We previously reported that a combination of 4 monoclonal antibodies (mAbs) (cocktail A) to type II collagen (CII), including immunoglobulin G (IgG)2b (C2B-9 and C2B-14) and IgG2a (C2A-7 and C2A-12), induced arthritis in DBA/1J mice. In this study, we found that C2B-9 and C2A-7 as well as C2B-14 and C2A-12 recognized the same or similar epitopes on CII. Based on these data, we hypothesized that the combination of more than 3 mAbs recognizing different epitopes on CII may more efficiently induce arthritis. Therefore, in addition to C2B-9 and C2B-14, which show high binding activity to CII compared with C2A-7 and C2A-12, we developed two more mAbs including IgG2b (C2B-17) and IgG2a (C2A-16), to make a new cocktail (cocktail B) consisting of these 4 mAbs. To compare the ability of cocktail B to induce arthritis with cocktail A, DBA/1J mice were injected with these cocktails. The results showed that cocktail B was able to induce more severe arthritis than cocktail A, especially more markedly affecting rear paws. Histologically, there was more marked proliferation of synovial tissues, massive infiltration by inflammatory cells, and severe destruction of cartilage and bone in mice treated with cocktail B than with cocktail A. Collectively, the new combination of 4 mAbs recognizing different respective epitopes appears to effectively induce arthritis in mice. Thus, the results may provide insights into the selection of mAbs associated with the development of arthritis.

Key words  arthritogenity; collagen antibody-induced arthritis; epitope; type II collagen; monoclonal antibody; rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints associated with elevated levels of autoantibodies to type II collagen (CII).13 The most commonly used animal model for studying the pathophysiology of RA is collagen-induced arthritis (CIA), induced by immunizing rodents with CII,2–4 as antibodies to CII play a role.5,6 It is also known that the classical complement-activating pathway through antigen-antibody immune complexes regulates arthritis,7,8 and these responses lead to chronic inflammation.

Similar arthritis can be induced in naïve mice by transferring sera from arthritic mice and partially purified antibodies from patients with rheumatoid arthritis.9,10 As evidence of inflammatory arthritis induced by serum transfer, collagen antibody-induced arthritis (CIA) has been developed by using anti-type II collagen antibody.11–13 The initial event in the development of CIA is the formation of collagen-antibody immune complexes on the cartilage surface or in the synovium, and subsequent activation of complement may induce arthritis.11,14 Therefore, to induce arthritis effectively, it is important that these antibodies recognize a wide range of epitopes on the CII molecule and thereby allow the formation of large immune complexes on the cartilage; however, it has not been fully examined whether a combination of monoclonal antibodies (mAbs) showing different epitopes efficiently induces arthritis.

We previously developed immunoglobulin G (IgG)2a (C2A-7 previously named CII-7 and C2A-12) and IgG2b (C2B-9 previously named CII-3 and C2B-14) subtypes of anti-CII mAbs13,15 and demonstrated that a combination of these 4 mAbs (cocktail A) induced arthritis in mice. In the present study, we first examined whether C2B-9, C2B-14, C2A-7, and C2A-12 recognized a different or the same epitope of CII. Second, we developed two more new mAbs, IgG2b (C2B-17) and IgG2a (C2A-16), and mixed these mAbs with C2B-9 and C2B-14 to make another 4 mAbs-containing antibody cocktail (cocktail B), and then tested whether each mAb recognized a different or the same epitope of CII. Third, we compared the ability of cocktail B to induce arthritis with cocktail A.

MATERIALS AND METHODS

Animals  Male DBA/1J mice (8 weeks of age) were bred in the animal breeding unit of Kobe Pharmaceutical University, Kobe, Japan. The 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.

mAbs to CII  In this study, we developed IgG2a (C2A-14, C2A-15, C2A-16, and C2A-17) and IgG2b (C2B-17) subtypes of anti-CII mAbs from spleen cells of DBA/1J mice immunized with chicken CII (Sigma-Aldrich Fine Chemicals, St. Louis, MO, U.S.A.) emulsified with Freund’s complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) as described previously.13 Briefly, mice were given a booster injection of 0.1 mg chicken CII dissolved in 100 µL potassium phosphate buffer (0.13 M K2HPO4, 18 mM KH2PO4, pH 7.6) on days 11–13. Three days after the injection, spleen cells (1×10⁸) were obtained and fused with NS-1 myeloma cells (2×10⁵) using PEG1500 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

Hybridoma cells producing antibodies against chicken
CII were screened by enzyme linked immunosorbent assay (ELISA) using plates coated with chicken CII (10 µg/ml in JG buffer). The wells were blocked with 1% casein (Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS) at room temperature for 1 h. Fifty microliters of culture medium mixed with an equal volume of PBS containing 1% Tween 20 (Sigma-Aldrich) was reacted at 37°C for 1 h. mAbs bound to collagen were detected by phosphatase-labeled anti-mouse IgG (Fc) (Sigma-Aldrich). Color was developed by adding 100 µl of 3 mM p-nitrophenylphosphate (Bio-Rad, Richmond, CA, U.S.A.), and absorbance was measured at 405 nm using an IMMUNO-MINI NJ-2300 (Thermo Fisher Scientific, Roskilde, Denmark).

The selected hybridoma cells were cloned by limited dilution and cultured in a serum-free CM-B medium (Sanko Junyaku Co., Ltd., Tokyo, Japan) in Nunc 96-micro well plates (Thermo Fisher Scientific). mAbs were purified by HiTrap IgG Protein A (GE Healthcare, Uppsala, Sweden) affinity chromatography, and concentrated by Vivaspin-20 (Sartorius Stedim Biotech Gmbh, Goettingen, Germany) to 10 mg/ml in PBS based on an OD280 of IgG mAb at 1 mg/mL of 1.42.

Preparation of Anti-CII Fabs To prepare anti-CII Fabs, anti-CII antibodies were digested with immobilized papain (Thermo Scientific, Rockford, IL, U.S.A.) at 37°C for 22h according to the methods described previously.16 The Fab fraction was purified by using HiTrap IgG Protein A affinity chromatography and concentrated by Vivaspin-20 (Sartorius Stedim Biotech Gmbh, Goettingen, Germany) to 10 mg/ml in PBS based on an OD280 of IgG mAb at 1 mg/mL of I.42.

Analysis of Epitope Specificity To investigate epitope specificities of the mAbs, plates were coated with 1 µg/ml mouse CII. Fabs (500 µg/mL) mixed with 1 µg/ml of intact antibodies were then added to ELISA plates, and the cross-reaction was determined by adding phosphatase-labeled anti-mouse IgG (Fc) (Sigma-Aldrich). The plates were developed with p-nitrophenylphosphate and read at 405 nm using a microplate reader. Values for the cross-reaction of mAbs with mouse CII were expressed as absorbance units.

Cross-Reaction of mAbs with Mouse or Chicken CII

The cross-reaction of mAbs with mouse or chicken CII (1 µg/mL) was determined by ELISA with the affinity for collagen.15 Dilutions (0.001–1000 µg/mL) of mAbs were detected using plates coated with mouse or chicken CII and adding phosphatase-labeled anti-mouse IgG (Fc). The plates were developed with p-nitrophenylphosphate and read at 405 nm using a microplate reader. Values for the cross-reaction of mAbs with mouse or chicken CII were expressed as absorbance at 405 nm.

Fig. 1. Arthritis Induced by a 4-Clone Antibody Cocktail A and the Epitopes Recognized by the 4 Clones

(A) Changes in the arthritis score after the administration of cocktail A (C2B-9, C2A-7, C2B-14, and C2A-12). DBA/1j mice were given i.p. injection of cocktail A on day 0 (8 mg/mouse) followed by an injection of 50 µg/mouse LPS on day 3. Each value is the mean±S.E.M. of five animals. (B) The competitive binding assay of intact antibody and Fab to compare epitope recognition of each clone. Intact antibody was mixed with Fab at 1:500. Mixture was incubated with mouse CII-coated plates and assayed using mouse anti-Fc antibody-conjugated alkaline phosphatase. Binding activities are expressed as absorbance unit (OD 405nm). Each value is the mean±S.D. of four assays.
Induction of Arthritis  The 4-clone cocktail was prepared by mixing an equal volume of 10 mg/mL, and mice were given 0.8 ml of the cocktail (8 mg/mouse) by intraperitoneally (i.p.) injection on day 0, respectively, followed by an i.p. injection of lipopolysaccharide (LPS) (50 µg/mouse) on day 3. The mice were observed daily after the injection of mAbs for the development of arthritis until day 10. The severity of arthritis was scored as: 0 = normal; 1 = mild erythema or swelling of the wrist or ankle or erythema and swelling of any severity for 1 digit; 2 = more than three inflamed digits or moderate erythema and swelling of the ankle or wrist; 3 = severe erythema and swelling inflammation of the wrist or ankle; 4 = complete erythema and swelling of the wrist and ankle, including all digits.

Histological Analysis of Arthritis  Rear paw joints were dissected on day 10, fixed in 10% neutral buffer formalin, decalcified in decalcifying solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and embedded in paraffin. The rear ankle joints were sectioned at 4 µm and stained with hematoxylin and eosin (H&E) by the standard technique.

Statistical Analyses  Data are shown as the mean±S.E.M. Statistical analyses between the two groups were performed using Student’s t-test. A probability value of $p < 0.05$ was considered significant.

RESULTS

Analysis of 4-Clone mAbs Epitopes Specificity of Cocktail A  As previously reported, C2B-9 and C2B-14, C2A-7, and C2A-12 induced arthritis in DBA/1J mice (Fig. 1A). Next, we investigated the epitopes of the clones. C2B-9 binding was inhibited by C2B-9 and C2A-7 Fabs; furthermore, C2A-7 binding was completely suppressed by C2B-9 and C2A-7 Fabs (Fig. 1B). In addition, C2A-12 binding was inhibited by C2B-14 and C2A-12 Fabs, although C2B-14 binding was not decreased by C2A-12 Fabs (Fig. 1B); the inhibition of C2A-12 binding by C2B-14 Fabs was not strong. Thus, C2B-9 and C2A-7, and C2B-14 and C2A-12 recognized same or similar epitopes on CII. Furthermore, since it has been reported that C2B-9 and C2B-14 showed high binding activities compared with C2A-7 and C2A-12, we attempted to add two new clones to C2B-9 and C2B-14 for efficient induction of arthritis.
Cross-Reaction of New Developed mAbs with Chicken and Mouse CII We newly developed anti-CII antibodies, IgG2a (C2A-14, C2A-15, C2A-16 and C2A-17) and IgG2b (C2B-17). Figures 2A and B show cross-reaction with mouse and chicken CII, respectively, as binding activity (OD 405 nm). It has been reported that C2B-9 and C2B-14 strongly bound to mouse CII as 0.976 and 1.104 of OD 405 nm at 1 µg/mL, respectively. C2B-17 and C2A-16 showed 0.985 and 0.605; however, C2A-14, C2A-15 and C2A-17 had lower than 0.4 binding activity to mouse CII. On the other hand, for chicken CII, C2A-16 indicated similar binding activity to mouse CII; however, C2B-17, C2A-14, C2A-15 and C2A-17 bound to chicken CII less than to mouse CII at 1 µg/mL antibody. In terms of affinity activity with mouse CII, the order was C2B-9=C2B-14>C2B-17>C2A-16>C2A-15>C2A-17>C2A-14.

Analysis of 4-Clone mAbs Epitopes Specificity of Cocktail B We selected two new clones, C2B-17 and C2A-16, from 5 mAbs because the two mAbs showed high binding activities. Thus, we prepared cocktail B (C2B-9, C2B-14, C2B-17, and C2A-16); furthermore, we assessed whether these clones showed respective different epitopes. These 4 clones were inhibited by Fabs themselves only, suggesting that these mAbs show different epitopes (Fig. 3).

Arthritis Induced by Cocktail B We subsequently attempted to examine whether cocktail B induced more severe arthritis than cocktail A. As shown in Fig. 4A, the percent incidence of arthritis by both cocktails was 100% during days 5–10. Furthermore, although the percent incidence of arthritis in both cocktails showed no difference, cocktail B induced more severe arthritis than cocktail A during days 7–10 (Fig. 4B).

Fig. 4. Comparison of Arthritogenicity of Cocktail A and B

(A and B) Changes in arthritis incidence (A) and arthritis score (B) induced by cocktail A and B. DBA/1J mice were given i.p. injection of cocktail A (C2B-9, C2B-14, C2A-7, and C2A-12) or cocktail B (C2B-9, C2B-14, C2B-17 and C2A-14) on day 0 (8 mg/mouse) followed by an injection of 50 µg/mouse LPS on day 3. Each value is the mean±S.E.M. of five animals. *p<0.05 and **p<0.01 compared with the cocktail A group.

Fig. 5. Comparison of the Induction of Arthritis in Front and Rear Paws by Cocktail A and B

Changes in arthritis incidence (front: A and rear: B) and arthritis score (front: C and rear: D) induced by cocktail A and B. DBA/1J mice were given i.p. injection of cocktail A or B on day 0 (8 mg/mouse) followed by an injection of 50 µg/mouse LPS on day 3. Each value is the mean±S.E.M. of five animals. **p<0.01 compared with the cocktail A group.
Next, we compared the percent incidence of arthritis and the arthritis score of front and rear paws by the cocktails. Both cocktails induced the same percent incidence and arthritis score for front paws (Figs. 5A,C). Meanwhile, for rear paws, cocktail B induced a percent incidence and arthritis score higher than cocktail A (Figs. 5B,D).

Comparison of the Histological Changes in Joints of Mice with Arthritis Induced by Cocktail A and B

Histological examination of joints in mice was performed on day 10 after injection both cocktails. Figures 6A and B show naïve rear paw and ankle joints (H&E) as a control, respectively. Furthermore, mice given cocktail B developed severe arthritis and marked proliferation of synovial tissues, massive infiltration by inflammatory cells, and severe destruction of cartilage and bone in the ankle joints (H&E) compared with cocktail A (Figs. 6C–F).

DISCUSSION

The present study demonstrated that cocktail B (a new combination of C2B-9, C2B-14, C2B-17, and C2A-16) induced severe arthritis in comparison with cocktail A (combination of C2B-9, C2B-14, C2A-7, and C2A-12) in DBA/1J mice. Importantly, the 4 new anti-CII mAbs showed respectively different epitopes and strong binding activities to mouse CII; however, cocktail A showed that 2 mAbs of 4 clones had the same or similar epitopes, although all of these mAbs had strong binding activities to mouse CII. Thus, a combination of mAbs showing different epitopes is important for efficient induction of arthritis.

First, to examine whether cocktail A shows respectively different epitopes, we examined the cross-reaction of the antigen and antibody using Fab inhibition, with the result that C2B-9 and C2A-7, and C2B-14 and C2A-12 recognized the same or similar epitopes on CII. Based on these data, we hypothesized that the combination of mAbs showing different recognized epitopes may more efficiently induce arthritis, because it is important that these mAbs recognize a wide range of epitopes on the CII molecule and thereby allow the formation of large immune complexes on the cartilage. Meanwhile, we have reported that C2B-9 and C2B-14 showed high binding activity to CII compared with C2A-7 and C2A-12; therefore, we decided to select C2B-9 and C2B-14 as the main clones in this study.

Next, in addition to C2B-9 and C2B-14, we developed two new clones, C2B-17 and C2A-16, which showed higher binding activities compared with the other new mAbs, C2A-14, C2A-15, and C2A-17; furthermore, the 4 new clones (C2B-9, C2B-14, C2B-17, and C2A-16) showed respectively different epitopes. Therefore, we attempted to investigate whether cocktail B can induce more severe arthritis than cocktail A. As expected, the new 4-clone cocktail B produced more severe arthritis, suggesting that a combination of clones showing not only strong binding activity but also different epitopes is important for the efficient induction of severe arthritis.

The cartilage and synovial surface of rear paw are obviously bigger than the front paws. Arthritis pathology of the front paws showed marked severity in both cocktail A and B treatment groups; however, only cocktail B mediated strong arthritis in the rear paws. Thus, the induction of more severe arthritis by cocktail B was dependent on the severity of arthritis in the rear paw, indicating that a combination of clones showing different epitopes and strong binding activity effectively binds cartilage and synovial surfaces in the rear paw and induces the severe arthritis.

It is thought that complement fragments binding to immune complexes, tissue damage, and/or Fc-gamma receptor crosslinking can activate local mononuclear cells that in turn release proinflammatory cytokines17–19 in or near the joints inducing neutrophil and macrophage recruitment.14,20,21 In this study, in cocktail B-treated mice, more massive infiltration by inflammatory cells and more severe destruction of cartilage and bone in the rear ankle joints were observed in comparison with cocktail A-treated mice. It has been reported that tissue-degrading enzymes from macrophages and neutrophils can cause cartilage and/or bone damage,22–24 suggesting that the destruction of cartilage and bone in cocktail B-treated mice is associated with the accumulation of inflammatory cells.

In conclusion, the new combination of 4 mAbs showing different epitopes effectively induced arthritis in DBA/1J mice; therefore, the results may provide insights into the selection of mAbs associated with the development of arthritis.

REFERENCES