The Type II Collagen-Specific T Cells Mediate the Onset of Arthritis

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Abstract **Objective** To establish the type II collagen-specific T cell line of Wistar rat and observe its effect on transferring arthritis. **Methods** The Wistar rats were immunized with the emulsified chicken type II collagen (CCII) in complete Freund´s adjuvant by intradermal injection to induce the rat model of collagen-induced arthritis (CIA). The lymphcytes were obtained from the mesenteric lymph nodes of CIA rats, and in the presence of antigen-presenting cells, the type II collagen-reactive T cell line was selected and expanded by CCII stimulating in vitro. The proliferation response and phenotype were analyzed by ^{3}H-TdR incorporation and fluorescence-activated cell sorter(FACS). The onset of arthritis and the pathological characteristic in ankle joints of recipient rats were observed with naked eye and by histochemical examination. Anti-CCII antibody in serum was assayed by enzyme-linked immunosorbent assay (ELISA). **Results** A T cell line was successfully established. The results of FACS analysis with fluorescent labeled antibodies showed that 98.2% of the line cells were T cells, 89.7% of which were CD_4^+ T cells. The results of adoptive transfer showed that the incidence of arthritis was 50% when the injected cell number was 5×10^7, meanwhile the level of anti-CCII antibodies in serum was elevated more than that of the control. **Conclusion** A T cell line has been successfully established. The result of arthritis transferring by T cell line shows that the T cell plays a great role in the pathogenesis of CIA and provides a research datum for rheumatoid arthritis therapy with T cell vaccination.

Key Words Arthritis experimental; Collagen-induced arthritis; Adoptive transfer; T-lymphocyte; Antibody anti-CCII; Collagen type II

Collagen-induced arthritis (CIA) is a polyarthritis in rodents induced by immunization with type II collagen (CII). Because the resulting symptom and joint pathology resemble the rheumatoid arthritis(RA), with synovial proliferation, cell infiltration, cartilage erosion, bone resorption and remodeling, CIA is regarded as a unique animal mode for human rheumatoid arthritis, and is widely applicated in researching the mechanism of RA [1,2]. It has been shown that autoimmune response against CII is involved in the pathogenesis of CIA. The autoreactive T cells mediate the onset of the CIA, but its role is unclear, however, transferring of the disease with T cells and immunohistochemical finding of activated T cell in the synovium of arthritis suggest that the autoreactive T cells are essential for the development of disease. The studies on T cells in experimental autoimmune models, such as in experimental autoimmune encephalomyelitis [2], have mostly involved the establishment of autoreactive T cell lines which have been used to transfer the disease. Thus, in order to address the role of the T cells in CIA , we use a similar approach to establish the T cell line reactive to CII, characterize the phenotype of the cells, and examine the ability of these cells in transferring the disease to naive rat.

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MATERIALS AND METHODS

Rats and Reagents

Inbred female Wistar rats (4~6 weeks of age and weight in 180±10 grams) were obtained from Shanghai Center of Experimental Animals and bred at the Center of Experimental Animals in Shanghai

Second Medical University. Chicken type II collagen (CCII) was the product of Sigma Co. Recombinant human IL-2 was purchased from GIBCO-BRL (invitrogen) and ^{3}H-TdR was purchased from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences.

Immunization

Induction of CIA was performed as described previously [2]. Briefly, CCII was dissolved in 0.01mol/L acetic acid at 5 mg/ml, for 16~24 hours prior to use, and stored at 4 ℃ overnight. The CCII solution was mixed and emulsified with equal volume of complete Freund´s adjuvant (CFA), with 2 mg/ml of the final concentration of BCG. Each rat was injected intradermally with 500 μg of CCII in 0.2 ml, divided by multiple sites at the back and the base of the tail. CFA diluted with equal volume of acetic acid solution was as the control.

Assessment of the arthritis

Rats were assessed 3 times per week for the presence of distal joint swelling and erythema. The rats were graded according to severity of arthritis by the method of Wood et al[3]. Lesions of the distal extremities to the elbow or knee were graded on a subjective scale ranging from 0~4 for each paw, based on the number of joints involved and the degree of erythema and swelling: 0-normal; 1-redness; 2-swelling; 3-digit deformity; 4-ankle deformity, unable to afford the body weight. Arthritis index(AI)=sum of the four paw scores; Mean arthritis index(MAI)=total arthritis scores/total number of rats per group. Same method was used to evaluated the CIA and transferring arthritis.

Establishment of T cell line

T cell lines reactive to CCII were established with a semi-limiting dilution method as described previous [4]. Briefly, 14 days after immunization, mesenteric lymph nodes of rat with early sign of arthritis were excised under the sterilized condition, and a single cell suspension was prepared by being filtered through a nylon mesh. The cells were washed 2 times with serum-free RPMI1640, then a little amount of red blood cells were separated and removed by rat lymphocyte separating solution, with the density of 1.088. The lymphocytes were adjusted to 5×10^{6} cells/ml in RPMI 1640 supplemented with 5% autologous rat serum (inactived at 56 ℃ for 30 min). In 96 well U-bottom microtiter plate, 100 μl of cell suspension was added into each well, and the cells were cultured with CCII (final concentration of 40 μg/ml) at 37℃ in 5% CO_2-air for 3 days, the culture medium was replaced by 100 μl of fresh RPMI 1640 containing IL-2 (20 U/ml) and 5% autologous rat serum, followed by changing medium every 3 days, lasting 2 weeks. All culture medium were disposed, and 150 μl of RPMI1640 without IL-2 were added. The suspended cells were splited equally into 3 aliquots and were stimulated with CCII cultured with 30Gy ^{60}Co-irradiated syngeneic spleen cells (2×10^{6} cells) as antigen-presenting accessory cells. In these 3 wells, the well not added CCII was as the negative control, the others were added CCII as the experimental group and backup group, with the final volume of 200μl per well. The plates were cultured at 37 ℃ in 5% CO_2-air for 72 hours. 12 hours prior to ending the culture, 1.85×10^{4} Bq [^{3}H]-TdR was pulsed. The cells of experimental wells and control wells were harvested and the value of cpm was measured in a β-liquid scintillation counter, and the stimulation index (SI, experimental/control) was calculated. The experimental wells with SI more than 3 were selected and the parallel backup wells were seeded into the new wells. IL-2 contained in RPMI1640 was added to stimulate and expand the cells, and the medium was semi-replaced every 3 days until 2 weeks. Then, the medium was totally disposed, the cells were suspended in RPMI1640 without IL-2, and 3 aliquots were made. The above procedures were repeated, and after 3 cycle of stimulation, the CCII-specific T cell lines were generated.

Proliferation assay

Line cells were incubated in microtiter plate in triplicate wells. Each well contained 5×10^{5} line cells and 1×10^{6} accessory cells (30Gy-irradiated) in medium with antigen CCII at the optimal concentration. After incubation for 72h, each well was plused with 1.85×10^{4} Bq [^{3}H]-thymidine at the last 12 h. The culture cells were then harvested and the incorporation of thymidine was measured in a liquid scintillation counter. The result of proliferative response was expressed as the mean cpm.

Phenotyping of the T cell line

3 days after antigen stimulation in vitro, the line cells (10^{6}/sample) were washed with 1% BSA 0.1% NaN_3 PBS 3 times, and incubated with 10 μl FITC-anti-rat CD3, 10μl PE-anti-rat CD4, 5μl

Cy5-anti-rat CD8 for 30 min at 4℃. After washed 3 times with 1% BSA 0.1% NaN_3 PBS, the cells were resuspended in PBS (PH 7.2). Analysis of phenotypes of T cell line was performed with a FACScan (Becton Dickinson).

Adoptive transfer of T cell line and identification of the arthritis

After antigen stimulation and proliferation in vitro, the T cell line was harvested and washed 3 times. The line cells were adjusted to various concentration and injected into the tail vein in naive Wistar rats. The recipients were examined daily for the development of arthritis. At the 15th days after transferring, the rats were sacrificed by cervical dislocation, and the diseased paws were immersed in 10% formaldehyde. After decalcification, they were embedding in paraffin wax, and were sectioned. Then, they were stained with hematoxylin and eosin and the pathological changes of synovial membrane, cartilage and bone were observed under the light microscope.

Determining the level of serum anti-collagen antibody

The sera were obtained on various days from rats injected with line cells or naive age-matched animals. Serum samples were stored at -20℃ and were heat-inactivated before ELISA testing. Briefly, 96-well flat-bottom microtiter plates were coated with 100 μl/well (25 g/ml) at 37 ℃ for 1 h and washed 3 times with PBS containing 0.05% Tween-20. The wells were then blocked by incubation with 200 μl of PBS containing 1% BSA at 37 ℃ for 1 h. After washing 3 times, the plates were incubated with 100 μl of 1:500 diluted rat sera at 37 ℃ for 1 h. The plates were washed 3 times, and 100 μl/well of a 1: 2500 dilution of sheep anti-rat IgG labeled with horseradish peroxidase was added and incubated at 37℃ for 1 h. After washing, 100μl of peroxidase substrate (o-phenylenediamine, OPD) was added per well and the plates were incubated in the dark at room temperature for 30 min. The absorbence(A value) was measured at 490 nm in ELISA reader (modle 550; Bio-Rad).

Statistical analysis

Data were expressed as $\bar{x}\pm s$. The significance of difference was performed by one-way ANOVA and Duncan´s test.

RESULTS

The incidence and the severity of CIA.

12~45 days after immunization, 54 rats developed arthritis in 60 cases of experimental group (CCII+CFA). The incidence of CIA was 90%; while no rat developed arthritis in 40 rats of control group. The ankles and the digital joints were the more easily involved, and the hind paws were the firstly involved. At the 27th day after immunization, the mean arthritic index of the diseased rats was the highest, and the MAI was 8.1.

Proliferation response of T cell line to CCII.

The cell line (5×10^5/well cells) was proliferated with stimulation of various concentration of CCII. The maximal response was obtained at the concentration of 40μg/ml of CCII, and culture for 3 days was required for the maximal response. Thus, the 5×10^5/well line cells were cultured with 40 μg/ml of CCII for 3 days throughout the experiment.

Establishment and analysis of T cell line.

Long term antigen specificity of T cell line was examined. As shown in the table 1, the proliferation response of line cells reactive to CCII increased significantly and the result reactive to PPD was in a decreased manner, after stimulation in vitro with antigen CCII and IL-2 for 2 cycles. After 3 cycles, the line cells showed the CCII-specific proliferation response and approached to the posi-

Table 1 Proliferation response of CCII-specific T cells ($\bar{x}\pm s$)

Stimulation cycle	Number of cells/well	Proliferation response to(cpm)			
	10^4	Medium	CCⅡ	PPD	Con A
0	50	3584±422	6466±629	11477±1031	18866±2126
1	50	1998±216	8684±981*	6445±829	16556±1763
2	50	1240±157	9986±965*	1876±194	13364±1512
3	50	887±93	11145±1057*	762±96	11676±1275

note: * compare with the prior one $P<0.05$

tive control (stimulated with mitogen Con A), and while the proliferation incubated with PPD showed a negative response.

Phenotype of the cell line.

The results of FACS analysis with fluorescent labeled antibody showed that, after 3 cycle of stimulation, 98.2% of line cells were T cells and 89.7% of them were $CD4^+$ T cells.

Induction of arthritis by adoptive transfer of the cell line.

The T cell line was inoculated i.v. into naive Wistar recipient rats at the concentration of 10^5~10^7 cells/rat (Table 2). 9 out of 60 inoculated rats developed the clinical arthritis (Fig.1). The onset of arthritis was about 7~9 days after injection of T cell line, and the clinical expression continued for about 15 days and then subside gradually. Histologically, infiltration of inflammatory cells and hyperplasia of synovial membrane were accompanied by destruction of the joint cartilage and bone(Fig.2).

The level of serum anti-collagen antibody.

As shown in the table 2, when the line cells were 1×10^6, the level of anti-CCII antibody in the recipient rats showed a slightly higher than that of the naive rat (15th day, $P<0.05$), but no clinical expression appeared. When the line cells were over 5×10^6, the level of anti-CCII antibody showed a significantly higher than that of the naive rat ($P<0.01$), and the clinical arthritis could be seen.

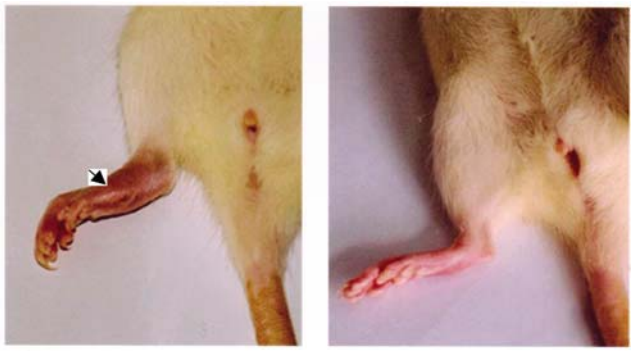

Fig.1 A female rat that received CCII-specific T cell line (5×10^7 cells) developed the arthritis (15th day, arthritis score 3) compared with a normal control rat on the right

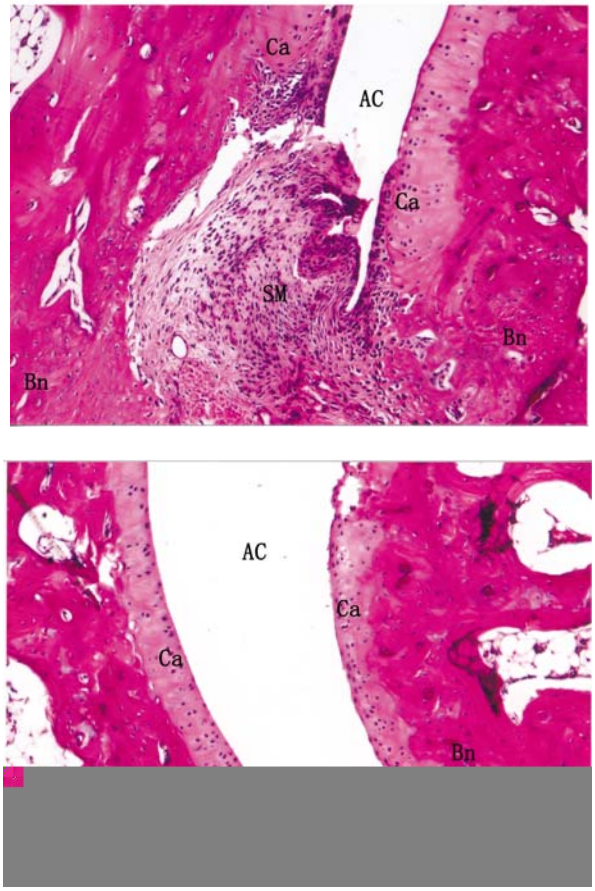

Fig.2 Histologic evidence of arthritic ankle joint in the transferring arthritic rat(15th day).
Note: the synovial membrane hyperplasia, mononuclear cell infiltration and destruction of cartilage and bone compared with normal control rat on the right. SM: synovial membrane; Ca: cartilage; Bn: bone; AC: articular cavity Hematoxylin and eosin. ×100.

Table 2 Arthritis transferring by CCII-specific T line cells

Dose	No.of recipient rats	Incidence of arthritis	MAI	The level of anti-CCII antibody
1×10^5	10	0/10	0	0.12±0.06
5×10^5	10	0/10	0	0.14±0.06
1×10^6	10	0/10	0	0.26±0.07*
5×10^6	10	1/10	0.5	0.39±0.05△
1×10^7	10	3/10	1.8	0.43±0.07△
5×10^7	10	5/10	4.0	0.50±0.09
naive rat	10	0/10	0	0.13±0.07

△$P<0.01$ vs naive rat; *$P<0.05$ vs naive rat.

DISCUSSION

CII is a tissue-specific protein, that mainly present in the joint cartilage and the tissue of eye ball. It is the major constitute protein of cartilage in diarthrodial joints, which are the predominant sites of inflammation in CIA. Joint injury can induce the release of CII, which will stimulate the immune system to produce an immune attach, that is thought a mechanism of RA occurring. CII has received considerable attention as a potential antigen in RA, and also the immunity to CII can be detected in RA patients. Following immunization with chicken type II collagen (CCII), the rats developed an erosive, polyarticular arthritis mediated by an autoimmune response to CCII called collagen-induced arthritis (CIA). The manifestation of CIA is in many ways similar to that of human rheumatoid arthritis (RA). Histologically, both CIA and RA are characterized by an intense synovitis, a pannus-like tissue, accompanied by erosions of cartilage and subchondral bone. So, the CIA can be regarded as a unique animal mode of RA to investigate the pathogenesis of RA[1]

Much evidence suggests that CIA and RA are type-1 immune response mediated disease. Synovial tissue from RA patients expresses IFN-γ but not IL-4 transcript and increased IFN-γexpression correlates with the severity of the disease. Recent studies are also consistent with the notion that the CIA is mediated by a type 1 immune response and showed that the establishment of a type 1 immune response specific for CII is essential for the induction and pathogenesis of CIA[5].

Autoreactive T cells are thought to be involved in several experimental autoimmune disease model. The most common model is the experimental allergic encephalomyelitis (EAE). Autoreactive T cells were readily isolated from rats and mice immunized with myelin basic protien (MBP), and these T cells will induce disease upon transferring to naive recipients[6]. In experimental autoimmune thyroiditis, the similar data have been obtained[7]. In this study, we established the animal model of CIA by intradermally immunizing with CCII, and then we isolated the mononuclear cells. Under the presence of antigen-presenting cells, we established the CCII-reactive T cell line after 3 cycles of stimulating with CCII and IL-2.

Phenotype analysis by the FACS showed that 98.2% of line cells were T cells, 89.7% of which were $CD4^+$ T cells. Intravenous injection of the line cells through tail vein could induce the arthritis in naive recipient rats, that were validated by the pathological examination. The incidence of CIA was 50% when the injected cells were 5×10^7, and the ankles and digital joints of diseased recipient rats showed a typical arthritic pathological characteristics by histochemical examination. While the injected cells were 1×10^6, the rats do not had the clinical arthritis, but showed a higher level of anti-CCII antibody than that of the naive rats.

In conclusion, the results of our study indicated that type II collagen--induced arthritis can be adoptive transferred by the CII-specific T cell line and supported the notion that the CIA is mediated by the immune response of T lymphocyte and an immunologic response specific to type II collagen could be the main arthritogenicity in CIA. Thus, our research data provided a strong basis for therapy of RA with T cell vaccination.

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