Identification of Clonally Expanded T Cells in Rheumatoid Arthritis Using a Sequence Enrichment Nuclease Assay

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Abstract

Identification of expanded clones engaged in immune and autoimmune responses is still imperfect, since they are often diluted by irrelevant cells expressing diverse specificities. To efficiently characterize T cell receptors expressed by clonally expanded lymphocytes in rheumatoid arthritis (RA) and other inflammatory conditions, we developed an assay system, termed sequence enrichment nuclease assay (SENA). Key elements of SENA are the efficiency of heat-denatured DNA strand reassociation, which increases exponentially with concentration, and the elimination of unhybridized sequences by single-strand–specific DNase. T cell clonal expansions were identified primarily in synovial fluids, but also in peripheral blood of RA patients. Synovial fluids had more prominent expansions in the CD8 than the CD4 subset, whereas clonal expansions in the CD4 subset predominated among peripheral blood lymphocytes. Dominant clones exhibited diverse sequences with no clear conservation of junctional motifs, although the same amino acid sequence was identified in two patients. In most instances, dominant clones in the blood were discordant to those in the corresponding synovial fluid, suggesting local stimulation or preferential sequestration of T cells displaying particular specifities. (*J. Clin. Invest.* 1996. 97:1335–1343.) Key words: T cell receptor • clonal expansion • rheumatoid arthritis • sequence enrichment nuclease assay • selective cloning

Introduction

A desirable, but often difficult, goal in immunopathology is the characterization of B and T cell clonotypes engaged in specific responses. Molecular analysis of immunoglobulins (Ig) and T cell receptors (TCR)[1] encoded by particular variable-diversity-joining (V-D-J) gene combinations can reveal important information on foreign and self-antigens. Such studies hold particular promise for the development of therapeutic strategies to increase immune responses to pathogens and malignant cells or to inhibit autoimmune and other inflammatory diseases.

Rheumatoid arthritis (RA) is a chronic joint disease of unknown etiology characterized by inflammation of synovium, destruction of cartilage and bone, and systemic sickness. Several lines of evidence, including the massive T cell infiltration in inflamed joints and the association with particular MHC class II molecules, clearly implicate autoreactive T cells in the pathogenesis of this disease (1). Previous studies on TCR usage by synovial T cells, although sometimes conflicting, often indicated clonal expansions, either documented by TCR cloning and sequencing or suspected on the basis of altered V gene expression profiles (2–7). Generally, however, polyclonal T cell repertoires were observed, probably due to large cell populations secondarily attracted to the affected joints by inflammatory factors. Based on these considerations, assay systems that discriminate between expanded, presumably antigen-specific, T cell clones and irrelevant polyclonal cells without resorting to approaches that are time consuming, affected by in vitro induced artifacts, or limited in scope (e.g., development of T cell clones and library construction) appear to be particularly useful.

Herein, we describe a new approach, sequence enrichment nuclease assay (SENA), that eliminates infrequent sequences and allows selective amplification of highly represented V-D-J sequences that subsequently can be analyzed by conventional methods like the RNase protection assay (8, 9), complementarity determining region 3 (CDR3) size analysis (10), and sequencing. Applied to RA patients, SENA revealed accumulation of diverse T cell clones in both synovial fluid (SF) and peripheral blood. Because of its relative simplicity, specificity, and sensitivity, SENA might significantly facilitate and expedite antigen receptor analysis in RA and other diseases characterized by in situ clonally expanded T and/or B cells.

Methods

Cells and RNA preparations. 25 individuals, classified as chronic RA patients based on American Rheumatism Association criteria, were obtained at the Department of Medicine of Northwestern University Medical School and the Health Sciences Center of Virginia Univer-

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Received for publication 27 June 1995 and accepted in revised form 12 December 1995.

J. Clin. Invest.

0021-9738/96/03/1335/09 $2.00
Volume 97, Number 5, March 1996, 1335–1343

1. *Abbreviations used in this paper:* CDR, complementarity determining region; SENA, sequence enrichment nuclease assay; SF, synovial fluid; TCR, T cell receptor.

sity. 50 normal blood donors were used as controls. RNA was purified from mononuclear cells (16–50 × 10^6) and single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) lymphocytes from SF and peripheral blood lymphocytes (PBL) and analyzed by RNase protection assay as described (8, 9). The TCRBV probes used in this assay are available to interested investigators upon request.

SENA. cDNA was synthesized from RNA (0.5–1 μg) using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), dG-tailed by TdT (5 U/μl) in 1 mM dGTP, 0.1 M potassium cacodylate (pH 7.2), and 2 mM cobalt chloride, and amplified by nested anchor-PCR, as described (11), with the modification of using an anchor-primer (5′-GCCAGTGAATTGTAATACGACTCACTATAGGAGATCCCCCCCCCCCCCC-3′) that included the T7-RNA-polymerase promoter and TCRBC primers (external 5′-CGAGGTAAGCCACAGTCTGCTCTA-3′ and internal 5′-AGAAGCCTGTGGCCAGGCACACCAGT-3′). For SENA, a fraction of anchor-PCR product was dissolved in 4 μl of hybridization buffer (33 mM Pipes, 3.3 mM EDTA, pH 8.0), overlaid with 30 μl of mineral oil and denatured by heat; 1 μl of 5 M NaCl solution was added and DNA was rehybridized for 16–20 h at 67°C. Unhybridized sequences were degraded by 1–2 h of incubation at 30°C with 25 U S1-nuclease in 50 μl of 50 mM Na-acetate (pH 4.5), 0.28 M NaCl, and 4.5 mM $ZnSO_4$. The reaction was stopped by adding 30 μl of a mixture containing 0.8 M ammonium acetate, 133 mM EDTA (pH 8.0), and 1.5 μg tRNA. The DNA retained after SENA, containing V-D-J sequence(s) expressed by the clonotypes enriched in the analyzed sample, was purified by Gene Clean (BIO 101, Inc., Vista, CA). A fraction (1/20) of the purified product was amplified (15 cycles) under the same conditions used for anchor-PCR. For analysis, synthetic RNA was generated (before and after SENA) by transcription with T7-RNA-polymerase in a 2.5-μl riboprobe system reaction (Promega, Madison, WI), containing 470 μM of each of the four ribonucleotides and 2 nM (6 μCi) of ^{35}S-UTP. After purification, the amount of synthetic RNA corresponding to 1 × 10^3 ^{35}S cpm, determined through titration experiments as being appropriate for analysis, was subjected to the RNase protection assay (8, 9).

CDR3 size analysis. A fraction (1/100 to 1/1,000) of anchor-PCR product (before or after SENA) was amplified by 20 PCR cycles in a 10-μl reaction using TCRBV and TCRBC primers labeled with ^{32}P using T4 kinase (Boehringer Mannheim Corp., Indianapolis, IN) as described (12). 5 μl of the product was loaded on a 6% acrylamide sequencing gel and bands were visualized by overnight exposure to Kodak AR films.

Sequence analysis. For sequencing, 1 μl of the anchor-PCR product (before or after SENA) was amplified by 30 PCR cycles using TCRBV and TCRBC primers, and 1 μg of the product was directly sequenced by an automated DNA sequencer (Applied Biosystems, Foster City, CA). For BV13S1 of patient RA-17, the anchor-PCR product (after SENA) was cloned in the pCRII vector (Invitrogen, San Diego, CA). Plasmids from individual colonies were sequenced as above and two sequences were identified.

Results

Principles of SENA. The enrichment step of SENA is based on second-order reassociation kinetics of heat-denatured complementary DNA strands, which is proportional to the square of DNA concentration. In essence, if the concentration of a double-stranded DNA species A is n times that of B, the ratio of A to B will increase to n^2 after denaturation and renaturation in appropriate conditions. This principle has been used to determine the complexity of prokaryotic and eukaryotic genomes (13) and, more recently, to create DNA subtraction libraries and identify gene mutations (14). A subsequent treatment with single-strand–specific nuclease, such as S1, will eliminate all unhybridized or partially hybridized molecules, thus allowing reassociated (i.e., enriched) sequences to be selectively amplified by PCR.

A schematic representation of SENA applied to the analysis of TCRβ chain cDNA from clonally expanded T cells is illustrated in Fig. 1. The same strategy can be adapted by the use of appropriate PCR primers to the analysis of V-(D)-J sequences encoding TCRα, TCRγ, TCRδ, and Ig molecules. RNA is initially isolated from a cellular population suspected to contain antigen-specific T cell clonal expansion(s) and amplification of V-D-J cDNA is then achieved by anchor-PCR (11), which avoids the use of multiple primers that might exhibit different amplification efficiencies, and is particularly suitable for somatically mutated Ig V genes. The amplified

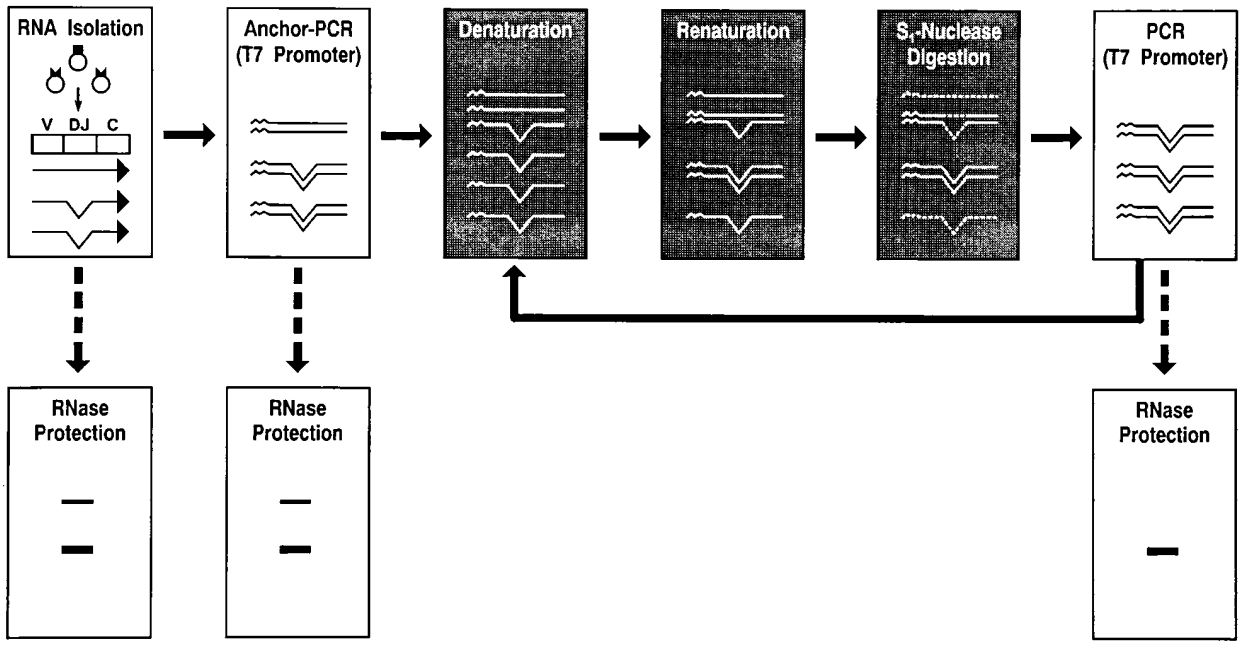

Figure 1. Schematic representation of SENA. See text for details.

cDNA is then denatured and renatured. Based on second-order reassociation kinetics, V-D-J sequences derived from expanded clonotypes preferentially reassociate, remain protected from digestion by single-strand–specific S1-nuclease, and are subsequently amplified by anchor-PCR. In contrast, sequences from the majority of nonexpanded clones, represented by one or few cells, reassociate with lower efficiency and are eliminated by nuclease digestion. The use of a 5′ anchor-primer that includes the T7-RNA-polymerase promoter allows production of synthetic RNA, which can be analyzed before or after SENA by a TCRBV-multiprobe RNase protection assay system previously developed in this laboratory (8, 9). Alternative methods, such as quantitative PCR (15) and CDR3 size analysis (10), can also be used to monitor the efficiency of enrichment (see below). Finally, if enrichment is insufficient, the PCR product can be subjected to additional rounds of SENA.

Sensitivity and specificity of SENA. To determine the sensitivity of SENA, increasing numbers of Jurkat T cells that express a TCRBV8S1-D-J sequence (16) were added to 10^6 total thymocytes from a cardiovascular surgery patient. As indicated by RNase protection analysis, before SENA (Fig. 2 *A*, *left* and *middle*) only small increases in BV8S1 expression compared with other thymocyte-contributed BV genes were noted. Similar repertoires were obtained with natural and synthetic RNAs, indicating that the cDNA library amplified by anchor-PCR represents the original transcript's diversity. In contrast, even after a single cycle of SENA (Fig. 2 *A*, *right*), the signal of BV8S1 was significantly increased compared with other genes, indicating that Jurkat-derived V-D-J sequences were efficiently enriched. The intensity of specific signals increased proportionally to the number of cells added, indicating that this parameter can be used to approximate the level of expansion. Similar results were consistently obtained in three in-

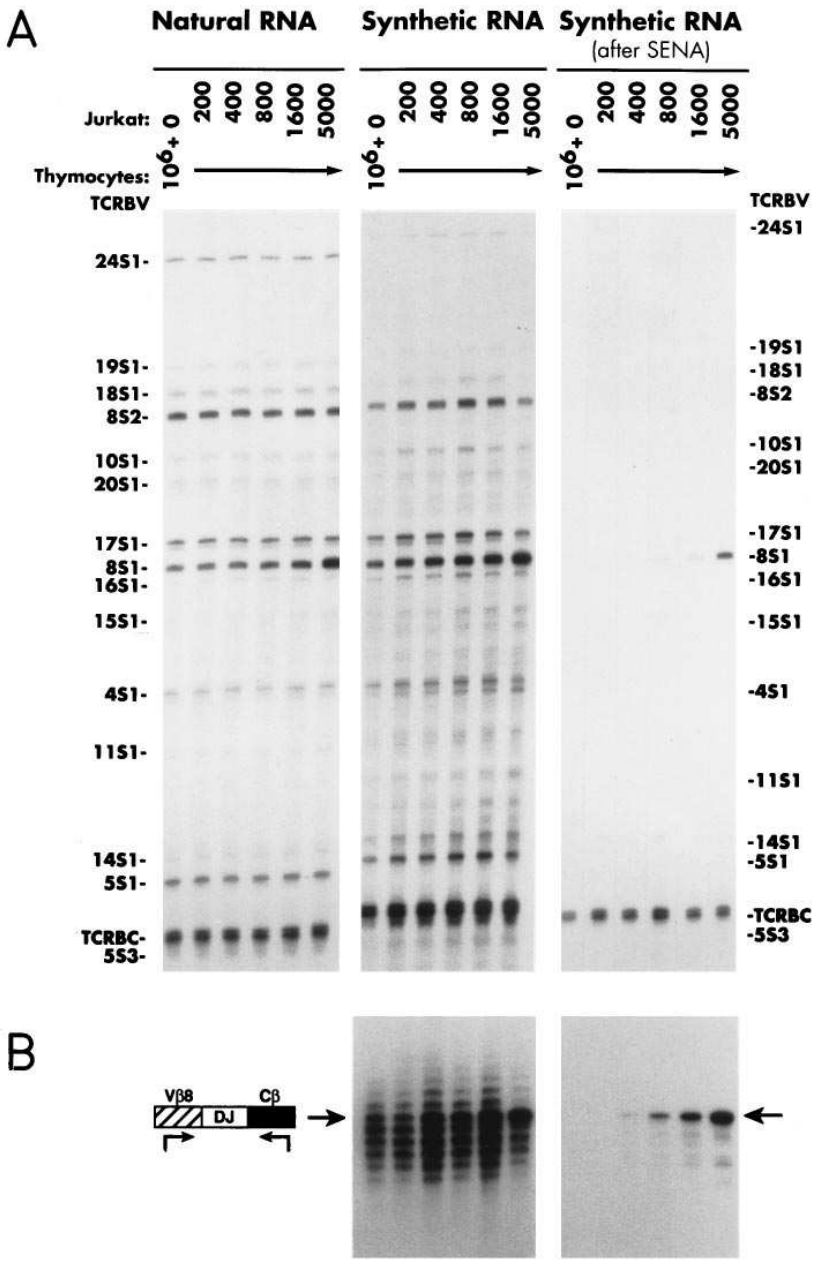

Figure 2. SENA sensitivity and specificity. (*A*) RNAs from 10^6 thymocytes mixed with increasing numbers of Jurkat T cells (expressing a particular TCRBV8S1-D-J combination) were isolated and submitted to a single cycle of SENA. Natural cell RNA (*left*) and amplified synthetic RNA, before SENA (*middle*) and after SENA (*right*), were prepared and analyzed by TCRBV multiprobe RNase protection assay (8, 9). (*B*) CDR3 size analysis of the different thymocyte-Jurkat cell populations. A fraction of cDNA product obtained by anchor-PCR before and after SENA was amplified using a TCRBV8-primer and a TCRBC-primer labeled with ^{32}P, as described (10, 12).

dependent assays and, although two SENA cycles appeared more efficient in eliminating thymocyte-derived background sequences, a single cycle was usually sufficient to detect clones of 400–800 cells diluted in 10^6 polyclonal cells (0.04–0.08%).

To further verify efficiency and specificity of enrichment, the heterogeneity of the TCRBV8S1-positive sequences selectively retained after SENA was compared with the original cDNA populations. Comparisons were performed by CDR3 size analysis, which consists of amplifying V-D-J junctional sequences by PCR using primers for V and C regions and product separation on high-resolution sequencing gels (10). As previously reported (10), polyclonal T cells give 8–12 bands differing in three nucleotides (i.e., one codon) and corresponding to the different V-D-J sizes (Fig. 2 *B*, *center*, *first line*). By increasing the number of monoclonal Jurkat cells, the band intensity corresponding to the specific rearrangement increases proportionally. Bands contributed by the other rearrangements are, nevertheless, not eliminated. In contrast, after SENA (Fig. 2 *B*, *right*), only the band corresponding to the clonal rearrangement is retained, as confirmed by sequence analysis. In fact, the exact V-D-J rearrangement of Jurkat cells (16) could be sequenced without previous cloning in bacterial vectors from a mix of 800 cells in 10^6 thymocytes after, but not before, SENA.

Overall TCRBV gene expression profiles in RA patients. To determine the overall TCR repertoires in RA patients, T cell RNA from SF and PBL of 25 patients was initially analyzed by RNase protection assay using 28 TCRBV-specific riboprobes (8, 9). The results indicated, in agreement with previous reports (2–7), polyclonal repertoires for both SF and PBL from most of these patients, with expression levels close to those displayed by thymocytes (8) and PBL from > 50 normal individuals (9, and results not shown). In only one case (patient RA-9) was a clear dominance of BV13S1 in SF observed by this assay. Additionally, increased expression was observed in the SF of patients RA-10 (BV1S1 and BV23S1), RA-22 (BV11S1), and RA-18 (BV15S1), and in the PBL of patients RA-22 (BV8S3, BV11S1, and BV13S1), and RA-20 (BV13S1 and BV13S2).

T cell clonal expansions in RA patients. Based on availability, cell numbers, and repertoire diversity, 14 of these 25 RA patients were selected for T cell clonal analysis by SENA. For six patients, sufficient cell numbers could be obtained to separate CD4 and CD8 T cells before analysis. Fig. 3 shows the results for a representative patient, RA-23, initially analyzed with 15 TCRBV riboprobes. As indicated above, and similar to most individuals, both PBL and SF showed heterogeneous T cell repertoires, as shown by the large number of TCRBV genes detected before SENA (Fig. 3, lanes *1* and *3*). However, in SF, but not PBL, of this patient, T cell clones expressing BV8S2 and BV17S1 were significantly expanded, as shown by selective retention of the corresponding signals after SENA (Fig. 3, lanes *2* and *4*). The BV17S1 band appeared to be of higher intensity than the BV8S2 band, suggesting a higher level of clonal expansion. CDR3 size analysis indicated that at least two clones expressed BV8S2, one displaying a junctional sequence four codons longer than the other, whereas expansion of the BV17S1-positive population appeared monoclonal. Examples of SENA patterns obtained from SF and PBL of two additional patients assessed by three TCRBV probe sets are shown in Fig. 4.

The results obtained with all 14 RA patients using 28

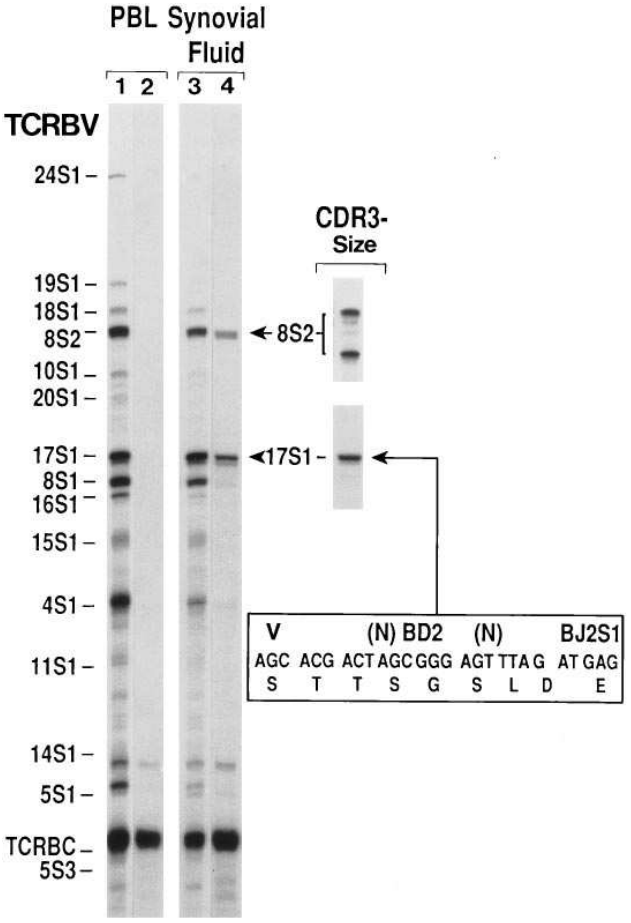

Figure 3. SENA analysis of T cell clones expanded in RA patient RA-23. T cell RNA from PBL and SF was analyzed by RNase protection assay, either directly (lanes *1* and *3*) to determine the total TCRBV repertoire, or after one cycle of SENA (lanes *2* and *4*) to identify the TCRBV genes expressed by expanded clonotypes. Clonal expansions of T cells expressing BV8S2 and BV17S1 were detected in the SF. CDR3 size analysis (10) showed a minimum of two clones expressing BV8S2 and one clone expressing BV17S1. The V-D-J junctional sequence of the BV17S1-positive clone was determined and is indicated.

TCRBV probes are summarized in Table I and Fig. 5. The identity of preferentially expressed TCRBV-D-J genes was confirmed in all 14 patients by SENA, RNase protection assays, and CDR3 size analysis using two independently produced cDNAs for each sample. In selected cases, sequence results were also confirmed in duplicate experiments. T cell clonal expansions were identified in each patient, although the minimal number of clones predicted by CDR3 size analysis of SENA-retained sequences ranged in SF from 1 (RA-9 and RA-11) to 23 clones (RA-20), and in PBL from 1 (RA-23) to 11 clones (RA-22). With the exception of BV5S1, BV5S3, BV5S6, BV10S1, BV13S3, BV14S1, BV16S1, and BV24S1, all TCRBV genes analyzed were used by the expanded T cell clones (Table I). It is noteworthy that all TCRBV overexpressions detected in the repertoire analysis by direct RNase protection assay (see above) were found by SENA to be due to clonal expansions, although they represent only a small proportion of all T cell clones identified.

Earlier studies have documented enhanced expression of

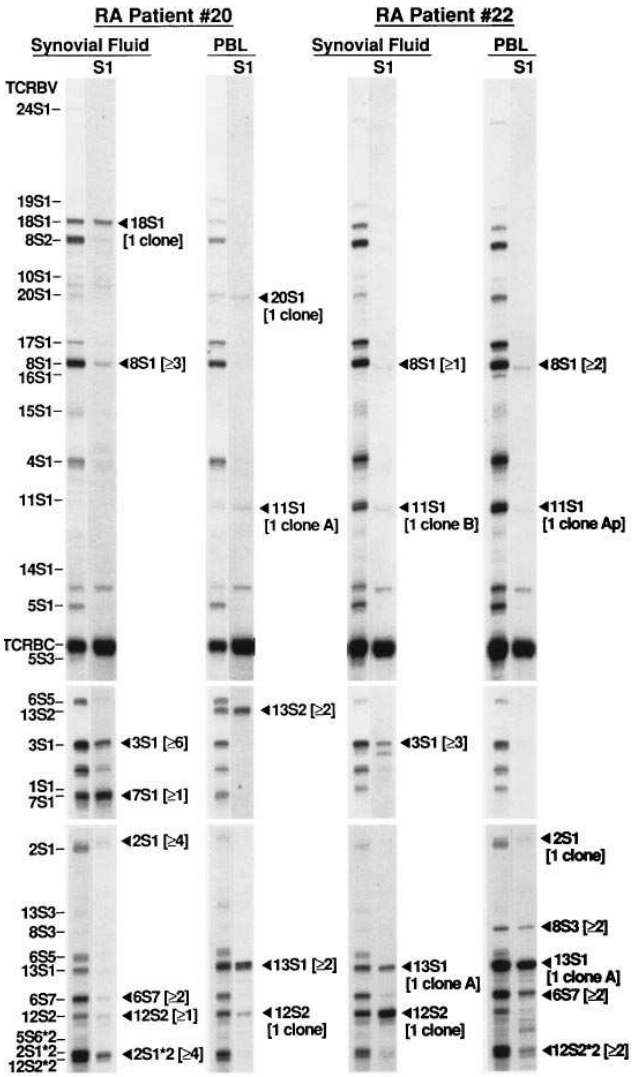

Figure 4. SENA analysis of T cell clones expanded in RA patients RA-20 and RA-22. T cell RNA from SF and PBL was analyzed by RNase protection assay (three probe sets), either directly (*left lane*) to determine the total TCRBV repertoire, or after one cycle of SENA (*right lane*, labeled with *S1*) to identify the TCRBV genes expressed by expanded clonotypes. Clonal expansions are indicated by arrows, and the minimum number of clones predicted by CDR3 size analysis is given in parentheses. V-D-J sequences for monoclonal T cells are characterized and are displayed in Fig. 5.

certain TCRBV gene families in SF compared with peripheral blood, including BV2, BV3, BV6, BV14, and BV17 (2, 5–7). In our samples of SF from 14 RA patients, clonal expansions were noted within cell subsets expressing several of these TCRBV families, including BV2 (7 patients), BV3 (4 patients), BV6 (4 patients), and BV17 (1 patient). Additionally, clonal expansions involving BV1 (3 patients), BV8 (6 patients), and BV12 (5 patients) were repeatedly observed in the 14 SF samples analyzed. In some, but not all cases, expanded clonotypes expressing the same TCRBV were also observed in the matched PBL. In general, more clones were detected in joint samples: a minimum of 103 clones in SF vs. 40 in PBL. In particular, eight clones giving strong signals after SENA, presumably composed of large numbers of cells, were found in SF vs. only two in PBL. In patients where CD4 and CD8 T cells could be separated, SF showed more clones in the CD8 than the CD4 subset (16 vs. 3 clones), whereas PBL showed more clones in the CD4 subset (8 vs. 4).

As a control, CD4 and CD8 T cells were isolated from the peripheral blood of seven healthy donors and analyzed for clonal expansions by SENA (not shown). No evidence for clonal accumulations was observed in the CD4 subset. In contrast, clonal expansions were clearly identified in the CD8 T cell subset of five of these donors. Based on band intensity observed after SENA, such T cell clones appeared to be composed of fewer cells compared with those identified in RA patients. Of interest and in agreement with results obtained previously in other laboratories (17–19), the number of T cell clones in normals appeared to correlate with donor age, since one clone was detected in three individuals 20–30 yr old, two clones in one individual 29 yr old, and three clones in one individual 48 yr old.

TCRBV-D-J sequences preferentially expressed in RA patients. Of the 68 TCRBV-positive sequences identified by SENA as being enriched in the 14 patients (Table I), 33 gave a single band in CDR3 size analysis and were therefore sequenced directly. For 27 such products, sequence analysis was successful and the results are displayed in Fig. 5. Since BV13S1 appeared to be dominantly expressed in the SF of patient RA-9 (see above), direct sequencing of BV13-D-J before SENA was also performed and the same was done for BV1-D-J of patient RA-10. Identical sequences were obtained in both instances, confirming the accuracy of SENA in selecting dominant V-D-J sequences. Generally, diverse sequences were identified, with 9 of the 13 TCRBJ and both TCRBD gene segments being used with no clear predominance. CDR3 sizes were variable, and no obvious amino acid motif was recognized. Interestingly, patient RA-22 showed the same BV13S1 sequence in both SF and PBL. Since an identical CDR3 size band was also observed in PBL of patient RA-17, both BV13S1 rearrangements of this individual were characterized by cloning in bacterial vectors and sequencing. However, the results indicated that the two BV13S1-expressing clones of patient RA-17 differed from that found in SF and PBL of patient RA-22. In contrast, the BV11S1-positive clones identified in PBL of patients RA-22 and RA-20 were identical at the amino acid level but differed at the nucleotide level, excluding the possibility of PCR contamination.

Discussion

Current characterization of TCR molecules includes sequence determination of mRNA or cDNA obtained from T cell clones or hybridomas selected for particular antigen reactivity. However, when the antigen is unknown, as in most autoimmune diseases (20), the relative contribution of defined TCR can only be inferred from their frequency in affected individuals compared with normals. Therefore, several methods have been developed to analyze antigen receptor V gene repertoires expressed in peripheral blood or inflammatory sites, including FACS® (21, 22), RNase protection assay (8, 9), and quantitative PCR (15). While such methods efficiently detect major repertoire modifications caused, for example, by superantigens (9, 15, 21, 22) or by particular peptide antigens that engage an almost uniform population of TCR (23, 24), they

PATIENT	SUBSET	TCRBV	V	(N) D (N)	J
RA-23	SF	V17S1 D2 J2S1	AGT AG S	C ACG ACT AGC GGG AGT TTA G S T T S G S L D	AT GAG CAG E Q
	PBL	V3S1 D2 J2S7	AGC AGT S S	TTA GGC CTC CA L G L H	C TAC GAG Y E
RA-22	SF	V12S2 D1 J1S5	GCC ATC A I	GGT ACA GGG GAT G T G D	AGC AAT CAT S N Q
	SF	V13S1 D2 J2S3	AGC AGT S S	TAC TCG GGG GAT Y S G D	GAT GCA CAG D T Q
	SF	V11S1 D2 J2S7	AGC AGT S S	GAA TTG AGC GGG AGG AG E L S G R S	C TCC TAC S Y
	PBL	V13S1 D2 J2S3	AGC AGT S S	TAC TCG GGG GAT Y S G D	GAT ACG CAG D T Q
	PBL	V2S1 D2 J2S7	TGC AGT C S	ACC GTG CAG GGG GCT ACG GGT GCT CT T V Q G A T G A L	C GAG CAG E Q
	PBL	V11S1 D1 J1S1	AGC AGT S S	GAT GGT GGC AAA G D G G K D	AC ACT GAA T E
RA-21	SF	V11S1 D1 J1S6	AGC AGT S S	TTA GGG ACC L G T	AAT TCA CCC N S P
RA-20	SF	V7S1 D2 J2S7	AGC AGC S S	CAA GGA ACA GGG GGA GGA Q G T G G G	TAC GAG Y E
	SF	V18S1 D2 J2S2	AGC TCA S S	CCA TGG GAC AGG AGA P W D R R	ACA GGG GAG T G E
	PBL	V11S1 D1 J1S1	AGC AGT S S	GAT GGG GGG AAG G D G G K D	AC ACT GAA T E
	PBL	V20S1 D1 J1S6	TGG AGT W S	ACC GGA CAG GCC A T G Q A N	AT AAT TCA N S
RA-17	PBL	V3S1 D1 J1S4	AGC AG S	G CAA CAC AGG GGA GTA GC R Q H R G V A	T GAA AAA E K
	PBL	V2S1 D2 J2S3	AGT GC S	C GAG ACA GGC GAA GAG A E T G E E	AGC ACA S T
	PBL	V13S1 D1 J2S1	AGC AGT S S	GAC ACC CCC GCG GGG GCC D T P A G A	ACC GGG T G
	PBL	V13S1 D1 J1S1	AGC AG S	G AAC TCA CAG GGG R N S Q G	ACT GAA T E
RA-14	SF	V2S1 D2 J2S7	GCT AG A	G GCA NCA GCG GGA G R A X A G A	CC TAC GAG Y E
	SF	V4S1 D2 J2S3	GTT GAA V E	GAG GGG ACT AGC GGG GTG G E G T S G V A	CA GAT ACG D T
RA-2	SF	V1S1 D1 J2S1	AGC AGC S S	GTA GAC ACA GGG AAA V D T G K	AAT GAG CAG N E Q
RA-18	SF-CD8	V15S1 D2 J2S2	ACC AG T	A GGG CTT R G L	ACC GGG GAG T G E
	SF-CD8	V3S1 D1 J1S5	AGC AGT S S	TTC AGG GGA GAT L S G D	CAG CCC CAG Q P Q
	PBL-CD4	V19S1 D2 J2S1	GCC ATG A M	AAT AGC GGG AGG AGA N S G R R	TAC AAT GAG Y N E
RA-19	SF-CD8	V4S1 D1 J1S6	GTT G V	GA TAC GGG GGG AC G Y G G T	T AAT TCA N S
	PBL-CD4	V4S1 D2 J2S2	AGC GT S	C CAC CTC CGG GGC GCC GGC C V H L R G A G Q	AG ACC GGG T G
RA-9	SF-CD8	V13S1 D1 J1S5	AGC AGT S S	TAC TCA GCT CAG GGC C Y S A Q G H	AT CAG CCC Q P
RA-10	SF-CD8	V1S1 D2 J2S1	AGC AGC S S	TCT ACC TCG GGT G S T S G A	CC TAC AAT Y N
	SF-CD8	V23S1 D1 J1S1	AGC AGC S S	TTA GTG G L V D	AC ACT GAA T E
RA-11	SF-CD8	V3S1 D1 J1S2	AGC AG S	C TCA TCC AGG GCG GAC AGG GC S S S R A D R A	C TAT GGC Y G

Figure 5. Junctional TCRB sequences expressed by expanded T cell clones in RA patients. V-D-J sequences enriched in SF or PBL from RA patients were selectively amplified by SENA, as described in Table I, and sequenced.

are not sensitive enough to assess perturbations induced by most conventional antigens, nor do they provide information on the clonal characteristics of the expanded populations. Recently, more sophisticated procedures have been used to analyze the heterogeneity of PCR-amplified V-D-J sequences in the attempt to identify clonal cell populations. These procedures rely on variations in electrophoretic mobility determined by V-(D)-J fragment length (CDR3 size analysis) (10), nucleotide sequence (single-strand conformational polymorphism) (25), or the relative instability of heteroduplex molecules (denaturing-gradient gel electrophoresis) (26). Possible drawbacks of these techniques include the fact that not every sequence change induces electrophoretic mobility shifts, and that different sequences may comigrate, which complicates banding patterns and, particularly, isolation and characterization of clonal sequences. This report describes a method, SENA, that efficiently eliminates infrequent V-(D)-J sequences contributed by nonspecific cells and selectively amplifies highly represented sequences derived from clonally expanded lymphocyte populations. Based on its adaptability to the RNase protection assay, quantitative PCR, CDR3 size analysis, or direct cloning and sequencing, SENA can be used for in vivo and in vitro studies addressing clonal evolutions and fluctuations induced by specific antigens (23, 24) or disease (27)

Table I. TcRBV Genes Expressed by Expanded T Cell Clones in PBL and SF of 14 RA Patients

Patient	Subset	TCRBV, CDR3 heterogeneity and expression levels							
RA-23	SF	**17S1(1)+++;**	8S2(2)++						
	PBL	**3S1(1)+++**							
RA-22	SF	**12S2(1)+++;**	**13S1(1)++;**	**11S1(1)++;**	3S1(3)++;	8S1(1)+			
	PBL	**13S1(1)+++;**	8S1(2)++;	8S3(2)++;	6S7(2)++;	12S2*2(2)++;	**2S1(1)+;**	**11S1(1)+**	
RA-21	SF	6S7(1)++;	**11S1(1)+;**	2S1(4)+					
RA-20	SF	**7S1(1)+++;**	**18S1(1)++;**	2S1(4)++;	2S1*2(4)++;	3S1(6)++;	8S1(3)+;	6S7(3)+;	12S2(1)+
	PBL	13S1(2)+++;	13S2(2)+++;	12S2(1)++;	**11S1(1)+;**	**20S1(1)+**			
RA-17	SF	12S2*2(3)++;	2S1(2)++						
	PBL	12S2*2(2)+++;	**3S1(1)++;**	8S1(1)++;	4S1(2)++;	**2S1(1)+;**	13S1(2)+		
RA-15	SF	2S1(4)++;	13S1(2)++;	6S7(5)++;	8S1(5)+				
RA-14	SF	**2S1(1)+;**	**4S1(1)+;**	6S5(1)+;	8S1(3)+;	7S1(4)+			
RA-2	SF	2S1(4)++;	12S2*2(7)++;	**1S1(1)+**					
RA-18	SF-CD8	**15S1(1)+++;**	**3S1(1)++**						
	PBL-CD4	**19S1(1)+**							
	PBL-CD8	3S1(4)+							
RA-19	SF-CD4	8S1(3)+							
	SF-CD8	**4S1(1)++**							
	PBL-CD4	**4S1(1)++;**	6S5(2)++;	6S7(2)++;	8S2(2)+				
RA-8	SF-CD8	1S1(4)+							
RA-9	SF-CD8	**13S1(1)+++;**							
RA-10	SF-CD8	**1S1(1)+++;**	**23S1(1)+++;**	2S1(3)++;	12S2*2(2)++				
RA-11	SF-CD8	**3S1(1)+++**							

SF and PBL RNA samples from 14 RA patients were analyzed as described in Fig. 3, but using 28 TCRBV probes. CDR3 size heterogeneity associated with each TCRBV is indicated in parentheses. Based on the titration shown in Fig. 2, clonal expansions were tentatively classified as being > 400 cells (+), > 800 cells (++), and > 1,600 cells (+++) per 10^6 polyclonal cells. Junctional V-D-J sequences of TCRB giving single CDR3 size (in bold) were analyzed by direct sequencing, while the two BV13S1 rearrangements of patient RA-17 were characterized upon cloning in bacterial vectors, and the results are displayed in Fig. 5. TCRBV designation is according to Wei et al. (34).

and for identifying TCR engaged in responses to known and unknown antigens.

SENA is based on two principles widely used in molecular biology, i.e., hybridization of complementary sequences, which is both quantitative and specific, and cleavage of nucleotide mismatches and loops. A potential problem in this method is that anchor-PCR, while limiting the number of primers needed, can create size-heterogeneous cDNA molecules at their 5′ ends. Such size heterogeneity may significantly decrease the efficiency of SENA, since only rehybridized molecules of the same length will be protected from nuclease digestion. Nevertheless, the sensitivity determined by the Jurkat cell titration experiments indicates that sufficient numbers of molecules of identical size are produced by anchor-PCR to correctly rehybridize. A second possible limitation is the use of S1-nuclease, an enzyme that is not always very efficient in detecting single nucleotide mismatches. Recently, another class of enzymes, the bacteriophage resolvases, was found to be more specific and efficient in mutation analysis (28, 29). It is probable that, in the future, the use of resolvases will improve the specificity of SENA, particularly for analysis of Ig somatic mutations, often characterized by single-nucleotide changes.

Previous reports indicated T cell clonal expansions in the CD8 subset of normal individuals (17–19). Control studies by SENA of CD4 and CD8 T cells from seven normal donors also revealed expanded clonotypes confined to the CD8 subset that increased numerically with age. The clones detected in normals were, nevertheless, clearly fewer (only one to three per individual) and, as defined by band intensity, composed of a smaller number of cells than in RA patients. However, the possibility exists that some of the clones found in RA patients are related to those observed in normals. In fact, the BV3S1-D-J sequence enriched in PBL of patient RA-23 is strikingly similar, showing a single (minor) amino acid difference (L for V), to those expressed by expanded clonotypes of two normal individuals included in the study of Fitzgerald et al. (19). Interestingly, longer exposures of RNase protection assay gels of SENA-treated samples showed several additional bands of minimal intensity in both normals and RA patients (not included in Table I). Although these bands could be due to inefficient S1 nuclease digestion, they probably represent small clones that, according to the titration shown in Fig. 1, consisted of < 0.04–0.08% of the total T cell population. The significance of T cell clonal expansions in normals remains unclear but, as suggested previously (17–19), could be related to proliferation of regulatory or effector cells specific for ubiquitous microbial antigens.

The fact that clonal T cells were more frequently observed in RA patients versus normals suggested that at least some clonal expansions are disease related. In addition, the presence of more clonal expansions in SF than PBL of RA patients indicates local stimulation or preferential sequestration of T cells displaying particular specificities. Consistent with this conclusion is the observation that the majority of TCRB sequences enriched in SF differed from those in PBL. Nonetheless, in RA-22, the same BV13S1-D-J sequence was found in both SF and PBL (Figs. 4 and 5), while in five additional cases (BV8S1 in RA-22, BV12S2 in RA-20, BV12S2 and BV2S1 in RA-17,

and BV3S1 in RA-18), the presence of the same clone in SF and PBL was suspected on the basis of CDR3 analysis showing identical bands (Table I and not shown). Similar evidence for recirculation of T cell clones in synovia and blood of RA patients has been obtained previously (2, 4). Interestingly, analysis of patients with early disease, although restricted to CD4 cells, showed that expanded clones were primarily found in PBL (4). The differences with our results, showing accumulation of CD8 clones in SF, of which only a few appear in PBL, might be related to the chronic state of the patients we analyzed. T cell clonal expansions in the CD8 subset in patients with advanced disease, also found by others (19, 30), could be connected to the enrichment of these cells, particularly those expressing activation markers in both PBL and SF. Therefore, it is possible that initial expansion occurs outside the joints, involves primarily MHC class II–restricted CD4 T cells, and is followed by gradual accumulation of CD8 T cell clones in the synovia. The timing of transition between helper functions provided by CD4 clones and effector functions contributed by CD8 clones is probably critical for disease manifestation and joint destruction. Additional longitudinal studies following patients from early disease to chronicity will significantly clarify this issue.

Obviously, the major questions are the basis of T cell clonal accumulations in RA, whether they are antigen dependent, and whether environmental or endogenous antigens are implicated. The diverse TCR expressed by expanded clones suggests that the stimulus is rather heterogeneous, although in vitro studies have shown that even single peptide–MHC complexes may induce diverse responses (31, 32). Nevertheless, the fact that the same TCRB amino acid sequence is enriched in both RA-20 and RA-22 raises the possibility of a common stimulus, at least in some patients. It would be interesting to determine whether these clones also express the same TCRα chain and whether the expansion is related to RA or to the MHC of these patients. Another observation that does not support random clonal expansion is that not all genes analyzed were found to be used by the expanded clonotypes, particularly BV5S1, as also noted by others (5, 19), which is normally highly expressed and might account for 5–20% of total TCRBV expression (8, 15, 33). Moreover, the repeated observation of T cell clones expressing members of particular TCRBV gene families, such as BV1, BV2, BV3, BV6, BV8, and BV12, could be connected to the increased expression of several of these genes identified previously in the synovia of RA patients (2, 5–7). Clearly, functional in vitro studies with T cell clones and synovial antigens or synthetic peptide libraries will be required to characterize the specificity of the clonotypes expanded in the inflamed joints and to determine the nature of the stimulus. It can be predicted that SENA will significantly facilitate isolation of clonotype-specific probes and thus selection of enriched and, therefore, potentially pathogenic T cell clones to be used in such functional studies.

Acknowledgments

This is publication No. 9347IMM from the Department of Immunology, The Scripps Research Institute.

The authors thank Drs. Shu Man Fu, Vincent J. Giuliano, and Greg S. Pudhorodsky for providing specimens, and Drs. Jonathan Sprent, Jonathan Kaye, Wendy Havran, and Nicholas Gascoigne for critical review.

The work reported herein was supported, in part, by National Institutes of Health grants AR31203, AR39555, AI34215, CA52539, and P60-AR30692, and the VA Research Service.

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