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Characterization of monoclonal antibody specific for human type II collagen: possible implication in collagen-induced arthritis

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SUMMARY

We obtained monoclonal antibodies specific for human type II collagen and characterized them using human collagen type I, II, III and V and tropocollagen A (3/4) (TC^A) and tropocollagen B (1/4) (TC^B) fragments of type II collagen which were obtained by digestion with tadpole collagenase. These antibodies were of the IgG2a class and specific for the conformational determinant of TC^A fragment of type II collagen. When injected intravenously into DBA/1J mice, one of the monoclonal antibodies induced arthritis, which was characterized by early onset, mildness in severity and preferential localization mainly in the peripheral joints of the lower extremities. These results suggest that, at least, one of the arthritogenic determinants of type II collagen for collagen-induced arthritis of mice exists in the three quarter region from the N-terminus of type II collagen.

Keywords monoclonal antibody type II collagen

INTRODUCTION

Connective tissues contain multiple collagen types (Bornstein & Sage, 1980). In biochemical and histochemical studies, preparation of monoclonal antibody against each collagen type would be very useful (Linsenmayer, Hendrix & Little, 1979; Fitch *et al.*, 1982; Mayne *et al.*, 1983). Type II collagen (IIC), which exists in cartilage, is known to induce polyarthritis in rats and mice injected intracutaneously (Trentham, Townes & Kang, 1977; Courtenay *et al.*, 1980). It has been suggested that antibodies to IIC play an important role in the induction of arthritis (Stuart *et al.*, 1982; Stuart & Dixon, 1983).

In this study, we prepared monoclonal antibodies against human IIC and characterized the antibodies using various types of human collagen and IIC fragments obtained by tadpole collagenase digestion. This enzyme, in distinction to the reaction of bacterial collagenase, cleaves the collagen molecule through all three α-chains at a single locus three-quarters from the N-terminus, producing tropocollagen A (3/4) (TC^A) and tropocollagen B (1/4) TC^B fragments (Gross & Nagai, 1965; Kang *et al.*, 1966; Nagai, Lapiere & Gross, 1966). Arthritogenic activity of the monoclonal antibody was also examined by injecting into DBA/1J mice intravenously.

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MATERIALS AND METHODS

Animals. Male and female DBA/1J mice, 7–8 weeks old, were purchased from Gokita Breeding Company (Tokyo, Japan) and used throughout the experiments.

Collagen. Human type I (fetal skin), type II (costal cartilage) and type III (fetal skin) collagens employed were those used in recent studies (Konomi *et al.*, 1981, 1983). Human type V collagen was kindly donated through the courtesy of Dr T. Hayashi, Tokyo Medical and Dental University.

Collagenase. Highly purified tadpole collagenase was prepared according to the method reported by Hori & Nagai (1979). One unit of collagenase digested 1 μg of type I collagen per min at pH 7·6 and 37°C.

Conventional antibodies against individual collagens. Conventional antibodies specific for human IIC were prepared by intracutaneous injection into rats with 200 μg of collagen in Freund's complete adjuvant (Difco). The animals were boosted several times with the same antigen every 2 weeks and bled 2 weeks after the last immunization. Type-specific anti-IIC antibodies were purified by affinity chromatography using a IIC-Sepharose 4B column. Type-specific antibodies against human type I and type III collagens were those used in a recent study (Konomi *et al.*, 1981). Antibody against type V collagen was a gift from Dr T. Hayashi.

Preparation of monoclonal antibody against IIC. DBA/1J mice were immunized with human IIC. On the third day after the first booster injection, immune spleen cells were collected and fused with Balb/c myeloma cells (P3-X63-Ag8-U1), which were kindly donated by Dr T. Watanabe, Saga Medical College, using 45% polyethyleneglycol 4000 (Sigma) according to the method of Köhler and Milstein (1975). After selection with RPMI 1640 media (GIBCO) containing hypoxanthine/aminopterin/thymidine and cloning with limiting dilution, hybridoma cells secreting antibody to IIC were obtained. Hybridoma cells were transplanted into the peritoneal cavity of (Balb/c × DBA/1J)F_1 mice pretreated with 2, 6, 10, 14-tetramethylpentadecane, Pristane (Aldrich). Ascites was collected and assayed for the antibody activity with enzyme-linked immunosorbent assay (ELISA).

Antibody assay by ELISA. ELISA was done to study the specificity of conventional as well as monoclonal antibodies against type I, II, III and V collagens and two fragments of IIC by the method reported by Engvall & Perlmann (1971). Round-bottomed 96 well microtitre plates

Table 1. Specificity of monoclonal antibody for various types of collagen (ELISA)*

Antibody to type II collagen	Antigenic specificity for: Type I	Type II	Denatured† type II	Type III	Type V
Monoclonal‡					
H-1 (IgG2a)	0	>2·0	0	0	0
F-2 (IgG2a)	0	>2·0	0	0	ND§
K-2 (IgG2a)	0	>2·0	0	0	0
M-2 (IgG2a)	0	>2·0	0	0	ND§
Conventional¶	0	>2·0	0·2	0	0

Type-specific antibodies to type I, type III or type V collagen reacted only with corresponding type of collagen (Konomi *et al.*, 1983).

* Each type of collagen (2 μg/100 μl) was used to coat the wells for ELISA.

† Prepared by heating at 60°C for 30 min.

‡ Used after 10,000 fold dilution.

§ Not determined.

¶ Used after 100 fold dilution.

(Greiner) were coated by incubation with 100 μl each of individual collagens or fragments of IIC in sodium carbonate buffer, pH 9·6 (20 μg/ml) at 4°C overnight. After removal of collagen solution by aspiration, the wells were washed with phosphate-buffered saline (PBS), pH 7·4, containing 0·05% Tween 20 (PBS-Tween), filled with PBS-Tween containing 1% bovine serum albumin and incubated at 37°C for 1 h. After removal of the solution, the resulting wells were incubated with 100 μl each of ascites containing monoclonal antibodies or conventional antiserum optimally diluted at room temperature for 2 h. After washing, the wells were incubated for an additional 3 h at room temperature with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1/500 to 1/1000, which was then removed. The resulting wells were washed with PBS-Tween and incubated with 100 μl of 4-nitrophenylphosphate (Sigma) in 10% diethanolamine buffer for 30 min followed by termination of the reaction by addition of stopper (3M NaOH). The titres of antibodies were estimated by measuring the absorbance at 410 nm with an ELISA reader (Corona).

Analysis of IIC digestion by tadpole collagenase. Stock solution (6 ml) of 0·2% IIC in 5 mM acetic acid was neutralized by adding dropwise to an equal volume of 0·1 M Tris-HCl buffer, pH 7·6, containing 0·3 M NaCl, 10 mM $CaCl_2$ and 0·04% NaN_3 and incubated with 240 μl of purified tadpole collagenase (50 units/ml) at 20°C for 35 days with repeated additions of 240 μl each of the enzyme on day 5, 10, 15 and 20. At various intervals, 10 μl of the reaction mixture was withdrawn and enzyme digestion patterns were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7·5% gels) at pH 8·5.

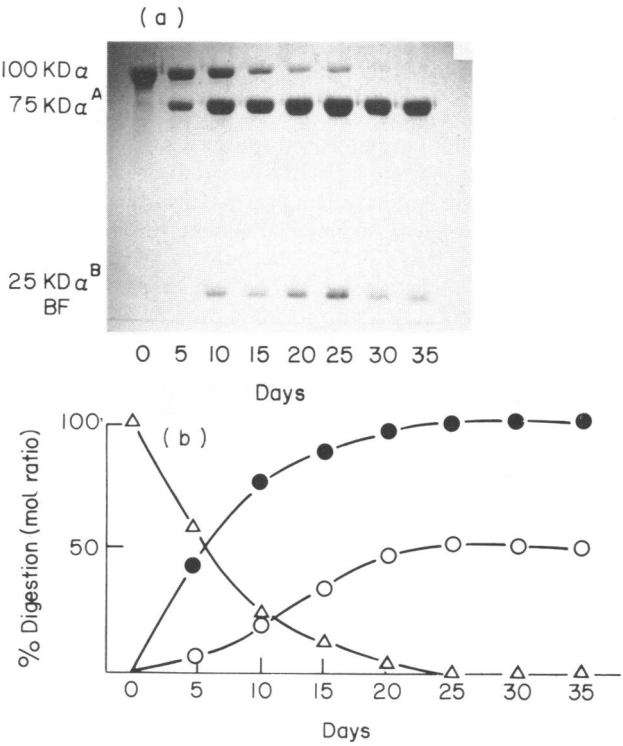

Fig. 1. Time course of type II collagen incubated with tadpole collagenase.

(a) SDS-PAGE pattern of human type II collagen incubated with tadpole collagenase at 20°C. At indicated time periods, 10 μl of the reaction mixture was withdrawn and electrophoresed on a 7·5% slab gel.

BF: buffer front. α: α1(II) chain of type II collagen.

(b) Relative amounts of α chain and α^A and α^B fragments were estimated from the integrated values of the densitometric curve of individual stained bands in Fig. 1a. Mole percents of residual α1 (II) chain (Δ) and the accumulated 75 KD, α^A (●) and 25 KD, α^B (○) fragments were estimated, assuming the initial amount of α1(II) chain to be 100%.

Purification of collagen fragments. After terminating the collagenase digestion by the addition of 20 mM EDTA, the reaction mixture was dialysed against 0·02 M acetate buffer pH 4·8, containing 0·03 M NaCl, 2 M urea and 0·5 M glucose and applied to a CM-32 cellulose column followed by elution with 0·03 M to 0·25 M NaCl gradient at 4°C. TC^A fragment-containing fractions were pooled and rechromatographed on a CM-32 cellulose column. The purity of TC^A and TC^B fragments was monitored by SDS-PAGE (7·5% gels).

Injection of monoclonal antibody to mice. Ascites fluid of normal mice pretreated with Pristane alone or ascites containing monoclonal antibody was salted out with 50% ammonium sulfate, dialyzed against 0·15 M phosphate-buffered saline, pH 7·2 (PBS) and adjusted to 25 mg/ml with PBS, according to the method described by Stuart *et al.*, (1982). A single intravenous injection of 200 μl of the concentrated antibody was given into the tail vein of normal DBA/1J mice. These mice were observed for clinical evaluation of the development of arthritis followed by termination for histological examination.

RESULTS

Specificity of monoclonal antibodies for various collagen types

Monoclonal anti-IIC antibodies were shown to be specific for native IIC, and no reactivity with type I, III, V collagen or heat-denatured IIC was observed (Table 1). The results suggests that these antibodies are specific for a conformational determinant of IIC. All of these monoclonal antibodies were of the IgG2a class.

Isolation of TC^A and TC^B fragments of IIC treated with tadpole collagenase

When the reaction products of IIC incubated with purified tadpole collagenase at 20°C were analysed by SDS-PAGE, an increasing amount of distinct products of molecular weight 75 KD and 25 KD accumulated with incubation time as seen in Fig. 1a (Hayashi *et al.*, 1980). These 75 KD and 25 KD fragments corresponded well with α^A and α^B fragments of type I collagen, although six to

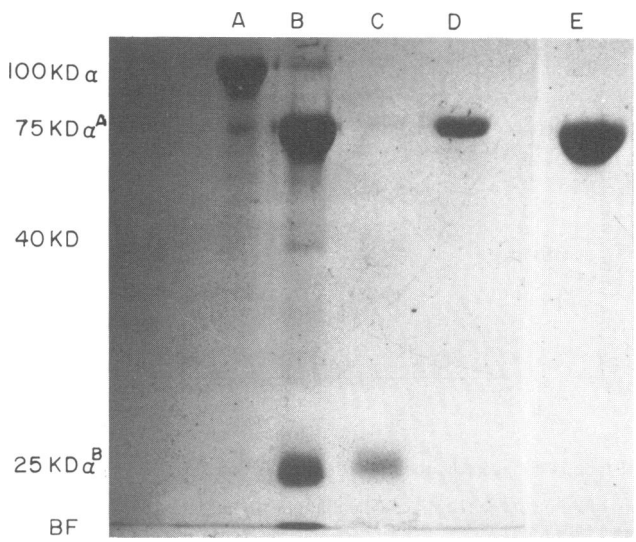

Fig. 2. Purity of isolated TC^A and TC^B fragments of human type II collagen examined by SDS-polyacrylamide gel electrophoresis. TC^A and TC^B fragments were isolated by CM-32 cellulose rechromatography. 40 K band corresponds to tadpole collagenase.

A: Zero hour digestion, B: 35 day digestion, C: purified TC^B fragment, D: 5 μg of purified TC^A fragment. E: 20 μg of purified TC^A fragment. BF: buffer front. α: α1 (II) chain of type II collagen.

Table 2. Reactivity of monoclonal antibodies with type II collagen and its TC^A and TC^B fragments before and after denaturation at 60°C for 30 min (ELISA)*

Antibody to type II collagen	Dilution	Antigenic specificity for: Type II	Denatured type II	TC^A	Denatured TC^A	TC^B	Denatured TC^B
Monoclonal							
H-1	$\times 10^4$	1·2	0	1·1	0	0	0
F-2	$\times 10^4$	1·8	0	1·8	0	0	0
K-2	$\times 10^4$	>2·0	0	1·7	0	0	0
M-2	$\times 10^4$	1·8	0	1·9	0	0	0
conventional†	$\times 10^2$	>2·0	0·2	1·8	0·2	1·3	0

* Two μg/100 μl of type II collagen and its fragments, TC^A and TC^B, was used to coat the wells for ELISA.

† Specific for human type II collagen.

seven fold digestion time was required for the complete digestion of IIC, compared to type I collagen.

Relative amounts of intact collagen and its digestion products estimated from the integrated values of the densitometric curve of individual stained bands at 570 nm showed that the yield of α^B fragment was lower (about 50%) than that of α^A fragment (Fig. 1b), probably due to the instability of the fragment because of its low denaturation temperature (28°–29°C) (Sakai & Gross, 1967). Fig. 2 shows typical SDS-PAGE patterns of TC^A and TC^B fragments isolated from the 35 day reaction mixture by CM-32 chromatography. Both fragments isolated were not cross-contaminated.

Specificity of monoclonal antibodies for IIC fragments

Monoclonal antibodies obtained in this study reacted with native IIC and its TC^A fragment, but not with TC^B or denatured TC^A and TC^B fragments as shown in Table 2. In order to confirm that isolated TC^A and TC^B fragments have native conformation, we studied the reactivity of these two fragments with conventional antibody which is directed to native IIC but little reactive to denatured IIC. As shown in Table 2, the antibody did react with TC^A and TC^B fragments, but much less with denatured TC^A and TC^B fragments, indicating that isolated TC^A and TC^B fragments maintain the integrity of conformation determinant of the molecule.

Table 3. Induction of arthritis by monoclonal anti-type II collagen antibody in normal DBA/1J mice.

Antibody injected	Incidence of arthritis (Arthritic/ Total recipients)*
Monoclonal antibody (H-1)†	10/10
Normal mouse Ig†	0/10

* Recipient DBA/1J mice were injected i.v. with 0·2 ml (5 mg/mouse) of each immunoglobulin.

† Ascites from normal mice pretreated with Pristane and ascites from hybridoma-carring mouse were salted out with 50% ammonium sulfate and adjusted to 25 mg/ml after dialysis against PBS.

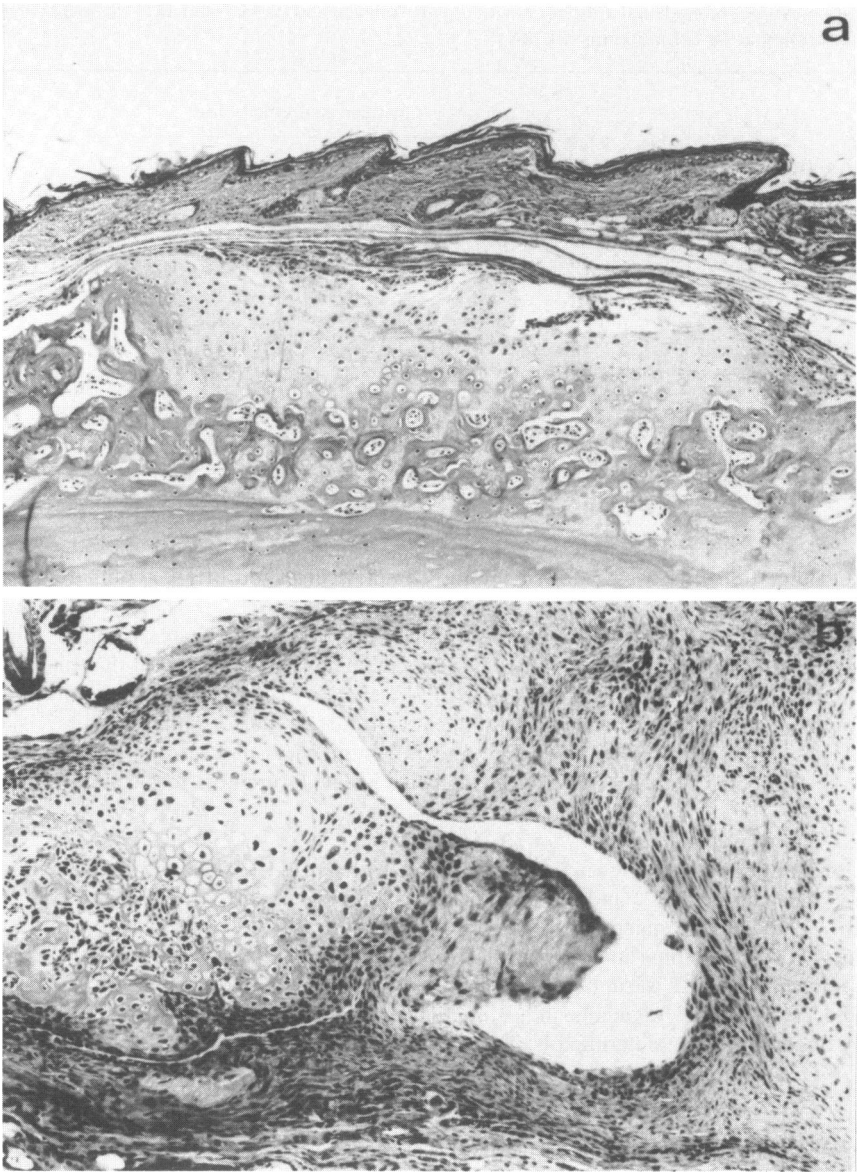

Fig. 3. Histological findings of the joints in mice with arthritis induced by the injection of monoclonal antibody. Joints were examined 2 weeks after the onset of arthritis. Granulation tissue with mononuclear cell infiltration and newly formed cartilagenous tissue are present. Fibrin exudate is noted in the joint space. a × 75, b: × 125 H.E.

Induction of arthritis by monoclonal antibody

To study the arthritogenic activity of the monoclonal antibody, we injected one antibody (H-1) intravenously into DBA/1J mice. H-1 antibody (IgG2a, k type) showed induction of arthritis with 100% incidence (10/10) (Table 3). Clinical manifestations such as erythema and swelling in the peripheral joints were mild but definite, appearing within 24 h after injection. Histological observations have shown that mononuclear and polymorphonuclear cell infiltration accompanied by proliferation of fibroblasts in the synovial tissue, destruction of the joint cartilage and pannus

formation associated with new cartilage formation were noted (Fig. 3a & b). These findings were very similar to those of passively transferred arthritis by conventional anti-IIC antibody reported by Stuart & Dixon (1983).

DISCUSSION

Linsenmayer and Hendrix (1980) reported preparation of monoclonal antibody specific for IIC which recognized the helical portion of native molecules. We further studied the antigenic site(s) on IIC with monoclonal antibodies using IIC fragments obtained by tadpole collagenase. Tadpole collagenase has been shown to cleave type I, II and III collagen molecules through all three α chains at a single locus to produce TC^A and TC^B fragments (Kang *et al.*, 1966; Nagai *et al.*, 1966; Hayashi *et al.*, 1980). Furthermore, we found an arthritogenic activity of the monoclonal antibody.

Monoclonal antibodies obtained in this study were specific for a conformational determinant of purified TC^A fragment but not of TC^B fragment. The yield of TC^B fragment by collagenase digestion was about a half of TC^A at the maximum (Fig. 1b). This finding suggests that TC^B fragment produced is labile and subjected to further degradation. Sakai *et al.* reported that temperature of denaturation for TC^A and TC^B fragments of type I collagen were 34–35°C, 28–29°C, respectively (Sakai *et al.*, 1967). Though denaturation temperature for these IIC fragments is not known exactly at the moment, we took care to prevent their denaturation. Digestion was done below denaturation point of TC^B fragment of type I collagen (20°C) and both TC^A and TC^B fragments were purified by CM-cellulose column chromatography at 4°C. In addition, as a control experiment, we studied the reactivity of conventional antibody directed to native IIC with denatured fragments in order to confirm that TC^A and TC^B fragments indeed hold native conformation (Table 2). This antibody reacted with each fragment, but lost their reactivity after heat denaturation of the fragments. This result suggests that TC^A and TC^B fragments prepared in this study maintain their conformational determinants.

We injected one of these monoclonal antibodies into normal DBA/1J mice, because the important role of humoral antibody to native IIC in induction of collagen-induced arthritis has been suggested by several investigators (Trentham *et al.*, 1977; Stuart *et al.*, 1982) and IgG antibody was active in transferring the disease (Stuart *et al.*, 1982; Stuart *et al.*, 1983). Monoclonal anti-IIC antibodies obtained did cross-react with heterologous IIC (rat or bovine). Therefore, it was anticipated that these antibodies are reactive to mouse IIC, although we did not check this reactivity due to the difficulty in obtaining mouse IIC. Injection of H-1 antibody induced definite arthritis with high incidence, though clinicopathological symptoms were mild. We are now studying arthritogenic activity of the other monoclonal antibodies (F-2, K-2 & M-2).

The collagen molecules prepared by pepsin digestion are known to have two different antigenic determinants, sequential and conformational ones (Beil, Timpl & Furthmayr, 1973). We have not yet obtained monoclonal antibody directed to denatured IIC or native TC^B fragment. Therefore, we could not compare the arthritogenic activity of monoclonal antibodies with different specificities. However, it is certain that monoclonal antibody alone directed to conformational determinant of TC^A fragment had arthritogenicity in DBA/1J mice without involvement of antibody specific for TC^B fragment or the sequential determinant of IIC. Since arthritogenic monoclonal antibody was directed to TC^A fragments, we are now studying whether or not isolated TC^A fragments is really arthritogenic in mice by active immunization.

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