Clonal heterogeneity of synovial fluid T lymphocytes from patients with rheumatoid arthritis

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ABSTRACT Although substantial evidence suggests that synovial T lymphocytes are critical in the pathogenesis of rheumatoid arthritis (RA), little is known regarding their antigenic specificities, antigen receptor gene rearrangements, and mechanisms of activation. To assess the extent of expansion of specific clones among RA synovial fluid T cells, Southern blot analyses of T-cell receptor (TCR) gene rearrangements were performed on 40 RA synovial fluid T-cell clones, as well as on both fresh and polyclonally activated T cells from RA synovial fluid, RA peripheral blood, and normal peripheral blood. Two of the clones had identical TCR rearrangement patterns, but the remaining were unique. The nonclonal RA T-cell samples showed the same pattern of TCR β-chain rearrangement that was observed among normal peripheral blood T cells, indicating no dominant clonal T-cell population in these samples. It was noted that with sufficient exposure of autoradiograms of the Southern blots, discrete TCR gene rearrangements, representing in some cases common D<sub>β</sub>J<sub>β</sub> (D, diversity; J, joining) rearrangements, were evident in T cells from peripheral blood of normal individuals and patients with RA, as well as T cells from RA synovial fluid. Taken together, the findings indicate that only a minor degree of oligoclonality can be demonstrated among T lymphocytes from RA synovial fluid.

In rheumatoid arthritis (RA), inflammation of the lining of multiple joints ultimately leads to destruction of cartilage and bone. Among the many cell types that participate in the pathogenesis of RA, T lymphocytes can be viewed as central to the chronic inflammatory processes ongoing in the rheumatoid joint (1–26). T cells, activated by either specific antigen or by antigen-independent mechanisms (27–29), can secrete multiple cytokines and recruit an array of effector cells of inflammation. T cells in the RA synovial compartment bear surface markers that reflect prior activation in vivo (3–5). Elimination of sufficient numbers of T cells in patients with RA reliably leads to amelioration of disease as long as T lymphopenia persists (6–10). Moreover, the linkage of RA to specific class II major histocompatibility complex determinants, which control antigen presentation to inducer T cells, further suggests that T-cell activation is central to the disease (11, 12).

Animal models of RA have also been useful in illuminating the role of T lymphocytes in inflammatory arthritis (13–26). In such models, measures that ablate T cells can prevent or retard the disease (19, 22). Moreover, in adjuvant arthritis, for example, disease can be transferred to naive animals by intravenous injection of cloned T lymphocytes derived from arthritic animals. These T cells recognize an epitope on one of the components of the adjuvant (mycobacterial) preparation and also respond to a cross-reactive epitope on cartilage proteoglycan (14–17, 25, 26). A role for this antigen in human RA has been proposed, in view of evidence for selective augmentation of responses to this antigen in RA synovial T cells (23, 24).

The adjuvant arthritis model is of additional interest because in vitro attenuation of arthritogenic cloned T cells endows such lymphocytes with the capacity to vaccinate against the disease, possibly by activating anti-idiotypic immune responses against the antigen receptor or the attenuated cloned T cells (14, 17, 25). Similar observations have been made in experimental allergic encephalomyelitis, an animal model of multiple sclerosis (30–34). If a clonally restricted T-cell response to an identifiable antigen were of similar importance in human RA, our understanding of this disease would be dramatically improved. New, specific forms of immunotherapy, such as have been used in animal model systems, might then be possible.

Due to uncertainty regarding the nature of target antigens in RA, attention is now being directed at defining the degree of T-cell oligoclonality in the rheumatoid synovial compartment (35). If synovial T-cell infiltrates were oligoclonal, this would suggest that recognition of a specific antigen, leading to expansion of an oligoclonal T-cell population, was in fact important in RA. Identification and characterization of such restricted T-cell populations could allow identification of the target antigen itself.

The present study was undertaken to characterize the extent of oligoclonality among RA T-cell populations, using Southern blot analysis of T-cell receptor (TCR) β- and γ-chain gene rearrangements. Fresh RA blood and synovial fluid T cells, control peripheral blood T cells, and two panels of RA synovial fluid T-cell clones were studied. The degree of clonal restriction detected among fresh RA synovial fluid T lymphocytes did not exceed that observed in normal peripheral blood T cells. Two of 40 synovial fluid clones had identical TCR gene rearrangements, but each of the remaining 38 clones was unique. The data suggest that the degree of oligoclonality or clonal dominance seen in RA synovial fluid T cells is negligible.

METHODS

Patient Population. Synovial fluid from therapeutic arthrocenteses was obtained from patients with RA who fulfilled at least four of the 1987 American Rheumatism Association (ARA) criteria for the classification of RA (36). The two synovial fluids used to generate T-cell clones were from patients who satisfied five ARA criteria and who had been treated only with aspirin and nonsteroidal anti-inflammatory drugs. The duration of disease was 6 months in one and 6 years in the other. Venous blood was obtained from patients with RA and from healthy normal controls.

Abbreviations: RA, rheumatoid arthritis; TCR, T-cell receptor; IL-2, interleukin 2; J, joining; C, constant; D, diversity.

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Preparation of Lymphocyte Populations and T-Cell Clones. Peripheral blood and synovial fluid mononuclear cells were isolated by centrifugation of blood or synovial fluid diluted in Hanks' balanced salt solution over Ficoll-Hypaque, followed by three washes. T cells were prepared by rosetting with sheep erythrocytes. Synovial T-cell clones were generated under conditions of limiting dilution using phytohemagglutinin (PHA) and autologous feeder cells as described (37-39). Clones were fed three times weekly with medium containing interleukin 2 (IL-2), and were restimulated every 2 weeks with fresh PHA and allogeneic feeder cells. For the two panels of synovial T-cell clones, the cloning efficiency averaged 10% (8% and 12%), lower than the cloning efficiency for peripheral blood T-cell clones generated in our laboratory and by others (37-40). T-cell lines were propagated in bulk cultures by methods otherwise similar to those described for the clones.

Southern Blot Analysis. The following cell populations were subjected to Southern blot analysis of TCR gene rearrangements: 40 RA synovial fluid T-cell clones; 8 RA T-cell lines (4 from synovial fluid, 2 from synovial tissue, 2 from peripheral blood T cells); 24 fresh RA synovial fluid T-cell samples, 16 fresh peripheral blood T-cell samples (8 RA, 8 normal control), and 1 control peripheral blood T-cell line. Genomic DNA extractions were performed according to standard methodology (41). DNA was digested with the restriction enzymes EcoRI, HindIII, or BamHI; size-fractionated by electrophoresis through 0.7% agarose gels; and transferred to nitrocellulose filters. The filters were hybridized overnight to nick-translated 32P-labeled probes of the TCR β- and γ-chain genes. The next day, the filters were washed at 65°C in 0.1% SDS/0.015 M NaCl/0.0015 M sodium citrate prior to autoradiography with intensifying screens. The filters were stripped with 0.5 M NaOH between hybridizations with different probes.

DNA Probes. The probes used are restriction enzyme DNA fragments of previously isolated human genomic clones (ref. 42; Fig. 1). Jp1 is a 2.6-kilobase (kb) HindIII/Nsi I fragment containing the Jp1 gene segment cluster (J, joining); Jp2 is a 4.4-kb EcoRI/EcoRI fragment containing the Jp2 gene segment cluster; Cα is a 2.8-kb HindIII/EcoRI fragment containing the Cα1 gene segment and hybridizes to the Cα1 and Cα2 gene segments (C, constant). Dg2 is a 2.3-kb EcoRI/ EcoRI fragment containing the Dg2 gene segment (D, diversity). The Jp1 and Jp2 probes do not hybridize to each other. Jp1, Jp2, 1.3 is a 0.8-kb HindIII/EcoRI fragment that recognizes Jp1, Jp2, and Jp3 and recognizes all rearrangements to known Jp gene segments on BamHI digestions (44).

RESULTS

To assess the degree of oligoclonality among RA synovial fluid T cells, T-cell clones were harvested under conditions of limiting dilution were subjected to Southern blot analysis of their TCR β-chain rearrangements. Cell populations with no more than two bands that hybridized with each of the Jp1 and Jp2 probes were considered to be clonal. For example, in Fig. 2, lanes a–n represent 14 of the 40 RA synovial T-cell clones included in the study. Lane o, in contrast, shows at least three bands that hybridized to the Jp2 probe, reflecting the presence of more than one clone, and this cell population was excluded.

Among 40 clones examined, 2, both from the same patient, had identical patterns of rearrangements of TCR β-chain genes after EcoRI digestion. These clones, both CD4+ CD8-, were further studied by Southern blot analysis of DNA digested with EcoRI, HindIII, and BamHI, then hybridized with Jp1, Jp2 as well as Jp1 and Jp2 probes. In each analysis, the two clones had the same pattern of TCR β- and γ-chain gene rearrangement (data not shown). In view of the complexity of the TCR β- and γ-chain loci and the number of potential patterns of rearrangement available (40), these results indicate that these two independently cultured clones are identical. The remaining 38 clones, however, had unequivocally unique TCR gene rearrangements.

These results indicate that RA synovial fluid T cells are heterogeneous, with only a negligible degree of oligoclonality. It has been previously shown that one or more monoclonal populations of T cells constituting <1% of a mixed T-cell population can be detected by Southern blot analysis (45, 46). We therefore investigated whether populations of T cells isolated from the peripheral blood and synovial fluid of RA patients contained β-chain gene rearrangements not normally found in T cells from unaffected individuals. The presence of such rearrangement(s) would be strong evidence of a clonal population of T cells.

Southern blot analyses of representative populations from each of these sources are shown in Figs. 3 and 4. In Fig. 3, lanes a–e contain DNA from peripheral blood T cells of five

![Fig. 1. Restriction enzyme map of the human TCR β-chain gene. The germ-line organization has been reported (43). The location of the 2 diversity (Dh), 13 functional joining (Jp), and 2 constant (Cp) gene segments is indicated by jagged vertical lines, vertical lines, and solid rectangles, respectively. The location of the EcoRI (R) and the distances between them in kilobase pairs (kb) is indicated. Probes are restriction enzyme DNA fragments. The size and location of the Jp1, Cα, Dg2, and Jp2 probes are indicated by stippled and hatched bars.](https://example.com/fig1)

![Fig. 2. Southern blot analysis of β-chain gene rearrangements in T-cell clones derived from the synovial fluid of a patient with RA. DNA from a representative group of T-cell clones (lanes a–o) and from the B-cell line, LA25B9 (lane p), were digested with EcoRI, fractionated on a 0.7% agarose gel, transferred to nitrocellulose, hybridized to the Jp1 and Jp2 probes, and analyzed by autoradiography. The autoradiograms are purposely overexposed to detect faint bands. Clones that exhibited more than two rearranged fragments (lane o) were not included in the analysis. Jp1 rearrangements vary in intensity because the rearrangement process deletes various amounts of DNA that hybridizes to the Jp1 probe (see Fig. 1 and text). The artificial band described in Fig. 3 and the text does not appear in these blots. The sizes of the fragments in kilobase pairs (kb) are indicated on the right.](https://example.com/fig2)
normal individuals and lane f contains DNA from RA synovial T cells. Rearrangements into the Jp1 gene segment cluster were detected with a Cbg1 probe (Fig. 4A), while rearrangements into the Jp2 gene segment cluster were detected with a Jp2 probe on the same EcoRI digestion (Fig. 4B). The 2.8-kb Cbg1 probe was used because it strongly hybridizes to 2.8 kb of the β-chain locus and detects all rearrangements with equal intensity on an EcoRI digestion. In contrast, the Jp1 probe, only 15% of which is 3' of the Jp1 gene segment cluster, detects rearrangements with variable intensity, as exemplified in Fig. 2. For example, Dp1 rearrangement to Jp1.6 would delete all the DNA 5' of Jp1.6, and would leave only 0.4 kb of DNA to hybridize to the Jp1 probe.

In Fig. 3 A and B, the germ-line β-chain EcoRI fragments are shown: the 10.8-kb fragment containing Dp1–Jp1–Cbg1, and the 4.0-kb fragment containing Cbg2, both of which hybridize to the Cbg1 probe (A); and the 4.4-kb fragment containing Jp2 (B). Many discrete rearranged bands are also seen in the peripheral blood T-cell samples. To investigate the origin of these bands, the Jp2 probe was stripped off the blot and a Dp2 probe was applied (Fig. 3C). This probe identifies a 2.3-kb germ-line band (Fig. 1), and four additional discrete rearranged bands in lanes a–f. These rearranged bands correspond exactly to bands 5, 7, 8, and 9 in Fig. 3B. Not all of the seven potential distinct bands, representing the possible Dp2–Jp2 rearrangements are seen, either because some Dp2–Jp2 rearrangements are uncommon and/or because 0.7% agarose gels cannot resolve Dp2–Jp2 rearrangements that are very similar in size. [The distances between the seven Jp2 segments vary from 117 to 284 base pairs (43).] The remaining rearranged bands in Fig. 3B may represent either Dp1–Jp2 rearrangements, common Vbg–Dp2–Jp2 rearrangements, or different Vbg–Dp2–Jp2 rearrangements that comigrate in an agarose gel. The greater abundance of Jp2 rearrangements (Fig. 3B) compared with Jp1 rearrangements (Fig. 3A) is consistent with the usage of Jp2 gene segments in the majority of T cells (47). It is important to note that all the rearranged bands were seen in multiple normal samples and that no unique bands were seen in the RA synovial fluid T-cell samples. The blots are deliberately overexposed, as evidenced by the intensity of the germ-line band. If less DNA is loaded into the gel (lanes b and c), all of the bands are less intense.

An artificial 8.4-kb band is identified by an asterisk in Fig. 3. It characteristically hybridizes more intensely to the Jp2 than to the Cbg1 probe and does not hybridize to a Jp1 probe (see Fig. 2). This band is not present in Figs. 2 and 4. A partial digestion of the EcoRI site between Jp2 and Cbg2 best explains these findings. The intensity of this band will vary depending on the resistance to digestion of this site.

Representative samples from the various T-cell populations analyzed are shown in Fig. 4. The nonclonal T-cell populations all demonstrate multiple rearranged bands, and the patterns seen with RA synovial fluid (lanes d–f) do not differ from those seen with RA peripheral blood (lanes i and
j) or normal peripheral blood (lanes k and l; see also Fig. 3, lanes a–e). We were consistently unable to demonstrate differences between matched RA blood and synovial fluid T cells. Discrete bands were best seen when the wells were overloaded with DNA, and the autoradiograms were overexposed. This can be appreciated by comparison of the germ-line Jg2 bands in Fig. 4 (lanes b–l) with the corresponding bands on a B-cell line (lane a) and a T-cell clone with one rearranged locus (lane m).

In Fig. 4, the normal (lane h) and RA (lanes b, c, and g) T-cell lines exhibit rearrangements similar to those seen in normal peripheral blood and in RA peripheral blood and synovial fluid. One RA synovial T-cell line (lane c) has several faint bands and two prominent bands hybridizing to the Jg2 probe, only one of which also hybridizes to the Dg2 probe. This pattern of bands is similar to the pattern exhibited by the T-cell clone in lane o, although the two T-cell populations are from different patients. These prominent bands may signify the emergence of a dominant clone in this line, derived from synovial tissue, which was in culture for 3 months at the time of analysis. The other five synovial T-cell lines (four from synovial fluid and one from synovial tissue) displayed no unique dominant bands.

**DISCUSSION**

To define the degree of clonal diversity in RA synovial fluid T cells, we examined TCR gene rearrangements in fresh RA synovial fluid T cells, RA synovial fluid T-cell lines, and 40 RA synovial fluid T-cell clones. T cells from normal and RA peripheral blood were examined concurrently. Two of 40 clones appeared to be identical, and the other 38 were all unique, suggesting that clonal restriction or “clonal dominance” is unlikely to be a prominent feature of RA synovial fluid T cells. This interpretation is supported by analysis of fresh RA synovial fluid T-cell samples, which did not differ from RA or normal peripheral blood T cells with respect to the pattern of TCR beta-chain gene rearrangements. When autoradiograms of these bulk populations were sufficiently exposed, individual rearranged bands could be detected, some of which were shown to represent Dg2–Ig rearrangements.

Previous Southern blot analyses of polyclonal T-cell populations have yielded “smears” of rearrangements. By adjusting the amount of DNA loaded, the size of the agarose gels, the concentration of agarose, the voltage applied, and the duration of exposure of the autoradiograms, we demonstrate that this smear of rearrangements can be resolved into discrete bands, many of which are Dg2–Ig rearrangements. As there are only six possible Dg1–Ig1, seven possible Dg1–Ig2, and seven possible Dg2–Ig2 rearrangements, different T-cell clones will often contain one or more rearrangements in common. It is therefore not totally unexpected that discrete bands corresponding to such rearrangements would be visible on Southern blot analysis of polyclonal populations. This finding emphasizes that caution is required in interpreting the significance of rearranged bands in bulk T-cell populations and that these may not necessarily indicate the presence of unique, pathologic T-cell clones or even an unusual degree of T-cell oligoclonality. The methods used in this study can detect oligoclonal T-cell populations constituting 1% of a mixed T-cell sample (45, 46). The absence of unique oligoclonal populations in any of the 24 fresh RA synovial fluid T-cell samples examined strongly suggests that oligoclonality ≥1% is rare, if ever, present among RA synovial fluid T cells.

During the course of these studies Stamenkovic and coworkers (35) published data from Southern blot analysis of TCR beta-chain genes of T-cell lines derived from synovial tissue of patients with RA and osteoarthritis. Our approaches, results, and interpretations differ substantively from those of Stamenkovic (35), who expanded synovial T cells in IL-2 alone prior to analysis of TCR gene rearrangements and demonstrated distinctive rearrangements of the TCR beta-chain gene. It is unclear whether these represent unique Vg2–Dg2–Ig rearrangements or common Dg2–Ig rearrangements shared by many T cells. It is also possible that adjusting the conditions of the Southern blot procedure as performed in the present study would have generated a complex pattern of beta-chain gene rearrangements, indicative of a diverse T-cell population. In addition, the culturing of synovial T cells in IL-2 could have led to preferential expansion of cells initially bearing high-affinity IL-2 receptors, producing a cell population that would not accurately reflect the composition of the original sample. The p55 IL-2 receptor subunit, necessary for the formation of high-affinity receptors, is expressed transiently during T-cell activation (48). Only low levels of p55 IL-2 receptor expression have been found on RA (49) and on juvenile chronic arthritis (50) synovial fluid T cells or on RA synovial tissue T cells (51). Culture of such cells in IL-2 would therefore be expected to cause significant skewing of cell lines, such as those previously examined (35). The pattern of clonal dominance in such cell lines was different in each patient (35), suggesting chance outgrowth of random clones. Furthermore, similar results were obtained in patients with osteoarthritis (35), a disease in which an important role for T lymphocytes is unlikely, casting doubt on the relevance of the findings to the pathogenesis of RA.

To avoid such difficulties we developed T-cell clones from single T cells under conditions of limiting dilution, using stimuli (lectin plus feeders, followed by IL-2) designed to permit the outgrowth of all T cells in the sample, not only those already bearing IL-2 receptors. Analysis of the clones revealed almost complete heterogeneity in the patterns of TCR gene rearrangements. In addition, we prepared bulk T-cell lines using the same stimuli. These lines generally remained polyclonal, although patterns suggesting the emergence of oligoclonality did appear in one line after 3 months in culture. This phenomenon is consistent with studies of the expression of glucose-6-phosphate dehydrogenase in human lymphoblast cultures, derived from heterozygous females. Even in the presence of polyclonal activators such cell populations can ultimately lose those lines without this marker (52). This indicates that rare cells can be amplified over time in culture in a disproportionate manner (52). One example of such skewing in culture of T cells over 2- to 4-week periods is the loss of T4+ cells and the outgrowth of T8+ cells (53).

One limitation of our studies is the relatively low cloning efficiency obtained for synovial fluid T cells (10%), despite use of methods optimal for cloning peripheral blood T cells. The reasons for this low cloning efficiency and possible modifications of cloning protocols to overcome this problem are not known. It is conceivable that dominant clones in vivo could be underrepresented among the panel of clones successfully propagated in vitro. However, the results obtained with fresh synovial T-cell samples and with bulk lines are consistent with the data from the clones. Furthermore, the exact methods used in our study have been successfully used to demonstrate oligoclonality in the cerebrospinal fluid in multiple sclerosis (40), which, like RA, appears to be a T-cell-mediated autoimmune disease.

An absence of dominant clones among synovial fluid T cells would not automatically exclude an important role for antigen-specific responses in RA. It is possible that responses to complex antigens in the synovial compartment may be polyclonal, both because of multiple epitopes on such antigens and because of the possibility that different combinations of TCR segments may be capable of recognizing indi-
T-cell activation is also possible that the specificities and degree of clonal restriction of synovial tissue T cells could differ from those present in synovial fluid T cells. Furthermore, important antigen-specific clones could constitute only a very small fraction of the total synovial T-cell population, making their detection by Southern blot analysis impractical and further confounding efforts to assess their biologic importance. Even if cells responding to specific antigen are uncommon, they may still be critically important in initiating recruitment of a polyclonal T-cell population to the site of an inflammatory lesion.

The many questions about the role of specific antigen in the activation of RA synovial T cells also serve to highlight the potential importance in RA of T-cell surface structures and activation pathways distinct from the TCR. Pathways of T-cell activation that can function in vitro in the absence of antigen, such as those mediated through CD2 (29) or through a recently described T-cell surface structure termed UMOD (38), may also be very important in RA. A role for such mechanisms is especially attractive in explaining long-term perpetuation of a T-cell-directed inflammatory response that, in RA, often lasts for decades, potentially long after the disappearance of specific antigens that could have initiated the disease. Elucidation of the relative importance of the various antigen-specific and antigen-independent pathways of T-cell activation in RA would not only shed light on the pathogenesis of this important disease, but would also likely lead to more rational approaches to its treatment.

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