Diversity of T-Cell Antigen Receptor Vβ Gene Utilization in Advanced Human Atheroma

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Abstract Human atheromata contain T lymphocytes, but knowledge of the function and receptor specificity of these cells is limited. Immunohistochemical studies have established that T cells in advanced human carotid plaques express predominantly the αβ form of the T-cell receptor (TCR). We then compared the use of variable region genes of the β-chain (Vβ) of the TCR for antigen by analysis of 14 carotid plaques and peripheral blood samples obtained at carotid endarterectomy. We used a direct approach that avoids isolation and culture of T cells. RNA extracted from lesions and peripheral blood mononuclear cells was reverse transcribed and amplified by polymerase chain reaction (PCR) to determine rearrangements of 18 Vβ sequences. PCR products were visualized on Southern blots using a probe internal to the PCR primers. Input cDNA from lesions and peripheral blood was adjusted to yield equivalent signals for a conserved region of the TCR Vβ-chain to permit comparisons. As expected, utilization of TCR Vβ genes in peripheral blood cells was nonselective: an average of 17 of 18 Vβ regions yielded signals (n=14). Frequency of variable-region gene usage in lesions and blood was highly concordant: of 252 sequences tested (14 samples, 18 sequences per sample), 240 were identified in peripheral blood versus 207 in plaques. Vβ genes 10 and 11 were not expressed in plaques, a significant difference when compared with peripheral blood (P=.0001 by χ2). However, the remaining 16 genes showed no significant differences. This analysis indicates that T cells generally express a diverse pattern of Vβ genes within complex human atheroma.

Key Words • immunology • polymerase chain reaction • atherosclerosis • T lymphocyte

It has only recently become appreciated that T lymphocytes constitute a major cell type in regions of advanced human atherosclerotic plaques.1-3 Recent evidence indicates that these cells are in a state of immunologic activation.4 For example, the majority of plaque-derived T cells express the low-molecular-weight form of leukocyte common antigen (CD45) and very late activation antigen-1, Ta-1 (CD26) and the major histocompatibility complex class II antigen HLA-DR.5,6 Plaque-derived T cells do not, however, express high levels of the interleukin-2 receptor β-chain (CD25).5 Furthermore, Hansson et al4 have localized interferon gamma, a product of T-cell activation, in atherosclerotic plaques. Smooth muscle cells in the vicinity of T cells also express class II histocompatibility antigens, indicating recent exposure to the TCR for antigen.6 Plaque-derived T cells propagated from advanced human atheroma using T-cell receptor β-chain variable (Vβ)-region sequences of the TCR for antigen.7,8 Although the presence of activated T cells in atheroma is well established, the nature of the potential antigen(s) that could provide activating signals remains undefined. It is not even clear whether the T cells in the atherosclerotic lesion respond to a restricted set of antigens or recognize a wide number of epitopes. Analysis of the clonality of the T-cell population resident in human atheroma should help to resolve this question. Each T cell bears on its surface a receptor for antigen (TCR), which is a heterodimer made up of an α-chain paired with a β-chain or, alternatively, a γ-chain paired with a δ-chain.9,10 In analogy with the generation of diversity in immunoglobulin molecules, the specificity of TCR for a given antigen depends on the sequence of a variable-region domain. Thus, analysis of the utilization of the different variable-region sequences in a T-cell population can provide information regarding the diversity of antigens they can recognize.

Stemme et al11 recently analyzed the utilization of β-chain variable (Vβ)-region sequences of the TCR in T cells propagated from advanced human atheroma using the T-cell growth factor interleukin-2 to amplify T cells in vitro. Southern blotting of restriction digests of DNA in T-cell populations cloned from lesions showed considerable heterogeneity, indicating a polyclonal population of cells in the original population. Because of the requirement for propagation in vitro and reliance on lymphokine rather than antigenic stimulation to expand the population of T cells, this method could select for or against various T-cell clones. In this study we applied an alternative approach involving polymerase chain reaction (PCR) analysis of the sequences encoding the Vβ region of the TCR in cDNAs prepared by reverse transcription of mRNA extracted directly from human atheroma. This method avoids any potential selection bias inherent in approaches that depend on in vitro propagation. Our analysis of 14 carotid endarterectomy specimens indicates that T cells in these advanced human lesions display considerable heterogeneity.
Methods

Patient Population

Fourteen patients selected for elective carotid endarterectomy at the Brigham and Women’s Hospital provided informed consent for providing venous blood (30 mL obtained by venipuncture) at the time of surgery. Immediately on removal, the carotid endarterectomy specimen was divided into portions, one for routine diagnostic use by the surgical pathologist, a second for extraction of RNA, and in some cases a third for immunohistochemical analysis. The protocols for blood drawing and the use of normally discarded tissues were approved by the institutional Human Investigation Review Committee.

Immunohistochemical Analysis

Four of the carotid endarterectomy specimens were studied by immunohistochemistry to determine the distribution of T cells bearing the α/β or γ/δ forms of the TCR. The portion of the plaque specimen used for cytochemical analysis was covered with embedding compound (optimal cutting temperature) and snap-frozen in 2-methylbutane with liquid nitrogen and stored at −70°C. Six-micrometer cryostat sections were mounted on polylysine-coated glass slides, fixed with acetone at −70°C, rehydrated with phosphate-buffered saline, treated with hydrogen peroxide (0.6% for 15 minutes) to inactivate endogenous peroxidases, and stained with either a monoclonal antibody that recognizes the β-chain of the TCR heterodimer (antibody TA 1151 βF1, T Cell Sciences), the β-chain of the TCR γδ complex (antibody TA 1061 TCRδ1, T Cell Sciences), or a pan-T cell antibody that recognizes CDS (catalog No. 92-0001, Becton-Dickenson). After washing to remove unbound primary antibody, the sections were incubated with a biotinylated secondary antibody (horse antimouse, ±5 µg/mL, Vector Laboratories; catalog No. BA-2000) and avidin-biotinylated horseradish peroxidase macromolecular complex (Vector PK6100). After more washing, the substrate (aminoethylcarbazole) was added, and the specimens were incubated for an additional 3 minutes at room temperature to develop the color. The slides were then washed again in sodium acetate buffer (0.05 mol/L, pH 5.2) and counterstained for 1 minute with Gill’s hematoxylin No. 3 (Sigma) diluted fourfold. After drying, the slides were coverslipped using a gel adhesive.

Peripheral Blood Mononuclear Cell Isolation

Mononuclear cells were isolated from the peripheral blood sample using the Ficoll extraction process. Blood samples were diluted 1:1 with RPMI-1640 medium and were then layered over Ficoll (density, 1.119) and centrifuged at 400g for 30 minutes. The hazy band containing the peripheral mononuclear cells was removed and resuspended in guanidinium isothiocyanate solution (GITC) (4 mol/L) containing 20% sarcosyl and immediately stored at −70°C.

RNA Preparation

The carotid endarterectomy specimens were washed carefully to remove blood and were homogenized in the presence of GITC; immediately after collection, 20% sarcosyl was added and the homogenate was pellets in a low-speed centrifuge at 3200g at 4°C for 10 minutes. The supernatant was then carefully layered over freshly filtered cesium chloride and centrifuged at 410,000g for 18 hours at 15°C. After discarding the supernatant, the pellet was removed, dried, and resuspended in 100 µL of Tris-EDTA buffer (10 mmol/L). This RNA was quantitated spectrophotometrically and visualized on a 1.5% agarose gel to evaluate its integrity. A similar procedure was carried out for the peripheral blood mononuclear cells extract prepared as described above.

cDNA Preparation and PCR Analysis

A PCR strategy was employed to analyze the use of different TCR β-chain variable-region sequences in the blood- and plaque-derived RNAs (Fig 1). Three-prime primers were based on sequences of the TCR β-chain common region. Nineteen different families of variable-region genes provided the sequences for 5' PCR primers.12 These 5' primers were used with a 3' primer denoted C5, which was chosen from exon 3 of common Cβ2 sequences (complementary to nucleotides between 1884 and 1854).14 In our study, sequences of variable-region gene family 13 did not amplify and were therefore not used. The strategy for amplification was based on the ability to selectively amplify sequences corresponding to the spliced and processed mature mRNAs rather than germline or genomic DNA because of the wide distances separating the complementary sequences in all but the mature mRNA. As internal controls, we performed two independent amplifications of Cβ2 sequences to yield distinct products denoted “H” and “C.” The H product resulted from amplification of common Cβ2 sequences using a 5' primer from exon 1 (denoted human common primer, HCP, from nucleotide position 1053 to 1085) and C5 as the 3' primer. The C product resulted from amplification of common Cβ2 sequences using another 5' primer further upstream in exon 1 (denoted C3, from nucleotide position 807 to 837) and the same 3' primer (C5) used to generate H.

The RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (200 U/µL per 1 µg of RNA) in the presence of bovine serum albumin, NTP (10 mm), RNAsin (40 U/µL), oligo-dT (0.1 µg/µL), and distilled water. The resultant cDNA (0.1 µg) was then amplified using the PCR for 35 cycles (5 µL of 10× PCR buffer, 1 µL of 10 mmol/L dNTP, and 1 U of Taq polymerase and distilled water in a final reaction volume of 50 µL) in the presence of a TCR Vβ gene or constant-region gene (HCP) as the 5' primer and a TCR β-chain constant-region gene (C5) as the 3' primer. The final concentrations of buffer constituents were Tris-HCl 10 mmol/L (pH 8.3), KCl 50 mmol/L, MgCl2, 2.5 mmol/L, and bovine serum albumin 100 µg/mL. Denaturation was carried out for 30 seconds at 95°C, reannealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds.

Paired blood- and plaque-derived products were visualized on a 3% agarose gel using ethidium bromide and transferred to a nylon membrane (Hybond N, Amersham) using the Southern technique. An internal probe (HCP) was then end-labeled with 80 µCi of γ-32P-labeled ATP (Dupont–New England Nuclear) using 10 U of T4 kinase (unincorporated precursor was removed by chromatography on a NENsorb column). The blot was prehybridized at 42°C for 4 hours (10 µL denatured salmon sperm DNA, 1000 µL 100× Denhardt’s solution, and 50 mL prehybridized solution: 30 mL 20× saline–sodium citrate (SSC), 67 mL distilled water, 2.5 mL of

FIG 1. Diagram shows strategy for polymerase chain reaction amplification of β chain variable (V) and constant (C) region genes. Arrows refer to the primers used for polymerase chain reaction. The specific V regions were amplified using a series of family-specific 5' primers and a 3' primer from exon 3 of Cβ2. The same 3' primer was used in conjunction with 5' primers from exon 1 to provide internal controls. We employed two different 5' primers for this purpose (see “Methods”); only one is depicted in this figure to illustrate the strategy. D indicates diversity; J, joining regions.
Figure 2. Photomicrographs show immunohistochemical analysis of carotid endarterectomy plaque (6-μm frozen sections). A, Section stained with T-cell receptor (TCR) δ1, an antibody against the δ-chain of the γδ TCR complex. Arrow designates the cell seen in B (original magnification ×100). B, Same as A (original magnification ×400). C, Similar section stained with βF1, an antibody that recognizes the β-chain of the αβ TCR complex (original magnification ×100). D, Same as C (original magnification ×400). E, Similar section stained with an anti-CD3 that recognizes all T cells (original magnification ×100). F, Same as E (original magnification ×400).

Results

Immunohistochemical analysis showed few cells reactive with the antibody that recognized the γδ form of the TCR in any of the four plaques studied with this technique (Fig 2A and 2B). However, all four of the specimens analyzed showed abundant cells staining for the αβ form of the TCR (Fig 2C). The staining for the αβ TCR was localized to cells with the appearance of small lymphocytes (Fig 2D). This evidence for predominance of αβ TCR-positive lymphocytes in advanced atheroma is consistent with results from other laboratories.14,15

Because γδ T cells were scarce in these specimens, we limited our analysis of the variable regions to the αβ...
heterodimer of the TCR. We used the common-region sequences as an internal standard to verify that we analyzed approximately equal levels of input T cell-derived cDNA in our PCR analyses. As the concentration of T cells bearing the $\alpha/\beta$ form of the TCR was higher in peripheral blood, we diluted the input cDNA to a degree shown in pilot amplifications from each specimen to yield approximately equal signals on the Southern blots for the TCR common-region sequences used to standardize the input T cell-derived RNA (Fig 3). A typical example (Fig 3) shows the similarity between the patterns of the $\beta$ sequences found in extracts of the plaque (top panel) and peripheral blood obtained from the same patient at the time of plaque removal (bottom panel).

Similar analysis of plaque and peripheral blood V$\beta$ utilization on all 14 patients yielded similar results (Fig 4). Although most of the V$\beta$ gene families were represented in both blood- and plaque-derived cDNA, plaque-derived cDNAs consistently showed no signals corresponding to V$\beta$-10 and -11 gene family sequences (Figs 3 and 4).

Of the 252 potential bands (18 V$\beta$ gene families analyzed in 14 patients), we found 207 in plaque-derived RNA and 240 in peripheral blood mononuclear cell-derived RNA (Table). $\chi^2$ Analysis established that this difference was highly significant statistically. On average, the plaque-derived cDNA contained 15 of a possible 18 V$\beta$ gene sequences, whereas blood-derived cDNA contained sequences from 17 of these families.

**Discussion**

We focused on analysis of the V$\beta$ region of the TCR for two reasons. First, our data, and that from other laboratories, established that the $\alpha/\beta$ TCR is the predominant form expressed by T lymphocytes in human atherosclerosis. Second, the $\beta$-chain of the TCR displays a greater diversity, encompassing almost 20 different families of variable regions,$^9,10$ in contrast to the $\gamma/\delta$ form of the TCR, which has fewer variable regions.$^{16,17}$ T cells expressing the $\gamma/\delta$ form of the TCR may have functions distinct from those expressing the $\alpha/\beta$ receptor. For example, the $\gamma/\delta$-positive T cells may be involved during ontogeny of the immune system or may respond in postnatal life to a distinct subset of antigens, for example, mycobacterial epitopes.$^{16,17}$

Our results show use of diverse V$\beta$ region families in plaque T cells. However, we found two families (V$\beta$-10 and -11) to be absent in plaque-derived T cells, while their use was sometimes detected in peripheral blood obtained simultaneously. Although this difference is highly significant statistically, its biological meaning is uncertain. It is well known that the efficiency of the PCR amplification varies widely as a function of the particular primers used to amplify different target sequences. We believe that the relative lack of products amplified from the V$\beta$-10 and -11 regions reflects lower efficiency of the primers used for PCR for these targets.
However, the difference between blood and plaque suggests that a technical limitation of PCR may not completely explain the lower signals derived from these two Vβ region families in plaque-derived T cells.

The analysis of 14 patients studied here using the PCR approach agrees well with the results obtained by Stemme et al.11 on cells cloned from four human atherosclerotic plaques. The PCR method used here is applied to RNA extracted from fresh atherosclerotic plaques and avoids any potential artifacts due to positive or negative selection of different T-cell clones during propagation in vitro. Nonetheless, it is striking that application of two distinct methods with different inherent limitations and advantages consistently shows that the T cells in advanced human atherosclerotic plaques bear structurally diverse TCR and are likely capable of responding to a wide variety of antigens. It should be noted that a single complex immunogen (eg, a modified lipoprotein or glycosylated protein) could have many antigenic determinants and thus could conceivably elicit a diverse T-cell response such as that observed in our study.

In some diseases with a probable immune pathogenesis, the utilization of variable regions of the TCR genes does appear restricted. For example, in experimental allergic encephalitis and in human multiple sclerosis, T cells infiltrating the central nervous system display enrichment in utilization of certain Vβ sequences.12 In human allografts undergoing rejection, there is likewise restriction in the use of Vβ regions.18-20 In Crohn’s disease and sarcoidosis, conditions with possible immune or autoimmune components, T cells also display some restriction in Vβ use.21-23 Nevertheless, in delayed-type hypersensitivity, a prototypic antigen-specific immune response, infiltrating T cells may display a polyclonal pattern of Vβ region gene utilization. This diversity could result from recruitment or retention of T lymphocytes not reactive to the initiating antigen because of cytotoxic cascades characteristic of this immunopathologic reaction.

In this regard, we must bear in mind that our current analysis examined only late-stage human atheroma that was severe or symptomatic enough to warrant surgical intervention. The genesis of these lesions presumably began decades before the specimen became available for this analysis. Thus, our finding of a polyclonal infiltrate does not exclude that a limited range of antigens contributed to the initial immune reaction in the earlier stages of atherosclerotic lesion development. Furthermore, T cells infiltrating the plaque might recognize a “superantigen,” as recognition of these structures does not require specific Vβ sequences. Many cells within atheroma can secrete cytokines that are chemotactic for T lymphocytes. Cytokines can also activate adhesion molecules on vascular wall cells that may recruit and retain T cells within lesions. Thus, cytokine gene expression at various stages in atherosclerosis may promote recruitment of T cells of a wide variety of antigenic specificities over time. Even if the T-cell infiltrate within the advanced human atheroma is recruited nonspecifically, the majority of these cells bear markers of chronic activation. Our results, and those of Stemme et al.,11 are consistent with polycloncal stimulation and an ongoing and chronic T cell–mediated immune response in even the late stages of human atherogenesis.

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References
