Clin. exp. Immunol. (1988) **73**, 443–448

Epitope specificity of antibody response against human type II collagen in the mouse susceptible to collagen-induced arthritis and patients with rheumatoid arthritis

H. IRIBE, H. KABASHIMA, Y. ISHII* & T. KOGA *Department of Biochemistry, Kyushu University School of Dentistry and *First Department of Internal Medicine, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan*

(Accepted for publication 10 May 1988)

SUMMARY

The presence of species-specific and species-non-specific (common) epitopes has been demonstrated on type II collagen (CII) using monoclonal antibodies. In this study, we investigated the role of antibody response to some species-specific and common epitopes in mice immunized with human CII for the induction of collagen-induced arthritis (CIA). Antibody responses to species-specific epitopes in arthritic mice appeared significantly higher than that in non-arthritic mice. However, no significant difference of antibody responses to common epitopes was found between arthritic and non-arthritic mice. Monoclonal antibody reactive with one of the common epitopes exhibited the ability to induce arthritis in mice previously given the primary injection of CII, indicating the involvement of this epitope in the induction of CIA. Finally, we investigated the epitope specificity of anti-human CII antibody present in serum samples of patients with rheumatoid arthritis and relapsing polychondritis, and found antibodies to some common epitopes.

Keywords epitope type II collagen collagen arthritis rheumatoid arthritis

INTRODUCTION

Type II collagen-induced arthritis (CIA) in susceptible rats and mice is known to be a useful model of human rheumatoid arthritis (RA) (Tentham, Townes & Kang, 1977; Courtenay *et al.*, 1980). Heterologous and homologus type II collagens (CII) have been used for the induction of CIA (Trentham *et al.*, 1977; Courtenay *et al.*, 1980; Holmdahl *et al.*, 1986a). Autoimmunity against self CII present in cartilage has been demonstrated to play a critical role in the development of CIA (Stuart, Townes & Kang, 1984). It was also suggested that both humoral and cell-mediated immunity against CII are involved in the induction of arthritis (Stuart *et al.*, 1984). However, the precise mechanism of induction of CIA is still unclear.

The molecular weight of CII is approximately 300,000 Daltons. Many antigenic epitopes on CII derived from chicken (Holmdahl *et al.*, 1986b) and man (Iribe *et al.*, 1988) have been demonstrated using monoclonal antibodies. However, the role of antibody response to each epitope in the induction of CIA has not been clarified. Wooley *et al.* (1985) postulated the existence of two arthritogenic and multiple non-arthritogenic epitopes on the CII molecule, based on an analysis of the genetic control of immune response against CII from various species. Terato *et al.* (1985) cleaved chicken CII with cyanogen bromide (CB) and isolated one of these CB peptides that has arthritogenicity. These findings suggest that the immune responses against some selected epitopes on CII are involved in the induction of CIA.

In the present study, we investigated the role of the antibody responses against several distinct epitopes on human CII in the induction of murine CIA. Furthermore, we analysed epitope specificity of anti-human CII antibodies derived from patients suffering from RA and relapsing polychondritis.

Correspondence: Dr. Hideaki Iribe, Department of Biochemistry, Kyushu University School of Dentistry, 3-1-1 Maidashi Higashi-ku, Fukuoka 812, Japan

MATERIALS AND METHODS

Animals

Seven to 8 week old male DBA/1J mice were purchased from Seiwa Experimental Animal Co. (Nakatsu, Japan).

Type II collagen (CII)

Human CII was purified from the costal cartilage according to Miller (1972) but with a slight modification as described (Kakimoto, Hirofuji & Koga, 1984).

Serum samples of patients

Serum samples of patients suffering from RA were collected at the First Department of Internal Medicine, Faculty of Medicien, Kyushu University. Serum samples of patients with relapsing polychondritis were provided by Dr. S. Sawada in the First Department of Internal Medicine, Faculty of Medicine, Nippon University.

Immunization

DBA/1J mice were intradermally injected in the right foot pad with 200 μg of human CII emulsified in complete Freund's adjuvant. The secondary immunization with CII emulsified in incomplete Freund's adjuvant was given to mice 3 weeks after the primary injection. Mice were inspected daily for the onset and the severity of arthritis. Arthritic index was scored as described elsewhere (Wood, Pearson & Tanaka, 1969).

Assay for anti-human CII antibody in enzyme-linked immunosorbent assay (ELISA)

The measurement of anti-human CII antibody in serum samples of mice and humans in ELISA was done according to the method described previously (Hirofuji *et al.*, 1985) with slight modifications.

Monoclonal antibodies to human CII

Eight monoclonal antibodies against human CII were developed in our laboratory and characterized (Iribe *et al.*, 1988). In this study we used six monoclonal antibodies designated as 2-56, 2-60, 1-5, 2-14, 2-15 and 2-25. All of them were of IgG2a.k subclass. In our previous study, 2-56 and 2-60 antibodies were found to recognize distinct epitopes specifically expressed on human CII (Iribe *et al.*, 1988). On the other hand, 1-5, 2-14, 2-15 and 2-25 antibodies were shown to recognize four distinct epitopes expressed on CII derived from rats and mice as well as man (Iribe *et al.*, 1988). To specify the epitopes recognized by monoclonal antibodies, we employed those names of monoclonal antibodies.

Conjugation of monoclonal antibody with alkaline phosphatase

Purified monoclonal antibody was coupled to alkaline phosphatase according to Voller *et al.* (1976). Briefly, 0·5 mg of monoclonal antibody was mixed with 1000 U of alkaline phosphatase (SIGMA, St. Louis, MO) and dialysed. Diluted glutaraldehyde solution was added to the mixture to give a final concentration of 0·2% and the preparation was incubated at room temperature for 2 h with vigorous shaking. After incubation, the sample was dialysed against PBS and finally against 0·05 M Tris-HCl pH 7·8 containing 1 mM $MgCl_2$. The antibody conjugated with alkaline phosphatase was diluted to a concentration of 100 μg/ml of antibody by adding Tris buffer supplemented with 1% BSA.

Competitive inhibition assay

To measure the titre of antibody to each epitope on human CII in serum samples, we performed a competitive inhibition experiment in ELISA. Serum samples from mice and humans were serially diluted and added to microtitre plates pre-coated with human CII (Greiner, West Germany). After incubation for 30 min at room temperature, we added alkaline phosphatase-labelled monoclonal antibody diluted 1:1000. The plates were then incubated for 2 h at room temperature, washed with PBS containing Tween 20 (PBS-Tween), and substrate, 4-nitrophenylphosphate, was added. The reaction was halted by adding 3 M NaOH. The absorbance at 410 nm was measured using an ELISA reader (Dynatech Lab., Alexandria, Virginia). The percent inhibition was calculated using the following formula.

$$\% \text{ inhibition} = \left(1 - \frac{\text{Absorbance with inhibitor}}{\text{Absorbance without inhibitor}}\right) \times 100$$

Antibody titre represents the reciprocal of the sample dilution giving 50% inhibition.

Purification of anti-human CII antibody on immunoadsorbent column

Human CII was coupled to cyanogen bromide activated Sepharose-CL 4B gel. The immunoadsorbent column was equilibrated with 0·15 M NaCl–0·05 M Tris-HCl, pH 7·5. Serum from mice or humans was applied to the column and antibodies bound to human CII were eluted by washing the column with 0·17 M glycine-HCl, pH 2·3.

RESULTS

Antibody responses to four distinct epitopes on CII

Previously we defined at least seven distinct epitopes on human CII including species-specific and common epitopes using monoclonal antibodies (Iribe *et al.*, 1988). Antibody response to each epitope in mice suffering from CIA was analysed and marked antibody responses to two species-specific epitopes and two common epitopes were found (data not shown). In this study we examined the time course of antibody responses to these four distinct epitopes, and analysed the relation between the magnitude of antibody response and the occurrence of arthritis. DBA/1J mice usually developed CIA two to four weeks after the secondary injection of CII. Some mice immunized with CII, however, failed to develop clinical signs of arthritis. As shown in Fig. 1a and 1b, the titre of antibody to common epitopes (2-60, 2-56) reached a maximum 3 to 5 weeks after the secondary immunization and then gradually decreased. As shown in Fig. 2a, the titre of antibody to one common epitope (1-5) reached a maximum 3 weeks after the secondary immunization, and then the level of antibody rapidly decreased. The production of antibody against another common epitope (2-15) is shown in Fig. 2b. High titre of antibody against this epitope was detected in two non-arthritic mice within 2 weeks after the secondary immunization. Statistical significance of antibody responses against these epitopes between arthritic and non-arthritic mice was evaluated using Student's *t*-test. As shown in Fig. 3, the titres of antibody against two species-specific epitopes in arthritic mice were significantly higher than that in non-arthritic mice. No significant difference in antibody response to common epitopes was found between arthritic and non-arthritic mice. Thus, the high antibody response to species-specific epitopes rather than to common epitopes correlated well with the occurrence of arthritis. Total antibody level to CII was not significantly different between arthritic and non-arthritic mice.

Antibodies against common epitopes rather than against species-specific epitopes on human CII should be involved in the induction of CIA. As described above, we noticed that the titre of antibody against one common epitope (1-5) in arthritic mice rapidly decreased after reaching the maximum. Since this may

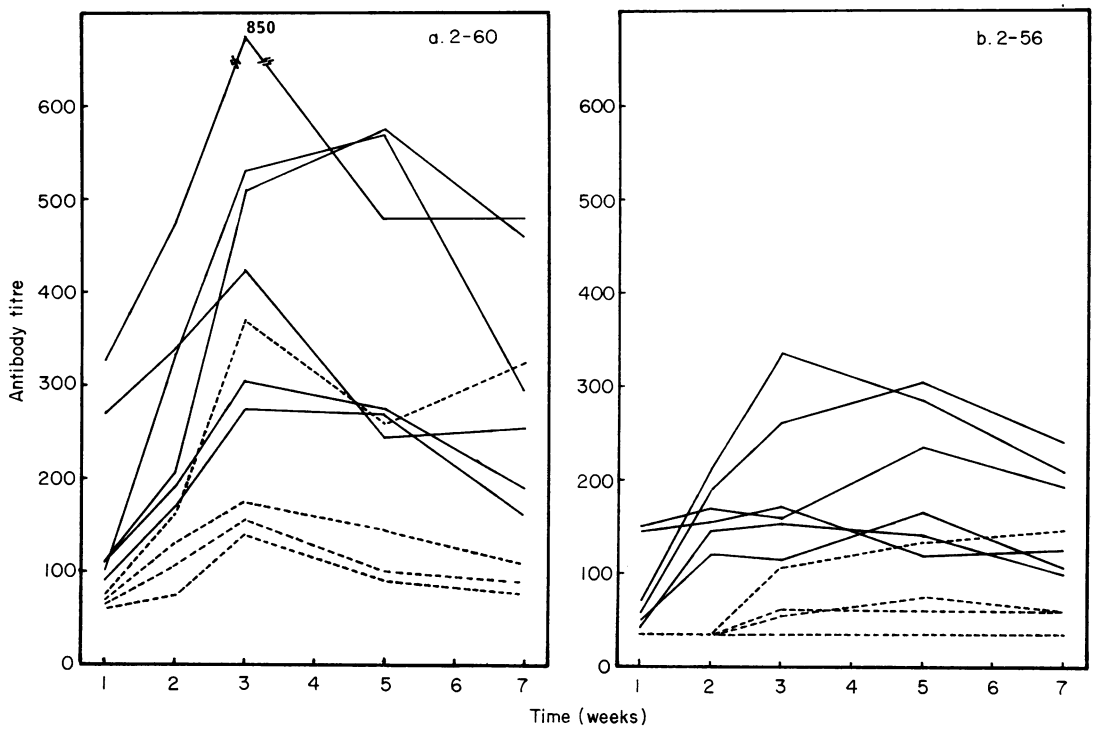

Fig. 1. The time course of the titre of antibody to species-specific epitopes on human CII in mice. Serum samples collected from mice immunized with CII as described were tested for antibody to species-specific epitopes on human CII in competitive inhibition of ELISA. (———), mice with arthritis; (– – –), mice without arthritis during the inspection. (a) 2-60 epitope; (b) 2-56 epitope.

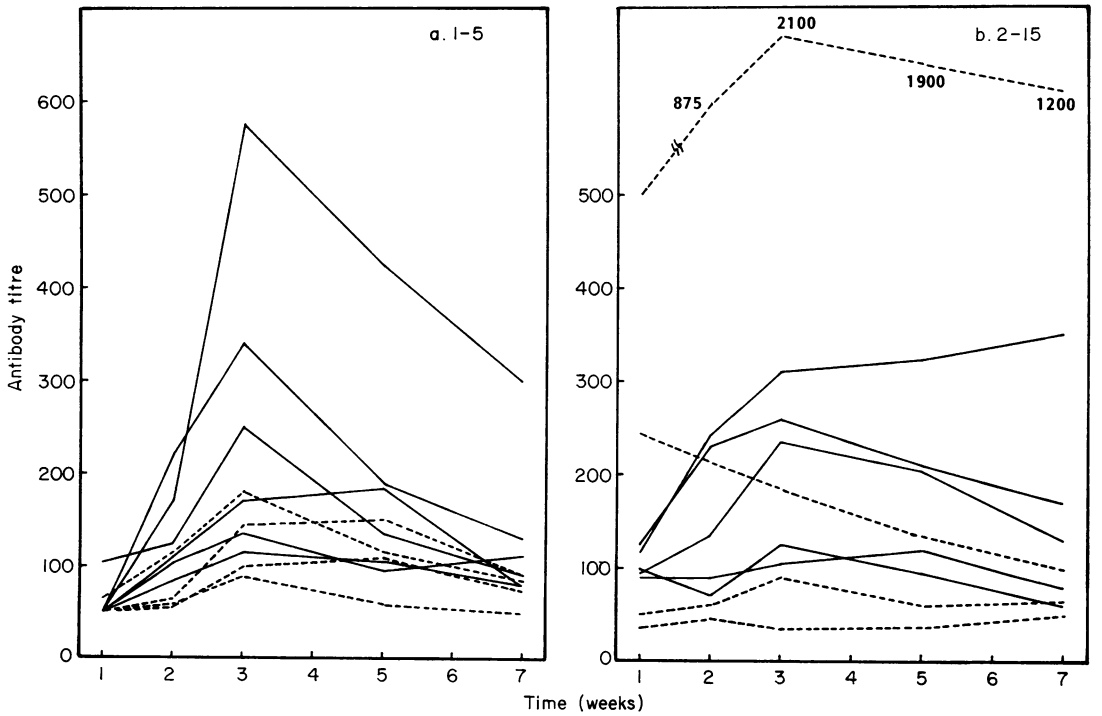

Fig. 2. The time course of titre of antibody to common epitopes on human CII in mice. Serum samples used in the experiment shown in Fig. 1 were tested for antibody to common epitopes on human CII in competitive inhibition of ELISA. (———), mice with arthritis; (– – –), mice without arthritis during the inspection. (a) 1-5 epitopes; (b) 2-15 epitope.

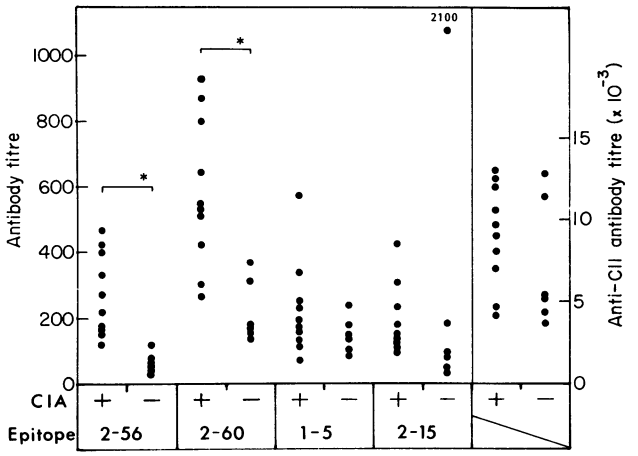

Fig. 3. Comparison of antibody titres to various epitopes on human CII between arthritic and non-arthritic mice. Antibody titres to various epitopes as well as to human CII were compared between arthritic and non-arthritic mice. Antibody titres of sera collected from mice 3 weeks after the secondary immunization are shown. The statistical significance of the data was evaluated with Student's *t*-test. * $P < 0{\cdot}01$.

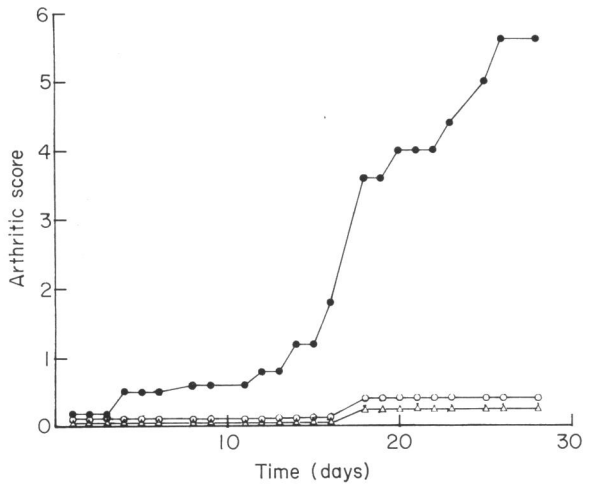

Fig. 4. Transfer of monoclonal antibody into mice. Monoclonal antibody (4 mg/mouse) was intravenously injected into mice previously given the primary immunization of human CII. The incidence and severity of arthritis were inspected daily after injection. Five mice were used for each group. (●——●), 1-5 antibody; (○——○), 2-56 antibody; (△——△), normal mouse IgG.

be due to the absorption of antibody by CII exposed *in vivo*, we examined the possible involvement of 1-5 antibody in the induction of arthritis.

Induction of arthritis by monoclonal antibody

When naive mice were given monoclonal anti-CII antibodies intravenously, they did not show any clinical sign of arthritis. Therefore, we used mice immunized with human CII three weeks previously as recipients of monoclonal antibodies. We gave these primed mice an intravenous injection of monoclonal antibody. As shown in Fig. 4, mice injected with 1-5 antibody developed mild arthritis 4 days after the transfer of antibody. Severe arthritis appeared 18 days after the injection of antibody. On the other hand, the injection of 2-56 antibody to species-specific epitope was not effective on the induction of arthritis. These findings indicate the pathogenic role of 1-5 antibody in the induction of CIA.

Epitope specificity of anti-CII antibody derived from patients

Several serum samples of patients with RA or relapsing polychondritis showed marked anti-CII antibody response in ELISA (Table 1). Epitope specificity of anti-CII antibodies of patients was examined in competitive inhibition. As shown in Table 1, some sera of these patients significantly inhibited the bindings of 1-5, 2-15 and 2-25 antibodies to human CII. However, the bindings of 2-60 and 2-14 antibodies were not significantly affected by the serum samples. Serum of one patient with RA showed high titre of anti-CII antibody and strongly inhibited the binding of 2-15 antibody to CII (98%). Antibodies to human CII were purified from serum of this patient using immunoadsorbent column coupled to human CII and tested for the epitope specificity. As shown in Table 2, purified antibodies of patients also exhibited strong inhibitory effect on the binding of 2-15 antibody to CII. On the other hand, anti-CII antibodies purified from arthritic mice had inhibitory activity on the binding of 2-56, 2-60, 1-5, 2-15 and 2-25 antibodies.

DISCUSSION

Heterologous CII has been used for the induction of CIA in mice. Many antigenic epitopes present on CII were defined by Holmdahl *et al.* (1986b) and by Iribe *et al.*, 1988, using monoclonal antibodies. It has been postulated that some selected epitopes among many epitopes on CII are involved in the induction of arthritis (Holmdahl *et al.*, 1986b, Wooley *et al.*, 1985). The immune response against species-specific epitopes expressed on CII derived from other species such as chick and man probably would not participate in the initiation of murine CIA. On the other hand, the immune response against common antigenic epitopes on CII could play a critical role in the induction of arthritis. However, it is possible that heterologous CII expressing species-specific epitopes on its surface can evoke a stronger immune response against CII than homologous CII when adminstered to mice. Indeed, it was reported that mice immunized with heterologous CII produced much more anti-CII anitbody than mice immunized with homologous CII (Holmdahl *et al.*, 1986a; Watson & Townes, 1985; Wooley *et al.*, 1981).

In this study, we investigated the antibody response against several distinct epitopes, including species-specific and common epitopes, on human CII in the DBA/1J mouse susceptible to CIA. We compared the antibody repsonse against four epitopes on human CII between arthritic and non-arthritic mice after the secondary immunization with CII. The antibody levels of arthritic mice against two species-specific epitopes (2-56, 2-60) appeared significantly higher than that of non-arthritic mice. On the other hand, the levels of antibody to two common epitopes (1-5, 2-15) were not significantly different between arthritic and non-arthritic mice. Thus the high antibody response against species-specific epitopes rather than that against common epitopes correlated well with the occurrence of arthritis. When we compared the total antibody level against human CII between arthritic and non-arthritic mice, we could not find a

Table 1. Epitope specificity of anti-human type II collagen antibodies derived from patients with RA and relapsing polychondritis

Serum samples†	Anti-human CII antibody in ELISA (OD at 410 nm)	% inhibition of binding labelled antibody				
		2-60	1-5	2-14	2-15	2-25
Normal human serum ($n=7$)	0·033±0·01	27±12	5±8	13±6	24±10	11±4
RA						
1	0·30	55	46*	7	33	19
2	0·66	63	53*	9	98*	25*
3	0·28	51	22	9	52	16
4	0·34	45	20	4	76*	35*
Relapsing polychondritis						
1	0·46	39	39*	23	70*	35*
2	0·40	35	29	7	55*	18
3	0·33	15	14	15	9	0

* Values exceeding the mean value of control sera by more than three s.d.
† Serum samples were diluted to 1/10 and used for assay.

Table 2. Epitope specificity of purified anti-human type II collagen antibodies derived from patient with RA and arthritic mice

Purified antibody to human type II collagen derived from	% inhibition of binding of labelled antibody					
	2-56	2-60	1-5	2-14	2-15	2-25
Mice immunized with human type II collagen*	89	96	93	0	93	68
Patient with RA†	0	9	18	0	88	0

* Antibody sample was diluted to 1/50 of the original volume of serum sample and used as inhibitor.
† Antibody sample was diluted to 1/5 of the original volume of serum sample and used as inhibitor.

significant difference between them. Previously, Klareskog *et al.* (1986) used monoclonal antibodies to three different epitopes on native CII for immunohistochemical analysis of antigenic determinants that are exposed in the cartilage and synovial tissue obtained from patients with RA and osteoarthritis (OA). They showed that two of the monoclonal antibodies reacted with cartilage from both OA and RA joints, but not with that from normal joints. Holmdahl *et al.* (1986a) used native murine CII for immunization of DBA/1J mice, and found that titres of anti-CII antibodies did not correlate well with arthritis development. The antibodies against common epitopes, but not antibodies against species-specific epitopes, may be partially absorbed from the circulation due to their binding property to murine CII, possibly exposed on the affected joints. Therefore, the antibody level against species-specific epitopes rather than common epitopes on human CII reflects the actual amount of antibody produced in the mouse. Antibody responses to species-specific epitopes on human CII may represent exactly the magnitude of antibody response against human CII in mice.

The antibody response against one common epitope (1-5) in arthritic mice reached the maximum just before the appearance of clinical signs of arthritis and then the antibody level rapidly decreased. Antibody against this epitope (1-5) might play some role in the initiation of arthritis. Transfer experiments with monoclonal antibody revealed the possible involvement of 1-5 antibody in the occurrence of arthritis. The 1-5 antibody exhibited the ability to induce arthritis in mice pre-immunized with human CII, suggesting that a common epitope recognized by 1-5 antibody is one of the arthtitogenic epitopes. The mechanism of the induction of arthritis by 1-5 antibody is not clear at present. However, it is possible that 1-5 antibody is involved in the induction of arthritis in cooperation with other antibodies and cell-mediated immunity against CII.

Autoantibodies against CII have been detected in the sera, synovial fluids, cartilage, and synovial tissue of some patients with RA (Adriopoulos *et al.*, 1976; Clague, Shaw & Holt, 1980; Stuart *et al.*, 1983; Jasin, 1985; Mostecky & Miller, 1975). Recently, purified anti-CII antibody from one patient with RA was found to passively transfer arthritis to naive mice (Wooley *et al.*, 1984), thereby providing evidence for the pathogenicity of this autoantibody in human arthritis. In the present study, we investigated the epitope specificity of anti-CII antibodies present in the sera of patients with RA or relapsing polychondritis. Autoantibodies reactive with common epitopes rather than species-specific epitopes on human CII were predominantly found in the serum samples of these patients. Thus, the spectrum of epitope specificity of human autoantibodies seems to be somewhat different from that of murine antibodies to human CII. With regard to antibodies to common epitopes, antibodies to some selected epitopes (1-5, 2-15, 2-25) were found in sera of both patients and arthritic mice. Antibody to one of the common epitopes (2-14) could not be detected in sera of either patients or arthritic mice. These findings indicate that some selected common epitopes of CII are recognized as autoantigen in both mice and humans. The specific immune response against CII, but not polyclonal activation of B cells is probably generated in some patients with RA, and the autoantibodies to CII produced would play some role in the development of disease.

ACKNOWLEDGMENTS

This work was supported by grants in aid of Scientific Research from the Ministry of Education, Science and Culture (No. 62770328) and for studies on Intractable Disease from the Ministry of Health and Welfare of Japan. We wish to thank Dr. K. Kakimoto for his useful comments and Ms. K. Higuchi for her secretarial work in preparing this manuscript.

REFERENCES

ANDRIOPOULOS, N.A., MESTECKY, J., MILLER, E.J., & BRADLEY, E.L. (1976) Antibodies to native and denatured collagens in sera of patients with rheumatoid arthritis. *Arthritis Rheum.* **19,** 613.

CLAGUE, R.B., SHAW, M.J. & HOLT, P.J.L. (1980) Incidence of serum antibodies to native type I and type II collagens in patients with inflammatory arthritis. *Ann. Rheum. Dis.* **39,** 201.

COURTENAY, J.S., DALLMAN, M.J., DAYAN, A.D., MARTIN, A. & MOSEDALE, B. (1980) Immunization against heterologous type II collagen induced arthritis in mice. *Nature* **283,** 666.

HIROFUJI, T., KAKIMOTO, K., HORI, H., NAGAI, Y., SAISHO, A., SUMIYOSHI, A. & KOGA, T. (1985) Characterization of monoclonal antibody specific for human type II collagen: possible implication in collagen-induced arthritis. *Clin. exp. Immunol.* **62,** 159.

HOLMDAHL, R., JANSSON, L., LARSSON, E., RUBIN, K. & KLARESKOG, L. (1986a) Homologous type II collagen induces chronic and progressive arthritis in mice. *Arthritis Rheum.* **29,** 106.

HOLMDAHL, R., ROBIN, K., KLARESKOG, L., LARSSON, E. & WIGZELL, H. (1986b) Characterization of the antibody response in mice with type II collagen-induced arthritis using monoclonal anti-type II collagen antibodies. *Arthritis Rheum.* **29,** 400.

IRIBE, H., KABASHIMA, H., KAKIMOTO, K. & KOGA, T. (1988) Induction of an anti-human type II collagen response by monoclonal anti-idiotypic antibody. *J. Immunol.* in press.

JASIN, H.E. (1985) Autoantibody specificities of immune complexes sequestered in articular cartilage of patients with rheumatoid arthritis and osteoarthritis. *Arthritis Rheum.* **28,** 241.

KAKIMOTO, K., HIROFUJI, T. & KOGA, T. (1984) Specificity of anti-type II collagen antibody response in rats. *Clin. exp. Immunol.* **57,** 57.

KLARESKOG, L., JAHNELL, O., HULTH, A., HOLMDAHL, R. & RUBIN, K. (1986) Reactivity of monoclonal anti-type II collagen antibodies with cartilage and synovial tissue in rheumatoid arthritis and osteoarthritis. *Arthritis Rheum.* **29,** 730.

MILLER, E.J. (1972) Structural studies on cartilage collagen employing cleavage and solubilization with pepsin. *Biochemistry* **11,** 4903.

MOSTECKY, J. & MILLER, E.J. (1975) Presence of antibodies specific to cartilage-type collagen in rheumatoid synovial tissue. *Clin. exp. Immunol.* **22,** 453.

STUART, M.J., HUFFSTUTTER, E.H., TOWNES, A.S. & KANG, A.H. (1983) Incidence and specificity of antibodies to type I, II, III, IV, and V collagen in rheumatoid arthritis and other rheumatic diseases as measured by ^{125}I-radioimmunoassay. *Arthritis Rheum.* **26,** 832.

STUART, J.M., TOWNES, A.S. & KANG, A.H. (1984) Collagen autoimmune arthritis. *Ann. Rev. Immunol.* **2,** 199.

TERATO, K., HASTY, K.A., CREMER, M.A., STUART, J.M., TOWNES, A.S. & KANG, A.H. (1985) Collagen-induced arthritis in mice. Localization of an arthritogenic determinant to a fragment of the type II collagen molecule. *J. exp. Med.* **162,** 637.

TRENTHAM, D.E., TOWNES, A.S. & KANG, A.H. (1977) Autoimmunity to type II collagen: an experimental model of arthritis. *J. exp. Med.* **146,** 857.

VOLLER, A., BIDWELL, D.E. & BARTLETT, A. (1976) Enzyme immunoassay in diagnostic medicine. Theory and practice. *Bull. WHO.* **53,** 55.

WATSON, W.C. & TOWNES, A.S. (1985) Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. *J. exp. Med.* **162,** 1878.

WOOD, F.D., PEARSON, C.M. & TANAKA, A. (1969) Capacity of mycobacterial wax D and its subfractions to induce adjuvant arthritis in rats. *Int. Arch. Allergy* **35,** 456.

WOOLEY, P.H., LUTHRA, H.S., STUART, J.M. & DAVID, C.S. (1981) Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. *J. exp. Med.* **154,** 688.

WOOLEY, P.H., LUTHRA, H.S., SINGH, S.K., HUSE, A.R., STUART, J.M. & DAVID, C.S. (1984) Passive transfer of arthritis to mice by injection of human anti-type II collagen antibody. *Mayo Clin. Proc.* **59,** 737.

WOOLEY, P.H., LUTHRA, H.S., GRIFFITHS, M.M., STUART, J.M., HUSE, A. & DAVID, C.S. (1985) Type II collagen-induced arthritis in mice. IV. Variations in immunogenetic regulation provide evidence for multiple arthritogenic epitopes on the collagen molecule. *J. Immunol.* **135,** 2443.