Effect of the C3a-Receptor Antagonist SB 290157 on Anti-OVA Polyclonal Antibody–Induced Arthritis

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Abstract. It was investigated whether the C3a-receptor antagonist (C3aRA) SB 290157 was involved in the suppression of anti-OVA pAb–induced arthritis because it is well known that anaphylatoxin C3a plays a crucial role in the development of an effective inflammatory response during complement activation. Anti-OVA pAb–induced arthritis was induced in DBA/1J mice by administration of anti-OVA pAb 0.5 h prior to intra-articular (i.a.) injection of OVA (0 h). Two peaks of joint swelling were observed at 0.5 and 3 h. The role of C3aRA in arthritis was investigated by injection of SB 290157 at concentrations of 10 and 30 mg/kg at 0 and 2 h. The antagonist was able to reduce joint swelling only at 3 h, and about 50% inhibition of joint swelling was observed with the concentration of 30 mg/kg. The C3 level was significantly decreased at 3 h compared with naïve mice showing complement consumption. Furthermore, the C3 activation was observed and increased corresponding to the graded concentration of anti-OVA pAb. The results also revealed that the C3aRA was able to reduce the expression of IL-1β in synovial tissue. Taken together, the results suggested that C3aRA may be effective in the inhibition of arthritis.

Keywords: rheumatoid arthritis, OVA, anti-OVA antibody–induced arthritis, C3a-receptor antagonist, inflammation

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by polyarticular joint inflammation with leukocytic recruitment into synovial fluid and tissue, eventually leading to cartilage and bone destruction (1). Neutrophil infiltration into the synovial membrane is one of the hallmarks of active RA (2) and plays a pivotal role in the pathogenesis of synovial inflammation (3). The infiltrated leukocytes can promote inflammation and cause joint tissue injury by releasing proinflammatory cytokines (2). Proinflammatory cytokine IL-1β is likely to have a primary role in the pathogenesis of RA because it can be detected at high levels in synovial fluid from patients with RA (4) and inflamed synovial tissue (5). In addition, blocking of IL-1 is able to effectively suppress the arthritic process (6 – 8).

Despite extensive studies, the pathogenesis of RA has remained elusive. Animal models of human RA have demonstrated that complement is involved in RA (9 – 12), with resultant elevations in serum concentrations of anaphylatoxins. Antibodies, particularly as constituents of antibody-antigen immune complexes (Ab-Ag IC), play a central role in inflammation in a number of autoimmune diseases (13). Administration of OVA and anti-OVA antibody comprises one model of human RA because its features are similar to those encountered in human RA (14 – 16). It has long been known that Ab-Ag IC–mediated activation of complement is the initial step leading to the release of chemotactic factors that cause neutrophil migration into the inflammatory reaction in arthritic joints and secret proinflammatory cytokines such as IL-1β and TNF-α (17, 18). Circulating C3 was necessary and sufficient for arthritis induction (19). The level of C3 product, C3a, was increased in the synovial fluid and peripheral blood of RA patients (20, 21) and involved in the mediation of immune responses and inflammatory processes (22, 23), including regulating the
synthesis of IL-1β (24). Several lines of evidence have reported the pathophysiological roles of C3a and C3aR in mediating inflammation (25, 26). Recently, several groups used a selective nonpeptide C3aR antagonist, N²-[(2,2-diphenylethoxy)acetyl]-L-arginine (SB 290157), that inhibited inflammation (27 – 31). It was revealed that SB 290157 was able to inhibit C3a-mediated ATP release from guinea-pig platelets (IC₅₀ of 30 nM) (27). The previous report has also shown that SB 290157 was a selective antagonist for rat basophilic leukemia cells expressing the human C3aR (IC₅₀ of 200 nM). Antagonism by SB 290157 was not only limited to the human C3aR (IC₅₀ of 12 nm), it also inhibited C3a-induced Ca²⁺ mobilization of cells expressing the mouse and guinea-pig C3aR (IC₅₀ of 7 and 30 nM, respectively). In animal models of inflammation, the C3aRA inhibitor SB 290157 inhibited neutrophil recruitment and accumulation in a guinea-pig LPS-induced airway neutrophilia model and significantly reduced paw swelling in a rat model of antigen-induced arthritis (27).

The establishment of anti-OVA antibody–induced arthritis and anti-inflammatory action of SB 290157 were reported previously. However, the effect of SB 290157 on antibody-mediated arthritis used in the present study has not been investigated so far. The present study investigated whether anti-OVA polyclonal antibody is involved in the induction of arthritis using a C3aR antagonist, SB 290157, in mice. The present data revealed that SB 290157 is anti-inflammatory agent that causes a significant reduction of joint swelling and it inhibits neutrophil recruitment and IL-1β expression. Neutrophil infiltration into synovium was correlated with joint levels of IL-1β expression. These findings indicate that antagonists targeted against C3aR may be effective for inhibiting arthritis.

Materials and Methods

Animals

LEW/Sea rats were purchased from CLEA, Inc. (Osaka). Male DBA/1J mice (8 weeks of age) were bred in the animal breeding unit of Kobe Pharmaceutical University, Kobe. Rats and mice were housed in a specific pathogen-free environment and fed standard rodent chow and water ad libitum. All procedures were performed with the approval of the Institutional Animal Care and Use Committee.

Induction of anti-OVA antibody–induced arthritis in LEW/Sea rats

A 100-µg sample of OVA (Sigma, St. Louis, MO, USA) was dissolved in 100 µl of PBS (phosphate-buffered saline) and emulsified with an equal volume of complete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA). The emulsion (200 µl) was injected intraperitoneally (i.p.) into LEW/Sea rats (day 0) and boosted 14 days later. Serum was collected on day 21. The anti-OVA pAb was isolated from rat serum by precipitation of the serum with saturated ammonium sulfate solution, dialyzed, and then passed through a HiTrap affinity column (Pharmacia Biotech, Uppsala, Sweden).

Induction of arthritis into DBA/1J mice

Mice were given 0.2 ml of rat anti-OVA polyclonal antibody (10 mg/ml) by intravenous (i.v.) injection 0.5 h before OVA administration (0.5 h). OVA (10 µg) was dissolved in 25 µl PBS and given by intra-articular (i.a.) injection (0 h). The OVA injection alone was used as the baseline. The net increase in joint thickness attributable to anti-OVA pAb injection was calculated by subtracting the joint thickness of OVA-injected nonimmunized mice from that of the anti-OVA pAb–injected mice. Administration of SB 290157 (Calbiochem, San Diego, CA, USA), a C3aR antagonist, (10 or 30 mg/kg) was injected i.p. two times, at 0 (right after OVA injection) and 2 h while 5% ethanol in PBS was used as a vehicle control. Joint swelling was measured using a dial thickness gauge (Ozaki MKG, Tokyo) before injection, at 0.5 h, and then every hour until 5 h after OVA injection.

Histology and immunohistochemistry assessment of arthritis

Hind ankle joints were dissected at 0.5 and 3 h. The hind ankle joints were fixed in 10% neutral-buffered formalin, decalcified in decalcifying solution (Wako, Osaka), and embedded in paraffin. The hind ankle joints were sectioned at 4 µm and stained with hematoxylin and eosin by standard techniques. For immunohistochemical staining, the sections were deparaffinized and hydrated through xylene and graded alcohol series. The sections were depleted of endogenous peroxidase by incubating the sections in 3% H₂O₂ in distilled water for 30 min. After blocking non-specific binding with diluted normal rabbit serum in PBS for 20 min, the sections were incubated for 1 h at room temperature with a primary antibody against IL-1β (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were developed using a VECTASTAIN Elite ABC Goat IgG kit and a DAB substrate kit for peroxidase (Vector Laboratories, South San Francisco, CA, USA). Counterstaining was done with hematoxylin. Scoring for each section was evaluated on scale of 0 – 4 with increments of 0.5 by a blinded observation (IL-1β) (31).

Complement C3 level in mouse sera

The levels of C3 were measured by a commercially
available ELISA kit as described by the manufacturer (Kamiya Biomedical Company, Seattle, WA, USA).

**C3 activation induced in vitro by OVA and anti-OVA pAb immune complexes**

The levels of C3 activation induced in vitro by anti-OVA pAb were examined by ELISA with a modification as described previously (32). Briefly, 96-well ELISA plates (Nunc, Rochester, NY, USA) were precoated for 24 h with OVA (Sigma) (25 µg/ml). The plates were washed six times with PBS / 0.05% Tween 20 and then blocked with 20% BSA (Sigma) in PBS / 0.05% Tween 20 for 24 h. The plates were washed again six times and incubated with graded concentrations (1 – 100 µg/ml) of anti-OVA pAb in 1% BSA / 0.05% Tween 20. The plates were washed and complement (Rockland Immunochimicals, Inc., Gilbertsville, PA, USA) diluted in veronal saline buffer added with 1 mM MgCl₂ and 2 mM CaCl₂ and incubated at 37°C for 1 h. HRP-conjugated goat IgG anti-mouse C3 Ab (MP Biomedicals, Solon, OH, USA) was added, and the color reaction was examined by adding TMB substrate (Pierce, Chicago, IL, USA). The reaction was stopped by adding H₂SO₄ solution. Absorbance was read at 450 nm.

**Statistic analyses**

Differences in values for various experimental groups were examined for significance using the two-tailed Student’s *t* test. Significance was defined as *P* < 0.05. All data are shown as the mean response ± S.E.M.

**Results**

**Induction of anti-OVA polyclonal antibody–induced arthritis**

To clarify the role of anti-OVA pAb in the induction of arthritis, mice were administered with anti-OVA pAb (2 mg) 0.5 h prior to i.a. injection of OVA (10 µg). Joint swelling was observed at 0.5, 1, 2, 3, 4, and 5 h as 0.51 ± 3.6, 0.41 ± 3.2, 0.61 ± 2.4, 0.77 ± 3.7, 0.53 ± 4.9, 0.37 ± 4.4 mm, respectively. The results revealed two peaks of joint swelling at 0.5 and 3 h (Fig. 1). Joint swelling started to decline after 3 h. There was no difference in joint swelling between the administration of OVA alone and the control (PBS) (P. Hutamekalin et al., unpublished data). Based on these data, it was assumed that administration of anti-OVA pAb was able to cause joint swelling.

**Investigation of the levels of C3 in serum**

To clarify that the complement system plays an important role in the induction of arthritis, the complement consumption of serum C3 was determined. The serum C3 level was significantly decreased at 3 h, while no change of C3 was observed at 0.5 h compared with naïve mice (Fig. 2). The results suggested that complement consumption was involved in joint swelling of the second peak (peak at 3 h).

**C3 activation induced by anti-OVA pAb**

Cleavage of the C3 complement releases the activated fragments C3a and C3b (33). To further investigate the role of anti-OVA pAb in induction of C3 activation, the level of C3b was measured since the production of C3b suggests C3a was also produced following the activation...
of C3. The complement component was significantly increased with graded concentrations of anti-OVA pAb (1 – 100 µg/ml) (Fig. 3). These results indicated that forming of the complex of OVA and anti-OVA pAb can induce complement activation which is observed from the increase in complement component level.

The effect of SB 290157 on the reduction of joint swelling

It appears that C3a plays an important role in inflammatory responses (34, 35). In order to investigate the role of complement activation in inflammatory arthritis, the C3aRA SB 290157 was injected into DBA/1J mice before both swelling peaks at 0 and 2 h. Administration of 5% ethanol in PBS was used as the vehicle control. Consistent with the observation above, the data presented here demonstrated that joint swelling was produced at 0.5 and 3 h in the vehicle-treated mice (Fig. 4). On the other hand, the injection of SB 290157 at concentrations of 10 and 30 mg/kg resulted in markedly diminished joint swelling by 32.14% and 48.80%, respectively, at 3 h but not at 0.5 h. Treatment with SB 290157 alone resulted in no effect on joint swelling (P. Hutamekalin et al., unpublished data). Based on these experiments, administration of SB 290157 at concentration of 30 mg/kg caused about 50% inhibition of joint swelling; therefore, 30 mg/kg SB 290157 was used in the following experiments.

Reduction of neutrophil infiltration in the synovial membranes by SB 290157

Because it is known that neutrophils are the hallmark of acute inflammation, the role of C3aRA in restricting neutrophil infiltration was investigated. Histopathological examination of the joints in DBA/1J mice was performed at 3 h. In naïve mice, a single-cell layer lining the membrane of synovial cells was observed in joints without inflammatory cells in synovial tissue (Fig. 5A). However, 3 h after i.a. challenge with OVA, most of the inflammatory cells infiltrated to inflamed synovial tissues were neutrophils (Fig. 5B). When mice were treated with SB 290157, few inflammatory cells including neutrophils were observed (Fig. 5C). Few inflammatory cells including neutrophils were seen 0.5 h after antigenic challenge (P. Hutamekalin et al., unpublished data). Treatment with SB 290157 alone did not affect the histological changes (P. Hutamekalin et al., unpublished data).

Reduction of IL-1β expression in synovial tissue with SB 290157

To investigate if SB 290157 could reduce the expression of proinflammatory cytokines, immunohistochemical staining of IL-1β was performed. Figure 6A showed normal joint staining. Cells expressing IL-1β were detected in one or two layers of superficial lining cells facing the joint cavity and scattered in the subsynovial region in vehicle-treated mice (Fig. 6B). In contrast, cells expressing IL-1β were reduced in 30 mg/kg SB 290157–treated mice (Fig. 6C). These results suggested that SB 290157 is able to reduce IL-1β expression. Treatment with SB 290157 alone had no effect on production of IL-1β (P. Hutamekalin et al., unpublished data).
Discussion

The present study indicated that the selective non-peptide C3aRA SB 290157 can reduce joint swelling and neutrophil infiltration in synovial tissue in a murine model of anti-OVA pAb–induced arthritis. In addition, administration of SB 290157 resulted in lower levels of the inflammatory cytokine IL-1β, indicating the pivotal role of SB 290157 in reducing inflammatory arthritis.

Injection of OVA alone did not show a significant difference in joint swelling (P. Hutamekalin et al., unpublished data). However, administration of anti-OVA pAb 0.5 h earlier than OVA (0 h) was able to cause acute inflammation, showing that the anti-OVA pAb was essential for the induction of inflammatory arthritis (10, 36). A previous report showed that anti-OVA pAb and OVA form complexes and cause acute synovitis (14) and induce acute inflammation (16, 37). Sensitization with anti-OVA antibody and challenge with OVA also activated histamine release from guinea-pig lung mast cells (38). Therefore, histamine may be involved in the first peak of arthritis since the H1-receptor blocker chlorpheniramine was able to reduce joint swelling, while there was no effect on the second peak (S. Yoshino et al., submitted manuscript). It is generally thought that H1 receptors are not only up-regulated by histamine, but also by large amounts of H1-receptor mRNA and are detected in rheumatoid synovial tissue (39).

The serum concentrations of complement component C3 was lower in RA patients, indicating complement consumption (40) and showing that C3 is necessary in the pathogenesis of RA (19). The C3 cleavage of the joint of RA patients was significantly higher than patients who had degenerative joint diseases (20). According to the complement consumption, it was revealed that the complement system played a role at 3 h (the second peak) but not at 0.5 h (the first peak). Once C3 was cleaved and C3a was generated (33), C3a reacted on neutrophils in an in vivo model and also contributed to the inflammatory response in arthritic joints (34, 35).

The joint swelling and infiltration of neutrophils were inhibited after SB 290157 administration. Prevention of neutrophil infiltration into the joints restricts disease
progression and in particular reverses the inflammatory swelling and tissue damage (2, 40). Recently, SB 290157 was shown to significantly decrease paw swelling in a rat RA model compared with control untreated animals, including inhibiting neutrophil recruitment in the lung neutrophilia model in guinea pigs (27). The previous report presented that C3a modulated LPS-induced IL-1β synthesis in peripheral blood mononuclear cells (24). Furthermore, several data supported that IL-1β recruits neutrophils (41, 42). Synovial lining cells appear to produce a large amount of IL-1β. Because synovial lining cells consist of macrophage-like cells and fibroblast-like cells, macrophage-like synovial lining cells may play an important role in the production of IL-1β. C3a is known as a chemotactic factor for inflammatory cells including neutrophils. Various mediators from activated neutrophils may have stimulated macrophage-like synovial cells, resulting in production of IL-1β from the cells. Production of IL-1β exacerbates joint inflammation. Taken together, these results indicated that the C3aR antagonist may be effective in the inhibition of acute arthritis, which inhibited neutrophil recruitment by regulation of IL-1β.

SB 290157 was selective for C3aR and did not antagonize other chemotactic G protein–coupled receptors (GPCR) on human neutrophils. The SB 290157 doses used in the present experiments (10–30 mg/kg) specifically antagonize C3aR (28, 31). Few side effects of SB 290157 appeared to be observed in mice treated with this antagonist when they were judged by their appearance and body weight gain. SB 290157 is able to inhibit neutrophil infiltration in a guinea-pig LPS-induced airway neutrophilia model (27). Our data (P. Hutamekalin et al., unpublished data) agreed with the previous report that C3a is a chemoattractant molecule for guinea-pig neutrophils (35). However, in vitro studies of C3a chemotactic activity are controversial (43, 44), which is likely to be a function only at high doses of the compound. It was reported that C3a is not chemotactic for human neutrophils (45), although they express C3aR (46, 47). This finding may be because of eosinophils contaminating the neutrophil preparation, showing that C3a stimulation of neutrophils is secondary to eosinophil activation (48). Nevertheless, this might indicate that anaphylatoxins are quite divergent between species because mouse and human C3aR share 65% identity (46, 47). In contrast, C3a may initiate signaling in neutrophils by a different mechanism, in particular, activation via a relatively new GPCR, C5L2 (49). For instance, phosphorylated ERK1/2 and AKT were detected in wild-type neutrophils, while the signals were reduced in C3a-stimulated c512−/− neutrophils. However, the effect of C3a on neutrophil chemotaxis might be partially independent of C3aR. A previous study showed that C3a is able to enhance CXCL12-induced chemotaxis of bone marrow from C3aR-knockout mice similar to wild-type mice (50).

In conclusion, the present data indicate that SB 290157 can inhibit the induction of arthritis by lowering the level of joint swelling, neutrophil migration, and IL-1β production. The current findings suggested that C3a may be involved in the aggravation of arthritis by using SB 290157. Therefore, SB 290157 may be effective in the inhibition of arthritis.

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