

Expression of CD44 in Synovium of Rabbits with Chronic Arthritis Induced by Immunization with *Escherichia coli*

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ABSTRACT. *Objective.* To investigate the expression of CD44 and its role in experimental chronic arthritis in rabbits.

Methods. Rabbits were immunized with *Escherichia coli* O:14 for short (4 mo) and long (8–10 mo) periods. Immunohistochemical staining was performed on knees, using anti-CD44 antibodies.

Results. Lymphocyte infiltration in the synovium was found in 30.0% of rabbits after short term immunization, and the rate increased to 58.3% after longterm immunization. CD44 was present in synovial lining cells in 30.0% of rabbits after short term immunization, and it increased significantly ($p < 0.05$) after longterm immunization (66.7%). CD44 was also observed in lymphocytes in knee synovium after longterm immunization (25.0%).

Conclusion. CD44 in lining cells might play a role in promoting chronic arthritis in rabbits immunized with *E. coli*. (J Rheumatol 2001;28:2579–82)

Key Indexing Terms:

ESCHERICHIA COLI O:14 RABBIT RHEUMATOID ARTHRITIS CD44

Rheumatoid arthritis (RA) has been characterized as chronic arthritis with hyperplasia and lymphocyte infiltration of synovium, and by appearance of rheumatoid factor (RF) in serum. To elucidate the etiology of RA and to find a therapeutic intervention, establishment of an animal model with these features is essential. Aoki, *et al* reported that immunization with *Escherichia coli* O:14 induced chronic arthritis in rabbits including pannus formation, ankylosis of joints, and serum rheumatoid factor-like substance (RFLS)^{1,2}.

Recently, upregulation of adhesion molecule CD44 in rheumatoid synovium^{3,4} and chondrocytes⁵ has been described, and its roles in lymphocyte homing and cytokine production in arthritis have been studied^{6,7}. Our studies revealed that CD44 appeared in the synovia and chondrocytes of mice with collagen induced arthritis⁸.

Using immunohistochemistry, we investigated expression of CD44 in synovium of rabbits immunized with *E. coli* O:14 for short (4 months) and long (8–10 months) periods.

MATERIALS AND METHODS

Animals. We studied 38 female New Zealand White (NZW) rabbits, bred in a closed colony, weighing 2 to 2.5 kg, and 4 to 6 months old. All rabbits were

caged individually and fed a commercial pellet diet (Oriental Yeast Company, RC4) and water ad libitum.

Antigen. *E. coli* O:14 strain was obtained courtesy of Dr. M. Yoshikawa (Department of Bacteriology, Institute of Medical Science, University of Tokyo). According to a method described by Aoki^{1,2}, *E. coli* was inoculated into tryptic soy broth (Difco, Detroit, MI, USA) and incubated at 37°C for 24 h on tryptic soy agar (Baltimore Biological Laboratories, Baltimore, MD, USA). After being harvested in saline, the suspension was heated to 100°C for 2 h and adjusted to a concentration of 1 mg/ml (wet weight) in saline.

Immunization. Twenty-two NZW rabbits were injected intramuscularly at multiple sites on the back at monthly intervals with 2 ml heat killed *E. coli* O:14 (1 mg/ml) suspended in an equivalent volume of Freund's incomplete adjuvant. The animals were divided into 2 groups, 10 rabbits in Group 1 and 12 rabbits in Group 2. The rabbits in both groups were sacrificed and both knees were resected 4 mo and 8–10 mo after the beginning of immunization, respectively.

As controls, rabbits were injected at the same sites and intervals as Groups 1 and 2 with 2 ml saline suspended in an equivalent volume of Freund's incomplete adjuvant and were divided into 2 groups: Group 3 and Group 4. Five rabbits (Group 3) were sacrificed at 4 mo and 6 rabbits (Group 4) at 10 mo after the beginning of immunization. Five untreated rabbits (Group 5) were sacrificed at 14 to 16 months of age.

All blood samples were taken from the marginal ear vein weekly for 15 weeks.

Histological examination. The hemilateral knees of sacrificed animals were fixed in 10% neutral formalin, decalcified in formic acid, and embedded in paraffin. Sections were stained with hematoxylin and eosin. For immunohistochemical examination, a Vecstain ABC-GO kit was used. Sections were treated with phosphate buffered saline (PBS) and 5% normal goat serum to reduce nonspecific background staining. Then they were incubated overnight with monoclonal rat anti-mouse CD44 antibody solution (20 µg/ml) (Pharmingen, San Diego, CA, USA). After 3 washes with PBS, the sections were incubated with biotinylated anti-rat IgG antibodies for 2 h. After 3 further washes with PBS, the sections were incubated with glucose oxidase labeled avidin for 1 h. Glucose oxidase labeling was visualized with ABC-GO substrate. Positive CD44 staining with the monoclonal antibodies was observed in lymphocytes in rabbit liver and kidney tissue fixed in 10% neutral formalin.

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Serological method. The hemagglutination (RAHA) test (RAHA kit, Fujizoki Co. Ltd., Tokyo, Japan) was performed to quantitate IgM-RFLS in the rabbits' sera⁹. Briefly, all sera were inactivated at 56°C for 30 min. Tanned sheep erythrocytes coated with aggregated rabbit gammaglobulin (heated at 70°C for 10 min) were added to 2-fold serial dilutions of the test serum. After incubation at room temperature overnight, agglutination of the erythrocytes was examined. A titer > 80 was considered positive.

Statistics. Mann-Whitney U test was used for comparison of group means of RAHA titers. Chi-square test was used for comparison of frequencies of pathological changes in independent groups. Statistical significance was set at the $p < 0.05$ level.

RESULTS

Lining cell hyperplasia in synovium of the knee was observed in rabbits after short term (4 mo) immunization with *E. coli* O:14 (70.0%). The rate increased to 91.7% after longterm immunization (8–10 mo). Lymphocyte infiltration in the synovium was found in 30.0% of rabbits after short term immunization, and the rate increased to 58.3% after longterm immunization (Figure 1, Table 1).

Immunohistochemically, CD44 was positive in synovial lining cells in 30% of rabbits after short term immunization, and it increased significantly ($p < 0.05$) after longterm immunization (66.7%). CD44 was also observed in lymphocytes in knee synovium after longterm immunization (25.0%) (Figure



Figure 1. Histological specimen from knee of a NZW rabbit after 10 month immunization with *E. coli* O:14. Note marked infiltration of lymphocytes in the synovium (original magnification $\times 100$).

Table 1. Pathological changes and expression of CD44 in synovium of knees of rabbits immunized with *E. coli* O:14.

	Immunization	
	4 Months, n = 10	8–10 Months, n = 12
Lining cell hyperplasia, percentage of rabbits positive for hyperplasia	70.0	91.7
CD44 positive lining cells	30.0*	66.7*
Lymphocyte infiltration	30.0	58.3
CD44 positive lymphocytes	0	25.0

* $p < 0.05$.

2, Table 1). In the longterm immunized group, 7 of 8 rabbits with CD44 positive lining cells showed lymphocyte infiltration. None of the above findings were seen in the controls (Groups 3, 4, 5).

RFLS appeared in the serum of rabbits 3 weeks after the initiation of immunization with *E. coli* O:14. The rates of RFLS positive rabbits after short and longterm immunization were 70.0% and 83.3%, respectively. The average titer of RFLS after longterm immunization was 1:400, significantly higher ($p < 0.05$) than that after short term immunization (1:244).

There was no significant difference in RFLS titer at the time of sacrifice between rabbits with CD44 positive lining cells and those with CD44 negative cells.

DISCUSSION

Hyperplasia of the lining cells and lymphocyte infiltration of synovium observed in this animal model are common features of patients with RA. In addition, RFLS also appeared in the serum of rabbits¹, suggesting that immunization with *E. coli* induced a kind of synovitis that closely resembled that of RA. Our previous studies also revealed that hyperimmunization with lipopolysaccharide (LPS) extracted from *E. coli* O:14¹⁰ or repeated injection with LPS¹¹ induced chronic arthritis in rats. Concerning the mechanism of developing arthritis of this model, LPS has been reported to work on polyclonal activation of B cells¹² and to stimulate various cell types to produce proinflammatory cytokines and enzymes^{9,13–15}. These findings suggested that prolonged presence of LPS might cause arthritis of this model.

Recently, upregulation of CD44 has been reported in joint tissue of patients with RA^{3–5}. CD44, an 85 kDa glycosylated molecule, was reported as a receptor for hyaluronate, with homology to cartilage link protein in the N-terminal sequence^{6,7}. CD44 in synovium may mediate cell to matrix interaction and may facilitate lymphocyte homing to inflamed tissue as an adhesion molecule. Further, signal transduction through the CD44 molecule was reported to promote production of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor- α (TNF- α)¹⁶. Synovial lining cells were found to synthesize IL-1 and TNF- α in the presence of LPS¹³. These cytokines were observed to stimulate CD44 expression in the lining cells^{17,18}. These findings suggested there may be positive feedback mechanisms in rheumatoid synovium that accelerate the inflammation. Our study revealed that CD44 expression in the synovial lining increased with a longer period of immunization with lymphocyte infiltration to synovium, suggesting that CD44 might play an important role in lymphocyte homing, through signal transduction for cytokine synthesis from lining cells.

Concerning the differences of animal models and human RA, *E. coli* induced arthritis in rabbits has 2 advantages compared to other models for RA — the appearance of serum RFLS and the relevance of the etiology of RA to enterobacte-

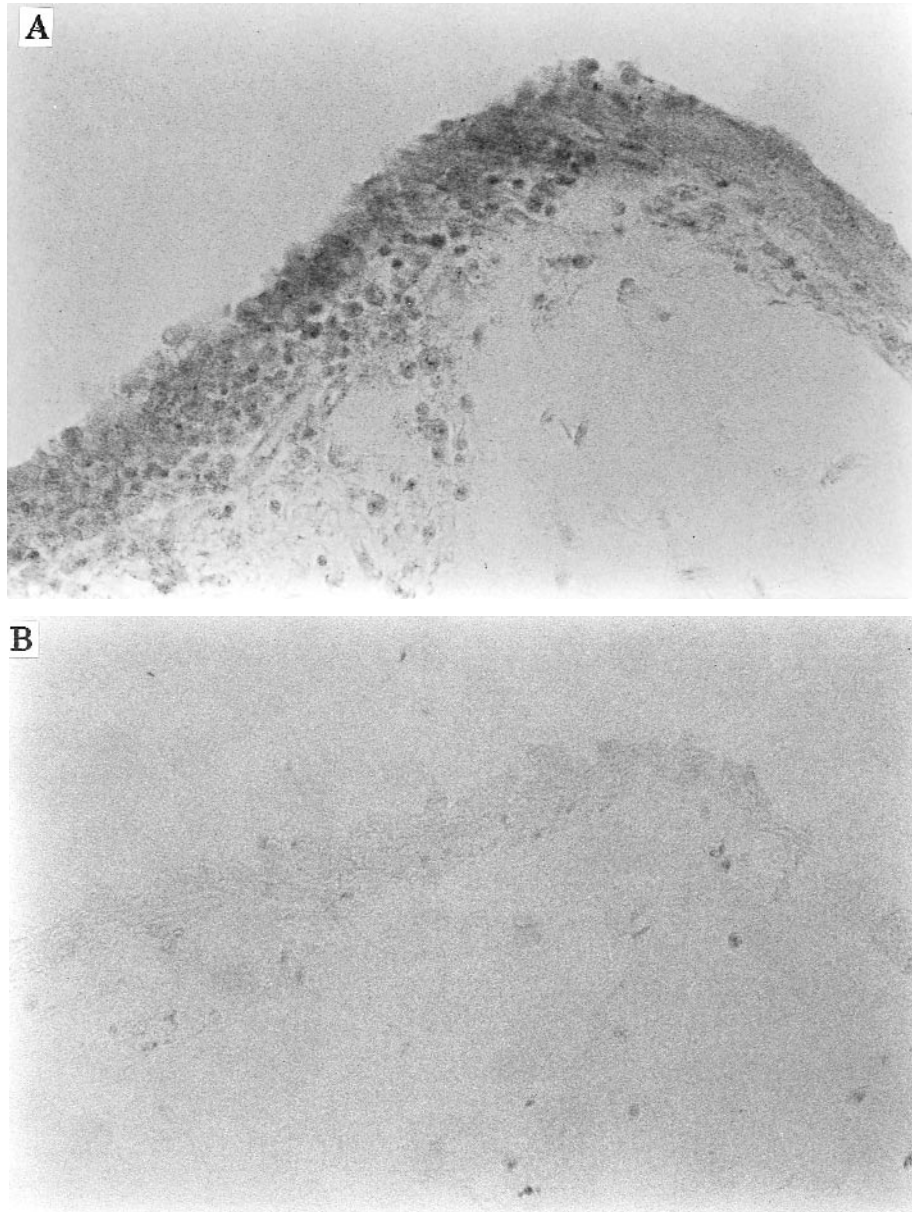


Figure 2. Immunohistochemical staining for CD44 in the knee of a NZW rabbit after 10 month immunization with *E. coli* O:14. A. Note CD44 positive hyperplastic lining cells and CD44 positive infiltrated lymphocytes (original magnification $\times 200$). B. Note negative staining of CD44 in the tissue treated with control rat IgG (original magnification $\times 200$).

rial antigens. Aoki, *et al* reported that anti-*E. coli* O:14 antibodies were detected in 39.8% of serum and 65.5% of synovial fluids of patients with RA, indicating that a significant number of RA patients have been sensitized with *E. coli* O:14¹⁹. Despite the prevalence of RF in patients with RA, its role in rheumatoid inflammation is not clear. Reports on the relevance of the serum level of RF and disease activity are controversial. In this study, there was no significant relationship between the level of serum RFLS and CD44 expression in the synovium; however, in our previous study using this model, lymphocyte infiltration in the synovium was observed

more frequently in the rabbits with early appearance of RFLS in serum (before 8 weeks after immunization)²⁰. Further investigation of the adhesion molecule, RFLS, and the mechanism of synovitis in this model is needed.

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