. Various times after the antigen challenge, the mice were anesthetized by ether and killed. Then, 6 mm diameter biopsy of the ears were taken and weighed as an index of tissue swelling. The specimens were homogenized in 50 mM potassium phosphate with 0.5% hexadecyltrimethylammonium bromide, pH 6.0, and myeloperoxidase (MPO) activities in the supernatants were measured by the method described by Bradley *et al*. (1982) as an index of neutrophil infiltration into tissue. In brief, the supernatants were mixed with MPO substrates buffer (0.167 mg ml^{-1} o-dianisidine (Sigma, St. Louis, MO, U.S.A.), 0.0005% H_2O_2, 50 mM potassium phosphate (pH 6)), and incubated for 20 min at 25°C. MPO activities were measured by determining the optical density (OD) at 450 nm. In addition, eosinophil peroxidase (EPO) activities in the supernatants were measured by the method described by Strath *et al*. (1985) as an index of eosinophil infiltration into tissue. In brief, the supernatants were mixed with EPO substrates buffer (1 mM o-phenylenediamine (Sigma, St. Louis, MO, U.S.A.), 0.5 mM H_2O_2, 0.1% Triton X-100, 50 mM potassium phosphate (pH 5)), and incubated for 30 min at 25°C. EPO activities were measured by determining the OD at 490 nm. For histological study, sensitized and non-sensitized mice were challenged with antigen, and the ears were removed 24 h later. The tissue was fixed in 10% neutral formalin solution and embedded in paraffin using a standard technique. The paraffin sections were stained with haematoxylin and eosin.

mRNA extraction and reverse transcriptase-PCR

At various times after the antigen challenge, ears were excised and immediately frozen in a liquid nitrogen, and stored at −80°C until mRNA extraction by oligo- (dT) magnetic particles (MPG Direct mRNA Purification Kit, CPG Inc. Lincoln Park, NJ, U.S.A.). The mRNA isolated from the ears were reverse transcribed into cDNA by SuperScript II Preamplification system (GIBCO−BRL, Gaithersburg, MD, U.S.A.). The E-selectin oligonucleotides primers were synthesized by Greiner Japan (Tokyo, Japan), and β-actin primers, were used as control, were purchased from Stratagene (La Jolla, CA, U.S.A.). Primer sequences and their corresponding locations in the nucleotide sequence of mouse E-selectin were as follows; E-selectin, forward: 5′-TTCTCCTCGCTGGAGAGAGCACAGC-3′ (193−217), reverse: 5′-TAGCTCCCAGGATTTGAGGAACATT-3′ (919−944). These sequence data are available under EMBL/GeneBank/DDBJ accession number MUSELAMIB. E-selectin cDNAs were amplified singly. The PCR mixtures of E-selectin cDNA and respective primers were amplified using a thermal cycler system (GeneAmp System 2400; Applied Biosystems, Foster City, CA, U.S.A.). One cycle of PCR consisted of 45 s 94°C, 45 s 58°C, and 60 s at 72°C, and a total of 35 cycles was performed. Each PCR reaction mixture was subjected to 2% agarose gel electrophoresis, and the amplified products were visualized by staining with ethidium bromide for 20 min at room temperature. To confirm the specificity of PCR, the bands were also identified by Southern blot analysis.

Statistical analysis

Results are expressed as mean ± s.e.mean. A one-way analysis of variance with Dunnett's test was used to determine statistical significance. Statistical significance was defined as $P<0.05$.

Results

Time course of the ear oedema and the expression of E-selectin

Sensitized Balb/c mice were challenged with 10 μg of OA, and the time course of the reaction was assessed by measuring ear weight and MPO activity, as indices of oedema and cell infiltration, respectively. The increase of ear weight was significant at 1 h after the antigen challenge, and then it decreased gradually (Figure 1). This reaction was regarded as immediate-phase response (IPR). On the other hand, MPO activity increased from 4 h after the antigen challenge and came up to plateau level (Figure 1), and this was regarded as LPR. These results indicate that the IPR and LPR were characterized by a rapid increase in capillary

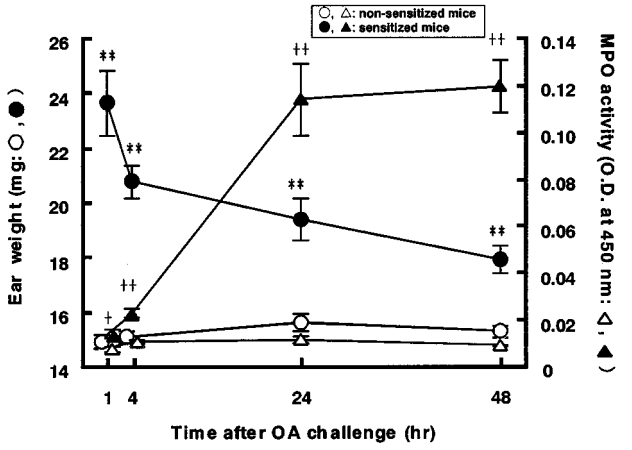

Figure 1 Time course of skin reaction mediated by IgE in ears of actively sensitized Balb/c mice. Ovalbumin (OA: 3 μg per animal) was given intraperitoneally with 4 mg per animal of alum 2 weeks before the challenge of 10 μg per ear of OA. Each point represents the mean of five animals. Vertical bars indicate s.e.mean. * $P<0.05$, ** $P<0.01$, significantly different from non-sensitized mice.

permeability and skin thickening with significant infiltration of inflammatory cells, respectively, in this model.

Histological analysis of the ears, excised at 24 h after the antigen challenge, is shown in Figure 2. No significant change was observed in non-sensitized mice. On the other hand, in sensitized mice, marked infiltration of neutrophils and eosinophils was shown as well as a significant increase in the MPO activity.

The expression of E-selectin mRNA was significantly increased to reach its highest level at 2 h after OA challenge, and decreased gradually (Figure 3). No amplified DNA fragments were observed when PCR performed with mRNA extracted from the ears of non-sensitized mice. Moreover, the sequences of these fragments were confirmed by Southern blot analysis (data not shown).

The effects of selectin-IgG chimeras and anti-selectin antibodies on IgE-mediated murine skin inflammatory model

Effects of E-, P- and L-selectin-IgG chimera, which bind sLe^x *in vitro*, on the IgE-mediated murine skin inflammatory model were studied. As shown in Figure 4, E-, P- and L-selectin-IgG chimera inhibited the increase in ear weight, MPO activity, and EPO activity in a dose-dependent manner at dose range of 0.1–10 mg kg^{-1}, i.v. Further, effects of anti selectin antibodies on the IgE-mediated murine skin inflammatory model were studied. Anti selectin antibodies did not inhibit the increase of ear weight. But anti E- and P-selectin

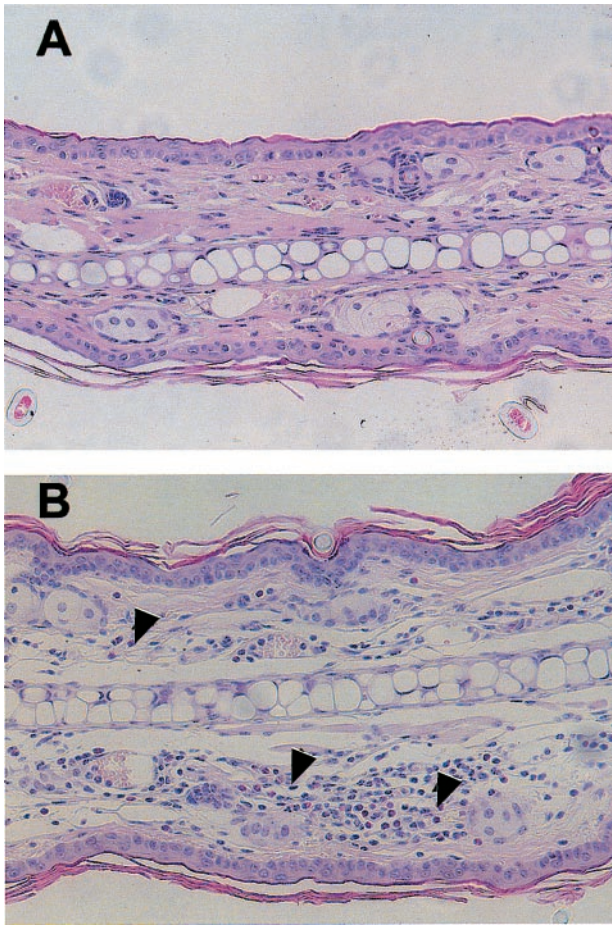

Figure 2 Histopathology of IgE-mediated skin lesion in the ears of actively sensitized mice. Skin sections were stained with haematoxylin and eosin. (A) Non-sensitized mice. (B) Sensitized mice. (Arrows) Infiltration of inflammatory cells (neutrophils and eosinophils). Magnification: × 200.

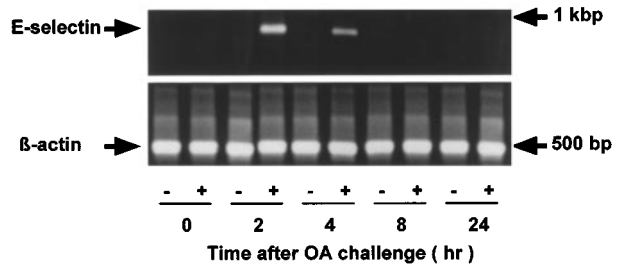

Figure 3 RT–PCR analysis of E-selectin expression in ear tissues of actively sensitized mice. −: non-sensitized mice.

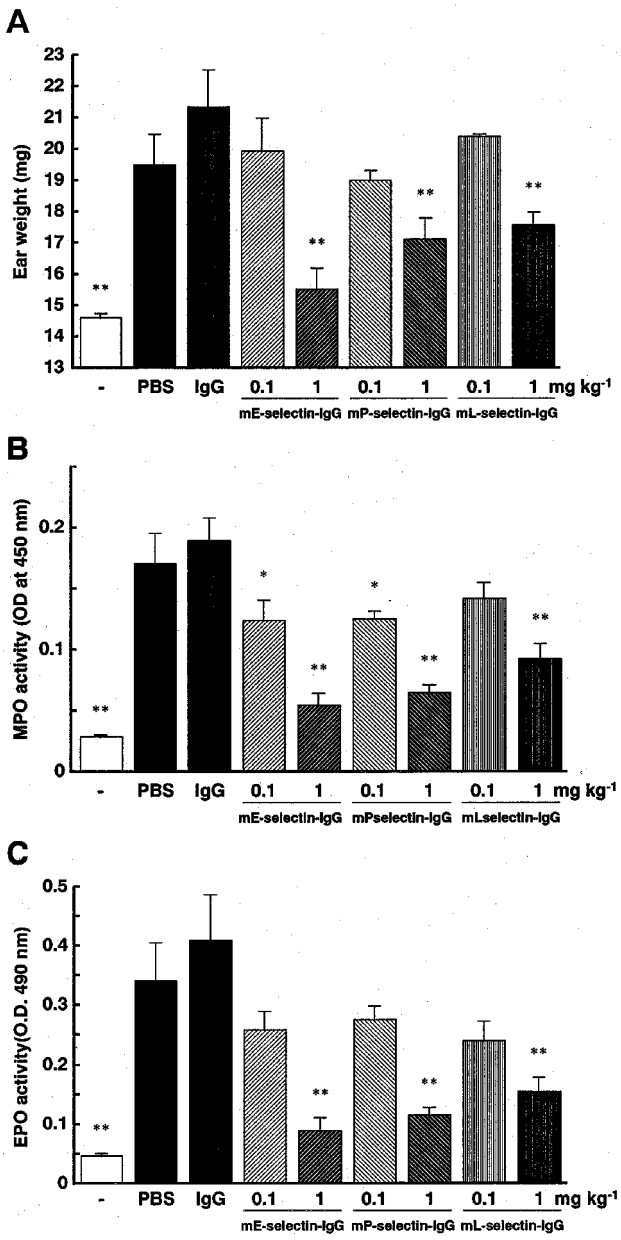

Figure 4 Effects of mouse E-, P- and L-selectin-IgG on IgE-mediated skin reaction in mice. (A) Effects on the increase of ear swelling. (B) Effects on neutrophil infiltration. (C) Effects on eosinophil infiltration. Each column represents the mean of five or six animals. Vertical bars indicate s.e.mean. * $P<0.05$, ** $P<0.01$, significantly different from IgG treated group. −: non-sensitized mice.

antibodies significantly inhibited the MPO and EPO activity (Figure 5) at dose range of 0.01–10 mg kg^{-1}, i.v. Anti L-selectin antibody significantly inhibited the MPO activity but it did not inhibit the EPO activity.

Discussion

Extravasation of leukocytes to sites of inflammation is thought to consist of at least three sequential processes (Butcher, 1991). Firstly, circulating leukocytes undergo margination, whereby they move from the centre to the periphery of the blood vessel, and begin to bind reversibly to the endothelium. This process is referred to as rolling, as they roll along the endothelial wall. This initial binding may be followed, during a second step, by the induction of firm adhesion (Luscinskas *et al*., 1991; Ebisawa *et al*., 1992), when the leucocytes stick to the endothelial wall. Subsequently, the leukocytes transmigrate through the endothelial cell monolayer into the tissue. *In vitro* studies indicate that the first stage consists of at least three sequential processes. One part of this process is mediated by the interaction of adhesion molecules on the leukocytes, such as L-selectin, with as yet unidentified counter structures on the endothelial cells (Spertini *et al*., 1991; Smith *et al*., 1991). A second process is mediated by the interaction between P-selectin on the endothelial cells and counter structures on the leukocytes, such as PSGL-1 (Moore *et al*., 1995). These two processes are thought to occur simultaneously. The third process is mediated by the interaction of E-selectin on the endothelial cells, synthesized *de novo*, with as yet unidentified counter structures on the leukocytes (Bevilacqua *et al*., 1987; 1989). In the present study, we have investigated the role of selectin molecules on the cell infiltration into skin tissue in IgE-mediated murine skin inflammatory model.

It has been previously reported that the IgE-mediated biphasic skin reaction in passively sensitized mice with monoclonal anti-DNP IgE antibody could serve as an animal model for atopic dermatitis (Katayama *et al*., 1990; Nagai *et al*., 1995). In this study, we demonstrate that the skin reaction model with actively sensitized mice could also serve as an animal model for atopic dermatitis. The IPR, which is characterized oedema, and LPR, which is characterized cell infiltration, were significantly observed in this actively

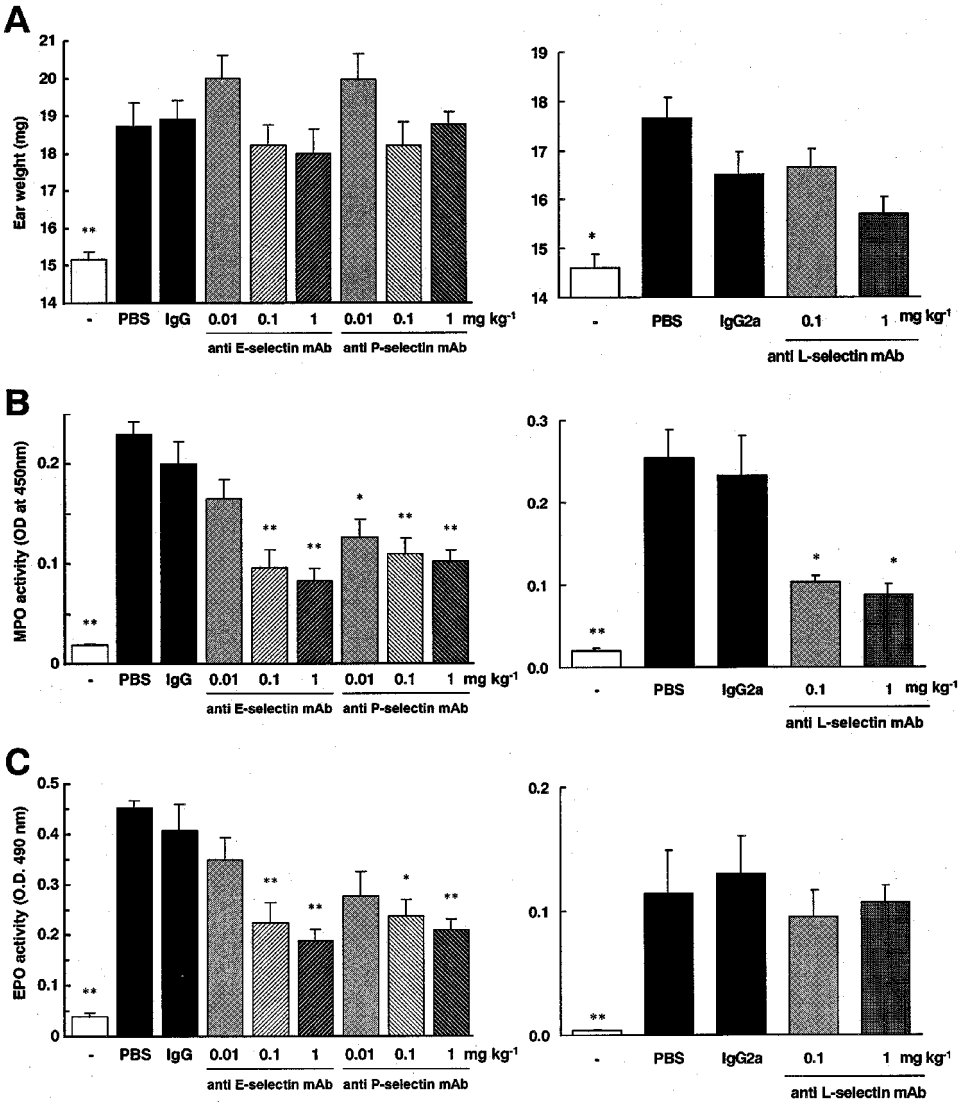

Figure 5 Effects of anti-mouse E-, P- and L-selectin antibodies on IgE-mediated skin reaction in mice. (A) Effects on the increase of ear swelling. (B) Effects on neutrophil infiltration. (C) Effects on eosinophil infiltration. Each column represents the mean of five or six animals. Vertical bars indicate s.e.mean. * $P<0.05$, ** $P<0.01$, significantly different from control immunoglobulin treated group. −: non-sensitized mice.

sensitized model used. In this study, we demonstrate that the skin reaction model with actively sensitized mice could also serve as an animal model for atopic dermatitis.

It was reported that marked vascular endothelial expression of E-selectin in association with inflammatory infiltration was observed in antigen-stimulated human skin (Cotran *et al*., 1986; Messadi *et al*., 1987) and atopic dematitis (Groves *et al*., 1991). E-selectin mRNA expression was also found in the skin tissue excised 2 h after the antigen challenge in our model. *In vitro*, E-selectin has been found to be induced on human endothelial cells after incubation with TNF-α, LPS, or IL-1 (Bevilacqua *et al*., 1987). It has been demonstrated that human dermal mast cells contained stores of TNF-α within granules, which can be released rapidly into the extracellular space upon degranulation (Walsh *et al*., 1991). Taken together, it might be suggested that in our model, E-selectin could be expressed by TNF-α released from skin mast cells induced by the interaction between antigen and IgE on mast cells.

In addition, neutrophil and eosinophil infiltration into skin tissue was significantly inhibited by selectin-IgG chimera and anti E- and P-selectin antibodies. Anti L-selectin antibody inhibited neutrophil infiltration, but it did not inhibit the eosinophil infiltration. The reason for the lack of effect of the anti L-selectin antibody in inhibiting the eosinophil infiltration is not clear. It is known that leucocytes express L-selectin constitutively, and the amount of neutrophils is extremely large compared with the eosinophils in peripheral blood. One possible explanation is that most anti L-selectin antibodies bind L-selectin molecules on neutrophils. If this is so, it could not bind eosinophils, and had no effect on infiltration of eosinophils. On the other hand, E-selectin and P-selectin were expressed by cytokine-activated endothelial cells, so anti E- and P-selectin antibodies bound endothelium and inhibited the inflammatory cells adhesion to endothelium. These results indicate that both selectins and their counter structures, which might be small sialylated oligosaccharides such as sLe^x which are displayed on a limited number of glycoproteins or glycolipids, play important roles on cell infiltration into inflammatory sites.

In conclusion, it is suggested that this murine IgE-mediated skin model of atopic dermatitis is a useful model for the study of mechanisms in dermatitis. The results indicate that selectins play important roles in the LPR of this model.

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