Brief Definitive Report

PREVENTION OF TYPE II COLLAGEN–INDUCED ARTHRITIS BY IN VIVO TREATMENT WITH ANTI-L3T4

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Type II collagen–induced arthritis (CIA) in mice and rats is an inflammatory polyarthritis that displays several characteristics similar to human rheumatoid arthritis (1, 2). The onset of CIA is associated with the development of both a cellular and humoral response to type II collagen (3) and is restricted in mice to animals of the H-2^q or H-2^r haplotype (4). The humoral response to type II collagen appears to be an essential element in the development of the disease although the role of cellular immunity is less clear.

The L3T4 antigen, defined by a rat anti-mouse monoclonal antibody GK1.5, is limited to T cells responsible for class II major histocompatibility complex (MHC)-restricted functions and is analogous to the OKT4/Leu-3 antigen on human T cells (5, 6). Recent reports (7, 8) have indicated that treatment of murine experimental autoimmune diseases with monoclonal anti-L3T4 antibody under either preventive or therapeutic protocols dramatically decreases disease expression. In this paper we demonstrate the prevention of murine CIA by in vivo treatment with monoclonal antibody to the L3T4 antigen.

Materials and Methods

Animals. DBA/1 male mice, 8–10 wk old, were obtained from The Jackson Laboratory, Bar Harbor, ME.

Type II Collagen. Bovine articular cartilage was processed through four 24-h extractions with 5 vol of 4 M guanidine HCl, 50 mM Tris, pH 7.0, at 4°C. The slices were washed twice for 2 h in 1 M NaCl, 50 mM Tris, pH 7.5, once for 2 h in distilled H_2O, and twice for 3 h with 0.5 M HAc, and then digested overnight with 10 vol of 200 μg/ml pepsin in 0.5 M HAc. The digest was centrifuged for 1 h at 10,000 *g*; solid NaCl was added to the supernatant to a concentration of 0.9 M and held overnight. After centrifugation, the pellet was resuspended in 5 vol of 1 M NaCl, 50 mM Tris, pH 7.5. The solution was adjusted to pH 8 by addition of 3 N NaOH and, after centrifugation as above, the supernatant was reprecipitated by the addition of solid NaCl to a final concentration of 4 M. After centrifugation, the pellet was suspended in 5 vol of 0.5 M HAc, dialyzed against 0.5 M HAc, and centrifugated at 27,000 *g* for 1 h. The purity of the type II collagen in the supernatant preparation was determined by sodium dodecyl sulphate gel electrophoresis, which showed a single band identical to a sample of pure bovine type II collagen (kindly provided by Dr. Paul Benya, University of Southern California School of Medicine).

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Monoclonal Antibodies. The hybridoma that produces monoclonal antibody GK1.5, a rat IgG2b monoclonal antibody recognizing L3T4, was passed in BALB/c mice irradiated with 500 rad. The antibody was isolated from the ascitic fluid by ammonium sulfate precipitation followed by passage over a PD-10 column and, finally, by passage of the protein over a QAE Sephadex A50 column (Pharmacia Fine Chemicals, Piscataway, NJ) and quantitation by ultraviolet absorption at 280 nm. The following rat anti-mouse monoclonal antibody–secreting hybridomas were developed by Dr. J. A. Ledbetter (9) and obtained from the American Type Culture Collection, Rockville, MD: 30-H12, anti-Thy-1.2; 53-6.72, anti-Lyt-2; 53-7.313, anti-Lyt-1. Antibody was obtained by growing the hybridoma cells in serum-free HB102 medium (DuPont Co., Wilmington, DE) for 24 h at 5×10^5/ml. Supernatants were harvested, filtered, and used undiluted for cell phenotype analysis.

Induction of CIA. DBA/1 mice were immunized with an intradermal injection of 100 μg native type II collagen emulsified in an equal volume of complete Freund's adjuvant (CFA), followed on day 21 by an intraperitoneal injection of 100 μg native type II collagen in phosphate-buffered saline (PBS). Control mice received injections of PBS. After the second immunization, animals were inspected every other day for clinical signs of arthritis until day 53, and twice weekly thereafter. The onset of arthritis was defined by the first signs of erythema and swelling of one or more limbs. In all instances it was subsequently confirmed by histologic examination.

Anti–Type II Collagen ELISA. Immulon II plates (Dynatech Laboratories, Alexandria, VA) were incubated for 4 h at 4°C with type II collagen in PBS, pH 7.4, at 10 μg/ml, 100 μl/well. The plates were washed with PBS, incubated for 2 h with PBS–1% bovine serum albumin (BSA), and washed repeatedly with PBS-Tween. Antisera were serially diluted in 1% BSA-PBS-Tween and dispensed at 100 μl/well. After overnight incubation at 4°C, the plates were washed and goat anti-mouse IgG–alkaline phosphatase conjugate (Tago, Inc., Burlingame, CA) was added for 2 h. After addition of *p*-nitrophenyl disodium (Sigma Chemical Co., St. Louis, MO), the reaction was stopped by addition of 50 μl 1 N NaOH to each well and the plates were read with an automatic enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Inc., Burlington, VT) at 405 nm. Titers were based on comparison to an anti–type II collagen antibody standard that was included with each assay.

Fluorescence Analysis of Lymphocyte Subpopulations. Single-cell suspensions from spleens and lymph nodes were washed and suspended at 5×10^6/ml in 1% BSA-PBS–0.1% NaN_3. 50 μl of cell suspension and 50 μl of monoclonal antisera were incubated for 30 min at 4°C, washed once, and the cells resuspended in 50 μl of fluorescein-conjugated monoclonal mouse anti-rat kappa chain antibody (Becton, Dickinson & Co., Mountain View, CA) for 30 min at 4°C. Control tubes were exposed only to the second antibody. After a final wash the labeled cells were enumerated using a cytofluorograph flow cytometer (model 2150; Ortho Diagnostic Systems, Raritan, NJ).

Results

In vivo effects of anti-L3T4 administration on T cell subsets. DBA/1 mice were given injections of 100 μg anti-L3T4 on day −1 and +1, followed by fluorescence-activated cell sorter (FACS) analysis of lymph node cells on days 3, 7, 14, and 30. The results shown in Table I indicate that such treatments resulted in a 90% depletion of $L3T4^+$ cells by day 3 associated with a decreased percentage of total T cells. The remaining T cells were predominantly of the $Lyt\text{-}2^+$ phenotype. After depletion, there was a gradual replenishment of the $L3T4^+$ population, which returned to normal by day 30. Similar results were observed with spleen cell populations (data not shown).

Effects of Anti-L3T4 on CIA. Two protocols were designed to determine the effects of anti-L3T4 on type II collagen arthritis induction (Table II). In the first protocol, DBA/1 mice were treated with 100 μg of anti-L3T4 1 d before and 1 d after immunization with type II collagen, and once every 2 wk thereafter until

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TABLE I

Changes in T Cell Subset Distribution after In Vivo Administration of GK1.5

	Day after GK1.5	Percent positive cells staining with:			
		Thy-1	Lyt-1	Lyt-2	L3T4
Untreated		68.7	79.4	19.6	51.9
GK1.5 Treated	3	51.1	53.3	45.7	6.9
	8	58.6	63.0	41.5	10.4
	14	59.0	65.3	33.6	16.1
	30	69.0	71.2	23.4	45.3

DBA/1 mice were given intraperitoneal injections of 100 μg GK1.5 on days −1 and +1. On days 3, 8, 14, and 30, axial and inguinal lymph node cells were removed and processed for FACS analysis. Each time point represents the average of three mice. Standard errors in each case were <5%.

TABLE II

Incidence and Mean Day of Onset of CIA

Exp.	Group A		Group B		Group C	
	CIA incidence	Mean day of onset (±SD)	CIA incidence	Mean day of onset (±SD)	CIA incidence	Mean day of onset (±SD)
1	9/10	44 ± 6	2/10	80 ± 5	5/10	45 ± 5
2	5/10	34 ± 7	1/15	45	5/12	38 ± 8
Totals	14/20		3/25 ($P < 0.005$)		10/22 ($P > 0.19$)	

CIA incidence is calculated as the number of mice displaying arthritis per number of mice in group. Group A, is control mice immunized with type II collagen and treated with PBS. Group B was immunized with type II collagen; injections of 100 μg GK1.5 on days −1, +1, 14, 28, 45. Group C was immunized with type II collagen; injections of 100 μg GK1.5 on days 28, 45 (Exp. 1) or on days 23 and every other day to day 50 (Exp. 2). *P* values, = chi values comparing groups B and C with group A.

day 45 (Table II, group B). In the second protocol anti-L3T4 treatment was delayed until a vigorous immune response to collagen was in progress, but before the clinical appearance of arthritis (Table II, Exp. 1, group C).

The onset of arthritis in the control group (A) began at day 31 and by day 53 reached a cumulative incidence of 90% (Table II, Exp. 1). The group of mice (B) receiving anti-L3T4 before immunization displayed no arthritis by day 53, but 2 of 10 mice developed arthritis by day 85, some 40 d after cessation of treatment. By day 140, none of the remaining animals from this group showed any signs of arthritis. The third group (C), which did not receive anti-L3T4 until 28 d after immunization, showed an arthritis incidence of 50% by day 54 with no further changes after that time. The results from group C were not statistically different from the control group (Table II).

Anti-L3T4 treatments were successful in preventing CIA when administered before immunization with type II collagen but, if treatments were delayed 28 d, the effect was equivocal. To determine if delayed but more intensive treatment with anti-L3T4 would have an effect on CIA, immunized mice were treated with anti-L3T4 beginning on day 23 and every other day thereafter for the remainder of the experiment (Table II, Exp. 2, group C). No difference in arthritis incidence or day of onset was seen between this group and the control group. Therefore, anti-L3T4 was effective in preventing CIA when given before immunization but was ineffective once a vigorous immune response to type II collagen was in progress.

Histologic evaluation of arthritic and nonarthritic limbs. To determine if some

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TABLE III
Summary of Anti-Type II Collagen Titers

	IgG titer ± SE			
	Day 14	Day 28	Day 45	Day 70
Group A control	767 ± 104	853 ± 74	1,585 ± 333	2,101 ± 755
Group B (GK1.5 days −1, +1, 14, 28, 45)	0	264 ± 73	412 ± 104	381 ± 172
Group C (GK1.5 days 28, 45)	759 ± 70	769 ± 117	863 ± 238	1,748 ± 605

IgG anticollagen antibody titers from control and GK1.5-treated mice (see Table II, Exp. 1). Sera was collected at days 14, 28, 45, and 70 after immunization with native type II collagen.

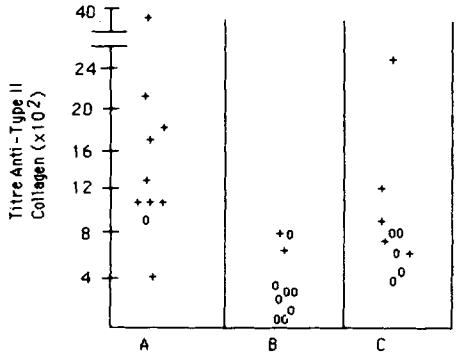

FIGURE 1. Day 45 IgG anti-type II collagen titers from individual mice immunized on day 0. Data from individual animals from Table II, Exp. 1. Group A, controls; group B, GK1.5 day −1, +1, 14, 28, 45; group C, GK1.5, day 28, 45. (+) Mice displaying arthritis; (0) mice without arthritis.

of the limbs from nonarthritic mice had subclinical evidence of arthritis, limbs were removed, fixed, sectioned, and stained with hematoxylin-eosin. Clinically involved and noninvolved limbs from arthritic mice were also examined histologically, by an evaluator who did not know the clinical status. There was complete correlation between the clinical and histologic evaluation. Thus, limbs from arthritic or nonarthritic mice that appeared free of clinical disease also showed no histologic evidence of arthritis.

Effect of anti-L3T4 on anticollagen antibody titer. Serum samples were obtained on days 14, 30, 45, and 70 and the antibody titers from control mice and treated animals are shown in Table III. Group A showed high IgG antibody titers to type II collagen by day 14, which were enhanced by a second injection of type II collagen. Titers from group B were not detectable on day 14 and remained significantly ($P < 0.005$) below the control titers through day 70. Group C had antibody titers equal to the control group on days 14 and 28, at which time they received their first injection of anti-L3T4. On day 45 the average titer of this group was significantly lower than the control ($P < 0.1$) but was equivalent by day 70. These data suggest that pretreatment with anti-L3T4 is associated with decreased anti-type II collagen IgG production in addition to decreased arthritis incidence. Anti-L3T4 treatment after a strong IgG response to collagen was underway appeared to delay the antibody response to the second injection of type II collagen, but did not affect the expression of the disease.

A comparison of antibody titer and arthritis incidence for individual animals revealed no strict correlation in groups A or C (Fig. 1). While all the mice in

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these groups had high titers to type II collagen, those with the highest titers did not in all cases show signs of arthritis. However, in group B, the two mice that eventually developed arthritis had the highest titers of that group. A possible explanation of these results is that a critical level of antibody must be reached to initiate arthritis development but that increases above this level do not necessarily influence disease expression or incidence. These findings also underscore the point that the presence of high antibody titer to type II collagen is not the only prerequisite for the development of arthritis.

Discussion

The effects of the in vivo administration of anti-L3T4 on T cell subset distribution and CIA expression can be summarized as follows: (*a*) treatment with anti-L3T4 resulted in a >90% deletion of the L3T4$^+$ population of T cells in lymph nodes and spleen, which gradually returned to normal numbers by 30 d after treatment; (*b*) administration of anti-L3T4 before immunization with type II collagen resulted in significantly decreased incidence and delayed onset of CIA and was associated with lower IgG antibody titers to type II collagen; (*c*) treatment with anti-L3T4 after the antibody response to type II collagen was already underway but before disease onset did not significantly affect disease expression; (*d*) while humoral response to type II collagen appears to be a necessary prerequisite for CIA development, the presence and intensity of such a response is not predictive of disease expression.

The mechanisms responsible for tissue damage in CIA are not fully resolved. Evidence for the involvement of both cellular and humoral immunity to type II collagen has been reported (10–12). Our finding that administration of anti-L3T4 before immunization with type II collagen resulted in a significantly decreased anticollagen IgG response and lower incidence of arthritis supports a role for anticollagen antibody in the induction of the disease, but does not answer the question of whether cellular mechanisms may be involved in full disease expression.

The precise mechanism by which the depletion of L3T4$^+$ cells reduces the anticollagen response is not known. However, since the response to collagen is T cell dependent (13), it is likely that the inhibition is due to some quantitative reduction in helper cell activity. It will be important to determine if the reduction in helper cell activity is due to simple depletion of helper cells, reversal of the helper to suppressor cell ratio, direct blocking effects upon helper cell function, or induction of suppressor cells.

Administration of anti-L3T4 antibody after the development of an antibody response to type II collagen but before the development of arthritis failed to inhibit the disease process. Although this might implicate the exclusive role of antibody to collagen in the development of the disease, it is also possible that the protocol adopted may not have been appropriate for suppressing intrasynovial T cell activity.

The results of these experiments suggest a critical role for L3T4$^+$ T cells in the pathogenesis of CIA with regard to helper activity in the development of the anticollagen response. Their role in the ongoing disease is less clear. The in vivo use of monoclonal antibody directed at distinct T cell subsets offers a powerful tool for dissecting this process and may lead to therapeutic modalities for autoimmune diseases in humans.

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Summary

The effect of in vivo administration of monoclonal anti-L3T4 antibody on the development of murine collagen-induced arthritis (CIA) was assessed. Treatment with anti-L3T4 resulted in a >90% depletion of $L3T4^+$ T cells in lymph nodes and spleen, an effect that appears entirely reversed 30 d after treatment. Administration of anti-L3T4 before immunization with type II collagen resulted in a significant decrease in arthritis incidence and delayed onset of the disease while treatment begun after a strong anticollagen IgG humoral response was underway was not effective in altering disease expression. These results suggest a prominent role for $L3T4^+$ T cells in the pathogenesis of CIA.

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