Persistence of collagen type II-specific T-cell clones in the synovial membrane of a patient with rheumatoid arthritis

(autoimmunity/pathogenesis/rheumatic disease/autoantigens)

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ABSTRACT  Rheumatoid arthritis is an autoimmune disease characterized by T-cell infiltration of the synovium of joints. Analysis of the phenotype and antigen specificity of the infiltrating cells may thus provide insight into the pathogenesis of rheumatoid arthritis. T cells were cloned with interleukin 2, a procedure that selects for in vivo-activated cells. All clones had the CD4 CDW29 phenotype. Their antigen specificity was tested by using a panel of candidate joint autoantigens. Four of 17 reacted against autologous blood mononuclear cells. Two clones proliferated in response to collagen type II. After 21 months, another set of clones was derived from synovial tissue of the same joint. One of eight clones tested showed a strong proliferative response against collagen type II. The uncloned synovial T cells of a third operation from another joint also responded to collagen type II. The persistence of collagen type II-specific T cells in active rheumatoid joints over a period of 3 years suggests that collagen type II could be one of the autoantigens involved in perpetuating the inflammatory process in rheumatoid arthritis.

Rheumatoid arthritis is a chronic inflammatory disease of synovial joints leading to destruction of cartilage and erosion of bone (1). Currently, the mechanisms underlying the perpetuation of the chronic inflammation and the consequent tissue damage are poorly understood. It is widely considered that the immune system is a major participant in these processes, perhaps through the activation of complement by immune complexes containing rheumatoid factor (2) or by activated immunocompetent cells (3) releasing protein mediators such as interleukin 1 (IL-1), tumor necrosis factor, and interferon-γ.

Study of cell-surface markers and cellular organization has shown that there is a predominance of T lymphocytes in the synovial infiltrate (4), with many T cells showing unequivocal markers of activation such as receptors for IL-2 and HLA class II molecules (5). The HLA class II-bearing tissue cells are intimately related to T cells, permitting the cell interactions for activation to occur (6). These features are shared with other autoimmune diseases and suggest that chronic stimulation of T helper cells by class II-bearing antigen-presenting cells may be one of the major steps involved in disease chronicity (3). It seemed logical therefore to investigate the pathogenesis of rheumatoid arthritis by phenotypic analysis of the infiltrating T cells and by cloning the activated infiltrating T cells by a procedure that we have used successfully in studying other autoimmune diseases (7).

Here we report the cloning of the activated T cells from a patient with active erosive rheumatoid arthritis. In two operative samples, 21 months apart, collagen type II-specific clones were detected at a frequency of ~12%. In a third sample, the uncloned joint T cells also responded to collagen type II. This persistent response suggests a possible role of these T cells in maintaining the chronic inflammatory state in rheumatoid arthritis, since in these joints there is abundant HLA class II and collagen type II available to restimulate the T cells.

MATERIALS AND METHODS

Patient. C.L., a Caucasian female (HLA DR-1,-6), developed polyarthritis diagnosed as rheumatoid arthritis in 1975. Laboratory investigations have shown a consistent lack of IgM rheumatoid factor. There was a low titer of anti-nuclear antibody with activity against DNA–histone and single-stranded DNA. She was treated with gold salts. After 4 years, she relapsed with severe symptoms. A right knee synovectomy was performed, which was the source of the first cloning procedure; 21 months later, because of severe pain, cartilage damage, and loss of mobility, a knee prosthesis was inserted, providing the source of synovium for the second cloning experiments. A third operation, to insert a prosthesis into the other knee was performed a year later.

Heparinized peripheral blood (50 ml) was obtained the day before surgical treatment and again after surgery.Peripheral blood mononuclear cells (PBM) were purified by Ficoll gradient. Synovial membrane was prepared by digestion with collagenase type IV (5 mg/ml) (Cooper Biomedical) and DNase (0.15 mg/ml) (Sigma) at 37°C (8). Some of the cells were cryopreserved and other cells were layered over a Ficoll gradient to purify the mononuclear cells.

Culture. Mononuclear cells purified as described above from the synovial membrane were cultured with purified human recombinant IL-2 (20 ng/ml) (IL-2, Ajinomoto, Kawasaki, Japan) in RPMI 1640 medium supplemented with antibiotics and 10% AB human serum (complete medium) and cloned by a procedure that selects for T cells activated in vivo (8). Briefly, after 1 week of culture with IL-2, the cells were purified over a Ficoll gradient and cloned by limiting dilution (0.3 cell per well) in Terasaki plates, with irradiated (4000 rad; 1 rad = 0.01 Gy) autologous PBM (10⁵ cells per ml), IL-2 (20 ng/ml), and the monoclonal antibody OKT3 (30 ng/ml). The expanding clones were transferred every week in fresh complete medium supplemented with IL-2 (20 ng/ml), OKT3 (30 ng/ml), and irradiated (4000 rad) autologous PBM.

Antigens. Collagen type II was prepared as described (9). The following antigens were generously provided: cartilage.

Abbreviations: AMLR, autologous mixed lymphocyte reaction; PBM, peripheral blood mononuclear cells; IL, interleukin.

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matrix protein and proteoglycans [M. T. Bayliss (10)]; mycobacterium tuberculosis soluble extract [Medical Research Council Tuberculosis Unit, Hammersmith Hospital (11)]; peptide P62 of the Epstein–Barr nuclear antigen [G. R. Rhodes (12)]; IgM rheumatoid factor and human IgG isolated from a rheumatoid patient [P. Venables (13)]; type I collagen (T. Hardingham, Kennedy Institute of Rheumatology).

Assays. The proliferative assay was performed with 10^4 cloned T cells in the presence of 2 × 10^4 irradiated (4000 rad) autologous PBM as a source of antigen-presenting cells and different antigenic preparations at various concentrations (1–100 μg/ml) in 200 μl of complete medium in a 96-microwell plate for 72 hr (8). Wells were pulsed with 1 μCi of [3H]thymidine (1 Ci = 37 GBq) (Amersharm) 10 hr before they were harvested onto glass fiber paper with a semiautomated cell harvester (Flow Laboratories, Titerkect 550). The strips were then assayed in a liquid scintillation spectrometer (LKB 1219 rack β). The results are expressed as the arithmetic mean cpm of triplicate wells. The standard errors were all within 10% of the means.

For fluorescence analysis, 2 × 10^5 cells were incubated for 30 min at 41°C with the optimal concentration of the monoclonal antibodies listed below. The cells were then washed twice with cold RPMI 1640 medium supplemented with 5% fetal calf serum. The samples in which unconjugated monoclonal antibodies were used were then incubated with goat fluorescein isothiocyanate-conjugated-anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) at a working dilution of 1:100, and all the samples were washed twice; 10^4 cells were analyzed with a FACStar (Becton Dickinson). The following monoclonal antibodies were used: Leu 4 (anti-CD3) (Becton Dickinson), Leu 2 (anti-CD8) (Becton Dickinson), Leu 3 (anti-CD4) (Becton Dickinson), 4B4 (anti-CDW29) (S. F. Schlossman, Dana–Farber, Boston), 2H4 (anti-CD45R) (S. F. Schlossman), 42/1C1 (anti-V65) (A. Boylston, Saint Mary’s Hospital, London), Mx9 (anti-V68) (S. Carrel, Ludwig Institute, Lausanne, Switzerland).

Anti-Collagen Antibody Assay. Collagen types I–V were diluted to 1 μg/ml in 0.05 M bicarbonate buffer (pH 9.6) and 100 μl was added to each well of a 96-well Titerkect PVC immunoasay plate (Flow Laboratories). The diluted collagen solutions were denatured by heating to 50°C for 15 min and then allowed to cool before coating plates at room temperature for 2 hr; then they were washed three times with phosphate-buffered saline (PBS)/0.1% Tween 20. Doubling dilutions in PBS/Tween were performed on the plates, which were incubated at room temperature for 2 hr before washing three times with PBS/Tween. An alkaline phosphatase-conjugated anti-human IgG (Sigma) was diluted 1:1000 and incubated for 1 hr. After exhaustive washing in PBS/Tween the substrate p-nitrophenyl phosphate (1 mg/ml) in 0.05 M bicarbonate buffer (pH 9.6) was added. After color development, the plate was read on an ELISA plate reader at 405 nm.

RESULTS

Antigen Specificity of Rheumatoid Arthritis T-Cell Clones. In principle, almost any joint constituent could be the relevant autoantigen(s). To maximize the possibility of detecting relevant antigens, T-cell clones were screened with autologous irradiated PBM as antigen-presenting cells and a panel of candidate antigens: rheumatoid factor, human IgG, human collagen types I and II, human cartilage matrix protein, and proteoglycan preparations. Based on results from other groups, mycobacterial antigen (14) and an Epstein–Barr virus-derived peptide (15) were also used. Of 17 clones tested initially, 11 did not show any reactivity in a 3-day proliferation assay, and 4 reacted against autologous PBM, demonstrating the so-called “autologous mixed lymphocyte reaction” (AMLR). Fig. 1 shows the data from one of the AMLR clones. One clone reacted specifically to collagen type II preparation (Fig. 2A), and another clone reacted against autologous peripheral blood lymphocytes but its proliferative response was significantly increased when collagen type II was introduced as antigen (Fig. 2B). These clones were tested on three or four occasions for its specificity, and similar results were obtained in all experiments.

From the second surgical specimen, the reactivity of eight T-cell clones has been assayed. None reacted against autologous blood mononuclear cells. One of these showed a strong response to collagen type II (Fig. 2C). From the third surgical specimen, the uncloned joint cells were assayed directly with antigen and a response to collagen type II was noted (Fig. 3).

Immunophenotyping the T-Cell Clones. Of the panel of clones (n = 65) derived from the first operative sample, a random selection of 20 was expanded and phenotyped by using the panel of monoclonal antibodies. All were CD3+CD4+ and CD8+. Four clones showed the cell sorter profile of clone 4 as an example. Staining with monoclonal antibodies recognizing the V65 and V8 gene products of the T-cell receptor was performed. Only one of the clones analyzed (clone 4) was recognized by the monoclonal antibody 42/1C1 (Fig. 4) directed against the products of the V65 gene family (16). Clones obtained from the second operative specimen have also been phenotyped and were all of the CD3+CD4+ phenotype (data not shown).

Antibody Response to Collagen Type II. The results revealed strong reactivity with native type II collagen (Fig. 5A) and somewhat less against denatured type II (Fig. 5B). Negligible reactivity was observed against any of the other collagen types tested.

DISCUSSION

We have generated and expanded T-cell clones derived from the activated T cells present in the two synovial membrane preparations of a single patient with active erosive RA to study in detail the infiltrating T lymphocytes. All the clones...
The third.. been well from either... directed autologous PBM were cultured with or without the antigen indicated. Abbreviations are as in Fig. 1. Antigens were used at 100 μg/ml, except MTSE was at 1 μg/ml. These were the optimal concentrations.

The lack of CD8 clones was due to the incapacity of this cloning method, as CD8 clones have been obtained with this method from other rheumatoid arthritis joints (unpublished data) and in Hashimoto's thyroiditis (8). One of the collagen-specific clones (clone 4) reacted with a monoclonal antibody recognizing the Vβ5 T-cell receptor gene family, but another collagen-specific clone did not, suggesting heterogeneity of Vβ gene usage. Analysis of the DNA obtained from the clones indicated a heterogeneous pattern of the T-cell receptor gene usage (M. Lipololove, M.L., M.F. & M. Owen, unpublished data) and confirms the clonal origin of the T-cell clones.

In rheumatoid arthritis, a number of autoantibodies have been well characterized (18). In contrast, there is little information about what antigens autoreactive T cells may recognize. In this study, we document that there are clones recognizing autologous blood cells, as in an AMLR. Clones of this type have been noted previously in rheumatoid arthritis (19). Since rheumatoid arthritis synovial membrane cells bear large amounts of HLA class II molecules (3, 6), such AMLR cells may be triggered in situ. These autoreactive T cells may have a physiological amplifier role in the immune response (20).

However, the most interesting result obtained in the analysis of the specificity of the clones is the reactivity against collagen type II found in 2 clones of 25 tested, a relatively high frequency for T cells not selected by antigen. A third clone that proliferated against autologous PBM, showed a 2-fold increased response when collagen type II was present. A similar phenomenon, of dual recognition of autologous class II, enhanced by addition of antigen, has been observed in a murine model (21). The frequency of autoantigen reactive T cell in these samples is high (≈12%). This is comparable to the frequency of thyrocyte-specific T-cell clones derived from the lymphocytic infiltrate in Graves disease operative specimens that we reported initially (7), and similar frequencies have been found in subsequent studies (unpublished data).

Collagen type II is a likely candidate autoantigen in rheumatoid arthritis for several reasons. First, there are autoantibodies against collagen type II in the serum and joint fluid of a significant percentage of patients with rheumatoid arthritis (22), including this one, with evidence for local production in joints (23). Serum taken at the time of the first operative sample was strongly reactive by ELISA against native collagen type II, less against denatured collagen type II, but not against collagen types I, III, IV, and V (Fig. 4). This indicates a strong specific immune response against collagen type II in this individual. Second, it is possible to induce with collagen type II in experimental animals (guinea pigs, rats, mice) a genetically determined chronic arthritis that resembles rheumatoid arthritis (22). Not all rheumatoid patients produce anti-collagen type II antibodies (22). Thus, collagen type II reactive T cells may be of importance in only a proportion of patients with rheumatoid arthritis, and patients like the one reported here may have a higher frequency.

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**Fig. 2.** Proliferative response of collagen type II-specific clones. (A) Clone 4 from the first synovial membrane. (B) Clone 55 with AMLR reactivity, from the first synovial membrane. (C) Clone B8 from the second synovial preparation. Results are the arithmetic means ± SEM of a representative experiment from each clone. In other experiments, collagen type I was also used, but there was no response from any of the clones. Cloned T cells (10⁷), 2 × 10⁴ irradiated autologous PBM were cultured with or without the antigen indicated. Abbreviations are as in Fig. 1. Antigens were used at 100 μg/ml, except MTSE was at 1 μg/ml. These were the optimal concentrations.

**Fig. 3.** Proliferative response of synovial membrane cells from the third operation. Cells (2 × 10⁴) were cultured for 7 days with either medium alone, purified protein derived (PPD) (1 μg/ml), phytohemagglutinin (PHA) and IL-2 together at 1% and 30 ng/ml, respectively, mycobacterium tuberculosis soluble extract (MT) at 1 and 10 μg/ml, denatured collagen (DC) type II at 10 and 50 μg/ml, or collagen type II (CII) at 10 and 50 μg/ml. [3H]Thymidine was added for the last 7 hr of culture. Results are expressed as the arithmetic mean ± SEM.
of collagen type II-specific clones than other patients. Recently, it has been reported that rhesus and cynomolgus monkeys immunized with bovine collagen type II develop a chronic polyarthritis (24).

The purity of the collagen type II preparation used (9) excludes the possibility that other cartilage-specific collagens such as type IX, X, or XI may be recognized by these clones. Of interest would be to determine which part of the collagen type II molecule is recognized and whether all clones recognize a single epitope. We have not yet investigated whether the minor cartilage-specific collagens are recognized by any of these clones.

We have analyzed the lymphokines released by these clones and have found that the majority can release a high level of interferon-γ (25). Of particular interest was the finding that these T cells, including the collagen-specific clones, can also produce tumor necrosis factors α and β (lymphotoxin) (25), molecules that can induce cartilage degradation (26). Moreover, there is evidence that these lymphokines (interferon-γ and tumor necrosis factor) may synergize in the induction of HLA class II (27) and in the production of other cytokines such as IL-1 (unpublished data), which have been found in high levels in the synovial fluid of rheumatoid arthritis patients (28).

Our observation of collagen type II-reactive T-cell clones in active rheumatoid arthritis joints reports antigen-specific clones recognizing a defined autoantigen in rheumatoid arthritis. That these cells remained present in vivo over a 3-year period during which the disease persisted and progressed is compatible with a role of these cells in the maintenance of arthritis. Although we have so far only analyzed clones from a single patient, this patient has provided a rare opportunity for a prolonged longitudinal study. Previous reports suggest that T-cell responses against collagen type II are present in the joints of many patients with rheumatoid arthritis but not in their blood (3, 29). At present, our results do not discriminate between a response to collagen type II being initiated after another destructive process within the joint or being responsible for initiating the destructive processes of rheumatoid arthritis. Nevertheless, their continuous presence and activation in vivo suggests that collagen type II-reactive T cells may be important in the pathogenesis of rheumatoid arthritis in this patient.

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![Fig. 4](image-url) Analysis of the antibody response in the serum of the patient C.L. against different collagen preparations (type I to type V). Native (A) or denatured (B) collagens were used as antigens.

![Fig. 5](image-url) Flow cytometric analysis of clone 4 specific for collagen type II. (A) Faint line, control; heavy line, UCHT1 (CD3). (B) Faint line, Leu II (CD8); heavy line, Leu 3 (CD4). (C) Faint line, 2H4 (CD45R); heavy line, 4B4 (CDW29). (D) Faint line, MX9 (V₆); heavy line, 42/IC1 (V₆).


