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# Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen

**(orally induced immunologic unresponsiveness/autoimmunity)**

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ABSTRACT **Although oral administration of protein antigens may lead to specific immunologic unresponsiveness, this method of immunoregulation has not been applied to models of autoimmune disease. Type II collagen-induced arthritis is an animal model of polyarthritis induced in susceptible mice and rats by immunization with type II collagen, a major component of cartilage. Intragastric administration of soluble type II collagen, prior to immunization with type II collagen in adjuvant, suppresses the incidence of collagen-induced arthritis. Administration of denatured type II collagen has no observable effect on the incidence or severity of the disease. The overall magnitude of the antibody response is not significantly reduced in collagen-fed mice as compared to controls. While the isotype distribution of the anti-collagen antibodies is similar in the two groups, there is a tendency toward reduced IgG2 responses in the collagen-fed mice.**

Type II collagen-induced arthritis (CIA) is an animal model of polyarthritis induced in susceptible mice and rats by immunization with type II collagen (1, 2). Type II collagen is the major matrix protein of hyaline cartilage. The similarity of the histopathologic changes observed in CIA to those seen in human rheumatoid arthritis has centered interest on the contribution of collagen autoimmunity to the pathogenesis of the human disease. Although humoral and cellular immunity to type II collagen have been shown in CIA, the precise contribution of each to the development of disease has not been established. While T cells have been shown to recognize undenatured and denatured type II collagen (3), the humoral response is restricted to the undenatured, nonrepeating helical antigenic determinants of the collagen molecule (3, 4). Development of disease after immunization with type II collagen in mice is restricted by the major histocompatability type (5). Although many mouse strains produce a vigorous humoral immune response to type II collagen, only mice of the H-$2^q$ haplotype develop arthritis. The induction of acute manifestations of CIA by the transfer of anti-type II collagen antibodies (hereafter referred to as anti-collagen antibodies) from arthritic to normal mice emphasizes the critical role of anti-collagen antibodies in the pathogenesis of CIA (6).

Several attempts to modulate the disease have led to antigen-specific suppression of collagen immunity and decreased incidence of arthritis. Induction of arthritis is suppressed by prior i.v. injection of type II collagen-coupled spleen cells (7). In rats, spleen cells from donors receiving type II collagen-coupled rat erythrocytes transfer antigen-specific suppression of CIA (8). Intravenous administration of soluble type II collagen suppresses induction of arthritis in rats and mice when given before primary immunization (9–11) or during the afferent phase of disease induction, 7–10 days after primary immunization (12).

Oral presentation of antigen is the earliest recorded method for experimentally inducing specific antigenic unresponsiveness (13, 14). This route of antigen administration can lead to immunity or tolerance, depending on the dose, number of feedings, and the form of antigen used (15). T-dependent, but not T-independent, antigens can lead to the induction of oral tolerance (16), and the immune response to collagen is T dependent (17, 18). The studies reported here show that intragastric administration of soluble type II collagen suppresses the induction of CIA in mice. It is, therefore, possible to suppress an experimental autoimmune disease by orally induced unresponsiveness.

## MATERIALS AND METHODS

**Antigens and Immunizations.** DBA/1 Lac J male mice were purchased from The Jackson Laboratories and immunized at age 8–14 weeks. Type II collagen was solubilized from fetal bovine articular cartilage by limited proteolysis with pepsin, essentially according to the technique of Trentham *et al.* (1). Collagen purity was assessed by analysis of amino acids by Genetic Design (Watertown, MA) and by $NaDodSO_4$/PAGE (19). Type II collagen was dissolved in 0.01 M acetic acid at 4°C prior to use. Denatured type II collagen was prepared by incubation at 56°C for 45 min. Intragastric feedings (0.5 ml) were administered with a ball-tipped feeding needle. Control animals were fed 0.01 M acetic acid (0.5 ml). Mice were immunized parenterally by intradermal injection of 300 μg of type II collagen emulsified in Freund's adjuvant containing heat-killed mycobacteria at 4 mg/ml (strains C, DT, and PN; Ministry of Agriculture, Fisheries and Food, Weybridge Surrey, England). Mice were boosted with 100 μg of type II collagen i.p. on day 21.

**Assessment of Arthritis.** Mice were observed two or three times each week for presence of distal joint swelling and erythema. Swelling was quantitated by measuring thickness of foot and width of ankle with a constant tension caliper (Dyer, Lancaster, PA). A mouse was considered arthritic when swelling and erythema were observed on consecutive measurement dates in at least one paw. In addition, clinical severity of arthritis was assessed by creation of an arthritic index. Each limb was subjectively graded on a scale of 0–3 (0, absence of arthritis; 1, mild swelling and erythema; 2, swelling and erythema of both tarsus and ankle; 3, ankylosis and bony deformity). A maximum arthritic index (MAI) was obtained for each mouse by summing the greatest score recorded for each limb (0, no disease; 12, highest possible

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Abbreviations: CIA, collagen-induced arthritis: MAI, maximum arthritic index.

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score) (9, 20). The MAI for each group was calculated according to the formula:

$$\frac{\text{mean MAI} \times \text{number of arthritic mice}}{\text{number of mice in the group}}.$$

**Measurement of Anti-Collagen.** Antibodies to type II collagen in immune sera were measured by ELISA (21). To obtain μg/ml values of anti-collagen antibodies of each isotype, a mouse immunoglobulin reference serum (Miles Scientific, Naperville, IL) containing known amounts of each isotype was used as a standard (22). A mouse anti-collagen standard immunoglobulin preparation was purified from the sera of arthritic mice on a type II collagen-Sepharose column (23). Immulon 2 plates (Dynatech, Alexandria, VA) coated with rabbit anti-mouse immunoglobulin at 100 μg/ml in 0.016 M boric acid/0.15 M NaCl, pH 8, were blocked with the same buffer containing 2% (vol/vol) horse serum. The plates were washed and incubated with serial dilutions of affinity purified mouse anti-collagen and reference serum. The assay was developed by the addition of peroxidase-conjugated rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Miles Scientific), and the substrate ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Zymed Laboratories, South San Francisco, CA]. The cross reactivity of these sera was tested with purified mouse myeloma subclass proteins (Litton Bionetics, Organon Teknica, Charleston, SC) and found to be <10%. Absorbances at 405 nm were determined with a vertical beam spectrophotometer (Artek Systems, Farmingdale, NY). The absorbance values obtained were used to construct a standard curve for each isotype using a computer program that does a least squares fit correlating absorbance with concentration. Curves were generated through a third-order equation in which absorbance is the independent variable and, after subtracting background, the intercept is assumed to be zero. The μg/ml values obtained for anti-collagen antibodies of each isotype in the affinity-purified mouse anti-collagen standard were then used to create standard curves with type II collagen-coated plates. Anti-collagen antibodies of each isotype in immune sera were quantitated by titration on type II collagen-coated plates developed with the rabbit anti-mouse isotyping reagents described above.

Anti-collagen antibodies in immune sera were also measured by solid-phase RIA (24). Polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were coated with type II collagen at 100 μg/ml, blocked with 2% (vol/vol) horse serum, and incubated with dilutions of immune sera. The plates were developed with $^{125}$I-labeled rabbit anti-mouse Fab. Individual wells were cut out and counted in a γ counter. Affinity-purified mouse anti-collagen antibodies were used as a standard.

**Statistical Analysis.** Comparisons of means were performed by Student's *t* test. Arthritis incidences were compared with the Fisher exact test. *P* values given are for two tailed tests.

## RESULTS

**Resistance to Arthritis Induction After Feeding of Soluble Type II Collagen.** Soluble type II collagen was administered intragastrically to CIA-susceptible DBA/1 Lac J mice prior to intradermal immunization with type II collagen in complete Freund's adjuvant. Twelve feedings (500 μg each) of type II collagen in 0.5 ml of 0.01 M acetic acid during 6 weeks significantly reduced the incidence of arthritis (Fig. 1*A*; $P < 0.004$ for each time point beyond day 30). For the group, the MAI on day 58 was 1.9 as compared to 3.9 in controls (Fig. 1*B*). For those mice in the type II collagen-fed group that did become arthritic the mean day of onset (day 44 ± 4) was not

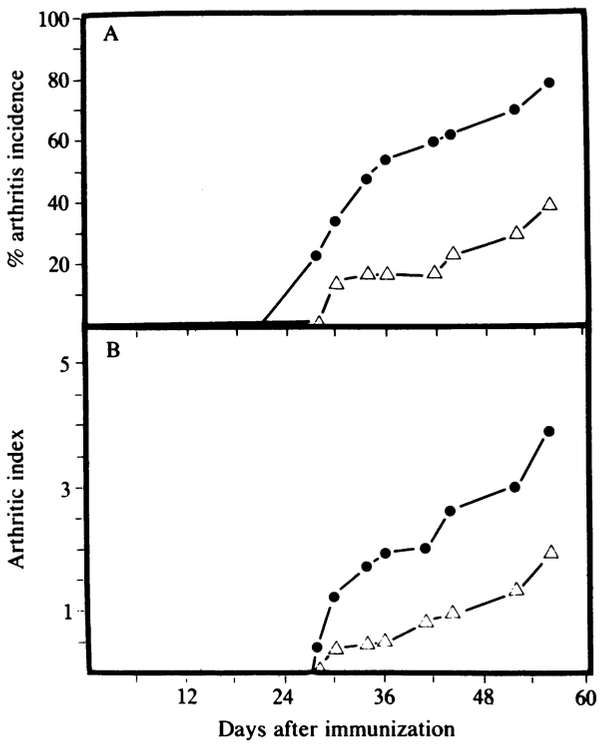


FIG. 1. (*A*) Incidence of arthritis after intragastric administration of 500 μg of type II collagen in 0.5 ml of 0.01 M acetic acid 12 times during 6 weeks (summary of six experiments). △, type II collagen ($n$ = 32); ●, controls ($n$ = 51). (*B*) Clinical severity of arthritis. △, type II collagen ($n$ = 32); ●, controls ($n$ = 51).

significantly delayed compared to controls (day 41 ± 2). The clinical severity of the arthritis in these animals also did not differ significantly from that in the arthritic controls (mean MAI ± SEM on day 58 of type II collagen fed animals, 5.6 ± 0.76; mean MAI controls ± SEM, 4.8 ± 0.36; *P*, 0.34).

When both the interval and the number of feedings were decreased, it was found that eight intragastric administrations of type II collagen given over a 2-week period were sufficient to reduce the incidence of CIA significantly (Fig. 2*A*). Administration of denatured type II collagen, however, had no effect on the incidence (Fig. 2*B*) or the severity of the arthritis (MAI for the group fed denatured type II collagen, 3.4; MAI controls, 3.3). When each intragastric administration consisted of 3 mg, rather than 0.5 mg, of type II collagen no suppression of CIA was observed (Fig. 2*C*). The clinical severity of the arthritis observed in these animals was slightly higher (MAI, 4.4) than that observed in the corresponding controls (MAI, 3.7).

**Serum Antibody Levels in Type II Collagen-Fed and Control Mice.** Table 1 shows the distribution of immunoglobulin isotypes in the anti-collagen response in sera taken during the onset of disease on day 35 after primary immunization. The results are presented separately for animals with and without arthritis. The total antibody levels given are the sums of those for the individual isotypes. None of the isotypes showed statistically significant differences between arthritic and nonarthritic mice in the control group but IgG2a and IgG2b tended to be higher in the arthritic mice. IgG2b levels in type II collagen-fed mice were significantly lower than those in arthritic control mice, while IgG2a levels were also reduced, but not significantly different from those in control mice. The IgG1, IgG3, and IgA responses were lower but did not differ significantly in fed and control mice.

Since IgG2a and IgG2b were most prominent in the anti-collagen response, it was of interest to determine the

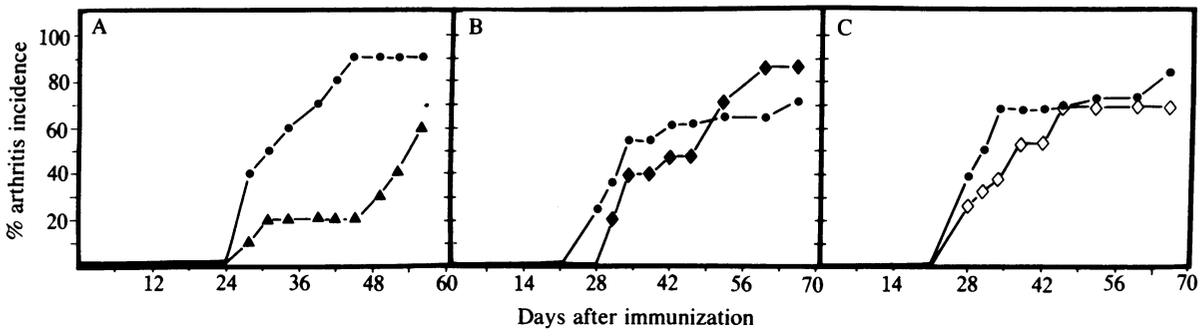


FIG. 2. Incidence of arthritis after intragastric administration of type II collagen. (*A*) type II collagen (500 μg) in 0.5 ml of 0.01 M acetic acid eight times during 2 weeks (▲, $n = 10$). Controls 0.5 ml of 0.01 M acetic acid (●; $n = 10$). (*B*) Denatured type II collagen (500 μg) in 0.5 ml of 0.01 M acetic acid 12 times during 6 weeks (◆, $n = 15$). Controls (as in *A*) (●, $n = 28$). (*C*) type II collagen (3 mg) in 1 ml of 0.01 M acetic acid 12 times during 6 weeks (◇, $n = 19$). Controls had 1 ml of acetic acid (●, $n = 18$). Mice were immunized with type II collagen in complete Freund's adjuvant 3 days after the last dose of type II collagen.

level of these antibodies during the afferent phase of disease induction prior to onset of arthritis, as well as later in the course of the disease (Table 2). The IgG2a and IgG2b levels measured on day 20 after immunization were similar in all of the mice. In spite of the resistance to disease induction in type II collagen-fed mice, their IgG2a antibody levels were not significantly lower than those of control mice. However, the tendency to reduced IgG2a and IgG2b antibody levels observed during the onset of the disease (day 35) persisted, even in sera taken on day 60 after immunization. Measurement of total serum anti-collagen antibodies by RIA gave similar results. In a group of control ($n = 4$) and type II collagen-fed ($n = 7$) mice, anti-collagen levels on day 20 were 78 ± 21 and 99 ± 35 μg/ml (mean ± SEM), respectively. Sera from arthritic control mice ($n = 9$) taken on day 60 measured 244 ± 46 μg/ml, while nonarthritic type II collagen-fed mice measured 156 ± 19 μg/ml (mean ± SEM; $n = 10$; $P = 0.17$).

## DISCUSSION

It has been postulated that immunologic unresponsiveness is induced after intragastric administration of antigen by the separation in the gut of tolerogenic, monomeric forms of antigen from immunogenic large molecular weight aggregates (25). After intragastric administration of soluble bovine serum albumin (25) or ovalbumin (26), only monomeric antigen could be detected in the sera of fed animals. Very small quantities of intact native proteins or small fragments bearing antigenic determinants of the native protein appear to be absorbed. In the present experiments, intragastric administration of undenatured, but not denatured, type II collagen leads to suppression of CIA. This is consistent with evidence that the immune response to type II collagen in mice is directed against the undenatured helical antigenic determinants (4, 27) and that only immunization with undenatured type II collagen readily induces arthritis (23, 27).

Eight feedings of soluble type II collagen over a 2-week period are as effective in suppressing CIA as 12 feedings over 6 weeks. In contrast, administration of large doses of type II collagen in each feeding does not result in suppression of CIA. Results from other investigators (reviewed in ref. 15) also suggest that the induction of unresponsiveness by the oral route is strikingly dose dependent. Continued feeding reduces the absorption of antigen, probably as a result of local immunity (15). It is possible that the high dose of type II collagen used here may have immunized the recipient, although no anti-collagen antibodies, even of the IgA isotype, have been detected in sera taken after feeding. It is of interest that the severity of the disease observed in the fed animals that do become arthritic does not differ from that in controls.

It has been reported that feeding of antigen subsequent to parenteral immunization either has no suppressive effect or boosts antibody production (16). Others suggest, however, that continued feeding of small doses of antigen may lead to systemic unresponsiveness in spite of initial priming (15). Repeated oral administration of ovalbumin can prevent a secondary antibody response in primed mice (28). In the present studies (data not shown) eight intragastric administrations of type II collagen given between days 10 and 29 after immunization with type II collagen in complete Freund's adjuvant did not result in decreased incidence or severity of CIA.

It has been suggested that CIA is an autoimmune disease initiated by the binding of antibody to autologous type II collagen in the joint (29). Complement is required for the development of CIA (30). Onset of disease in susceptible mouse strains is associated with a predominance of IgG2a anti-collagen whereas resistant strains mount a relatively deficient IgG2a response (22). This is in agreement with the fact that IgG2 is the most efficient member of the mouse IgG class in the fixation of complement by the classical pathway (31). The IgG2 anti-collagen response is, therefore, of par-

Table 1. Effect of type II collagen feeding on isotype distribution of serum anti-collagen on day 35 after immunization

| Pretreatment | Arthritis | No. of mice | Anti-collagen isotype, μg/ml | | | | | Total immunoglobulin, μg/ml |
|---|---|---|---|---|---|---|---|---|
| | | | IgG1 | IgG2a | IgG2b | IgG3 | IgA | |
| Acetic acid-fed control | + | 6 | 52 ± 14 | 437 ± 108 | 107 ± 17 | 10 ± 3 | 48 ± 14 | 653 ± 132 |
| | − | 4 | 49 ± 21 | 169 ± 31 | 42 ± 11 | 6 ± 3 | 25 ± 7 | 291 ± 53 |
| | Total | 10 | 51 ± 11* | 330 ± 77† | 81 ± 15‡ | 8 ± 2§ | 39 ± 9¶ | 508 ± 98‖ |
| Collagen fed | + | 2 | 47 ± 7 | 122 ± 32 | 30 ± 9 | 4 ± 3 | 16 ± 3 | 227 ± 18 |
| | − | 6 | 34 ± 16 | 136 ± 8 | 37 ± 23 | 5 ± 4 | 16 ± 1 | 219 ± 42 |
| | Total | 8 | 44 ± 7* | 126 ± 24† | 32 ± 9‡ | 4 ± 2§ | 16 ± 2¶ | 221 ± 31‖ |

Results are mean ± SEM. +, Mice with arthritis. −, Mice without arthritis.
*$P$, 0.64; †$P$, 0.09; ‡, 0.03; §$P$, 0.19; ¶$P$, 0.12; ‖$P$, 0.06.

Table 2. IgG2a and IgG2b serum anti-collagen prior to and late in the development of CIA in type II collagen-fed and control mice

| Day | Pretreatment | Arthritis | No. of mice | Anti-collagen isotype, μg/ml IgG2a | IgG2b |
|---|---|---|---|---|---|
| 20 | Acetic acid-fed control | − | 5 | 70 ± 8* | 19 ± 6† |
| | Collagen fed | − | 8 | 98 ± 36* | 16 ± 4† |
| 60 | Acetic acid-fed control | + | 9 | 448 ± 134‡ | 32 ± 7§ |
| | Collagen fed | + | 4 | 387 ± 143 | 59 ± 17 |
| | | − | 9 | 294 ± 166‡ | 12 ± 2§ |

Results are mean ± SEM. +, Mice with arthritis. −, Mice without arthritis.
*$P$, 0.6; †$P$, 0.7; ‡$P$, 0.49; §$P$, 0.06.

ticular importance for the development of CIA. The possibility exists that resistance to induction of CIA observed after intragastric administration of type II collagen results from a decrease in the magnitude of the IgG2 response or a switch in isotype predominance. We have found that while the overall anti-collagen response is slightly, but not significantly lower, IgG2a remains the predominant isotype in both fed and control mice. In addition, although much lower in magnitude than the IgG2a response, the IgG2b anti-collagen response is significantly lower in nonarthritic type II collagen-fed mice than in arthritic control mice.

There are at least two possible mechanisms for the resistance to arthritis induction observed after feeding of soluble type II collagen. Mattingly and Waksman (32) found that feeding sheep erythrocytes for 2 weeks resulted in systemic unresponsiveness. Within 2 days after feeding, suppressor T cells appeared in the Peyer's patches and mesenteric lymph node, but were undetectable in these locations and present in the spleen and thymus after 4 days. Suppressor T cells have also been shown, by adoptive transfer, in the mesenteric lymph node and spleen of animals suppressed by oral administration of soluble proteins or haptens in several other experimental systems (33–38). In the present system collagen-specific suppressor T cells could have suppressed antibody production, particularly of the IgG2 class, or might have prevented the sensitization of T cells directly involved in the initiation and maintenance of arthritis. Alternatively, production of anti-idiotypic autoantibody has been postulated to depress specificially the IgM and IgG immune response after systemic challenge of fed animals with an immunizing dose of antigen (39, 40). Although in the present study the effect of type II collagen feeding on the magnitude of the humoral anti-collagen response is not marked, it is still possible that anti-idiotypic regulation could have suppressed a part of the response critical for the induction of CIA by virtue of its specificity.

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