Suppression of Collagen-Induced Arthritis in DBA/1J Mice by Preimmunization with House Dust Mite Extract

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Collagen-induced arthritis (CIA) can be induced in DBA/1J mice by immunization with bovine type II collagen (bCII) and is a model of some types of human autoimmune rheumatoid arthritis. In this study we examined whether preimmunization of the mice with various antigens could inhibit the development of CIA. Preimmunization of the mice with an extract of the house dust mite Dermatophagoides farinae (mite antigen), chicken ovalbumin, or keyhole limpet hemocyanin strongly inhibited CIA development, but hen egg lysozyme, β-lactoglobulin from bovine milk or myelin basic protein from guinea pig brain did not substantially affect CIA development. Splenic T cells and serum antibodies specific for mite antigen did not cross-react with bCII. Preimmunization of the mice with mite antigen did not affect the IFN-γ and proliferative response of splenic T cells to bCII, nor serum antibody responses. The most inhibitory constituent had a molecular weight between 1,000 and 10,000.

Key words: house dust mite; collagen-induced arthritis; suppression

A large number of people suffer from rheumatoid arthritis. Although many drugs that can inhibit the symptoms of rheumatoid arthritis are known, effective methods for its prevention and a curative treatment have not been established yet. Collagen-induced arthritis (CIA) is a model of some types of human autoimmune rheumatoid arthritis. CIA can be induced in genetically susceptible primates, rats, and mice such as DBA/1 mice by immunization with heterologous type II collagen (CII) including bovine CII (bCII).1-3 An antibody response and a T cell response specific for CII have been detected in mice suffering from CIA.4,5

In this report, we examined whether the immune response to antigens other than CII could protect DBA/1 mice from CIA in a bystander manner. In spite of the fact that immune responses to various antigens should occur simultaneously in the body of a single animal, the effects of the response to one antigen on the response to another antigen are not well understood. Even if two antigens are recognized by different repertoires of T cells and antibodies without cross-reactivity, there is still the possibility of a bystander effect. As to the mechanisms responsible for the bystander effect of the immune response, regulatory functions of cytokines and lymphocytes are thought to be involved. IL-2 and IFN-γ released from Th1 type T cells in response to an antigen can downregulate the response of Th2 type T cells, while IFN-γ released from Th1 cells can upregulate the Th1 cell response. On the other hand, IL-4 and IL-10 released from Th2 cells can downregulate the Th1 cell response, while IL-4 released from Th2 cells can upregulate the Th2 cell response.6,7 Falcone et al. reported that immunization with keyhole limpet hemocyanin (KLH) can suppress Th1-driven experimental autoimmune encephalomyelitis (EAE) due to an autoantigen-specific response to the predominant Th2 type response.8 When lymphocytes in the immune network are stimulated as a result of the immune response to an antigen, the response to a different antigen may be regulated. In vivo stimulation of T cells expressing the BV828A1 T cell receptor by a superantigen has been shown to inhibit CIA development through downregulation of autoimmune pathogenic T cells by regulatory cells.9

In this study, first we examined whether preimmunization with antigens different from CII could inhibit CIA development. The results showed that some of the tested antigens inhibited CIA development and that, among those antigens, an extract from house dust mite had the strongest inhibitory activity. Mite antigen inhibited CIA development in a dose-dependent manner, although the T cell and antibody responses to CII were not downregulated.

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Abbreviations: bCII, bovine type II collagen; CIA, collagen-induced arthritis; CFA, complete Freund’s adjuvant; EAE, experimental autoimmune encephalomyelitis; IFA, incomplete Freund’s adjuvant; KLH, keyhole limpet hemocyanin; β-Lg, β-lactoglobulin; MBP, myelin basic protein; PBS, phosphate-buffered saline; OVA, ovalbumin; S. l., stimulation index
Materials and Methods

Animals. DBA/1J mice were purchased from Charles River Japan (Yokohama, Japan) and Jackson Laboratories (Bar Harbor, ME). All experiments were done in accordance with the guidelines for the care and use of laboratory animals of The University of Tokyo.

Antigen preparations. bCII was purified by the salting-out method after digestion of bovine joint cartilage with pepsin. Mite extract-Df, the PBS (phosphate buffered saline)-soluble fraction of an extract of *Dermatophagoides farinae*, was purchased from LSL (Tokyo, Japan) and used as mite antigen. The mite antigen was dialyzed against water, using a molecular sieving membrane (molecular weight cutoff; 1,000) (Spectro/Pol 7, Spectrum Laboratories, Rancho Dominguez, CA), and lyophilized before use. We separated the mite antigen extract into three fractions on the basis of molecular weight by FPLC on a Sepharose 12 column (Amersham Pharmacia Biotech, Uppsala, Sweden): 1) less than 10,000, 2) 10,000 to 100,000, and 3) more than 100,000. Each fraction was desalted by dialysis and lyophilized. Chicken lysozyme (HEL; hen eggwhite lysozyme) and chicken ovalbumin (OVA) were purchased from Seikagaku-Kogyo (Tokyo, Japan), and myelin basic protein of guinea pig brain (MBP) was obtained from Sigma (St. Louis, MO). β-Lactoglobulin (β-Lg) was obtained through fractionation of raw skim milk from a Holstein cow of the genotype AA by the method of Aschaffenburg et al. and purified by DEAE-Sephacel ion-exchange chromatography as described in our previous report. Preimmunization with antigens. DBA/1J mice were preimmunized by injection of 100 μl of an emulsion intradermally at the base of the tail. The emulsion was composed of the antigen dissolved in 10 mm acetic acid or PBS and complete Freund's adjuvant (CFA) (Difco, Detroit, MI) or incomplete Freund's adjuvant (IFA) (Difco). As a control group, mice were preimmunized with an emulsion made with a solution that did not contain the antigen. As another control group, some mice were not preimmunized.

Induction of CIA. Twenty-one days after the first immunization, DBA/1J mice were injected intradermally at the base of the tail with 100 μl of an emulsion containing 200 μg of bCII dissolved in 10 mm acetic acid or 10 mm citric acid solution plus IFA. The clinical severity of arthritis was assessed by scoring arthritis for each limb on a scale of 0 to 3: 0 for absence of arthritis, 1 for one finger swelling or mild swelling, 2 for two fingers swelling or swelling of tarsus and ankle, 3 for hard swelling or bony-deformity. We calculated the total scores for four paws of all mice in a group and obtained the arthritic index by dividing this total by the number of mice in the group. The cumulative value for all paws of a mouse was analyzed with the Mann-Whitney U test for each group, while the number of arthritogenic mice and legs was analyzed with Fisher's exact test.

ELISA for detecting antibody specific for bCII and mite antigen. The relative amount of anti-bCII antibody in serum was measured by ELISA as described in our previous paper. Antibody specific for mite antigen was also assayed by a method similar to that for anti-bCII antibody. Specific antibody was detected upon binding to immobilized antigen using alkaline phosphatase-labeled anti-immunoglobulin isotype antibody and its substrate. Standard titration curves were prepared based on the absorbance observed in analysis of serially diluted standard serum. The ELISA value for relative antibody amounts specific for bCII was analyzed with the Mann-Whitney U test for mite-administered groups by comparing with the PBS-IFA administered group.

T cell proliferation assay of lymph node cells and splenocytes. DBA/1J mice were preimmunized by injection of mite antigen dissolved in PBS and emulsified with IFA at a dose of 0, 20 or 200 μg/head. Twenty-one days later they were immunized with bCII in IFA at a dose of 100 μg/head. Fourteen days after injection of CII, splenocytes were obtained and cultured with bCII or mite antigen in X-vivo medium (BioWhittaker, Walkersville, MD). Seventy-two hrs later, 1 μCi of [3H]-thymidine was added to the cultures and incubated overnight. The incorporation of thymidine into the cells was measured by solid or liquid scintillating counting.

ELISA to detect IFN-γ. IFN-γ in the supernatant of splenocyte cultures mentioned above was detected by the method described in our previous paper with minor modifications. Anti-IFN-γ antibody (R4-6A2) (PharMingen, San Diego, CA) dissolved in 0.1 M phosphate buffer (pH 9.0) was incubated in Nunc Maxisorp plates overnight at 4°C. After washing and blocking, the samples were incubated in the wells overnight at 4°C. Biotin-labeled anti-mouse IFN-γ antibody (XMG1.2) (PharMingen) was incubated for 1 hr to bind captured cytokine. Binding of this secondary antibody was detected by incubating 100 μl per well of a 1/2000 dilution of horseradish peroxidase-conjugated avidin D (Vector, Burlingame, CA) in PBS containing 3% bovine serum albumin for 30 min. The plates were washed 8 times and then 100 μl of tetramethylbenzidine substrate solution containing 0.1% hydrogen peroxide was added. To stop the reaction, 100 μl of 2 M sulfuric acid was added. The absorbance was read at 450 nm.
Results

Inhibition of CIA by preimmunization with antigens

We preimmunized DBA/1J mice with 6 kinds of antigens before the induction of CIA and examined whether the antigens inhibited CIA development. Four to 8 DBA/1J mice in each group were injected with a dose of 200 μg/head of HEL, MBP, KLH, β-Lg, OVA or mite antigen in 10 mM acetic acid plus CFA, or a mixture of 10 mM acetic acid solution and CFA, or not treated. Twenty-one days later, bCII was injected in each case to induce CIA. The mice were monitored to assess intensity of CIA after immunization with bCII (day 0). Statistical analysis for arthritic index with the Mann-Whitney U test and for arthritic mice and legs with the Fisher’s exact test indicated that injection of acetic acid and CFA before induction of CIA exaggerated the symptoms and incidence of CIA, compared with the group of mice that received no preimmunization (points with a significant difference are not shown in Fig. 1). Therefore, the effect of preimmunization with antigens was mainly compared with the mice that received no preimmunization. Data points with a significant difference compared with the mice that received no preimmunization are shown in Fig. 1. Data points with a significant difference compared with the mice preinjected with acetic acid plus CFA are not shown, although many points for mice preinjected with various antigens are significantly different from those for the acetic acid-administered mice. The Fisher’s exact test for the number of arthritic mice and legs revealed that preimmunization with KLH, OVA or mite antigen, in acetic acid solution plus CFA inhibited the development of CIA, compared with both 1) the mice that were not preimmunized and 2) the mice that were injected with acetic acid and CFA before induction of CIA. The incidence of CIA in the mice preimmunized with HEL, β-Lg or MBP was lower than that in the mice that were injected with acetic acid and CFA prior to induction of CIA (significantly different at $P<0.05$, by Fisher’s exact test). However, compared with the mice that were not pretreated, the inhibition was not significant.

Effects of preimmunization of the mice with various doses of mite antigen on CIA and the antibody response.

We examined the inhibitory effect of various doses of mite antigen on the development of CIA. Ten to 12 mice in each group were injected with a dose of 0, 2, 20 or 200 μg/head of mite antigen in PBS plus IFA. The results of CIA are shown in Fig. 2. Statistical analysis indicated that preimmunization with mite antigen at 20 or 200 μg/head but not 2 μg/head decreased the number of arthritic legs, compared with the group pretreated by injection of a mixture of PBS and IFA. Although the significance in differences was below the statistical level, inhibition was observed by arthritic index for the groups of 20 and 200 μg mite antigen and arthritic mice for 200 μg mite.

Fig. 1. Clinical Evaluation of CIA in DBA/1J Mice Preimmunized with Various Antigens.

DBA/1J mice were preimmunized with mite antigen (○), KLH (●), OVA (△), β-Lg (●) MBP (●) or HEL (△) dissolved in 10 mM acetic acid plus CFA, or not preimmunized (▲). Mice were also injected with 10 mM acetic acid plus CFA (△). CIA was induced 21 days later (day 0) by injecting mice with bCII plus IFA. The arthritic index is expressed as the cumulative value for all paws of a mouse. The maximum score per mouse was 12. The arthritic index was statistically analyzed by the Mann-Whitney U test, while the number of arthritic legs and arthritic mice was done by the Fisher’s exact test. Each group had 4 to 8 mice. "*" and "**" indicate significant increase and decrease, respectively, in the arthritic index at $P<0.05$, compared with the group that did not receive preimmunization before induction of CIA. "***" and "****" indicate significant increase in the number of arthritic legs at $P<0.05$ and $P<0.01$, respectively, compared with the group that did not receive preimmunization. "#" indicates a significant decrease in the number of arthritic legs at $P<0.05$, compared with the group that did not receive preimmunization.
Fig. 2. Clinical Evaluation of CIA in DBA/1J Mice Preimmunized with Various Doses of Mite Antigen.

DBA/1J mice were preimmunized with 0 μg (△; control), 2 μg (○), 20 μg (●) or 200 μg (○) of mite antigen in PBS plus IFA. CIA was induced 21 days later (day 0). The number of tested mice in each group was 10. * indicates a significant difference at P < 0.05 by Fisher’s exact test for the number of arthritic legs, compared with the control group.

Fig. 3. Relative Amounts of Antibodies Specific for Mite Antigen and bCII in the Sera of Mice Preimmunized with the Mite Antigen before the Induction of CIA.

Amounts of anti-mite antibody and anti-bCII antibody in the sera of mice immunized with the mite antigen and bCII were measured by ELISA. DBA/1J mice were preimmunized with 0 μg (△; control), 2 μg (○), 20 μg (●) or 200 μg (○) of mite antigen in PBS plus IFA and immunized with bCII 21 days later (day 0). The mice in each group are those in Fig. 2. Averages and standard deviations of the amount of specific antibody for individual mice per each group were plotted. Detection limit for anti-mite IgG1, and IgG2a antibodies and anti-bCII IgG1 and IgG2a antibodies were all 13.717. Points for the relative amount below this detection limit were plotted as zero.

Proliferative response and IFN-γ response of splenocytes from DBA/1J mice immunized with mite antigen plus IFA

We examined the effects of preimmunization with mite antigen on the proliferation and cytokine response of T cells. DBA/1J mice were preimmunized by injection of a dose of 0, 20 or 200 μg/ head of mite antigen plus IFA. Twenty-one days later they were administered a dose of 200 μg/head of bCII plus IFA. Splenocytes were isolated and examined in a proliferation assay. The results are presented in Fig. 4. The proliferative response to mite antigen observed was dependent on the amount of mite antigen used for preimmunization. The group of mice administered a mixture of PBS and IFA without mite antigen (0 μg/head mite antigen plus IFA) also showed a weak response to mite antigen. This response probably resulted from B cell mitogenic activity of the mite antigen. A proliferative response to bCII was seen in the case of all 3 groups, and there was no significant difference among the groups (Fig. 4). As shown in Fig. 5, the secretion of IFN-γ from the splenocytes was measured. Also, an IFN-γ response to bCII was observed in the case of splenocytes from all 3 groups, and there was no significant difference among the groups (Fig. 5). These data indicate that the inhibition of CIA development was not accompanied by a lowered response of T cells to bCII.
Suppression of CIA by House Dust Mite Extract

**Fig. 4.** Proliferative Response of Splenocytes to bCII and Mite Antigen

Three mice per group were preimmunized with 0 μg (□ and ■; control), 20 μg (△ and ●) or 200 μg (○ and •) of mite antigen in PBS plus IFA and 21 days later, immunized with bCII. Fourteen days later, splenocytes were isolated and tested for [³H]-thymidine incorporation. The proliferative response is expressed as stimulation index (S. L.), where the cpm obtained for a given culture in the presence of various concentrations of bCII (closed symbols) or mite antigen (open symbols) was divided by the cpm obtained in the absence of antigen. The amount of radioactivity incorporated in the absence of antigen was 1223.2 cpm for splenocytes from mice immunized with 0 μg of mite antigen, 1033.1 cpm for those immunized with 20 μg and 1475.5 cpm for 200 μg. The similar results were obtained by repeated experiments.

**Fig. 5.** Cytokine Secretion Responses to bCII and Mite Antigen

Cytokine secretion in response to various concentrations of bCII and mite antigen during a 72-hr culture period was measured. Splenocytes were obtained from mice preimmunized with 0 μg (□; control), 20 μg (△ and ●) or 200 μg (○ and •) of mite antigen in PBS plus IFA and immunized with bCII on day 21. Splenocytes were cultured in the absence of antigen (closed symbols), or the presence of bCII (closed symbols) or mite antigen (open symbols).

*The active mite antigen fraction*

Mite antigen was separated into three fractions by gel permeation chromatography. CIA was induced in four mice of each group after preimmunization of mice with the individual fractions or the unfractionated mite antigen extract. CIA of mice injected with mite antigen or its fractions was compared with mice immunized with PBS plus IFA. As shown in Fig. 6, the unfractionated mite antigen extract decreased the arthritic legs after day 40, similarly to the previous data in Figs. 1 and 2, and also decreased the arthritic index on only days 43 and 50. The fraction with constituents having a molecular weight smaller than 10,000 was the most effective in inhibition of CIA development. Inhibition by this fraction was observed by all three CIA parameters of the arthritic index, arthritic mice and arthritic legs with statistical significances on almost all days after the control group developed CIA. The fraction with constituents having a molecular weight larger than 100,000 also inhibited CIA development slightly, shown by arthritic index on day 50 and arthritic index on day 40 and all days later. On the other hand, the fraction with constituents having a molecular weight between 10,000 and 900,000 did not inhibit the development of CIA (Fig. 6)

**Discussion**

In this study, we found that preimmunization with particular antigens could inhibit CIA development strongly, whereas other antigens were not substantially effective. We tested an extract of the house dust mite (mite antigen) and five purified proteins, OVA, KLH, HEL, β-Lg, and MBP, for preimmunization. These antigens were selected as nominal antigens having no relationship with the immune responses involved in the development of CIA. Mite antigen, OVA and β-Lg are known as allergens involved in induction of human allergies to the house dust mite, eggs, and milk, respectively. Since these allergies are mediated by Th2 responses, we could expect that these antigens might induce a preferential Th2 response. The Th2 cytokine IL-10 is known to be effective to inhibit the development of CIA. In the early phase of CIA development a predominant Th1 response to CII is observed. A shift to a Th2 response from a Th1 response may be effective in inhibiting CIA development. It was reported that induction of Th2 response to allergens and other proteins can shift response to another antigen into Th2 response. Comoy et al. showed that immunization with Der p 1, one of the major antigens of the house dust mite Dermatophagoides pteronyssinus, shifted the response to S-transferase of the parasite Schistosoma mansoni into a Th2 type response in BALB/c mice. MBP is known to induce EAE in susceptible strains of mice by inducing a Th1 type autoimmune response. Falcone et al. reported inhibition of EAE in a bystander manner by preimmunization with KLH through a shift of a Th1 response to MBP into a Th2 response. KLH may inhibit both EAE and CIA by a bystander shift of a pathogenic Th1 response to autoantigens into a Th2 response. Among the tested antigens, mite antigen, OVA, and
KLH were highly effective to inhibit CIA development, while HEL, β-Lg and MBP were not effective. Inhibition of CIA development by OVA and KLH may result from a shift from a Th1 response to a Th2 response.

We further studied the inhibition of CIA development by mite antigen, since this antigen showed the strongest inhibitory effect. By ELISA, an antibody response to mite antigen was not detected in the mice immunized with bCII but not preimmunized with mite antigen. These data suggest that there is no cross-reactivity between bCII and mite antigen at the B cell level. Assays of T cell proliferation and IFN-γ secretion showed that the response to bCII was independent of that to mite antigen, suggesting that there is no cross-reactivity between bCII and mite antigen at the T cell level. No cross-reaction between mite antigen and bCII suggest that if an immunological mechanism was responsible for inhibition of CIA development, response to mite antigen inhibited arthritogenic response by some bystander regulatory mechanism.

The inhibition of CIA development by mite antigen did not decrease the antibody response, the T cell proliferative response nor the IFN-γ response to bCII. Thus, the downregulation of the B cell and T cell responses to bCII is not involved in the inhibition of development of CIA, although the responses to a limited number of antigenic determinants in bCII may have been inhibited as a result of preimmunization of the mice with mite antigen. We need to further study the immune response to each determinant in bCII in mite antigen-treated and non-treated mice. No change in the IFN-γ and IgG2a response to bCII suggest that a Th1/Th2 shift in response to whole bCII molecules did not occur in mice preimmunized with mite antigen.

It is possible that CIA development was inhibited by the regulatory mechanism which involved active downregulation of arthritogenic T cells and B cells by regulatory T cells. Immune response to mite antigen may have initiated activation of such regulatory T cells. Kumar et al. reported that regulatory T cells specific for the B5 peptide derived from T cells receptor BV8S2A1 could protect B10.PL and (SJL × B10.PL)F1 mice from EAE or DBA/1LacJ mice from CIA.9,20 In preliminary experiments we have found that splenocytes from DBA/1 mice immunized with mite antigen proliferate in response to the B5 peptide, in a manner dependent on the amount of mite antigen used for immunization. We would like to further study the novel inhibition pattern in which the response to some non-self antigen such as mite antigen activates the anti-B5 response, resulting in suppression of autoimmune responses responsible for the induction of CIA. On the other hand, it has been reported that CIA development can be inhibited by treatments resulting in both downregulation of co-stimulation for T cell activation and inhibition of inflammation.25–28 Therefore, the inhibition of CIA development by mite antigen may be driven by an antigen-non-specific immunological mechanism or a non-immunological, anti-inflammatory mechanism.

The mite antigen used in this study, an extract from the whole mite body, is considered to be a mixture of proteins and some other molecules with molecular weights greater than 1,000. We have found that the inhibitory constituent has a molecular weight between 1,000 and 10,000. However, some other constituents with molecular weights greater than 100,000 also showed a weak inhibitory effect. Der f 1, 2, 3, 5,
6, 7, 10 and 11 antigens from *Dermatophagoides farinae* have been identified: Der f 1, 3 and 6 are known to be proteases. There is a report indicating that Der p 1, homologous to Der f 1, can modify the immune response to S-transferase of the parasite *Schistosoma mansoni* in BALB/c mice, causing a shift from a Th1 type response to a Th2 type response as previously mentioned. However, we found that purified Der f 1, with a molecular weight of 25,000, was not effective to inhibit CIA development (data not shown). Therefore, even if a Th2 type response to Der f 1 was induced, it is evident that this response is not related to inhibition of CIA development. All identified Der f antigens have molecular weights between 10,000 and 100,000. It has not been shown that mite component(s) causes an anti-inflammatory effect. Thus, further study of the constituents of the mite extract may lead to the discovery of a novel molecule effective in inhibiting CIA development.

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