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Effects of VLA-1 Blockade on Experimental Inflammation in Mice

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VLA-1 (very late antigen-1) is implicated in recruitment, retention and activation of leukocytes and its blockade has been referred as a potential target of new drug discovery to address unmet medical needs in inflammatory disease area. In the present study, we investigate the effects of an anti-murine CD49a (integrin α subunit of VLA-1) monoclonal antibody (Ha31/8) on various experimental models of inflammatory diseases in mice. Pretreatment with Ha31/8 at an intraperitoneal dose of 250 μg significantly (P<0.01) reduced arthritic symptoms and joint tissue damage in mice with type II collagen-induced arthritis. In addition, Ha31/8 at an intraperitoneal dose of 100 μg significantly (P<0.01) inhibited airway inflammatory cell infiltration induced by repeated exposure to cigarette smoke. In contrast, Ha31/8 failed to inhibit oxazolone-induced chronic dermatitis and OVA-induced airway hyperresponsiveness at an intraperitoneal dose of 100 μg. These results show that VLA-1 is involved, at least partly, in the pathogenesis of type II collagen-induced arthritis and cigarette smoke-induced airway inflammatory cell infiltration in mice, indicating the therapeutic potential of VLA-1 blockade against rheumatoid arthritis and chronic occlusive pulmonary disease.

Integrins are heterodimeric trans-membrane receptors composed of non-covalently associated subunits, referred to as the α and β subunits. It is widely acknowledged that these molecules regulate a wide variety of biological processes, such as embryonic development, immune response, bone resorption, platelet aggregation, wound healing, angiogenesis, and tumor metastasis, through bridging extracellular matrix (ECM) proteins and intracellular actin cytoskeleton or signaling proteins (1-4). The integrin-mediated transmembrane crosstalk is responsible for various cellular processes including cell adhesion required for appropriate cell movement toward the destinations. Recently, much evidence has been accumulated, indicating that integrin-mediated cell adhesion is involved in the pathogenesis of inflammation associated with leukocyte infiltration (1, 5). The infiltration of leukocytes is known to be a multistep process involving tethering and rolling of leukocytes on vascular surfaces, followed by firm adhesion and transmigration into the inflammatory tissues (6). Among these processes, the dynamic interaction of integrins with ECM is essential for leukocytes to pass through the gap between adjacent endothelial cells and to reach tissue sites of inflammation.

VLA-1 (very late antigen-1), known as α1/β1 integrin or CD49a/CD29, is a member of the integrin family and functions as a receptor for certain types of ECM, i.e., type IV collagen and laminin, which are constituents of the basement membranes and interstitium. It is reported that the binding of VLA-1 to these ECMs plays important roles in adhesion and activation of $CD4^+$ and $CD8^+$ T lymphocytes, macrophages and eosinophils in inflammation (7, 8). In particular, VLA-1 on T cells has been widely examined and shown to participate in collagen-dependent adhesion, proliferation and cytokine production in vitro (9, 10), and in T cell-mediated delayed-type hypersensitivity and resistance to viral infection in vivo (11, 12). Furthermore, Suzuki et al. have provided new evidence to indicate that VLA-1 is also a crucial receptor for Semaphorin 7A (Sema7A), and that Sema7A on activated T cells stimulates IL-6 and TNF-α production from macrophages through VLA-1 (13). Thus, VLA-1 plays a critical role in the pathogenesis of variety of inflammatory disorders, and is expected to be a potential new therapeutic target for treatment of acute and chronic inflammation. Several studies have indicated that depleting α1 integrin (CD49a) gene or blocking functions of VLA-1 with monoclonal antibodies against CD49a significantly reduced inflammatory responses in animal models of colitis, psoriasis, arthritis and asthma (8, 11, 14-18).

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In the present study, in order to confirm and further extend the therapeutic usefulness of VLA-1 blockade in treatment of inflammatory diseases, we investigated the effects of an anti-CD49a monoclonal antibody on the experimental inflammation models that were not tested in previous studies, i.e., mouse models of chronic arthritis, cigarette smoke-induced lung inflammation, atopic dermatitis and asthma.

MATERIALS AND METHODS

Animals

Male BALB/c mice, male C57BL/6N mice, female A/J mice and male DBA/1J mice were purchased from Japan SLC (Shizuoka, Japan) or Japan Charles River (Yokohama, Japan). Mice were housed under controlled temperature (23 ± 3°C), humidity (55 ± 10%) and photoperiod (12/12-h light/dark cycle), and allowed unrestricted access to food and water. Experiments on animals were conducted in compliance with protocols approved by the Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation.

Antibodies

Antibodies (Abs) were purchased from BD Biosciences (San Diego, CA): purified anti-CD49a mAb (Ha31/8, hamster IgG2), purified anti-CD49b mAb (Ha1/29, hamster IgG2), purified anti-CD29 mAb (Ha2/5, hamster IgM), the hamster isotype control mAbs (Ha4/8, hamster IgG2a and G235-1, hamster IgM), FITC- or APC-labeled anti-CD3 mAb (145-2C11, hamster IgG1), APC-labeled anti-CD4 mAb (RM4-5, rat IgG2a), PerCP-Cy5.5-labeled anti-CD45 mAb (30-F11, rat IgG2b), FITC- or APC-labeled isotype control mAbs (A19-3, hamster IgG1), FITC-labeled anti-hamster IgG mAb (G70-204/G94-56, mouse IgG1/ IgG2b), FITC-labeled anti-hamster IgM mAb (G188-2, mouse IgG1) and purified anti-CD16/CD32 mAb (2.4G2, rat IgG2b).

Cell preparation

Cell preparation was performed according to the method of de Fougerolles et al (11). In short, splenocytes were prepared from BALB/c mice and cultured with 20 ng/ml of IL-2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (Gibco BRL, Grand Island, NY). After 14 days, cells were washed and re-suspended in RPMI 1640 supplemented with 0.2% BSA (Sigma Chemical Co., St. Louis, MO) for cell adhesion assay and flow cytometry.

Flow cytometry

Splenocytes were first incubated for 10 min with anti-CD16/32 mAbs to avoid nonspecific binding of mAbs to FcγR. Cells were then washed with PBS(-) with 0.1% bovine serum albumin (BSA) and 0.1% NaN3 (FACS buffer), incubated for 30 min with anti-integrin mAbs, washed and re-suspended in the appropriate FITC-labeled secondary antibody, followed by staining with APC-labeled anti-CD3 mAb and PerCP-Cy5.5-labeled anti-CD45 mAb. After washing, stained cells were analyzed by flow cytometry on FACSCalibur™ flow cytometer using CellQuest software (BD Biosciences).

Cell adhesion assay

Each well of 96-well microtiter plates (BD Biosciences) was coated with mouse type IV collagen (BD Biosciences) at concentrations ranging from 0.01 to 10 µg/ml overnight at 4°C, and washed with D-PBS (+). The plates were then blocked with 1% BSA in D-PBS (+) for 2 hours at room temperature and washed with D-PBS (+) for cell adhesion assays. Control wells were treated with BSA alone. In the collagen-coated wells, IL-2-activated splenocytes (2.5×10^5 cells/well) were pre-incubated with or without antibodies at 37 °C for 15 min. and then incubated for 60 min. Each well was washed twice with RPMI 1640 supplemented with 0.2% BSA to remove non-adherent cells. The number of the adherent cells was quantified by measuring the activity of endogenous N-acetyl-hexosaminidase by the method of Landegren (19).

Type II collagen-induced arthritis model

Type II collagen-induced arthritis was made in mice by the method of Quinones et al. (20). Male DBA/1J mice (6 weeks of age, Japan Charles River) were immunized subcutaneously with 100 µg of bovine type II collagen (Collagen Research Center, Tokyo, Japan) emulsified with an equal volume of Freund's complete adjuvant (FCA) containing 100 µg H37Ra Mycobacterium tuberculosis (Sigma-Aldrich, St. Louis, MO). On day 21, mice were boosted with a subcutaneous injection of 100 µg of type II collagen in the same manner as for the initial immunization. Just before the second immunization, mice were treated with mAbs (250 µg) intraperitoneally, and the treatment with mAbs was repeated every three day from day 24 to 39. Mice were monitored at specified time intervals up to day 42 for the severity of arthritis symptoms. A clinical score assigned for each hindpaw and forepaw was based on the degree of swelling and redness as follows: grade 0 = no swelling or erythema, grade 1 = obvious swelling and redness in a single digit, grade 2 = swelling and erythema in several digits, grade 3 = severe swelling in several digits, grade 4 = severe swelling with joint rigidity or deformity. The score of each paw was added together so that a maximal attainable score per mouse was 16 (4 paws × 4 grades). At the end of the experiment (day 42), skeletal changes were radiographically examined with a soft X-ray unit (Ohmic, Tokyo, Japan). The severity of bone erosion was ranked using a

modified version of the Larsen scoring method as described by Seeuws et al. (21). : 0 = normal; 1 = slight abnormality with any one or two of the exterior metatarsal bones showing slight bone erosion; 2 = definite early abnormality with any of the metatarsal or tarsal bones showing bone erosion; 3 = medium destructive abnormality with the metatarsal bones or any one or two of the tarsal bones showing definite bone erosions; 4 = severe destructive abnormality with all the metatarsal bones showing definite bone erosion and at least one of the tarsometatarsal joints being completely eroded, leaving some bony joint outlines partly preserved; 5 = mutilating abnormality with no bony outlines that can be deciphered. The score of bone erosion was added together so that a maximal attainable score per mouse was 20 (4 paws × 5 grades).

Cigarette smoke-induced lung inflammation model

Experiments were carried out by modified method of Quémentet et al. (22). Female A/J mice (11 weeks of age, Japan SLC) were placed in the chamber of a cigarette smoke inhalation experiment system for small animals (INH06-CIGR02A; MIPS, Osaka, Japan), and were exposed to cigarette smoke (CS) of five 2R4f reference cigarettes (University of Kentucky, Lexington, KY), which was diluted to 10% with compressed air, twice daily from day 0 to 2. As a control, air was delivered to mice under the same conditions described above instead of CS. Mice were treated with 100 μg of mAbs intraperitoneally once daily from day 0 to 2. Twenty-four hours after the last CS challenge, animals were sacrificed, and bronchoalveolar lavage fluid (BALF) was collected and measured for the total leukocyte counts using hemocytometer. Cytospin slides were prepared, stained with Diff-Quick reagents (Kokusai Shiyaku, Kobe, Japan), and differentiated by standard hematological procedures.

Oxazolone-induced dermatitis model

Chronic dermatitis was induced by repeated cutaneous application of oxazolone according to the method of Kitaguchi et al. and Tamura et al. (23-25). BALB/c mice (6-8 weeks of age, Japan Charles River) were sensitized by applying with 10 μL of 0.5% oxazolone (Sigma-Aldrich St. Louis, MO) in acetone to each side of the right ear (20 μL in total). On days 7, 9, 11, 14, and 16, mice were challenged with the topical application of 10 μl of 0.25% oxazolone onto both sides of the same ear (20 μl in total) to induce dermatitis. Abs (100 μg) were administered intraperitoneally 1 hour before oxazolone challenge on days 7, 9, 11, 14, and 16. The thickness of the right ear was measured using a dial thickness gauge (Mitutoyo, Tokyo, Japan) before each oxazolone application from day 0 to 16 and 24 hours after the final application. On day 16, scratching behavior was counted automatically using MicroAct (Neurosciense Inc., Tokyo, Japan) for 2 hours after oxazolone challenge.

Allergic airway inflammation and hypersensitivity model

Allergic airway inflammation with ovalbumin (OVA) was made in mice by the method of Takeda et al. (26). Male BALB/c mice (6 weeks of age, Japan SLC) were sensitized by intraperitoneal injection of 20 μg of OVA (Seikagaku corporation, Tokyo, Japan) emulsified in 2.25 mg of Al $(OH)_3$ (Nakarai Tesque, Kyoto, Japan) in a total volume of 100 μl on days 0 and 14. After the second immunization, animals were challenged with aerosolized 1% OVA or saline (blank) using an ultrasonic nebulizer NE-U17 (Omron, Kyoto, Japan) for 20 min on days 28, 29, and 30 in a closed chamber. Abs (100 μg) were administered intraperitoneally at 1 hour before OVA challenge on days 28, 29, and 30. Twenty-four hours after the last OVA challenge, airway hyperresponsiveness (AHR) was measured noninvasively using a whole-body plethysmograph (Buxco, Sharon, CT) as previously described (27). Shortly, enhanced pause (Penh) was calculated based on inspiratory and expiratory time and pressure, and used as the measure of airway responsiveness in this study. The average Penh over every 10 min was determined after exposing the mice for 1 min to aerosolized saline or methacholine chloride (methacholine, Sigma-Aldrich, St. Louis, MO) at increasing concentrations (1.56, 3.125, 6.25, and 12.5 mg/ml in normal saline). Airway reactivity was expressed as a percent increase for each concentration of methacholine compared with Penh values after saline challenge. After experiments, mice were sacrificed, and BALF was collected and measured for the total leukocyte counts using hemocytometer. Cytospin slides were prepared, stained with Diff-Quick reagents (Kokusai Shiyaku, Kobe, Japan), and differentiated by standard hematological procedures. In addition, $CD4^+$ T cells in each BALF sample were counted by flow cytometry. Briefly, cells were re-suspended in FACS buffer, incubated with anti-CD16/32 mAbs and stained with fluorochrome-labeled anti-CD3, CD4 and CD45 mAbs. The percentage of $CD4^+$ T cells was measured by using a FACSCanto™ flow cytometer. The cells stained by the isotype antibody (rat IgG2A) were also measured as the negative control.

Statistics

The results are expressed as the mean ± SEM. Statistically significant differences were determined by Student's unpaired two-tailed t-test or Dunnet's multiple comparison tests. Values of $p < 0.05$ were considered significant.

RESULTS

In vitro profiles of anti-CD49a mAb (Ha31/8)

As shown in Fig 1, expressions of CD49a and CD29, but not CD49b, were detected on the surface of the IL-2-activated mouse splenocytes by flow cytometry. In addition, adhesion of the IL-2-activated splenocytes to type IV collagen was observed in a concentration dependent manner (0.01-10 μg/ml) (Fig 2), and the response reached a plateau at 3 μg/ml. Thus the following blocking study was carried out with 3 μg/ml of type IV collagen. Pre-treatment of the IL-2-activated splenocytes with 10 μg/ml of anti-CD49a mAb (Ha31/8) and 10 μg/ml of anti-CD29 mAb reduced the number of adherent cells to background level (Fig 3). In contrast, mAb against CD49b, which is the α chain of VLA-2, had no inhibitory effect on the type IV collagen-dependent cell adhesion. On the basis of these results, we utilized Ha31/8 in the following experiments to produce VLA-1 blockade in vivo.

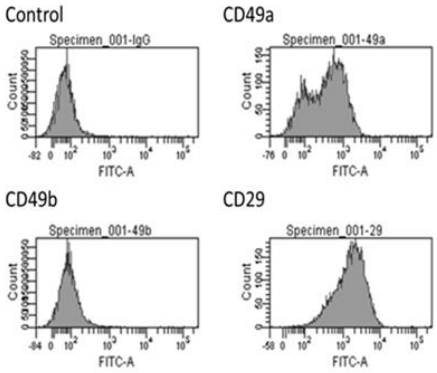

Figure 1. Flow cytometry of IL-2-activated mouse splenocytes stained with anti-CD49a (Ha31/8), anti-CD49b and anti-CD29 mAbs followed by FITC-labeled secondary Abs. The X axis reflects log fluorescence intensity, and the Y axis reflects cell number.

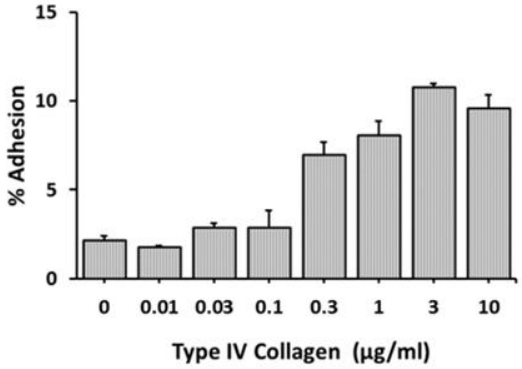

Figure 2. Type IV collagen-mediated adhesion of IL-2 activated mouse splenocytes in vitro. IL-2 activated mouse splenocytes were incubated in wells coated with various concentrations (0.01~10 μg/ml) of mouse type IV collagen for 60 min. The number of the adherent cells was quantified by measuring the activity of endogenous N-acetyl-hexosaminidase. Assays were done in triplicate and data are expressed as mean ± SEM.

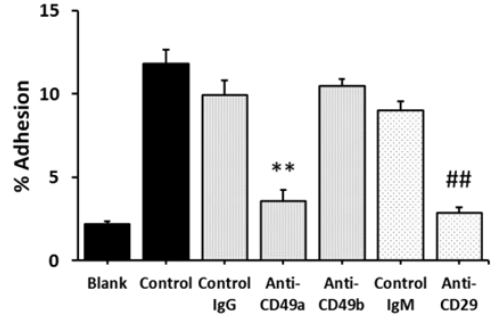

Figure 3. Effects of anti-CD49a (Ha31/8), anti-CD49b and anti-CD29 mAbs on the type IV collagen mediated cell adhesion. IL-2 activated mouse splenocytes were pretreated with the indicated antibodies at 37 °C for 15 min, and incubated for 60 min in wells coated with 3 μg/ml of mouse type IV collagen. The number of the adherent cells was quantified by measuring the activity of endogenous N-acetyl-hexosaminidase. Assays were done in triplicate and data are expressed as mean ± SEM. **: $p<0.01$ versus isotype control IgG, ##: $p<0.01$ versus isotype control IgM.

Effect on collagen-induced arthritis

Figure 4 shows the effect of Ha31/8 on the development of CIA in mice. The first sign of arthritis development, i.e., paw swelling and redness, was seen on day 27 in disease control animals (Fig 4A). Thereafter CIA progressed rapidly and the clinical score as the index of the disease severity reached a maximum on day 33. Mice treated with Ha31/8 showed significant decrease in the clinical score from day 29 to 42, compared with the disease control animals treated with control hamster IgG2a mAb. Figure 4B illustrates the development of the

skeletal changes associated with joint inflammation that were examined with a soft X-ray unit at the end of the experiment. Blockade of VLA-1 by treatment with Ha31/8 also resulted in a significant reduction in the severity of bone erosion.

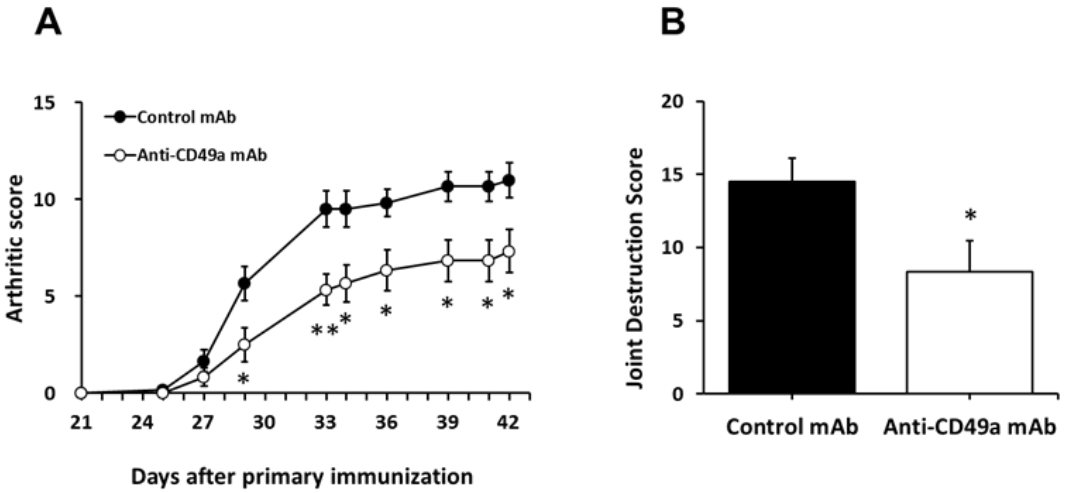

Figure 4. Effects of anti-CD49a mAb (Ha31/8) on the development of collagen-induced arthritis in DBA/1 mice. Animals were immunized with bovine type II collagen and boosted on day 21. Mice were treated with Ha31/8 at 250 μg intraperitoneally every three days from day 21 to 39. (A) clinical score. (B) joint destruction score on day 42. Data are shown as means ± SEM (n = 6). *; $p<0.05$, **; $p<0.01$ versus control mAb treated mice.

Effect on cigarette smoke-induced pulmonary inflammation

CS exposures twice a day for 3 days induced marked increase in neutrophil count of the BALF at 24 hours after the last CS exposure, although there was no significant change in mononuclear cell count, as shown in Figures 5A and 5B. Intraperitoneal treatment of mice with Ha31/8 once daily from day 0 to 2 prior to CS exposures significantly ($p<0.01$) reduced neutrophil count of the BALF.

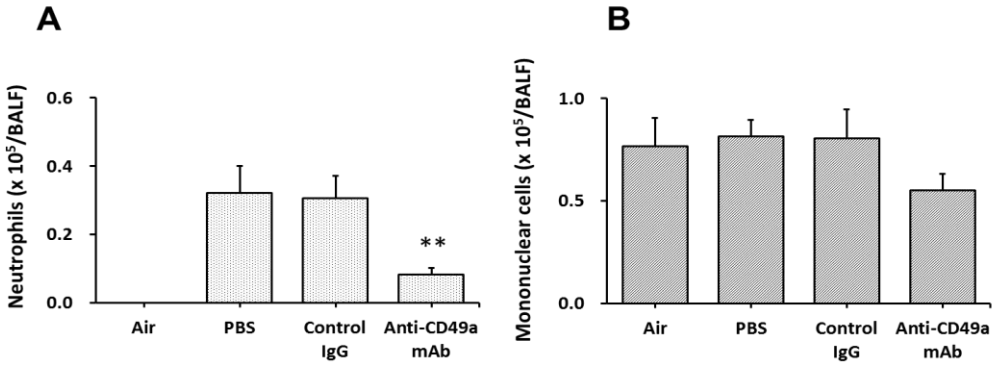

Figure 5. Effects of anti-CD49a mAb (Ha31/8) on lung inflammation induced by cigarette smoke (CS) in BALB/c mice. Animals were exposed to CS twice daily for three consecutive days, and intraperitoneal treatment with 100 μg of Ha31/8 was carried out once daily from day 0 to 2. Bronchoalveolar lavage fluid was taken at 24 hours after final CS exposure. (A) neutrophils. (B) mononuclear cells. Each bar represents the mean ± SEM (n=4~8). **; $p<0.01$ versus control mAb-treated mice.

Effect on oxazolone-induced chronic dermatitis

Figure 6A shows the chronological change in the severity of oxazolone-induced dermatitis evaluated by measuring ear thickness with a dial thickness gauge. Treatment with 100 μg of Ha31/8 before oxazolone challenge significantly ($p<0.05$) inhibited the early contact hypersensitivity response on day 11. However, ear thickening of chronic phase, i.e., day 14, 16 and 17, was not affected at all by treatment with Ha31/8. Also, the

scratching behavior measured just after the last oxazolone application on day 16 was not affected by treatment with Ha31/8 and the control mAb (Fig 6B).

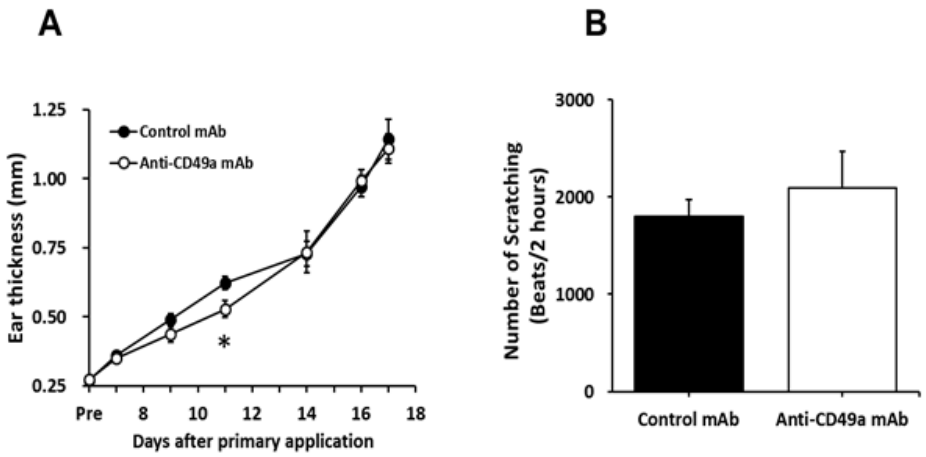

Figure 6. Effects of anti-CD49a mAb (Ha31/8) on oxazolone-induced chronic dermatitis in A/J mice. Application of oxazolone to ear skin was carried out once daily on days 7,9,11,14 and 16, and Ha31/8 was administered intraperitoneally at a dose of 100 μg one hour before each oxazolone application. (A) ear thickness. (B) number of scratchings on day 16. Data are shown as the mean ± SEM (n=5). *; $p<0.05$ versus control mAb-treated mice.

Effect on allergic airway inflammation and hypersensitivity

Airway inflammation was induced by OVA aerosol challenges to OVA-sensitized mice on three consecutive days, which exhibited inflammatory cell accumulation to airway and AHR. Twenty-four hours after the last OVA challenge, there were significant increase in the number of total cells, eosinophils, mononuclear cells and $CD4^+$ T cells in BALF compared with those from saline challenged mice (Figs 7A and 7B). Intraperitoneal administration of 100 μg of Ha31/8 at 1 hour before each OVA challenge significantly inhibited the increase in total cells, eosinophils and $CD4^+$ T cells by 59%, 74% and 48%, respectively. No difference, however, was seen in AHR between anti-CD49a mAb- and control mAb-treated mice (data not shown).

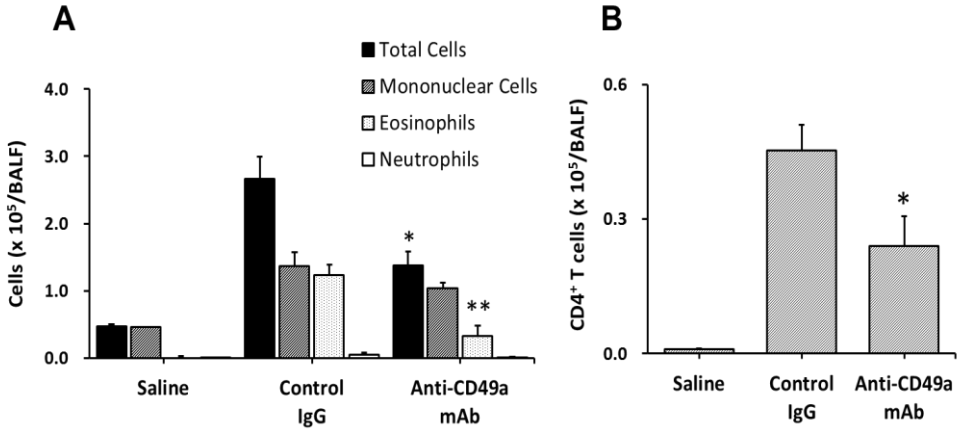

Figure 7. Effects of anti-CD49a mAb (Ha31/8) on allergic airway inflammation in ovalbumin (OVA)-sensitized BALB/c mice. Sensitization was established by intraperitoneal injection of ovalbumin (20 μg) on days 0 and 14. Animals were intraperitoneally treated with Ha31/8 at 100 μg at 1 hr before each OVA challenge that was carried out to elicit allergic airway inflammation on days 28, 29 and 30. Bronchoalveolar lavage fluid was taken at 24 hrs after the final OVA challenge on day 30. (A) total cells, eosinophils, neutrophils and mononuclear cells. (B) $CD4^+$ T cells. Each bar represents the mean ± SEM (n = 4~7). *; $p<0.05$, ** $p<0.01$ versus the control mAb treated mice.

DISCUSSION

VLA-1 (very late antigen-1) is implicated in recruitment, retention and activation of leukocytes and its blockade has been referred as a promising target of new drug discovery to address unmet medical needs in inflammatory disease area (3, 28). Recent studies have demonstrated that leukocytes with increased VLA-1 expression migrated in the affected organs and tissues in psoriasis, RA and asthma patients (3, 29), and that VLA-1 blockade significantly inhibited animal models of inflammation, including psoriasis, colitis, arthritis and asthma (11, 16-18). These findings suggest that VLA-1, like other integrins, plays an important role in the pathogenesis of various inflammatory diseases. In contrast, Ridger et al. have reported that VLA-1 blockade by its monoclonal antibody was without significant inhibitory effect on the development of LPS-induced pulmonary inflammation in mice (30). Thus the therapeutic usefulness of VLA-1 blockade remains to be fully elucidated. In the present study, in order to confirm and further extend the therapeutic potential of VLA-1 blockade in the treatment of inflammatory diseases, we investigated the effects of a monoclonal antibody against murine VLA-1 (Ha31/8) on mouse models of arthritis, CS-induced lung inflammation, chronic dermatitis and asthma.

Firstly, we confirmed that treatment of mouse primary cultured splenocytes with IL-2 resulted in marked CD49a expression in the flow cytometry using hamster anti-CD49a mAb (Ha31/8), and that both Ha31/8 and anti-CD29 mAb completely blocked adhesion of IL-2-activated mouse splenocytes to the plastic well surface coated with 3 μg/ml of type IV collagen which induced maximum adhesion response in this study. These results are essentially comparable to those of a previous report (11), validating the hamster anti-CD49a mAb as a tool to produce functional VLA-1 blockade in the following in vivo experiments.

It is widely acknowledged that immunization of DBA1/J mice with type II collagen elicits development of severe chronic, destructive polyarthritis mediated by autoimmune responses against cartilage type II collagen (31). The collagen-induced arthritis (CIA) shares a number of clinical, histologic and immunological features with human rheumatoid arthritis (RA), and has been widely used as the most clinical-predictive animal model for testing various immuno-suppressors and anti-inflammatory agents (31-33). No experiment, however, was carried out to evaluate the effect of VLA-1 blockade using CIA model, although several studies have reported protective effects of VLA-1 blockade by Ha31/8 against rat adjuvant-induced arthritis and mouse anti-type II collagen antibody-induced arthritis (11, 17). In the present study, VLA-1 blockade by repeated treatments with 250 μg of Ha31/8 significantly inhibited the development of CIA in DBA1/J mice. This result is essentially consistent with previous studies (11, 17), suggesting possible involvement of VLA-1 in the pathogenesis of chronic arthritis associated with bone damage. The precise mechanism by which VLA-1 blockade resulted in inhibition of CIA remains unknown in this study. Several in vivo studies, however, showed that depletion of T cells resulted in significant inhibition of CIA (34, 35). In addition, it is reported that T cells express VLA-1 in response to antigenic activation (9), and that interaction between VLA-1 and collagen enhanced T cell receptor-mediated proliferation and cytokine secretion of T cells (36, 37). Thus it is reasonable to understand that functional inhibition of activated T cells via VLA-1 blockade is responsible, at least partly, for significant suppression of CIA in this study.

Chronic obstructive pulmonary disease (COPD) is another chronic inflammatory disorder characterized by progressive lung destruction (38, 39). Many clinical and experimental studies indicate that cigarette smoking is a major risk factor responsible for exaggerated inflammation and progressive lung destruction in patients with COPD (40, 41). Little evidence, however, is available to demonstrate the involvement of VLA-1. In the present study, exposure to CS, twice daily on three consecutive days, led to marked increase in neutrophil counts of bronchoalveolar lavage fluid (BALF), in A/J mice. These data are essentially comparable to those of the earlier report by Yao and Morris who examined CS-induced pulmonary inflammation in multiple strains of mice, including A/J (42, 43). Our data also show that pretreatment with Ha31/8 produced a significant decrease in neutrophil counts of BALF, indicating the involvement of VLA-1-dependent mechanisms in the CS-induced lung inflammation model. This finding provides the first pharmacological evidence suggesting that VLA-1 blockade may be useful for preventive treatment of COPD. In 2001, Ridger et al. reported that VLA-1 is expressed on non-activated murine neutrophils by flow cytometry (30). More recently, however, Becker et al. reported no expression of VLA-1 on non-activated normal murine neutrophils (44). Thus the expression of functional VLA-1 on neutrophils is still a matter of controversy. On the other hand, much evidence has accumulated, demonstrating that activated-macrophages express VLA-1 (7-9, 11, 14, 45) and may initiate CS-induced pulmonary leukocyte infiltration in mice (46, 47). Further studies are necessary to elucidate whether Ha31/8 suppressed lung neutrophil infiltration through its direct effect on neutrophils and/or indirect effect via suppression of activated macrophage in this study.

There are several studies suggesting a pathogenic role of VLA-1 in cutaneous inflammatory diseases. Recent studies show that psoriasis, a typical chronic relapsing T-cell-mediated dermatitis, is associated with increased levels of VLA-1 expression in psoriatic lesions, and that development of psoriasis was significantly prevented by VLA-1 blockade in a xenotransplantation mouse model (16, 48). Little attention, however, has been paid to the

roles played by VLA-1 in development of atopic dermatitis (AD). We therefore investigated the effect of Ha31/8 on experimental AD model induced by repeated application of oxazolone in BALB/c mice. This AD model is characterized by increased levels of hapten-specific serum IgE and tissue Th2 cytokines, and widely used for evaluating the efficacy of various drugs and clinical development candidates for AD (23, 25, 49). In this study, repeated topical application of oxazolone onto the ear skin resulted in skin thickening and increased itch-associated scratching behavior in BALB/c mice. Repeated administration of Ha31/8 at 100 μg significantly inhibited early contact hypersensitivity phase (day 11) of dermatitis. This finding is essentially comparable to the report of the earlier investigators who demonstrated that VLA-1 blockade was capable of suppressing early effector phase of contact hypersensitivity response induced by dermal application of fluorescein isothiocyanate (FITC) in FITC-primed BALB/c mice (11). Ha31/8, however, was without any significant effects on skin thickening on days 14, 16 and 17. The reason why VLA-1 blockade was without effect on the chronic phase of skin inflammation is unknown. Previous reports demonstrated that repeated epicutaneous application of a contact sensitizing agent 2,4,6-trinitro-1-chlorobenzine induced marked skin swelling in association with large numbers of mast cells in BALB/c mice, but smaller response in $S1/S1^d$ mice devoid of mast cells (23)**.** Furthermore, a histamine antagonist olopatadine hydrochloride is reported to inhibit skin swelling and itching induced by repeated application of oxazolone in mice (24). These findings suggest that mast cells are also deeply implicated in the development of chronic skin inflammation in mice. Since VLA-1 is not significantly expressed on human mast cells (50), it is reasonable to assume that mast cells were not affected by Ha31/8 treatment in this study. Further studies on the effect of VLA-1 blockade on the late phase of the experimental dermatitis, with particular focus on coordination of $CD4^+$ T cells and mast cells, are warranted.

Our data also showed that pretreatment with Ha31/8 produced no protective effect against airway hyperresponsiveness (AHR) to methacholine in OVA-induced asthma mice, although infiltration of eosinophils and $CD4^+$ T cells into the airway was significantly suppressed. Abraham et al. reported that direct lung inhalation of a monoclonal antibody to human α1 blocks antigen-induced AHR in sheep (18), although systemic one failed to produce significant protection. In a separate study, we found that $CD4^+$ T cell depletion by an anti-CD4 monoclonal antibody GK1.5 completely diminished both AHR to methacholine and lung eosinophil infiltration in OVA-induced asthma mice (unpublished data), as Gonzalo et al. reported previously (51). In addition, a potent immunosuppressor FK-506 was reported to effectively inhibit AHR to methacholine, infiltration of eosinophils and IL-5 production of $CD4^+$ T cells in OVA-induced asthma mice without affecting infiltration of $CD4^+$ T cells in the lung (52). These reports may all indicate that $CD4^+$ T cells play an important role in the pathogenesis of the OVA-induced asthma model. The reasons for the apparent discrepancy between the present data and the previous findings remain unknown, but the possibility cannot be excluded that the more profound or complete inhibition of $CD4^+$ T cells infiltration was necessary for producing significant inhibition of AHR in OVA-induced asthma mice. Further studies are necessary to elucidate the effect of the larger doses of Ha31/8 and the possible involvement of other cells such as mast cells that are thought to contribute, in concert with $CD4^+$ T, to the development of OVA-induced AHR.

In summary, the present study demonstrated that VLA-1 blockade significantly inhibited CIA and CS-induced pulmonary inflammation, but not in oxazolone-induced chronic dermatitis and OVA-induced airway hypersensitivity in mice, new findings not reported previously. Over the last decade, drug therapy of RA has been improved by development of novel biological agents such as anti-human TNF-α monoclonal antibodies. However, the fact that these biological agents are not effective in all patients indicates the heterogeneity of RA, and implies that there still remains the unmet medical need in RA therapy to be addressed by new therapeutic agents (53). On the other hand, the current management of COPD depends on the use of long-acting bronchodilators that does not achieve the causative therapy, i.e. suppression of lung inflammation underlying the chronic pulmonary disease. Recently, roflumilast, an oral PDE4 inhibitor, has been introduced as the first anti-inflammatory therapy for COPD, but its utility is limited by the gastrointestinal side effects of nausea, emesis and diarrhea (54). The present data that VLA-1 blockade produced marked suppression of CIA and CS-induced COPD models provides a new strategic insight into new drug discovery to address the unmet medical needs associated with RA and COPD.

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