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Type II Collagen-induced Murine Arthritis

I. Crucial Role of L3T4$^+$ T Cells in Arthritis Induction

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Abstract A chronic perpetuating arthritis was induced not by humoral immunity alone but by synergy between humoral and cell-mediated immunity. The cells capable of transferring cell-mediated immunity are Thy-1$^+$ and L3T4$^+$ Lyt-2$^-$ T cells. They mediate anti-collagen DTH responses in vivo and production of IL-2 and IL-6 *in vitro* Enhanced production of IL-6 in sera (which is produced by L3T4$^+$ T cells) was found in arthritic DBA/1 mice, but not in nonresponder mice (C57BL/6,BALB/c and C3H/He). These results indicate that L3T4$^+$ T cells play a crucial role in collagen-induced murine arthritis.

INTRODUCTION

Type II collagen-induced arthritis is an experimental model of chronic inflammatory arthropathy that can be induced by the intradermal injection of type II collagen in susceptible rodents (Trentham et al., 1977; Stuart et al., 1979; Courtenay et al., 1980; Wooley et al.,1981) and primates (Cathcart et al., 1986). The resulting disease shares a number of histologic, immunologic, and genetic, as well as clinical, features in common with human rheumatoid arthritis and other related autoimmune diseases (Trentham ,1982; Caulfield et al., 1982; Terato et al., 1982; Steffen & Timpl ,1963; Andriopoulos et al., 1976; Trentham et al.,1987; Stuart et al., 1980).

The development of arthritis is associated with high levels of cell-mediated immunity as well as humoral immunity to type II collagen (Trentham et al., 1978b). Whereas the role of cell-mediated immunity in the pathogenesis of arthritis is debated (Trentham et al., 1978a; Klareskog et al., 1983), invariable association of arthritis with high level of antibody has focused on the importance of anti-collagen antibody responses in inducing arthritis. In fact, it has been reported that transfer of serum fractions or purified anti-collagen antibodies results in synovitis in both rats and mice (Stuart et al., 1982a; Stuart & Dixon, 1983). However, this antibody-mediated synovitis has been shown to be transient and the histpathology is somewhat different from the one observed in collagen-induced arthritis (Holmdahl et al., 1986). These studies thus suggested that anti-collagen antibodies are not sufficient to give rise to the lesions that characterize collagen-induced arthritis (Holmdahl et al.,1986). This has suggested the importance of anti-collagen cell-mediated immunity in the induction of arthritis. In this context, our

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studies has demonstrated that a chronic, progressive arthritis is established by synergy between anti-collagen antibody and L3T4$^+$ cell-mediated immuniy (Seki et al., 1988).

In the present study, it has been confirmed that L3T4$^+$ T cells function to induce arthritis in synergy with anti-collagen antibody. We demonstrate that a subset of L3T4$^+$ T cells are responsible for anti-collagen DTH response and production of IL-2 and IL-6.The results indicate that L3T4$^+$ T cells play a crucial role in anti-collagen immune response and the induction of arthritis. Moreover, in relation to the mechanism by which the L3T4$^+$ T cell-mediated immunity contributes to the development of arthritis, we also investigete the enhanced production of IL-6 in arthritic DBA/1 mice.

MATERIALS AND METHODS

Mice

DBA/1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and C3H/He, C57BL/6, and BALB/c mice were obtained from Japan SLC Inc. (Shizuoka, Japan). All mice were used at 6 to 8 wk of age.

Collagen. Bovine type II collagen was obtained from Cosmo-Bio, Co. Ltd. (Tokyo, Japan) and dissolved in 0.1 M acetic acid. Denatured collagen was prepared by placing collagen solutions ina boiling water bath for 5 min (Seki et al.,1988). For *in vitro* studies, collagen solution was dialyzed against RPMI-1640 medium.

Sensitization to collagen

Mice were sensitized at tail base with 200 μg native or denatured collagen emulsified in 40 μl of complete Freund's adjuvant containing *Mycobacterium tuberculosi* strain $H_{37}R_V$ (Wako Pure Chemical Industries Ltd., Osaka, Japan), and boosted with the same preparations of antigen plus complete Freund's adjuvant 3 weeks later.

Incidence and scoring of arthritis

Incidence of arthritis was determined 5 weeks after the first immunization with collagen unless otherwise indicated. An animal was judged to be arthritic if one or more joint regions (digitus, wrist, or ankle) were red and swollen. In some experiments, severity was graded visually by using a method adapted from that previously described for rats (Rogers et al.,1980), and expressed by the arthritic index which was obtained by scoring each limb from 0-3 severity-grade and by summing up scores of four limbs.

DTH responses to collagen

Assay system was essentially the same as described for viral protein (Takai et al.,1985). Briefly anti-collagen DTH response was assessed by an adoptive transfer system in which 1 x 10^7 spleen cells from immunized mice, mixed together with 100 μg native collagen, were inoculated into hind footpad of syngeneic normal mice. Twenty-four hours after the adoptive transfer, the increase in the footpad thickness was measured by using a microcaliper. Footpad swelling was expressed as the mean increment $\pm$ S.E. of five mice/group.

Immunoassay of antibody to collagen

An enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies to

collagen. The method was adapted from previously described (Stuart & Dixon,1983; Fujitsu et al.,1986). Briefly, wells of flat-bottomed microplates (Dynatech Corp., Chantilly, VA) were coated with 50μl of native collagen(50μg/ml) in phosphate-buffered saline (PBS; 10mM phosphate, 150 mM NaCl, pH7.6), at 37°C for 1 hr. The wells were then washed three times with PBS containing 0.05% Tween 20 and 0.1% BSA, and 50 μl of sample diluted with PBS containing 0.05% Tween 20 and 0.5% BSA was added. After a 1-hr incubation at room temperature, the wells were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween), incubate with 50 μl of a 1:200 dilution of goat anti-mouse IgG coupled to horseradish peroxidase (Sigma Chemical Co., St.Louis, MO) at room temperature for 30 min, washed three times with PBS-Tween, and developed at room temperature for 20 min in the dark with 0.2ml of 0.4mg/ml o-phenylenediamine−0.003% H_2O_2 in 0.05M citrate−0.1M phosphate buffer at pH 4.5. The reaction was stopped by adding 50μl of 6.6% sulfuric acid, and the OD at 490 nm was read using Micro ELISA Mini Reader MR 590 (Dynatech Corp.). As reference, a serum pool from DBA/1 mice exhibiting typical arthritis was given arbitarily a value of 100 U/ml.

Production of IL-2 and IL-6

Lymph node cells (4 x 10_6/well) were cultured with 10-100 μg/ml collagen in RPMI-1640 medium supplemented with 0.5% normal mouse serum and 2-mercaptoethanol in a 1 ml volume in 24-well culture plates for 1 day. IL-2 or IL-6 activity was measured by using the respective lymphokine-dependent CTLL-2 (Baker et al.,1979) or PIL6 cell line (Takai et al.,1989). It was previously confirmed that CTLL-2 responds to IL-2 but not to IL-4 (Ogata et al.,1987). Briefly, 4 x 10_3 CTLL-2 or 10_4 PIL6 cells were cultured in a volume of 200μl containing the above culture supernatant. After 1 (CTLL-2) or 5 days (PIL6) of incubation at 37°C, cells were pulsed with 0.5μCi/well of ^{3}H-TdR and harvested 6 hr later. The incorporated radioactivity was measured with a scintillation spectrometer (Aloka,Co.,Ltd., Tokyo,Japan). The results of the ^{3}H-TdR incorporation assay were expressed as the mean cpm of triplicate cultures.

Serum samples

Blood was were obtained from all mice, coagulated, centrifuged, and the serum was prepared. The sera were diluted with complete culture medium and were sterilized by filtration through 0.45 μm diameter filters. Samples were stored at-40°C until use.

Preparation of immunoglobulin for transfer studies

Immune sera were obtained from arthritic DBA/1 mice 5 weeks after primary immunization with collagen. The sera were pooled, and an equal volume of saturated ammonium sulfate solution was added to it to precipitate the immunoglobulin. The precipitate was collected by centrifugation, dissolved in a small volume of water, and dialyzed against 0.15M NaCl buffered by 0.01M sodium phosphate (PBS) at pH 7.2. The volume was adjusted to 1/3 that of the orignal serum volume. Immunoglobulin solution thus prepared was sterilized by filtration through a 0.45-μm porosity filter, and stored at-80°C until use.

Preparation of T cell-depleted mice

The procedure was similar to that described previously (Seki et al.,1988; Sugihara et al.,1988; Fujiwara et al.,1984). Briefly, DBA/1 mice was thymectomized at 6 weeks of age, and administered i.p. 2 days later 0.25 ml rabbit anti-mouse thymocyte serum. Three weekes later, these mice were whole body X-irradiated at 750 R and received i.v. inoculation of 3 x 10^6 syngeneic adult bone marrow cells pretreated with anti-Thy-1.2 plus rabbit complement (C). These mice were used after an additional 3 weeks. T cell depletion in the spleen and lymph nodes of these mice was confirmed by fluorescence-activated cell scanning analysis and by the lack of response to T cell mitogens, but not to B cell mitogen i.e. LPS (Fujiwara et al.,1984).

Treatment of immune lymphoid cells with antibody plus complement

Monoclonal antibodies to Thy-1.2, L3T4, or Lyt-2 antigens were used. Anti-Thy-1.2 was purchased from New England Nuclear Co. (Boston,MA). Anti-L3T4 GK1.5 monoclonal antibody-producing hybridoma cell line (Dialynas et al., 1983) was a gift from Dr. F. Fitch, University of Chicago (Chicago,IL), and anti-Lyt-2 3.155 monoclonal antibody-producing hybridoma cell line was obtained from ATCC (Rockville, MD). Spleen and lymph node cells (5 x 10^7) from immunized mice were incubated at room temperature for 40 min with one of these monoclonal antibodies at a dilution 1:1000 in anti-Thy1.2, 1:100 in anti-L3T4, or 1:10 in anti-Lyt-2. The treated cells were washed and incubated at 37°C for 45 min with rabbit C, preabsorbed with syngeneic mouse spleen cells, at a final dilution 1:10.

RESULTS

Requirement of synergy between humoral and L3T4$^+$ T cell-mediated immunities for inducing chronic arthritis

We have confirmed the fact that chronic perpetuating arthritis can be induced by synergy between humoral and cell-mediated immunities to type II collagen (Seki et al., 1988). In the present experiment, T cell-depleted DBA/1 mice were injected with anti-collagen antiserum, spleen and lymph node cells capable of selectively mediating cell-mediated immunity (cells from denatured collagen-sensitized mice), or both, and incidence of arthritis was determined 1 and 2 weeks after the injection (Table 1). It was previously demonstrated that immunization with native collagen mounted both vigorous antibody and cellular immune responses, whereas sensitization with heat-denatured collagen resulted in selective generation of anti-collagen cell-mediated immunity without inducing effective antibody production (Seki et al., 1988). As shown in Table 1, injection of antiserum from arthritic mice alone or with unsensitizedcells into T cell-depleted mice elicited only transient joint swelling but did not induce typical chronic arthritis (Group 2 or 3), which is compatible with the previous results (Seki et al., 1988; Holmdahl et al., 1986). The adoptive transfer of spleen and lymph node cells from denatured collagen-sensitized mice without antiserum also failed to elicit arthritis (Group 4). In contrast, transfer of such lymphoid cells together with antiserum was capable of inducing a typical arthritis (Group 5). The results of Table 1 also demonstrate that L3T4$^+$ T cell subset functions to induce arthritis in synergy with antiserum (Group 8).

Since L3T4$^+$ T cells are responsible for the induction of a chronic, progressive

arthritis, we next investigate the precise role of L3T4$^+$ T cells in expressing cell-mediated immunity against type II collagen.

Various role of L3T4$^+$ T cell subset in anti-collagen immune responses

Spleen or lymph node cells from DBA/1 mice sensitized with native or denatured collagen were tested for their ability to elicit DTH response *in vivo* and to produce various

Table 1. Synergy between antibody and L3T4$^+$ T cells from denatured collagen-sensitized mice for inducing chronic arthritis

Group	Cells transferred[a]		Antiserum transfer[c]	Incidence of arthritis	
	immune to :	treated with[b] :		1w	2w
1	(no transfer)		−	0/7 (0.0)[d]	0/7 (0.0)
2	(no transfer)		+	2/7 (28.6)	1/7 (14.3)
3	none	−	+	1/7 (14.3)	1/7 (14.3)
4	d-collagen[e]	−	−	0/7 (0.0)	0/7 (0.0)
5	〃	−	+	7/7 (100.0)	7/7 (100.0)
6	〃	α-Thy-1.2 + C	+	2/7 (28.6)	1/7 (14.3)
7	〃	α-L3T4 + C	+	2/9 (22.2)	1/9 (11.1)
8	〃	α-Lyt-2 + C	+	9/9 (100.0)	9/9 (100.0)

[a] T cell-depleted mice (7–9 mice/group) were untransferred (Groups 1 & 2) or transferred with spleen and lymph node cells from normal (Group 3) or denatured collagen-immunized mice (Groups 4–8) on day 0.
[b] Cells from denatured collagen-immunized mice were treated with monoclonal antibodies (indicated) pluc C before the cell transfer.
[c] Concentrated antiserum (0.2 ml) from arthritic mice was i.v. transferred on days 1 and 2.
[d] percentage of incidence
[e] DBA/1 mice were immunized with 200μg heat-denatured collagen (d-collagen) and boosted 3 weeks later. These mice were used as cell donors 5 weeks after the first immunization.

Table 2. Capacity of lymphoid cells from native or denatured collagensensitized mice to mediate DTH response and to produce IL-2 and IL-6

Cells from mice immunized with collagen[a] :	Stimulation with native collagen	DTH response[b] Footpad increment (x 10^{-2} mm)	Lymphokine production[c] 3H-TdR uptake (cpm) of	
			CTLL-2	PIL6
−	−	0.0 ± 0.0	354 ± 66	519 ± 245
−	+	13.0 ± 5.4	260 ± 59	1564 ± 274
native	−	10.4 ± 1.6	590 ± 89	2709 ± 193
native	+	63.8 ± 4.9	3814 ± 248	15136 ± 1596
denatured	−	19.6 ± 1.5	538 ± 129	4047 ± 453
denatured	+	68.8 ± 6.7	5421 ± 209	15277 ± 614

[a] Spleen or lymph node cells were obtained from mice 5 weeks after immunization with 200μg native or denatured collagen.
[b] Spleen cells (10^7) from normal mice or those from native or denatured collagen-immunized mice were inoculated into footpads of normal syngeneic recipient mice alone or together with 100μg native collagen.
[c] Lymph node cells (4 x 10^6) from unsensitized normal or collagen immunized mice were stimulated *in vitro* with 100μg/ml of native collagen for 1 day. Culture supernatants obtained were added to cultures of CTLL-2 (4 x 10^3/well) or PIL6 (1 x 10^4/well) for the assay of IL-2 or IL-6 activity.

Table 3. Nature of cells responsible for producing IL-2 and IL-6

Lymph node cells		^{3}H-TdR uptake (cpm)b of:	
from mice immunized with	treatment[a)]	CTLL-2	PIL6[c)]
denatured collagen	−	9435 ± 119	8169 ± 920
〃	anti-Thy-1.2 + C	1006 ± 28	4125 ± 911
〃	anti-L3T4 + C	201 ± 32	3425 ± 863
〃	anti-Lyt-2 + C	15992 ± 346	11526 ± 536

a) Lymph node cells from denaured collagen-immunized DBA/1 mice were treated with various monoclonal antibodies plus C.

b) Untreated or antibody-treated lymph node cells (4 x 10^6) were stimulated *in vitro* with 100 μg/ml of denatured collagen. Culture supernatants obtained 1 day later were assayed on CTLL-2 or PIL6 for IL-2 or IL-6 activity.

c) ^{3}H-TdR uptake of PIL6 was in the range of 3000-4000, when culture supernatants were obyained from each group of denatured collagensensitized cells without in vitro stimulation with denatured collagen.

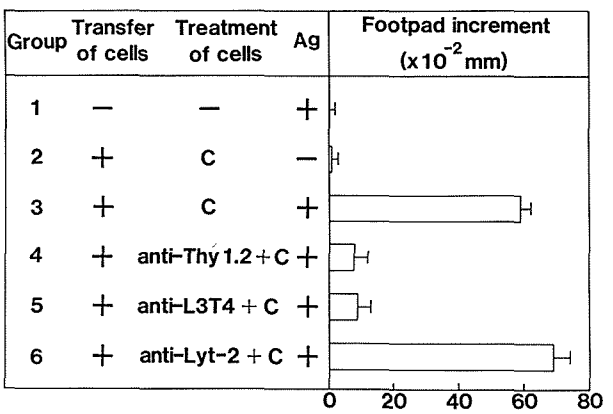

Fig. 1. Nature of cells required for inducing DTH responses. Spleen cells from denatured collagen-sensitized DBA/1 mice were treated with various monoclonal antibodies plus C. These treated or untreated cells (10^7) were inoculated into footpads of normal syngeneic recipient mice together with 100 μg native collagen. Footpad swelling was expressed by the mean footpad increment ± S.E. of 5 mice/group.

types of lymphokines *in vitro*. Results of Table 2 demonstrate that lymphoid cells from denatured collagen-sensitized mice were capable of mounting potent DTH response and producing IL-2 and IL-6 on the stimulation with native collagen and that such magnitude was almost comparable to that obtained by cells from native collagen-sensitized mice.

In Fig. 1, it is shown that anti-collagen DTH response was mediated by L3T4$^+$ T cells, because treatment of denatured collagen-sensitized cells with anti-Thy-1.2 or anti-L3T4 antibody plus C but not with anti-Lyt-2 antibody plus C resulted in almost complete abrogation of the response.

In vitro experiments were also performed to try to determine which component(s) of lymph node cells is responsible for the production of IL-2 and IL-6. Lymph node cells were treated with various monoclonal antibodies and rabbit complement before culturing with collagen. Treatment with anti-Lyt-2 antibody plus complement did not affect the

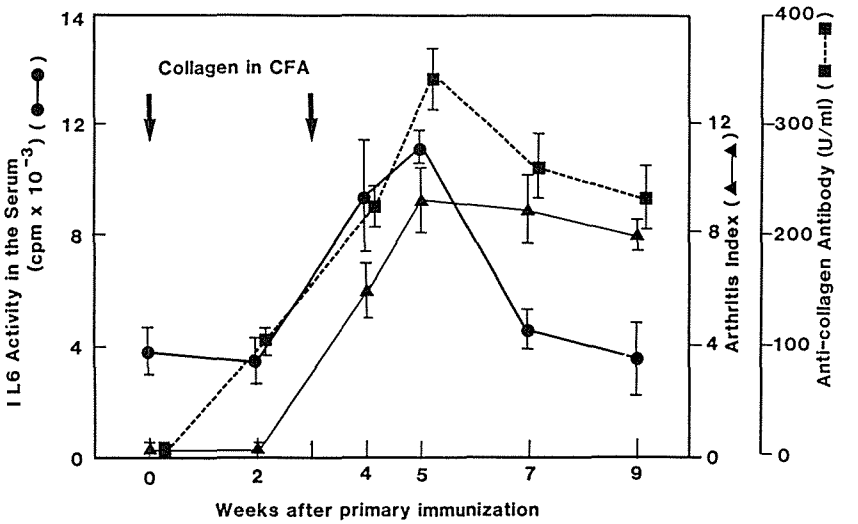

Fig. 2. Time course of collagen-induced arthritis in DBA/1 mice. Ten DBA/1 mice were immunized with native collagen twice on weeks 0 and 3. Serum samples were obtained individually various weeks after primary immunization. IL-6 activity (● — ●) was determined by proliferation of PIL-6 cells (triplicate cultures,at 1:16 dilution of test sera). Anti-collagen antibody (■---■) was determined by ELISA. Arthtitic index (▲—▲) was determined by scoring each limb from 0-3 severity-grade and by summing up scores of four limbs. The results were expressed by the mean ± S.E. of 10 mice.

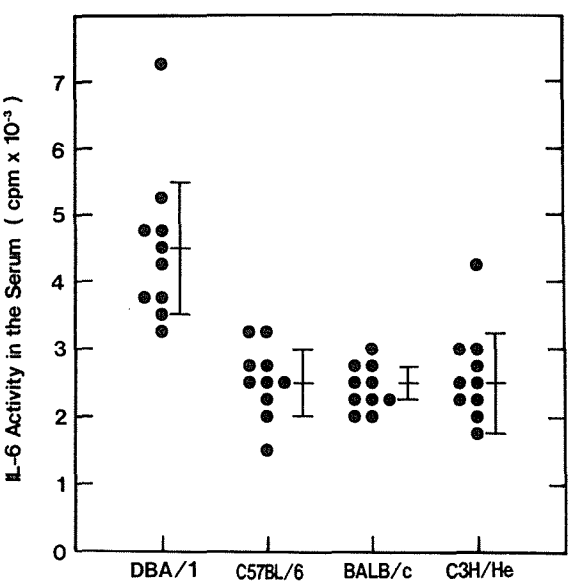

Fig. 3. Strain difference in serum IL-6 activity after immunization with native collagen. Variou strains of mice were immunized with native collagen twice. Five weeks after the first immunization,serum was obtained from each mouse. IL-6 activity was determined as described in Fig. 2. Each point represents the mean IL-6 activity in serum from 1 mouse (the S.E. were $<10\%$ of the mean values and were omitted from the fig.). Bars show group means ± S.E. .

production of IL-2 and IL-6 (Table 3). However, the production of these lymphokines were almost completely abrogated by treatment with anti-Thy-1.2 or anti-L3T4 antibody plus complement. These results indicate that the L3T4$^+$ T cell subset is responsible for the production of IL-2 and IL-6 in mice with collagen-induced arthritis.

The potential role of IL-6 produced by L3T4$^+$ T cell subset in the pathogenesis of collagen-induced arthritis

We finally investigated the relation of serum IL-6 level with arthritis manifestation in the native collagen-immunized mice. First, we examined the time course of IL-6 level, that of anti-collagen antibody titer and arthritic index in the serum of native collagen-immunized DBA/1 mice (Fig. 2). Mice were immunized twice at an interval 3 weeks. IL-6 level was elevated probably in response to the second immunization, and reached the peak 2 weeks after that. Both Anti-collagen antibody titer and arthritic index parallely changed with the kinetics of IL-6 level until 5 weeks after the primary immunization. Then, however, IL-6 level rapidly declined, although anti-collagen antibody titer and arthritic only slightly decreased.

Second, we examined the serum IL-6 level in several strains of mice immunized twice with native type II collagen. All serum samples were obtained 5 weeks after the first immunization. As shown in Fig. 3, only a marginal IL-6 level was detected in mouse strains (C57BL/6, BALB/c, C3H/He) that failed to develop arthritis after immunization with native collagen. The results demonstrate that enhanced production of IL-6 is observed only in DBA/1 mice which manifest overt arthritis.

DISCUSSION

The immune response to type II collagen has been used to establish an arthritis in mice (Courtenay et al., 1980) and rats (Trentham et al., 1977) that bears a close resemblance to human rheumatoid arthritis in the histlogic and clinical manifestations of the disease.

Incidence of collagen-induced arthritis correlates with the level of immune response to type II collagen (Tretham et al., 1978b; Stuart et al., 1982b). Studies concerned with the genetic control of arthritis induction have been, therefore, performed by examining the ability to mount a strong anti-collagen antibody response. However, resent studies have revealed that injection of anti-collagen antiserum results in only a transient swelling of joint but fails to induce a typical, chronic arthritis (Holmdahl et al., 1986). We have also confirmed this and further demonstrated that the induction of a typical, chronic arthritis requires synergy between humoral and cell-mediated immunity (Seki et al., 1988). Moreover, we demonstrated the nature of cells capable of transferring cell-mediated immunity was of Thy-1$^+$ and L3T4$^+$ Lyt-2$^-$ (Table 1). These observation are in accordance with the results of Hom et al. (1988) that the depletion of L3T4$^+$ T cells by *in vivo* administration of anti-L3T4 antibody prevents the induction of arthritis by the subsequent immunization with collagen.

It has become evident that L3T4$^+$ T cells have an important role in autoimmune diseases (Maron et al., 1983; Hanafusa et al., 1986). Then we investigated the role of L3T4$^+$ T cells in anti-collagen cell-mediated immunity. As shown in Figure 1 and Table 3, both anti-collagen DTH response and production of lymphokines (IL-2,IL-6) are mediated by L3T4$^+$ T cells. It is possible that L3T4$^+$ T cells also function as T helper cells

for B cells forming antibody to collagen, because L3T4$^+$ T cells are responsible for the production of IL-6 which induces final maturation of B cells into antibody producing cells (Kishimoto & Hirano; 1988). Therefore, L3T4$^+$ T cells play an important role in type II collagen-induced murine arthritis.

Which function(s) of L3T4$^+$ T cell-mediated functions is the most crucial for the development of arthritis remains to be defined. In this context, it should be noted that IL-6 exerts its multifunctional effect (Hirano et al., 1986; Zilberstein et al., 1986; Gauldie et al., 1987; Haegeman et al.,1986: Poupart et al., 1987; Takai et al., 1988; Wong & Clark, 1988; Muraguchi et al., 1988; Garman et al., 1987; Lotz et al., 1988; Nordan et al., 1986; Van damme et al., 1987). Although IL-6 (B cell stimulatory factor-2) has been considered to be involved in the differentiation of B cells, it has become evident that this lymphokine has an important role in the development and/or perpetuation of chronic inflammatory lesions (Kishimoto & Hirano, 1988; Al-balaghi et al., 1984). We also reported that IL-6 activity was elevated in the serum of arthritic DBA/1 mice, and that the elevated level of serum IL-6 activity was associated with high levels of IL-6 produced when lymph node cells from arthritic mice were stimulated *in vitro* with type II collagen. Then we investigated the time course and the strain difference of IL-6 level in the serum in the present study. IL-6 activity in sera from DBA/1 mice immunized with collagen was elevated in parallel with the increase of anti-collagen antibody titer and arthritic index. It was also revealed that an appreciable difference in IL-6 activity in sera between high-responder (DBA/1) and low-responder (C57BL/6, C3H/He, BALB/c) mice. The same genetic control is found in the production of IL-6 by L3T4$^+$ T cells on the stimulation with collagen *in vitro* (the accompanying paper). These findings indicate that IL-6 is one of the most crucial factors in the developement of collagen-induced murine arthritis.

In the next paper, cellular and molecular mechanisms underlying genetic control in the collagen-induced arthritis are analyzed on each component of these humoral and L3T4$^+$ T cell-mediated immunities.

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