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Therapeutic potential of a novel synthetic selectin blocker, OJ-R9188, in allergic dermatitis

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1 We investigated the ability of a newly synthesized sugar derivative, OJ-R9188, {N-(2-tetradecylhexadecanoyl)-O-(L-alpha-fucofuranosyl)-D-seryl}-L-glutamic acid 1-methylamide 5-L-arginine salt, to block binding of selectins to their ligands *in vitro* and inhibit the infiltration of leukocytes *in vivo*.

2 OJ-R9188 prevented the binding of human E-, P- and L-selectin-IgG fusion proteins to immobilized sialyl Lewis^x (sLe^x)-pentasaccharide glycolipid, with IC_{50} values of 4.3, 1.3, and 1.2 μM, respectively.

3 In a mouse model of thioglycollate-induced peritonitis, OJ-R9188 at 10 mg kg^{-1}, i.v. inhibited neutrophil accumulation in the peritoneal cavity. In the IgE-mediated skin reaction, OJ-R9188 at 3 and 10 mg kg^{-1}, i.v. significantly inhibited extravasation of neutrophils and eosinophils into the inflammatory sites and at 10 mg kg^{-1}, i.v. also inhibited infiltration caused by picryl chloride-induced delayed-type hypersensitivity in mice. These results suggest that OJ-R9188 may be a useful selectin blocker, with activity against human and mouse E-, P- and L-selectins *in vitro* and *in vivo*, and that blocking selectin-sLe^x binding is a promising strategy for the treatment of allergic skin diseases.

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Abbreviations: EPO, eosinophil peroxidase; MPO, myeloperoxidase; OA, ovalbumin; PBS, phosphate-buffered saline; sLe^x, sialyl Lewis x

Introduction

The selectins are cell adhesion molecules known to support the initial attachment of leukocytes to inflamed vascular endothelium through their recognition of carbohydrate ligands such as sialyl Lewis^x (sLe^x) (Bevilacqua & Nelson, 1993; Lasky, 1992; Phillips *et al*., 1990; Rosen *et al*., 1993; Walz *et al*., 1990). There are three types of selectins: E-selectin (ELAM-1), P-selectin (GMP-140) and L-selectin (LECAM-1). E-selectin and P-selectin are expressed on endothelial cells and upregulated during inflammation (Bevilacqua *et al*., 1989; Bonfanti *et al*., 1989; Isenberg *et al*., 1986; McEver *et al*., 1989; Stenberg *et al*., 1985). A ligand for E-selectin on human lymphocytes was identified as an antigenic determinant selectively expressed by the majority of T cells in skin. The epitope was called the cutaneous lymphocyte antigen (CLA), and it selectively and avidly binds to E-selectin (Berg *et al*., 1991; Picker *et al*., 1933). E-selectin expression on vascular endothelial cells is observed in inflammatory skin diseases, such as contact dermatitis, atopic dermatitis and psoriasis (Groves *et al*., 1991; Leung *et al*., 1991). Increased expression of E-selectin is also observed in chronic skin diseases (Picker *et al*., 1991).

P-selectin was initially found on activated platelets, and its expression on the surface of endothelial cells is induced within a few minutes by appropriate stimuli (Bonfanti *et al*., 1989; Isenberg *et al*., 1986; McEver *et al*., 1989; Stenberg *et al*., 1985). In P-selectin-deficient mice, a significant reduction of CD4+T cell accumulation in the skin in oxazolone-induced delayed-type contact hypersensitivity (DTH) has been demonstrated (Subramaniam *et al*., 1995; Staite *et al*., 1996).

L-selectin is constitutively expressed by most leukocytes. In addition to mediating leukocyte/endothelial cell interactions, L-selectin can also mediate intercellular interactions between leukocytes (Butcher *et al*., 1992). Binding of selectins to their carbohydrate ligands appears to be required for neutrophil rolling and extravasation and plays a major role in lymphocyte recirculation (Berg *et al*., 1991; Picker *et al*., 1991; Lorant *et al*., 1993; Symon *et al*., 1994; Akbar *et al*., 1991). Thus, blockade of the functions of selectins may become a strategy for suppressing inflammation.

In previous studies, we have demonstrated that the selectins play important roles in the late phase response of the IgE-mediated skin reaction in mice (Wada *et al*., 2000). We have been studying low molecular weight selectin blockers that prevent the inflammatory skin reaction. We have identified a sugar derivative, {N-(2-tetradecylhexadecanoyl)-O-(L-alpha-fucofuranosyl)-D-seryl}-L-glutamic acid 1-methylamide, OJ-R9188, from studies using computer modelling of the relationship between selectins and glycolipids, including sLe^x, and we have reported the effects of OJ-R9188 on the binding of human E-selectin-IgG fusion protein to immobilized sialyl Lewis^x neoglycoprotein *in vitro*

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(Hiramatsu *et al*., 1998). In the present paper, we investigated the effects of OJ-R9188 on extravascular infiltration of leukocytes caused by the IgE-mediated skin reaction and by picryl chloride-induced delayed type hypersensitivity (PC-DTH) in mice.

Methods

Animals

Balb/c mice (7 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The mice were acclimatized in the animal facility of the R&D Laboratories, where they were kept at a temperature of 22–26°C and a relative humidity of 35–70% under a light period of 12 h (0700 to 1900 h) for more than a week until use for experiments. During the experiments, the mice were fed in the animal facility. All animal experiments were performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Drugs

OJ-R9188, {N-(2-tetradecylhexadecanoyl)-O-(L-alpha-fucofuranosyl)-D-seryl}-L-glutamic acid 1-methylamide 5-L-arginine, sLe^x-pentasaccharide and TBC-1269 were synthesized at the R&D Laboratories, Nippon Organon. Bovine serum albumin (BSA), dexamethasone, *o*-dianisidine dihydrochloride, fucoidin, hexadecyltrimethylammonium bromide (HTAB), *p*-nitrophenylphosphate, ovalbumin (OA), fucoidin and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diethanolamine, H_2O_2 and *o*-phenylenediamine dihydrochloride were from Wako Pure Chemical (Osaka, Japan). Biotinylated goat $F(ab')_2$ anti-human IgG (Fc) was from Biosource (Camarillo, CA, U.S.A.). Streptavidin-alkaline phosphatase was from Zymed (San Francisco, CA, U.S.A.).

Table 1 Blocking activities of OJ-R9188 and sLe^x pentasaccharide against sLe^x glycolipid binding to human E-, P-, and L-selectin-IgG chimeras

Compound	*IC50 (μM) E-selectin*	*P-selectin*	*L-selectin*
OJ-R9188	4.3	1.3	1.2
sLe^x penta	580	>1000	>1000
Fucoidin	60*	0.014*	0.042*
TBC-1269	>500	97	130

Blocking activities were measured by ELISA. *; $\mu g\ ml^{-1}$.

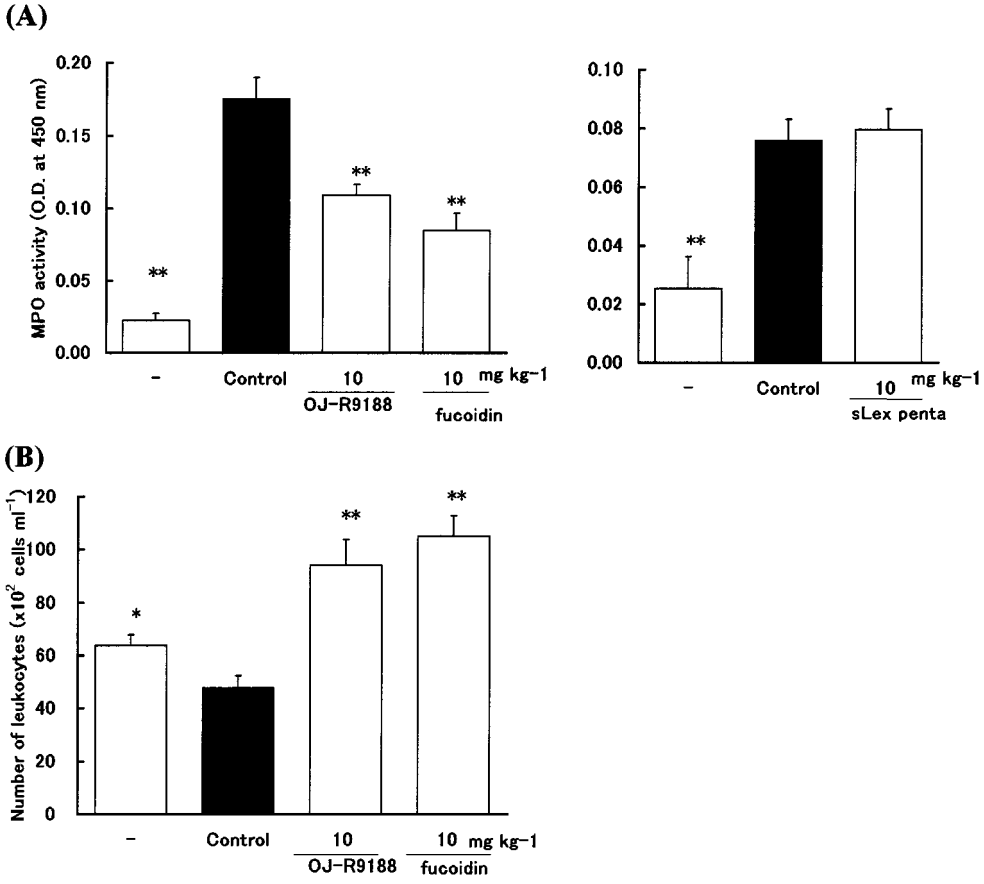

Figure 1 Effects of OJ-R9188, fucoidin and sLe^x-pentasaccharide on thioglycollate-induced peritonitis in mice. OJ-R9188, fucoidin and sLe^x-pentasaccharide were administered intravenously just before the thioglycollate injection. Each column represents the mean for five or six animals. Vertical bars indicate s.e. $*P<0.05$, $**P<0.01$, significantly different from control. -: Saline was injected instead of thioglycollate.

In vitro *binding of selectin-IgG chimeras to immobilized sLe^x-pentasaccharide ceramide*

Selectin blocking activities were tested by inhibition of binding of human E-, P- and L-selectin-IgG chimeras (selectin Ig) to sLe^x-pentasaccharide ceramide (Kiyoi *et al*., 1996) according to the method described by Foxall *et al*. (1992). The construction, expression and purification of human and mouse E-, P- and L-selectin Ig were carried out as described previously (Ohmoto *et al*., 1996). Aliquots (50 μl; 100 pmol) of sLe^x-pentasaccharide ceramide in 50% methanol were placed into microplate wells (96-well plates; Falcon Pro-Bind (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and the solvent was evaporated. The wells were washed twice with distilled water, blocked with 5% BSA in 50 mM imidazole buffer (pH 7.2) supplemented with 1 mM $CaCl_2$ for 1 h at room temperature, and washed three times with 50 mM imidazole buffer (pH 7.2). Separately, 0.2% biotinylated goat F(ab′)$_2$ anti-human IgG (Fc), 0.2% streptavidin-alkaline phosphatase and selectin Ig (E-selectin, 2 μg ml^{-1}; P-selectin, 20 μg ml^{-1}; L-selectin, 20 μg ml^{-1}) in 50 mM imidazole buffer (pH 7.2) containing 1% BSA and 1 mM $CaCl_2$ were incubated at room temperature for 30 min to form a complex. This solution containing selectin Ig was mixed with test compound solution (1 : 1) and allowed to stand for 30 min at room temperature. This mixture was then added to the above microplate wells at 50 μl well^{-1} and allowed to react for 45 min at 37°C. The wells were washed three times with 50 mM imidazole buffer (pH 7.2) followed by distilled water. Then, 50 μl of 1 M diethanolamine (pH 9.8) containing 1 mg ml^{-1} of *p*-nitrophenylphosphate and 0.01% of $MgCl_2$ was added to each well. The plate was incubated for 1–2 h at room temperature and the absorbance at 405 nm was measured with a spectrophotometer. The test compounds at 10 mM were dissolved in distilled water, which was diluted with 50 mM imidazole buffer (pH 7.2) containing 1 mM $CaCl_2$. Percentage inhibition of binding was calculated as:

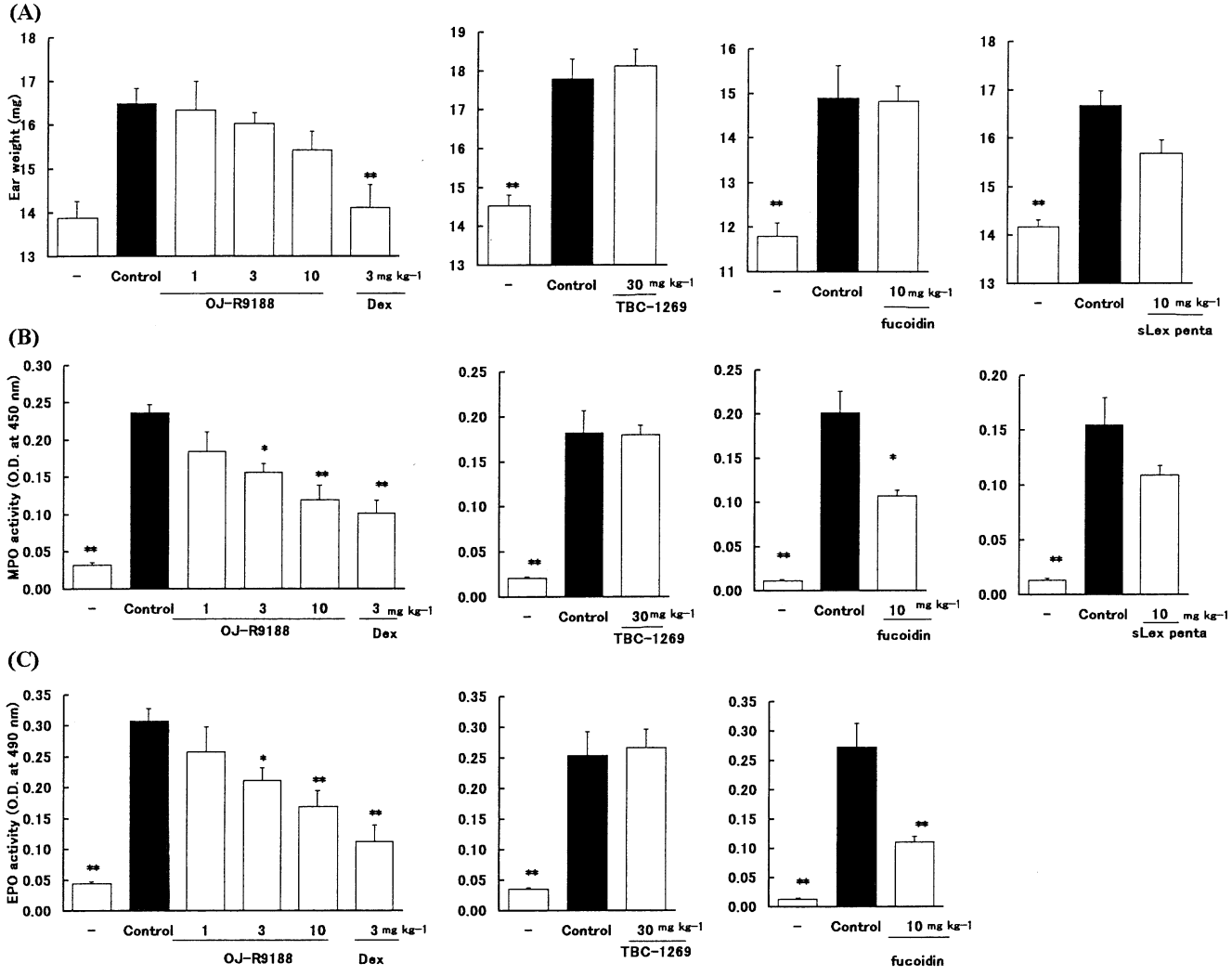

Figure 2 Effects of OJ-R9188, TBC-1269, fucoidin and sLe^x-pentasaccharide on the IgE-mediated skin reaction in actively sensitized mice. (A) Ear weight, (B) MPO activity, and (C) EPO activity. OJ-R9188, TBC-1269, fucoidin and sLe^x-pentasaccharide were given intravenously just before the antigen challenge. Dexamethasone (Dex) was given orally 2 h before the antigen challenge. Each column represents the mean of five to seven animals. Vertical bars indicate s.e. $*P<0.05$, $**P<0.01$, significantly different from control. -: unsensitized group.

$$\text{Inhibition } (\%) = 1(\text{-X/A}) \times 100$$

Where X is the absorbance of wells containing the test compound at each concentration, and A is the absorbance of wells not containing the test compound. The IC_{50} value was calculated by Probit analysis.

Thioglycollate-induced peritonitis in mice

Peritonitis was induced by injection of 1 ml of 3% thioglycollate into the peritoneal cavity of male Balb/c mice (8 weeks old). Two hours later, the mice were sacrificed and peritoneal cells were harvested by lavage with 7 ml of PBS containing 0.1% BSA, 0.5 mM EDTA, and 10 u ml^{-1} heparin. The cells were collected by centrifugation ($180 \times g$, 10 min, 4°C) and treated with hypotonic solution to cause haemolysis. After centrifugation ($180 \times g$, 10 min, 4°C), the cells were lysed with 1 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% HTAB. After centrifugation ($10{,}000 \times g$, 10 min), myeloperoxidase (MPO) activities in the supernatants were measured by the method described below. The test compounds were dissolved in saline and administered intravenously just before the thioglycollate injection.

IgE-mediated murine skin inflammatory model

The procedure for the IgE-mediated skin inflammatory model was described previously by Nagai *et al.* (1995). Briefly, female Balb/c mice (8 weeks old) were sensitized by intraperitoneal injection of 3 μg of OA and 4 mg of alum. Two weeks later, the mice were challenged by intracutaneous injection of 10 μl of OA (1 mg ml^{-1}) into each ear. At 24 h after the challenge, the mice were sacrificed and 6-mm diameter biopsies of both ears were taken and weighed. The specimens were homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% HTAB with the use of an HG30 homogenizer (Hitachi, Tokyo, Japan). After centrifugation ($10{,}000 \times g$, 10 min), MPO and eosinophil peroxidase (EPO) activities in the supernatants were measured by the method described below. OJ-R9188 was dissolved in distilled water and given intravenously just before the challenge with antigen. Dexamethasone was suspended in 0.5% CMC and given orally 2 h before antigen challenge.

Picryl chloride-induced delayed-type hypersensitivity (PC-DTH) in mice

Male Balb/c mice (8 weeks old) were sensitized by topical application of 1% PC in acetone/olive oil (4 : 1) on the shaved abdomen. A week later, the same PC solution was applied topically to both ears to induce a DTH reaction. At 24 h after the challenge, the mice were sacrificed and 6-mm diameter biopsies of both ears were taken and weighed. The ear tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% HTAB and centrifuged (12,000 r.p.m., 10 min). The supernatant was stored at −20°C until determination of MPO and EPO activities. The test compounds, except dexamethasone, were dissolved in saline and given intravenously just before the challenge. Dexamethasone was suspended in 0.5% CMC and given orally 2 h before the challenge.

Determination of MPO activity

MPO activity was determined by the method described previously by Bradley *et al.* (1982). Briefly, an aliquot (25 μl) of the supernatant and 225 μl of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg ml^{-1} *o*-dianisidine and 0.0005% hydrogen peroxide were placed into 96-well plates and incubated for 30 min at room temperature. The absorbance at 450 nm was measured with a microplate reader (Spectra Max 250; Molecular Devices, Sunnyvale, CA, U.S.A.).

Determination of EPO activity

EPO activity was determined by the method described previously by Strath *et al.* (1985). Briefly, an aliquot (50 μl) of the supernatant and 100 μl of 50 mM Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100, 1 mM *o*-phenylendiamine and 0.5 mM hydrogen peroxide were placed into 96-well plates and incubated for 30 min at room temperature. Sulphuric acid (50 μl of 1 M) was added to the solution to stop the reaction. The absorbance at 490 nm was measured with a microplate reader.

Statistical analysis

Data are presented as means ± s.e.means. Statistical analysis between two groups was performed by Student's *t*-test. In the case of multiple comparison, statistical differences were determined by one-way ANOVA followed by Dunnett's test. *P* values less than 0.05 were considered statistically significant. The IC_{50} values of test compounds against the binding of the selectins to their ligands were calculated by Probit analysis.

Results

Effects on in vitro *binding of selectin-IgG chimeras to immobilized sLe^x-pentasaccharide ceramide*

The effects of OJ-R9188, TBC-1269, fucoidin and sLe^x-pentasaccharide on the binding of human E-, P- and L-selectin-IgG chimeras to immobilized sLe^x-pentasaccharide ceramide are shown in Table 1. OJ-R9188, TBC-1269, fucoidin and sLe^x-pentasaccharide showed a concentration-dependent blockade of the binding of human E-, P- and L-selectin-IgG chimeras to sLe^x-pentasaccharide. On a molar concentration basis, the potency of OJ-R9188 to prevent the binding of human E-, P- and L-selectin-IgG chimeras was greater than those of TBC-1269 and sLe^x-pentasaccharide ceramide. Although fucoidin is a heterogeneous molecule with a high molecular weight, on a weight basis it was more potent than the other compounds tested in preventing the binding of human E-, P- and L-selectin-IgG chimeras.

Effects on thioglycollate-induced peritonitis in mice

In mice, peritoneal inflammation induced by injection of thioglycollate is accompanied by accumulation of neutrophils in the peritoneal cavity. In this model, intravenous injection of OJ-R9188 (10 mg kg^{-1}) and fucoidin (10 mg kg^{-1})

significantly reduced neutrophil infiltration into the peritoneal cavity 2 h after injection of thioglycollate (Figure 1). Conversely, a known ligand for selectin, sLe^x (10 mg kg^{-1}), did not reduce neutrophil infiltration. Fucoidin, which consists of highly sulphated macromolecules, is known to suppress thioglycollate-induced infiltration of neutrophils (Shimaoka *et al*., 1996) as a result of interference with the function of L-selectin. Treatment with OJ-R9188, as well as fucoidin, increased the systemic neutrophil count by F-800 microcell counter (Sysmex corporation, Kobe, Japan).

Effects on IgE-mediated skin reaction in mice

In the IgE-mediated skin reaction in ears of actively sensitized mice, ear swelling appears in the early phase (1 h after antigen challenge) and the late phase (24 h after antigen challenge), and infiltration of neutrophils and eosinophils into the tissue occurs in the late phase. We have reported that selectins play an important role in the late phase response in this model (Wada *et al*., 2000). When OJ-R9188 (1–10 mg kg^{-1}), TBC-1269 (30 mg kg^{-1}), fucoidin (10 mg kg^{-1}) and sLe^x (10 mg kg^{-1}) were given intravenously just before OA challenge, they slightly inhibited ear swelling in the late phase reaction. OJ-R9188 and fucoidin significantly inhibited neutrophil and eosinophil infiltration into the tissues. Furthermore, the activity of OJ-R9188 was dose-dependent (Figure 2). Dexamethasone, a glucocorticoid, also significantly inhibited this reaction at a dose of 3 mg kg^{-1}, p.o.

Effect on picryl chloride-induced delayed-type hypersensitivity (PC-DTH) in mice

At 24 h after the challenge, a significant increase in ear weight and infiltration of neutrophils and eosinophils were observed (Figure 3). OJ-R9188 and fucoidin showed significant inhibition of cell infiltration at a dose of 10 mg kg^{-1}, i.v. OJ-R9188 also significantly reduced tissue oedema. Dexamethasone also significantly inhibited this reaction at a dose of 3 mg kg^{-1}, p.o

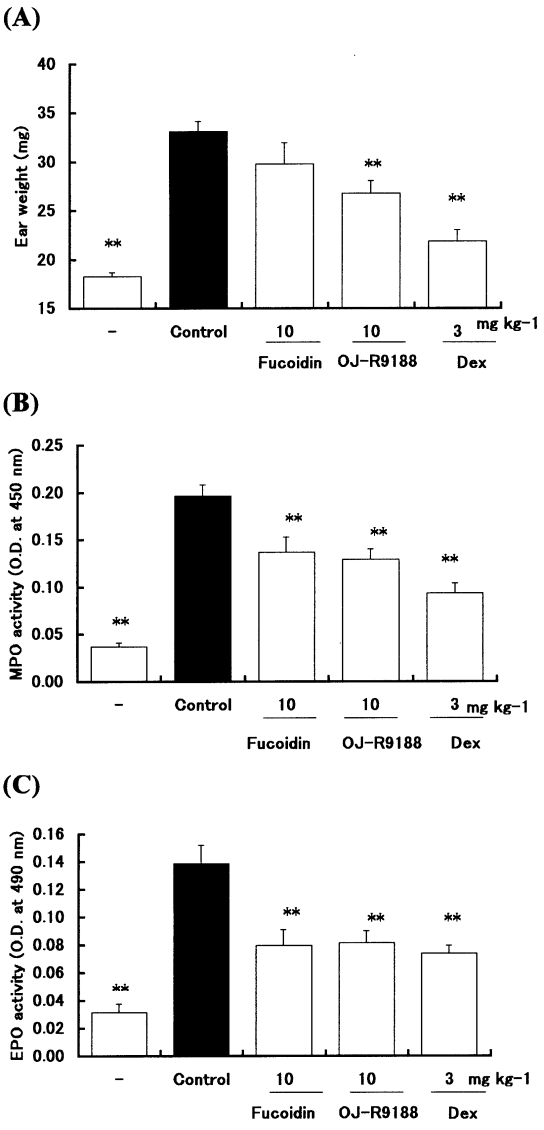

Figure 3 Effects of OJ-R9188 and fucoidin on PC-DTH in mice. (A) Ear weight, (B) MPO activity, and (C) EPO activity. OJ-R9188 and fucoidin were given intravenously just before the antigen challenge. Dexamethasone (Dex) was given orally 2 h before the antigen challenge. Each column represents the mean of six or seven animals. Vertical bars indicate s.e. $^{**}P<0.01$, significantly different from control. -: unsensitized group.

Discussion

OJ-R9188 inhibited the binding of human E-, P- and L-selectin-IgG fusion proteins to immobilized sLe^x neoglycoprotein. OJ-R9188 is a stronger inhibitor of binding than a known ligand for selectin, sLe^x. OJ-R9188 has a long hydrophobic chain. In preliminary experiments, another synthetic compound that has a shorter hydrophobic chain showed weaker activity, suggesting that the hydrophobic moiety may be important in binding (data not shown). E-, P- and L-selectins have been demonstrated to bind to sLe^x and related oligosaccharides, although the natural ligand for each selectin has not been completely characterized. Under physiological conditions, carbohydrate alone may be insufficient to bind to selectins with high affinity, and the existence of protein or lipids attached to the carbohydrate may be important for its function (Lasky *et al*., 1992; Norgard *et al*., 1993).

Fucoidin is a natural sulphated glycan that prevents binding of P- and L-selectin to sLe^x but has less inhibitory activity on the binding of E-selectin (Skinner *et al*., 1991; Varki *et al*., 1994; Yoshida *et al*., 1994; Shimaoka *et al*., 1996). In the present study, the weaker effect of fucoidin on the binding of E-selectin to sLe^x neoglycoprotein was confirmed.

A low molecular weight selectin blocker, TBC-1269, has been demonstrated to inhibit binding of selectin-IgG fusion protein to sLe^x-bearing HL-60 cells (Kogan *et al*., 1997). This compound shows the most potent inhibition against P-selectin-mediated binding *in vitro*. This observation was consistent with our results. TBC-1269 has been demonstrated to have beneficial effects in experimental asthma and cardiac ischaemia/reperfusion injury (Abraham *et al*., 1999).

Selectins are thought to play a critical role in leukocyte infiltration by controlling the initial attachment of leukocytes to activated vascular endothelium (Berg *et al*., 1991; Picker *et al*., 1991; Lorant *et al*., 1993; Symon *et al*., 1994; Akbar *et*

al., 1991). In the early stages of thioglycollate-induced peritoneal inflammation in mice, anti-P-selectin and anti-L-selectin, but not anti-E-selectin, antibodies prevent neutrophil infiltration into the peritoneal cavity. These results indicate that P- and L-selectins are involved in the neutrophil extravasation in response to thioglycollate (Arbones *et al*., 1994; Mayadas *et al*., 1993; Labow *et al*., 1994; Briggs *et al*., 1996; Bosse & Vestweber., 1994). The intravenous injection of OJ-R9188 significantly reduced thioglycollate-induced neutrophil infiltration in the peritoneal cavity. The intravenous injection of fucoidin, a natural selectin blocker, also reduced neutrophil infiltration, as described previously (Skinner *et al*., 1991; Yoshida *et al*., 1994). In addition, OJ-R9188 and fucoidin increased the number of circulating leukocytes 2 h after injection. These results suggest that OJ-R9188 may effectively prevent P- and L-selectin-mediated binding of neutrophils to vascular endothelium *in vivo*.

A skin-associated population of memory T lymphocytes, defined by expression of the cutaneous lymphocyte antigen (CLA), binds selectively and avidly to E-selectin. CLA itself is a lymphocyte homing receptor for E-selectin (Picker *et al*., 1993; Berg *et al*., 1991).E-selectin expression on the vascular endothelial cell surface is observed in inflammatory skin diseases, such as contact dermatitis, atopic dermatitis and psoriasis (Picker *et al*., 1991; Leung *et al*., 1991; Groves *et al*., 1991). Thus, the expression of E-selectin may be involved in the pathogenesis of such skin inflammation. We reported that the expression of E-selectin mRNA was significantly increased 2 h after OA challenge and that E-selectin may be involved in the leukocyte infiltration into the skin during the late phase response of the IgE-mediated skin reaction in mice (Wada *et al*., 2000).

Additionally, all the E-, P- and L-selectin-IgG chimeras inhibited the late phase response, ear swelling, and neutrophil and eosinophil infiltration 24 h after OA challenge. Intravenous administration of OJ-R9188 significantly inhibited late phase responses such as ear swelling and neutrophil and eosinophil infiltration in this model. Fucoidin also inhibited the late phase responses. These findings indicate that blockade of the functions of selectin may be a promising treatment strategy for allergic skin reactions.

The plasma level of OJ-R9188 was *ca*. 90 μg ml^{-1} at 1 h after intravenous administration of 10 mg kg^{-1} and the half-life was 1.2 h in female mice (unpublished data). These results indicate that the plasma levels of OJ-R9188 would be higher than the levels required to block the interaction between P- or L-selectin and their ligands for about 10 h after administration.

Intravenous administration of OJ-R9188 and fucoidin also inhibited the ear swelling and neutrophil and eosinophil infiltration in picryl chloride (PC)-induced delayed-type hypersensitivity (DTH) of mice (type IV allergy model). It has already been reported that fucoidin is effective on contact hypersensitivity in mice (Nasu *et al*., 1997). In the present study, we confirmed the effect of fucoidin on DTH. It has been reported that both Th1 and Th2 cytokines play important roles in the inflammation and hypertrophy of the skin in atopic dermatitis (Grewe *et al*., 1994; 1995; Thepen *et al*., 1996; Kapsenberg *et al*., 1991; Hamid *et al*., 1994). Thus, the treatment of atopic dermatitis should act on skin inflammation mediated by both Th1 and Th2 mechanisms. In this regard, OJ-R9188 has the potential to be an effective compound for treatment of inflammation in atopic dermatitis.

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