8-P28

INTRANASAL ADMINISTRATION OF DENATURED TYPE II COLLAGEN AND ITS FRAGMENTS CAN SUPPRESS COLLAGEN-INDUCED ARTHRITIS

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Backgrounds and Objectives:

Immunization with type II collagen (CII) induces severe multiple arthritis in genetically susceptible rats, primates, and mice such as DBA/1 [1]. This collagen-induced arthritis (CIA) system has been utilized as an animal model for not only human rheumatoid arthritis (RA) but also general autoimmunity. Among many experimental treatments for autoimmune diseases, oral administration of antigens can be considered as an effective and safe way to diminish autoimmunity [2, 3]. Actually, clinical trials for human autoimmune diseases such as RA, multiple sclerosis and uveitis have been performed by orally administering antigen protein to the patients [4]. However, the protocol of oral administration of intact CII has not been well optimized for all RA patients [5]. Administration via the intranasal mucosal tissue by inhalation of a soluble protein or peptide can induce systemic tolerance more effectively than oral administration in some case [6]. In this study we tried to find more effective protocols for inhibiting CIA on the mouse model by intranasally administering various forms of antigen such as native bovine CII, heat-denatured CII and trypsin-digested CII.

Methods:

Preparation and treatment of CII: CII was extracted from bovine articular cartilage by pepsin digestion, extensively purified by differential salt precipitation [7], and checked for purity by SDS-PAGE (designated as N.CII). D.CII (heat-denatured CII) was obtained by dissolving N.CII in 10 m*M* citric acid and heating for 15 min at 65°C. A.CII (autoclaved CII) was obtained by heating N.CII for 20 min at 121°C. Tryptic CII was obtained by digesting N.CII with TPCK-trypsin (Sigma, St. Louis, MO) for 1 hr at 37°C. Protein structure was analyzed by optical rotatory density (ORD).

Animals: Female DBA/1J mice, 6 to 8 weeks old, were purchased from Charles River Japan (Yokohama, Japan).

Induction of CIA: CII was dissolved in 10 m*M* citric acid at a concentration of 4 mg/ml and emulsified in an equal volume of CFA (Difco Laboratories, Detroit, MI). Mice were immunized i.d. at the base of the tail with 0.1 ml of the emulsion containing 200 μg of CII. On the 21st day after primary immunization, all mice were boosted with 200 μg of CII in IFA (Difco).

Assessment of CIA: The clinical severity of arthritis was assessed by criteria of the arthritic index. Each limb was subjectively graded on a scale of 0 to 3. The arthritic index was expressed as the cumulative value for all paws of a mouse. The severest score was 12.

Intranasal administration regimens: Seven days before primary immunization, DBA/1 mice were intranasally administered 200 μg of various CII preparations in 20 μl of 10 m*M* citric acid under light ether anesthesia.

Determination of anti-CII antibody production by ELISA: Anti-N.CII antibody production was determined by ELISA. At four weeks after the first immunization, sera were collected and analyzed for anti-N.CII specific IgG1, IgG2a, and IgG2b antibody production.

Cell culture and determination of cytokines by ELISA: The lymph node cells (LNC) from DBA/1 mice were cultured with various antigens. The supernatant was collected and the levels of secreted cytokines were assayed by sandwich ELISA method.

Statistical analysis: The statistical analysis was performed using the Mann-Whitney *U*-test for arthritic indexes, onset of CIA and antibody levels, or using the Fisher's Exact Test for values of arthritic mice and legs.

Results and Discussions:

CII preparation: ORD analyses of the CII solution showed that N.CII had a triple helix structure, but that D.CII and A.CII had lost the triple helix structure. SDS-PAGE analyses indicated that N.CII and D.CII were composed of undigested polypeptide chains, but that A.CII and tryptic CII contained fragmented polypeptides.

Effect of intranasal administration of N.CII, D.CII and A.CII on CIA: We determined the effect of intranasal administration of antigen via mucosal surfaces on development of CIA. The results indicated that intranasal administration of D.CII or A.CII decreased the intensity and incidence of CIA and delayed the onset of disease (Table 1). However, administration of N.CII scarcely inhibited CIA. Production of anti-CII IgG2a antibody rather than other subclasses has been considered to be related to arthritis due to its ability to bind with complement and cause inflammation [8]. Anti-N.CII IgG2a antibody levels of the D.CII- and A.CII-administered groups were significantly lowered, but those of anti-N.CII IgG1 antibody were not different from the control group. Levels of anti-N.CII IgG2b antibody of the D.CII- and A.CII-administered groups were also significantly low.

Effect of intranasal administration of the tryptic CII peptides on CIA: We evaluated the suppressive effects of CIA by tryptic digests of CII which contained fragments smaller than A.CII. The results indicated that intranasal administration of tryptic CII peptides inhibited the intensity and incidence of CIA and delayed the onset of disease (Table 2). Anti-N.CII IgG2a antibody levels of the tryptic CII peptides-administered mice were lowered. Anti-N.CII IgG1 antibody levels of the tryptic CII peptides-administered mice also decreased significantly. Anti-N.CII IgG2b antibody levels tended to decrease, although the difference was not significant.

Effect of intranasal administration of CII on antigen-specific cytokine secretion: Some reports have revealed that CIA development in DBA/1 mice is dependent on Th1-type cytokine production, in which IFN-γ production is related to arthritis

severity [9]. Compared with non-treated mice, decrease in IFN-γ secretion in response to stimulation with D.CII or A.CII observed in LNC from the A.CII-administered mice, while slight decrease in LNC from the N.CII-administered mice. No difference among the groups was found for the levels of secretion of various other cytokines such as IL-4, IL-10 and TGF-β.

Our finding of the strong activity of inhibiting CIA by denatured and digested CII can be a basis for developing an antigen-specific therapy for some types of RA that are related to anti-CII immune responses. It is difficult to prepare N.CII at both the laboratory and industry scale, since the triple helix structure is easily lost through careless treatment of the molecules and during procedures such as heating for processing and sterilization. On the other hand, the use of CII peptides possessing inhibitory activity is convenient, since heating and acidification can be applied in sterilization of this protein.

Conclusion:

Our results clearly indicated that intranasal administration of either denatured CII or digested CII inhibited CIA. Associated with this suppression of CIA by denatured CII and digested CII, there was shown to be a down regulations of IgG2a secretion and IFN-γ responses to CII but no IgG1 and IgG2b responses to CII were observed. No increase in Th2-type cytokines (IL-4 and IL-10) was detected. Our results have shown that inhibition of CIA was not caused by a shift of Th1/Th2-type profile.

Pertinent literature:

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Table 1 Effect of intranasal administration of N.CII, D.CII and A.CII on CIA

Group	Arthritic index	Incidence	Arthritic leg	Onset
Control	6.56 ± 2.70	8/9 (89%)	24/36 (67%)	34.1 ± 6.70
N.CII-administered	4.33 ± 2.83	8/9 (89%)	15/36 (42%)	33.2 ± 5.80
D.CII-administered	1.60 ± 2.12**	5/10 (50%)	8/40*** (20%)	41.9 ± 5.11*
A.CII-administered	2.50 ± 2.92**	5/10 (50%)	9/40*** (23%)	48.3 ± 11.1**

Before the induction of CIA, DBA/1 mice were intranasally administered 200 μg of specimen. Values of arthritic index, incidence and arthritic leg are results determined at six weeks after the immunization. Arthritic index and onset are indicated as mean ± SD. Asterisks of *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Table 2 Effect of intranasal administration of tryptic CII peptides on CIA

Group	Arthritic index	Incidence	Arthritic leg	Onset
Control	5.30 ± 2.58	10/10 (100%)	22/40 (55%)	37.6 ± 5.04
Tryptic CII-administered	1.40 ± 2.17**	4/10* (40%)	7/40*** (18%)	48.8 ± 10.2**

See the footnote of table 1.