# Anti-Human Type II Collagen CD19+ B Cells Are Present in Patients with Rheumatoid Arthritis and Healthy Individuals

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*ABSTRACT.* ***Objective.*** To determine the frequency and repertoire of CD19+ B cells capable of producing antibodies reactive to type II collagen (CII) in synovial fluid (SF) and peripheral blood (PB) of patients with rheumatoid arthritis (RA) and PB of healthy control individuals.

***Methods.*** CD19+ B cells were isolated and activated to secrete immunoglobulins (Ig) by CD4+ T cells. Frequencies of anti-CII B cells were determined by limiting dilution analysis. The isotype and cross-reactivity of the antibodies produced were determined by ELISA.

***Results.*** SF and PB from 5 patients and PB from 4 healthy controls were analyzed. Anti-CII CD19+ B cells were identified in all samples tested. In the RA SF, the percentage of activated B cells reactive to human CII was significantly higher than in the PB of patients with RA ($p < 0.05$) or controls ($p < 0.01$). A majority of anti-human CII B cells from patients' SF secreted IgG isotype, whereas most anti-human CII B cells in PB of patients and controls secreted IgM. The anti-CII B cells, regardless of source, are usually reactive to both native and denatured human CII, to different types of human collagens, and to type II collagens from different species.

***Conclusion.*** Anti-CII CD19+ B cells responsive to activated helper T cells are present in both patients with RA and healthy individuals. However, these B cells, especially those secreting the IgG isotype, accumulate in the inflamed joints of RA patients. (J Rheumatol 2001;28:2168–75)

*Key Indexing Terms:*
RHEUMATOID ARTHRITIS SYNOVIAL FLUID COLLAGEN
B LYMPHOCYTES IMMUNOGLOBULINS

Rheumatoid arthritis (RA) is an inflammatory disease of joints characterized by chronic synovitis. Although its etiology is unknown, chronic immune stimulation is felt to be involved in the pathogenesis. The source of antigens inducing and perpetuating the immune response remains unidentified. However, type II collagen (CII), the main component of hyaline cartilage, is a likely candidate.

Evidence that antibodies to CII can provoke inflammatory arthritis comes from studies of experimental arthritis in animals. Intradermal injection of native homologous or heterologous CII emulsified in complete Freund's adjuvant induces a chronic polyarthritis (CIA) in genetically susceptible strains of mice including animals bearing HLA-DR1 or DR4 as transgenes[1-6]. Arthritic animals develop high levels of anti-CII antibodies and passive transfer of the antibodies to naive recipients results in their deposition within the joint, producing an acute arthritis with cartilage destruction[7-11]. When B cells are deleted, or when T cell–B cell interaction is blocked by anti-gp39 or CTLA-4, the animals exhibit no measurable titers of anti-CII antibodies, show no signs of arthritis, and do not demonstrate the typical histopathological manifestations of the disease[12-14].

We and others have shown that patients with RA have autoantibodies to CII[15,16]. While circulating antibodies to CII are found only in a proportion of patients[17-19], they are almost universally present in cartilage removed from patients undergoing joint replacement surgery. It has also been shown that cells secreting anti-CII antibodies are present in synovial tissue, and synovial fluid (SF) in a majority of patients[20-24]. Antibody secreting cells consist mainly of plasma cells, which lack CD19 or CD20 molecules on their surface. In contrast, much less information is available regarding anti-CII CD19+ B cells in RA[25]. The CD19+ B cells coexpress CD20 and include both naive and memory B cells. These B cells do not spontaneously secrete immunoglobulin *in vitro*, but can be activated and become antibody secreting B cells with help from activated CD4+ helper T cells[26]. It has been reported that in the inflamed joints of patients with RA, a large majority of CD20+ B cells are longterm memory B cells[27,28]. The repeated chronic activation of the memory B cells might play a role in the chronicity and periodic relapse of RA.

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The studies reported here provide information about the anti-CII CD19+ B cells in RA. We developed a T cell–B cell coculture system with which we are able to stimulate the purified B cells using for support a selected CD4+ T cell line stimulated by immobilized anti-CD3 monoclonal antibody. Using this system, we compared the frequencies and the isotypes of anti-CII B cells in the SF and peripheral blood (PB) of patients with RA and the PB of healthy control individuals. The frequencies of the cells were determined by limiting dilution analysis, and the isotypes of the cells were analyzed at the clonal level.

## MATERIALS AND METHODS

*Study subjects*. Five RA patients ages 28–77, with disease duration of 6–20 years, were randomly identified from the outpatient clinics of the Section of Rheumatology, VA Medical Center and University of Tennessee. Full informed consent was obtained from all participants. All patients fulfilled the American College of Rheumatology diagnostic criteria for seropositive RA[29]. Three patients had anti-CII antibodies in their serum and all had antibodies to CII in their SF. Four healthy controls, who had no personal or family history of inflammatory joint disease, were also studied.

*Preparation of collagens*. Native collagens were obtained by limited pepsin digestion of human, bovine, and chicken cartilage as described[30]. The α1 chains of CII were purified from the native collagen by thermal denaturation and ion exchange chromatography on carboxy-methyl cellulose as described[31].

*Purification of B cells from PB and SF.* Mononuclear cells were purified from heparinized PB by Histopaque-1077 density gradient centrifugation. For B cell purification, $2 \times 10^7$ mononuclear cells were suspended in 1 ml of RPMI containing 2% fetal calf serum (FCS) and mixed with 50 $\mu$l Dynabeads M-450 Pan-B (CD19, Dynal Inc., New York, NY, USA). Samples were incubated at 2–4°C for 15–20 min with gentle tilting and rotation. Rosetted CD19+ B cells were isolated and washed 5 times by placing the test tube in the Dynal magnetic particle concentrator for 2–3 min and removing the supernatant containing the nonrosetted cells from the tube. To detach the CD19+ B cells, the rosetted cells were resuspended in 100 $\mu$l of RPMI containing 2% FCS and incubated with 10 $\mu$l DetachaBead (Dynal) for 45–60 min at room temperature. Detached CD19+ B cells were isolated by placing the test tube in the concentrator and collecting the supernatant. Collected cells were washed to remove the DetachaBead.

For purification of B cells from SF, the fluid was diluted 1:5 with RPMI and centrifuged. After washing, $5 \times 10^7$ cells were resuspended in 1 ml of RPMI containing 2% FCS and mixed with 50 $\mu$l Dynabeads M-450 Pan-B. Processing was as described above.

*Selection for a human CD4+ T cell line capable of providing B cell support*. The generation and maintenance of human CD4+ T cell lines at clonal level has been described[32]. To select a line to provide B cell support, 56 CD4+ T cell lines at $1 \times 10^5$ cells/culture were cocultured with the B cells at $1 \times 10^4$ cells/culture from PB of a healthy individual in the presence of immobilized anti-CD3 in 96 well flat bottom microtiter plates. Antibody to CD3 was purified from ascites fluid of nude mice injected with an anti-CD3 producing hybridoma cell line (American Type Culture Collection, Rockville, MD, USA). For anti-CD3 stimulation, the plates were coated with antibody to human CD3 (50 $\mu$g/ml in phosphate buffered saline) and incubated overnight at 4°C. Plates were used directly after washing twice to remove the unbound antibodies. After 14 days, the content of IgM and IgG in the supernatant of the cultures was analyzed. The T cell line that induced the highest amount of Ig production, H20, was selected for all T cell–B cell coculture experiments.

*T–B cell cocultures*. Purified B cells were cocultured with H20 at a concentration of $1 \times 10^5$ cells/culture in 96 well flat bottom microtiter plates in the presence of anti-CD3. Average number of B cells added per culture varied according to the experiment. For studies of the time course of antibody production, $1 \times 10^3$ B cells per culture were used. For studies of the anti-CII B cells at the clonal level, a limiting number of B cells was used, so that cultures producing anti-CII antibodies accounted for less than 30% of the total cultures established. For limiting dilution analysis, replicate cultures (32 cultures per B cell concentration) with serial dilutions of B lymphocytes at 1, 3, 10, 32, 100, 316, and 1000 cells/culture were established. In some analyses, additional B cell concentrations at 3162 and/or 5000 cells/culture were also included. Control cultures included replicate cultures with anti-CD3 activated T cells in the absence of B cells. Culture supernatants were harvested after 14 days and analyzed for production of IgM and IgG and antibodies against human CII by ELISA.

*Determination of concentrations of IgM, IgG, and antibodies against CII*. For measuring total IgM and IgG, Maxisorb plates were coated with a polyvalent goat anti-human Ig (Sigma Chemical Co., St. Louis, MO, USA) and blocked by 2% bovine serum albumin. Diluted culture supernatants were added and the plates were incubated at 4°C overnight. After washing, second antibody consisting of peroxidase conjugated goat anti-human IgM or IgG (Sigma) was added and incubation continued for 1–2 h. The plates were washed again and developed with o-phenylenediamine dihydrochloride in substrate buffer consisting of citric acid, $Na_2HPO_4$, $H_2O$, and $H_2O_2$. Optical densities were measured at a wavelength of 490 nm with a reference wavelength of 650 nm. Standard solutions of IgM and IgG were included in the assays.

To detect antibodies against CII, Maxisorb plates were coated with 5 $\mu$g/ml of CII in phosphate buffer, 0.4 ionic strength, pH 7.4. Subsequently, immunoassay plates were handled and incubated identically as in the IgM and IgG immunoassays. As a developing antibody, goat anti-human IgM and/or anti-human IgG coupled with biotin (Sigma) were added, followed by ExtrAvidin conjugated with peroxidase (Sigma). For each sample measured, parallel noncoated wells were used as controls for nonspecific binding.

In the limiting dilution analysis, microcultures were scored positive if the OD values in the ELISA measuring immunoglobulin or anti-CII antibody concentration in the culture supernatant exceeded the mean OD value in the control cultures by more than 3 standard deviations. The data were analyzed by linear regression analysis, assuming a Poisson distribution. Frequencies were calculated from the slope of the curves.

*Statistical analysis*. Statistical evaluation of differences in numeric values was performed using paired and unpaired T test. When the normality test in unpaired T test failed, Mann-Whitney rank sum test was used. For differences in numbers of observations in categories, Fisher's exact test was used. Data were judged statistically significant when $p < 0.05$.

## RESULTS

*Activation of anti-CII CD19+ B cells by anti-CD3 stimulated helper T cells*. To study the CD19+ B cells with potential to secrete anti-CII antibody, we cocultured B cells with H20 stimulated by immobilized anti-CD3 Mab. Using this system, B cells were stimulated nonspecifically. The stimulation is so efficient that a single CD19+ B cell, as identified by expression of a unique VH chain and a unique CDR3 region after clonal expansion, can be induced to proliferate and produce detectable antibodies[33]. The selected T cell line used in this study, H20, was a Th0 line that did not react to any alloantigens tested and that secreted high levels of interleukin 2 (IL-2) (3004 pg/ml), interferon-γ (12,672 pg/ml), IL-4 (694 pg/ml), IL-5 (952 pg/ml), and IL-10 (1018 pg/ml) upon anti-CD3 stimulation. It was selected from a panel of 56 CD4+ T cell lines established at limiting dilution condition according to their ability to provide T cell help in the presence of anti-CD3. When B cells were cocultured with CD4+ T cell line without additional T cell stimulation, no immunoglobulin

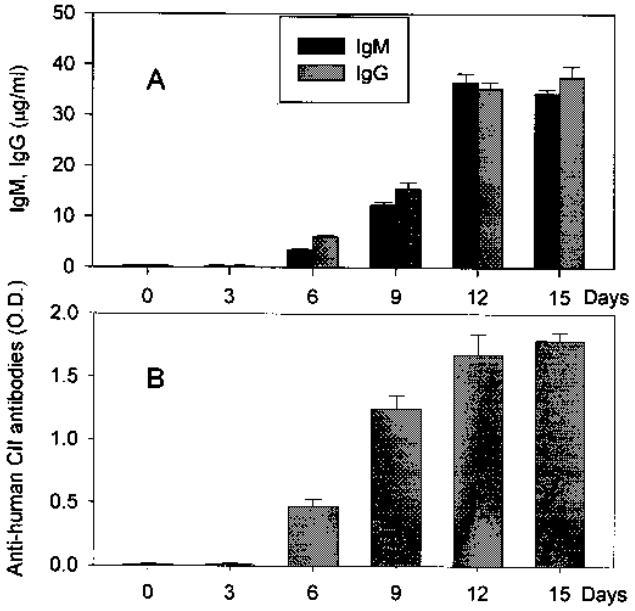


*Figure 1*. The time course of IgM, IgG, and anti-CII antibody production. A. CD19+ B cells ($1 \times 10^3$ cells/culture) from peripheral blood of a patient with RA were cocultured with a T cell line, H20, at $10^5$ cells/culture in the presence of anti-CD3 monoclonal antibody. Supernatants (10 ml) were taken from each culture at the days indicated and replaced with same amount of fresh culture media. The supernatants were assayed by ELISA for the presence of IgM, IgG using either goat anti-human IgM or anti-human IgG as developing antibodies (A) and anti-human CII antibodies by using a mixture of goat anti-human IgM and anti-human IgG as developing antibodies (B). The data are given as mean (± SD) of duplicated ELISA measurements. For anti-CII antibody production, multiple cultures were set up and assayed, but only one of the positive cultures is illustrated here.

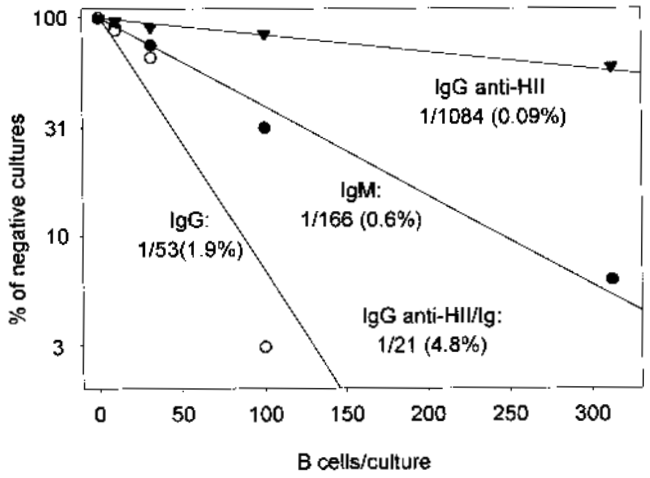


*Figure 2*. Frequency of anti-human CII CD19+ B cells of a patient with RA. A series of replicate cultures with serial dilutions of synovial CD19+ B cells were established in the presence of CD4+ T cell line at $10^5$ cells/culture stimulated by anti-CD3. After 14 days the cultures were analyzed for the presence of IgM, IgG, and anti-human CII antibodies by ELISA. Cultures were scored positive if OD values in the ELISA exceeded the mean OD value in the control cultures by more than 3 SD. The frequencies of IgM, IgG, and anti-human type II antibodies were calculated based on Poisson distribution. Both IgM and IgG anti-human CII antibodies were analyzed, but only IgG antibodies are shown. HII: human CII.

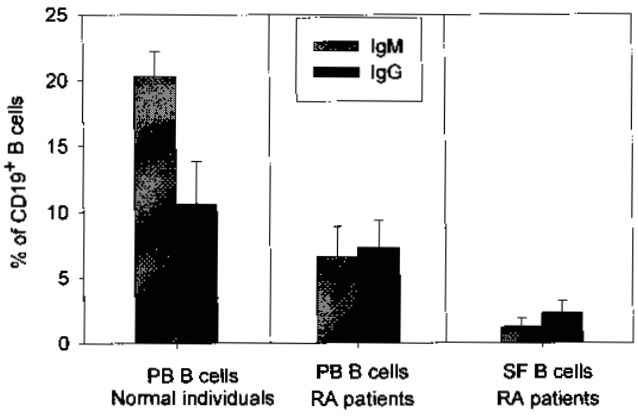


*Figure 3*. Comparison of frequencies of CD19+ B cells that could be stimulated to secrete IgM and IgG. CD19+ B cells were purified from the peripheral blood (PB) and synovial fluid (SF) of 5 RA patients, and from the PB of 4 healthy controls. Limiting dilution analysis was used to estimate the frequencies of IgM and IgG producing B cells activated by anti-CD3 stimulated helper T cells, as given by percentage of CD19+ B cells (mean ± SD). The frequencies were calculated based on Poisson distribution as in Figure 2.

secretion could be detected[34]. Upon activation of the T cells with immobilized anti-CD3 antibody, B cells began to proliferate and synthesize IgM and IgG (Figure 1A). In some of the cultures antibodies against type II collagen were detected (Figure 1B). Usually the antibody secretion could not be detected until after 5–6 days of culture. Antibodies accumulated rapidly at 6–12 days and reached a peak at 12–15 days.

*Frequencies of anti-CII B cells in patients and controls*. To determine the frequency of B cells that could be induced to secrete antibodies against human type II collagen, limiting dilution analysis was used. CD19+ B cells were purified from SF and PB of the 5 patients and the PB of 4 controls. Replicate cultures (32 cultures per B cell concentration) of serial dilutions of B cells were cocultured with H20 at a concentration of $1 \times 10^5$ cells/culture in the presence of anti-CD3. After 14 days, supernatants were collected, and the concentration of IgM, IgG, and anti-CII antibodies in aliquots of each culture was determined by ELISA. The frequencies of B cells that could be stimulated to secrete IgM and IgG and anti-CII antibodies were calculated based on Poisson distribution (Figure 2). The frequencies of those secreting IgM and/or IgG in RA patients and controls are summarized in Figure 3. In the RA SF only 1.2 ± 0.6% B cells secreted IgM and 2.3 ± 1.1% B cells secreted IgG after anti-CD3 stimulation. The percentages were significantly lower than those obtained from the PB from RA patients (6.6 ± 2.3%, 7.3 ± 2.1%; $p < 0.05$) and controls (20.3 ± 1.9%, 10.6 ± 3.3%; $p < 0.01$). The ratio of IgG to IgM producing B cells in SF was 2.2 ± 0.9, which was about 2 times higher than that in the RA PB (1.1 ± 0.2) and about 4 times higher than that in the control PB (0.5 ± 0.2; $p < 0.05$).

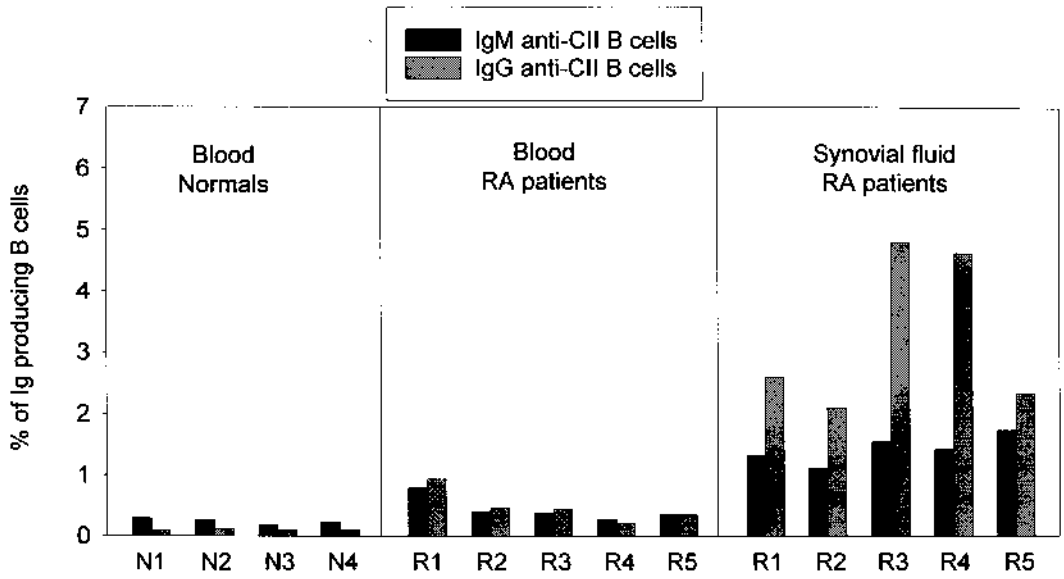


*Figure 4*. Accumulation of anti-human CII B CD19+ cells in the joints of RA patients. CD19+ B cells were purified from peripheral blood and synovial fluid of 5 RA patients (R1–R5), and from peripheral blood of 4 healthy controls (N1–N4). Limiting dilution analysis was used to estimate the frequencies of anti-human CII antibody producing B cells activated by anti-CD3 stimulated helper T cells, as given by percentage of Ig producing B cells after culturing with activated helper T cells. Percentages of cultures producing IgM (black bars) and IgG (shaded bars) antibodies reactive to CII are illustrated.

CD19+ B cells that could be stimulated to secrete anti-CII antibodies were found in all the SF and PB samples of 5 RA patients and in all PB samples of 4 healthy controls. The frequencies of the anti-CII B cells were estimated by percentage of Ig producing B cells after coculturing with anti-CD3 stimulated helper T cells. In the RA SF, 1.4 ± 0.2% and 3.3 ± 1.3% of the B cells produced IgM and IgG anti-CII antibodies, respectively. The percentages were significantly higher than in the PB from RA patients (IgM anti-CII B cells: 0.4 ± 0.2%, $p < 0.01$; IgG anti-CII B cells: 0.5 ± 0.3%, $p < 0.05$) and healthy controls (IgM anti-CII B cells: 0.2 ± 0.05%, $p < 0.01$; IgG anti-CII B cells: 0.1 ± 0.02%, $p < 0.01$). The percentages of both IgM and IgG anti-CII secreting B cells for each of the individuals are shown in Figure 4. In the RA SF, the ratio of IgG anti-CII B cells to IgM anti-CII B cells was 2.3 ± 0.8, which was significantly higher than that in RA PB (1.0 ± 0.2; $p < 0.05$) and in controls (0.4 ± 0.1; $p < 0.01$).

*Isotypes produced by human anti-CII B cells*. Antibodies produced by CII reactive B cell lines were analyzed for isotype (Figure 5). In the RA SF, 37% (7/19) of the B cells secreted IgM, or both IgM and IgG, while 63% (12/19) of the B cells secreted IgG isotype. In the RA PB, the anti-CII B cells secreting IgM, or both IgM and IgG, was increased to 74% (14/19), whereas the IgG from anti-CII B cells was increased 26% (5/19; $p < 0.05$). In contrast to RA patient results, 90% (18/20) of the antibodies secreted by anti-CII B cell lines from controls were IgM, or both IgM and IgG. Only 10% (2/20) of them were IgG ($p < 0.01$). These data indicate that IgG anti-CII B cells exist in both patients with RA and healthy individuals. However, they are significantly enriched in the synovial fluid of patients with RA.

*Reactivities of antibodies to CII*. To estimate the cross-reactivity associated with the anti-CII B cells, we used human type I collagen (CI), bovine CII, and/or purified α1 chain of human CII. The cross-reactivity was found to be common for anti-CII antibodies in both healthy individuals and patients with RA (data not shown). To investigate whether cross-reactivity exists in B cells at the clonal level, we used the antibodies secreted by B cell lines (see Materials and Methods). The lines were established from CD19+ B cells of PB and SF from RA patients and PB from controls. No difference in the cross-reactivities among anti-CII B cells from different sources was

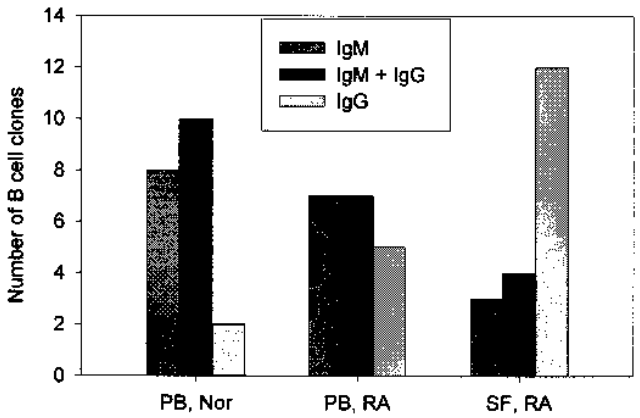


*Figure 5*. Isotype of antibodies reactive with CII. B cell lines were established as described in Materials and Methods. The anti-human CII antibodies secreted by 20 B cell lines from peripheral blood of normal individuals, 19 lines from peripheral blood of RA patients, and 19 lines from synovial fluid of RA patients were analyzed for IgM and IgG isotypes by ELISA. The lines that secreted both IgM and IgG were believed to have undergone a heavy chain switch *in vitro*, while those secreting only IgG an *in vivo* heavy chain switch.

identified. Some B cells recognized predominantly native or denatured forms of CII, others recognized both forms (Figure 6A). The recognitions were studied in 22 B cell lines by comparing the ratio of OD values for native CII to that for α1 chain of CII (Figure 6B). It was found that 17 of the 22 B cell lines (73%) reacted to both native and α1 chain of human CII, with the ratio < 8 and > 1/8. Among them, 11 lines (50%) reacted to both forms of collagen equally well, with the OD ratio ranging form 1/2 to 2. In comparison, 3 of the 22 B cell lines (14%) reacted predominantly to native CII, with the OD ratio > 8, and 2 of the lines (9%) reacted predominantly to purified α1 chain of CII, with the OD ratio < 1/8.

The cross-reactivity of human CII reactive B cells to other types of human collagens and type II collagens from other species were tested in 13 B cell lines. Eight lines (62%) found to be type II collagen-specific were cross-reactive to bovine and chicken CII, but did not react or reacted weakly to human CI and CIII. Three lines (23%) were human collagen-specific — they cross-reacted to human CI and CIII, but did not react or reacted weakly to bovine and chicken CII. The remaining 2 lines (15%) were also cross-reactive, but could not be classified into either of the categories. None of the lines cross-reacted to a control antigen, tetanus toxoid (Table 1).

## DISCUSSION

It has been reported that antibodies to CII are found in the sera of only a proportion of patients with RA[17-19]. However, they are found in the synovial fluid and cartilage of a majority of patients[20,21,24]. This observation has been supported by studies using Elispot assays, which showed that anti-CII antibody secreting cells could be identified in the SF and synovial tissue of a majority of RA patients, but not in any peripheral blood samples obtained in parallel[22,23]. In accord with these studies, our findings indicate that in addition to the anti-CII antibodies and anti-CII antibody secreting B cells, the CD19+ anti-CII B cells that can be activated by anti-CD3 stimulated T cells are selectively enriched in the inflamed joints of RA patients.

There is general agreement that self-reactive lymphocytes are either clonally deleted during development or are functionally inactivated. Studies in transgenic mice indicate both mechanisms are used for the B cell lineage[35,36]. However, we and others have found that immunization by CII in Freund's adjuvant leads to activation of anti-CII B cells and enhanced anti-CII antibody in mice, indicating that tolerance for anti-CII B cells, including clonal deletion, is not complete[7-11]. In humans, rheumatoid factors (RF), antibodies to thyroglobulin, and the cells secreting these antibodies have been observed in peripheral blood of healthy individuals[26,37-39]. But it is still not clear whether healthy individuals, who usually lack anti-CII antibodies in the circulation, harbor functionally competent CII reactive precursor B cells. This study indicated that CD19+ anti-CII B cells exist in the peripheral blood of healthy controls. Interestingly, the actual number of functionally competent peripheral CII reactive B cells that can be stimulated by CD4+ T cells in normal individuals seems to be similar to that

*Table 1.* Cross-reactivities of anti-human CII B cell lines. Anti-CII antibodies secreted by B cell lines established at limiting dilution concentration were tested in duplicate by ELISA for their reactivity to a panel of collagens as shown and a control antigen TET. The intensities of the readings are shown as estimated by OD values.

| B Cell Lines | HI | HII | HIII | ChII | BII | TET |
|---|---|---|---|---|---|---|
| ND1 | ± | + | ± | +++ | ++ | – |
| NF7 | + | +++ | + | +++ | ++ | – |
| PE7 | + | ++ | ± | +++ | ++ | – |
| PF1 | ++ | +++ | – | +++ | +++ | – |
| PF2 | + | ++ | + | +++ | ++ | – |
| PF3 | – | ++ | – | +++ | +++ | – |
| SD8 | + | +++ | + | ++ | ++ | – |
| SF4 | ± | +++ | ± | +++ | +++ | – |
| PC1 | + | ++ | +++ | – | – | – |
| PE9 | +++ | +++ | +++ | + | + | – |
| SD4 | +++ | + | + | – | ± | – |
| NE3 | +++ | + | +++ | ± | ++ | – |
| SE7 | + | +++ | ++ | ++ | ++ | – |

HI: human type I collagen; HII: human type II collagen; HIII: human type III collagen; BII: bovine type II collagen; ChII: chicken type II collgen; TET: tetanus toxoid.

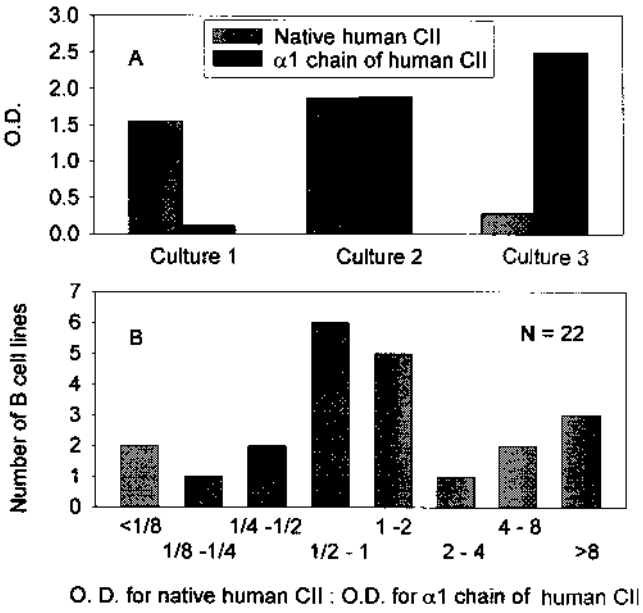


*Figure 6.* Specificity for native and denatured collagen. Anti-human CII antibodies from individual cultures were tested for their reactivity to native human type II collagen and purified α1 chain of human CII by ELISA. A. Three examples showing antibodies that recognized native form of collagen (culture 1), its α1 component (culture 3), or both (culture 2). B. The comparison of recognition by 22 B cell lines of native human CII with purified α1 chain of human CII, as illustrated by the ratio of OD for native human CII to OD for α1 chain of human CII.

in patients with RA. In RA patients, there are higher frequencies of CII reactive B cells, but lower frequencies of CD19+ B cells that can be stimulated to become antibody secreting cells.

The high frequency of CD19+ anti-CII B cells in inflamed RA joints determined by our current experiments may result from several mechanisms. First, the local inflammatory environment, including the presence of the autoantigens, may lead to the aberrant T cell support and abnormal proliferation and differentiation of anti-CII B cells. This concept is supported by the observation that in RA patients, especially those with long disease duration, germinal center-like structures evolve in the inflamed synovial tissue[28]. Because of their location, such germinal centers imply abnormal regulation of lymphocyte activation. B cells with identical sequences, including those in the CDR regions, have been identified within germinal centers in consecutive sections[27]. However, the specificity of these B cells is largely unknown. Second, the chronic inflammatory environment of the inflamed RA joint may facilitate inhibition of apoptosis and maintain the viability of autoreactive B cells. The mechanisms for this are not completely understood, but it has recently been reported that the interaction of synovial stromal cells and nurse-like cells can rescue B cells from apoptosis and promote their survival[40,41]. In addition, synovial CII reactive B cells from RA patients might have a distinct activation state, so that a higher proportion of synovial CD19+ anti-CII B cells are able to mature and produce antibodies when helper signals are provided by anti-CD3 stimulated T cells. This possibility is implied by our studies of RF+ B cell repertoires. We found that healthy subjects and patients with RA share a pool of B cells that secrete RF when activated in the presence of staphylococcal enterotoxin D and T helper cells. In comparison, the fraction of RF+ B cells responding to anti-CD3 stimulated T cells is expanded significantly in RA patients[26]. Thus the CII reactive B cells in RA that are present in the same inflammatory environments might also have become more sensitive to anti-CD3 driven helper T cells.

Schröde, *et al* have characterized the cells in the synovial tissue of RA patients. They reported that the B cells are CD20+. Sequence data show that most of these B cells are highly mutated. On average, 18 substitutions per variable region were seen, indicating that a large majority of them are memory B cells that have undergone multiple cell divisions[27,28]. We observed that the ratio of IgG to IgM B cells, especially the B cells reactive to CII, in SF is higher than that in PB of RA patients and healthy individuals. Analysis of the isotypes of B cell lines established at limiting dilution concentration showed that about 2/3 of the anti-CII B cells secrete IgG, while only 1/3 of the cells secrete only IgM or both IgM and IgG. The secretion of both IgM and IgG at limiting dilutions might result from an *in vitro* heavy chain switch, because the chance for 2 or more anti-CII B cells in the same culture is low, although the possibility cannot be totally ruled out. These data indicate that most CD19+ B cells in RA SF, especially those with anti-CII activity, have undergone heavy chain switching. Therefore, like those in the synovial tissue, most CD19+ B cells in synovial fluid are also memory cells.

Another interesting observation from our study is that the proportion of CD19+ B cells in RA patients, especially the proportion that can be activated to produce IgM and/or IgG, is significantly less than in normal individuals. The reasons for the reduced responsiveness of CD19+ B cells are not fully understood. It is possible that the B cells in RA patients may already be chronically activated, resulting in increased Fas expression[42]. If so, these B cells may be more susceptible to Fas-ligand on the T cells that are activated by anti-CD3 *in vitro*. Alternatively, B cells, especially the synovial B cells, might have some defect along their signal transduction pathways. It has been reported that the T cell receptor mediated signaling in synovial T cells is defective[43,44]. Thus, it is possible that B cells in RA, which are in the same inflammatory environment as the T cells, suffer from a similar defect.

Consistent with our studies, Rudolphi, *et al* reported that the frequency of anti-CII precursor B cells in synovial samples was higher than in PB. However, IgG anti-CII B cells were present in 3 of 7 PB samples (43%), as well as in 5 of 7 synovial fluid/synovial tissue samples (71%) analyzed. IgA anti-CII B cells were detected in 4/5 (80%) of the PB and synovial fluid/synovial tissue samples[25]. The differences might result from the particular cohort of RA patients participating in the studies. In our studies only patients with disease duration > 6 years were studied, while Rudolphi, *et al* included patients with disease duration less than one year. The differences might also be caused by the different B cell stimulation system. Rudolphi, *et al* stimulated the B cells with PMA, conditioned media from activated T cells, and irradiated EL-4 B5 cells. In contrast, we activated B cells with a selected CD4+ T cell line stimulated by anti-CD3, which we believe is closer to the stimulation of B cells *in vivo*.

It is known that some anti-CII antibodies are cross-reactive to different types of collagen and type II collagen from different species[16,22,45]. We investigated the cross-reactivity of antibodies produced by B cell lines. We were able to distinguish anti-CII B cells that recognized conformational epitope on native CII from those that recognized sequential B cell epitopes on the $\alpha$1 chain of collagen. Our data showed that most of the anti-CII B cells recognized both forms of collagen, suggesting that most sequential B cell epitopes are also accessible on native CII. In addition, we observed that the cross-reactivity to different types of human collagen and the CII from different species is a common characteristic of human CII reactive B cells. For most of the CII reactive B cell lines the strongest cross-reactivity was found among the type II collagen among different species rather than different types of collagen. This is consistent with the fact that collagen represents a large macromolecule with limited antigenic variation among species[46].

We observed that the anti-human CII 19+ B cells that can be activated by anti-CD3 stimulated T cells are present in both patients with RA and healthy individuals. These B cells, especially the memory B cells, are significantly expanded in RA synovial fluid. Our estimates of frequency may underestimate the total number of anti-CII B cells, since many synovial B cells cannot be nonspecifically stimulated to produce antibody. Further studies of the subsets of B cells, including their activation requirements, might help us understand the actual magnitude of our observations.


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