The antigen receptor diversity of pathogenic T cells in Sjögren’s syndrome (SS) may have important implications in the development of the disease; cytokines from these cells and other sources also play a role in the pathogenesis of this disease. Using a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique, we have attempted to correlate the presence of restriction in the T-cell receptor (TCR) repertoire with cytokine profiles. We have analysed TCR V\(\alpha\) family usage, and the expression of interleukin-1\(\alpha\) (IL-1\(\alpha\)), IL-1\(\beta\), IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, interferon-\(\gamma\) (IFN-\(\gamma\)) and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), in labial biopsies from 12 patients with SS and compared these with samples from three patients with chronic sialadenitis (CS). Only one of the SS biopsies showed evidence of V\(\alpha\) restriction (three out of 18 gene families). Apart from this, expression patterns were similar in both patient groups. Four of the 12 SS samples demonstrated a ‘limited heterogeneity’ of the V\(\alpha\) repertoire with 3–4 families predominantly expressed, in particular V\(\alpha\)1 and V\(\alpha\)3. Peripheral blood lymphocytes were unrestricted. The cytokine profiles of the SS and CS biopsies were generally similar. However both IFN-\(\gamma\) and IL-1\(\alpha\) were absent from CS, but present in SS samples. The expression of IFN-\(\gamma\) in the majority of the samples, together with a lack of IL-4 and IL-13 mRNA, suggests the predominance of a Th1 response in SS. There was no clear association between the repertoire of V\(\alpha\) genes expressed and the cytokine profile observed. However, the V\(\alpha\) restriction in one SS sample did correspond with a limited diversity of cytokines detected.

**KEY WORDS:** T-cell receptor, V\(\alpha\), Cytokines, Sjögren’s syndrome.

Sjögren’s syndrome (SS) is an autoimmune disease characterized by focal lymphocytic infiltration of salivary and lacrimal glands, in which patients present with dry eyes and mouth [1]. The resulting destruction of the acini and ducts of the glands is thought to be linked to the infiltrate; unlike many other autoimmune diseases, this infiltrate is formed predominantly of memory CD4\(^+\) T cells [2, 3]. The possibility of restriction of the TCR repertoire in human autoimmune disorders, as has been found in several animal models of autoimmune disease [4, 5], would have important therapeutic implications [6]. Despite early hopes, however, the detection of T-cell receptor (TCR) restriction in human autoimmune disease has remained elusive [7, 8].

The analysis of TCR usage in the salivary glands of SS patients has mainly been directed at the TCR\(\beta\) locus, typically showing limited heterogeneity in TCR family usage, with overexpression of V\(\beta\)2 and V\(\beta\)13, and evidence of biased TCR usage [9–12]. An analysis of TCR V\(\beta\) families showed restricted expression early in disease, with diversification in TCR V\(\beta\) usage later in disease [13]. A similar situation is known to occur rapidly in experimental autoimmune encephalomyelitis, due to ‘repertoire spreading’ and bystander lymphocyte accumulation [14, 15]. Other investigations have revealed a diverse V\(\beta\) repertoire in the lacrimal glands [16], and a more restricted V\(\beta\) repertoire in the kidneys than salivary glands of SS patients [11].

Cytokines are also important in the development of autoimmune diseases, both as mediators of the immune response and, potentially, as agents of tissue destruction [17]. Several cytokines have been detected in biopsies from SS patients, including interferon-\(\gamma\) (IFN-\(\gamma\)), interleukin-1\(\alpha\) (IL-1\(\alpha\)), IL-2, IL-6, IL-10 and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), with detection of cytokine mRNA in both infiltrating lymphocytes and salivary gland epithelial cells [18–20]. Other investigations have shown increased synthesis of IL-10 by T-cell clones from salivary gland biopsies [21], and increased expression of the immunosuppressive cytokine transforming growth factor-\(\beta\)2 (TGF-\(\beta\)2) in salivary gland biopsies [19, 22]; the lower production of TGF-\(\beta\)2 in biopsies with the highest levels of lymphocytic infiltration has led to the suggestion that it is acting as a local immunosuppressive agent in vivo [19, 22]. Contradictory results could be due to methodological differences in various studies, or may indicate discrepancies in the cytokine profile according to the stage and activity of the disease.

Despite these investigations into the TCR and cytokine patterns present in salivary gland biopsies, no previous studies have attempted to correlate the patterns of TCR usage, cytokine production and histological evidence of disease state in the same biopsies. In the present study, we have attempted to correlate TCR and cytokine gene expression in the same tissue samples. If TCR gene usage suggests the presence of a
clonally limited subpopulation, then the pattern of cytokine expression associated with this autoreactive T-cell fraction may be expected to be predominant in the pool of cytokine mRNAs expressed in the biopsy, and may represent the key cytokines involved in the pathogenesis of the disease. Using RNA derived from minor salivary glands from 12 patients with SS and three with chronic sialadenitis (CS), we have therefore carried out reverse transcription-polymerase chain reaction (RT-PCR) analysis of TCR V\textalpha family usage and for mRNA from a wide range of cytokines.

**MATERIALS AND METHODS**

**Patients**

Minor salivary glands were obtained following a lower labial salivary gland biopsy from 11 female patients and one man with SS, and one man and two women with CS, with informed consent. Blood samples (10 ml) were obtained from all patients. This study had local ethical committee approval and all subjects gave informed consent. All patients complained of persistent dry mouth and those with SS also complained of persistent dry eyes. Each biopsy comprised at least five lobules of minor salivary glands. One biopsy was fixed in 10% buffered formal saline, prepared for routine paraffin embedding, sectioned and stained with haematoxylin and eosin. The sections were assessed semiquantitatively for focal lymphocytic sialadenitis (FLS) using the focus scoring system described in 1974 by Greenspan et al. [23]. The finding of more than one focus of 50 or more mononuclear cells in 4 mm of glandular tissue was considered supportive of the diagnosis of SS. Since a focus score of 10 is generally the highest that can be counted before the foci become confluent, a value of 12 was assigned to those specimens where two or more gland lobules contained confluent lymphocytic infiltration [24]. FLS was defined as multiple, dense aggregates of 50 or more lymphocytes (usually several hundred or more) in perivascular or periductal locations, containing no more than a small proportion of plasma cells, and located adjacent to normal-appearing acini in gland lobules not showing duct dilation or fibrosis [24].

The diagnosis of primary SS was made when four of the six European classification criteria for SS were met in the absence of any evidence of other autoimmune disorders [25, 26]. In the group of patients with secondary SS, in whom there was an already established systemic autoimmune disorder, the symptoms of xerophthalmia and xerostomia were confirmed as being related to autoimmune exocrine dysfunction by Shirmer’s 1 test and Rose Bengal staining of the eyes, as well as by histological confirmation of focal lymphocytic sialadenitis. No patients in this study group had evidence of lymphoma or HIV infection.

The patients with CS did not satisfy the criteria for SS other than their complaint of dry mouth. Histologically, CS (or non-specific sialadenitis) is characterized by diffuse atrophy of glandular epithelium in lobes or entire glands, usually with ductal dilatation and interstitial fibrosis. It is further characterized by infiltration with lymphocytes, or lymphocytes and plasma cells, in scattered, interstitial or focal patterns. It occurs in people who have no history of connective tissue disease, increases with age and affects females at an earlier age than males. This form of sialadenitis is likely to occur secondary to gland obstruction or injury.

**Preparation of peripheral blood lymphocytes (PBL) and salivary gland RNA and cDNA**

PBL were prepared from heparinized blood using Ficoll-Hypaque density-gradient centrifugation. An aliquot of 5 \times 10^6 cells was used for RNA synthesis. Total RNA was prepared from PBL using TRIzol (Gibco-BRL, Paisley) according to the manufacturer’s protocol. Total RNA was extracted from whole minor salivary gland biopsies by homogenization of 1–2 mm^3 portions in 1 ml of TRIzol according to the manufacturer’s protocol. RNA pellets were resuspended in diethyl pyrocarbonate-treated water, and samples either used immediately to synthesize cDNA or stored at −70°C under ethanol.

First-strand cDNA was then synthesized from 1 μg of total RNA using 0.3 μg of random oligonucleotide hexamers, 1 U RNAGuard RNase inhibitor, 1 mM each of dNTPs (all from Pharmacia, Milton Keynes), 12 μl 5× reverse transcriptase buffer, 10 mM dithiothreitol (DTT) and 400 U MMLV reverse transcriptase (all Gibco-BRL). Reactions were overlaid with a drop of mineral oil and incubated at room temperature for 10 min, 37°C for 45 min, 95°C for 10 min and finally cooled to 4°C.

**TCR V\textalpha PCR amplification**

First-strand cDNA was amplified with V\textalpha family-specific primers (18 in total) and a common C\textalpha primer as described previously [27–29] for between 35 and 40 cycles (94°C, 1 min; 56°C, 2 min; 72°C, 3 min). Negative controls were carried out frequently to ensure that reagents were not contaminated. Amplified products were resolved on 1.5% agarose gels and blotted onto Hybond-N+ membrane (Amersham, Bucks.). Membranes were hybridized with a 32P-labelled internal C\textalpha oligonucleotide probe as described previously [28, 29]. Densitometry of hybridized bands was carried out using the Gelbase Image Analysis System (UVP, Cambridge). For each cDNA preparation, relative expression was calculated as the optical density for each hybridized band as a percentage of the total optical density for all bands; only semiquantitative analysis was possible with this type of data [29]. Positive expression was defined as being >0.5% of total expression of transcripts [29].

**Cytokine PCR amplification**

The primer sequences used in this study for IL-1\textalpha, IL-1\textbeta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IFN-\textgamma, TNF-\textalpha and β-actin have been described previously [30, 31]. PCR was performed in a 50 μl amplification reaction which contained 5 μl of 10× buffer [giving final concentrations of 1.5 μM MgCl2, 10 mM Tris–HCl
Control reactions without cDNA were carried out in predominant detection of only a few TCR VαGibco-BRL) for 24 h. Cells were pelleted by centrifugation of spurious products derived from contamination controls, in which reverse transcription was omitted, detected in most biopsies, and were dominant bands for the detection of IL-2, IL-4, IL-13 and IFN-γ cDNA.

Amplifications were carried out using cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. β-Actin cDNA amplifications were typically performed using 26 cycles; 35–36 cycles were used to amplify IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α cDNA, and 40 cycles for the detection of IL-2, IL-4, IL-13 and IFN-γ cDNA.

To provide a positive control for cytokine PCR amplifications, normal peripheral blood mononuclear cells (PBMC) from a laboratory volunteer were cultured in RPMI 1640 with 10% fetal calf serum, and stimulated with 1 μg/ml phytohaemagglutinin M (all Gibco-BRL) for 24 h. Cells were pelleted by centrifugation and used to prepare CDNA, as described above. Control reactions without CDNA were carried out in parallel, and were consistently negative. Additional controls, in which reverse transcription was omitted, were also performed in order to detect any amplification of spurious products derived from contamination of RNA samples with genomic DNA. Under the conditions described, no products were detected in PCR products were resolved on 1% agarose gels and blotted onto Hybond-N membrane (Amersham). Filters were pre-hybridized at 45°C for 2 h in hybridization buffer composed of 0.5% SDS, 6 × SSPE and 1 × Denhardt’s solution. Filters were then hybridized for 4–5 h with fresh buffer containing 32P-labelled oligonucleotide internal probes, the sequences of which are described elsewhere [30, 31]. After hybridization, filters were washed twice in 1 × SSC, 1% SDS at 55°C for 45 min. Filters were then subjected to autoradiography for 1–12 h.

RESULTS

Patient details

Table I summarizes the clinical and immunological features and histology of the patients reported in this study.

TCR Vα expression
The number of TCR Vα families present in salivary gland biopsies was assessed by RT-PCR using 18 Vα family specific primers followed by hybridization with an internal TCR Cα probe. There was no uniform pattern of expression in either patient group, with the majority of the samples from each group expressing over 9/18 families, whilst in several samples there was predominant detection of only a few TCR Vα families (Fig. 1; Table II). In particular, Vα1 and Vα3 were detected in most biopsies, and were dominant bands

![Image](https://via.placeholder.com/150)

FIG. 1.—Amplification of TCR Vα families from infiltrating T lymphocytes from salivary gland biopsies of patients with Sjögren’s syndrome. Eighteen Vα families were analysed for expression by RT-PCR. PCR amplification and Southern blot analysis with an internal Cα primer are shown from patient 6 (SS unrestricted) and patient 1 (SS restricted).

### TABLE I
Summary of the diagnostic (clinical, immunological, histological) profiles of the patients reported in the study

<table>
<thead>
<tr>
<th>Patient initials</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis (see key*)</th>
<th>Ocular KCS status</th>
<th>Labial gland focus score†</th>
<th>Ro &amp; La antibodies</th>
<th>Rh factor</th>
<th>Ig levels</th>
<th>Other autoimmune disease</th>
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<td>IgM†</td>
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<td>RA 40</td>
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*CS, chronic sialadenitis; 1° SS, primary Sjögren’s syndrome; 2° SS, secondary Sjögren’s syndrome.
†Number of foci of 50 or more mononuclear cells in 4 mm² of labial salivary gland (a score of 12 = confluent foci in two or more gland lobules).
TABLE II
PCR detection of T-cell receptor Vα families in salivary gland biopsies from patients with Sjögren’s syndrome and chronic sialadenitis

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<th>Sample number</th>
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</table>

SS, Sjögren’s syndrome; CS, chronic sialadenitis.
* +, strong expression detected; +, expression detected; +/−, weak expression detected; −, no expression detected.

in the majority of samples (Table II). There was no distinct differential pattern of expression detectable between the SS and CS biopsies. TCR Vα expression was also analysed in peripheral blood samples from a number of the patients as a formal control to ensure that they were not expressing a limited Vα heterogeneity generally; there was no evidence for this.

Cytokine gene expression

Cytokine mRNA from salivary gland biopsies from all 12 SS and three CS samples was detected using cytokine gene-specific RT-PCR analysis and probing with internal probes (Fig. 2). The results are summarized in Table III. IL-1 gene expression was restricted to patients with SS, with seven and three out of the 12 samples positive for IL-1α and IL-1β, respectively; none of the CS controls showed evidence of IL-1 expression. IL-2 expression was detected in two CS and six out of 12 SS samples. IFN-γ was not detected in the CS group, but was found in 10 SS samples. Eight SS samples were positive for IL-6 expression; in addition, a faint product was detected from one CS sample. IL-8 expression was detected in eight SS samples and two CS samples. IL-10 expression was detected in nine SS samples, and a faint product was detected in one CS sample. TNF-α was expressed in all except one SS samples; it was also detected in two CS samples. IL-13 was detected in one SS sample, whereas IL-4 was not expressed in any SS or CS sample.

Pattern of cytokine expression

Some similarities were observed between the expression of IFN-γ, TNF-α and IL-10 in SS samples, with four of 12 SS samples expressing IFN-γ strongly also expressing TNF-α and IL-10.

DISCUSSION

There have been several previous studies both of cytokine gene expression and TCR Vα and Vβ family expression in SS. However, previous studies have not attempted to correlate clinical features of the patients with cytokine gene and TCR expression. In the current study, we have analysed cytokine expression and TCR Vα family expression in minor salivary gland biopsies...
from patients with SS, and a control group with CS, and attempted to correlate these data with clinical features of the patients.

There was no consistent pattern of TCR Vβ expression detected in either patient group, but there was evidence of substantial restriction in the Vβ repertoire in a single patient who expressed only a limited number of cytokines which appeared as faint bands. This raises the possibility of artefactual restriction of TCR usage due to a limited number of infiltrating lymphocytes in this particular sample. Generally, there was predominant expression of a small number of families in most SS and CS patients, in contrast to our previous findings in Graves’ disease [28, 29]. The presence of such limited heterogeneity, rather than restriction, in Vβ gene usage has previously been described, but only two patients were studied [32]. In addition, several groups analysing the TCR V/β repertoire in SS patients [9–13] have also found limited heterogeneity. In particular, one study showed a correlation between the number of Vβ families detected and the stage of disease [13]. In these patients, there was evidence of monoclonal T-cell expansion early in disease, although the expanded TCR sequences differed between patients [13]. In contrast, another group has found overexpression of Vβ13 families in the absence of any detectable monoclonal expansion [10].

Studies on animal models of the disease have clearly shown restricted TCR Vβ usage according to the stage of the disease [33]. Therefore, it would appear that early in disease, restriction of the T-cell repertoire may be present, at which stage T cells are probably recognizing a single unknown self-antigen in the salivary gland.

As disease progresses, diversification of the immune response to antigenic epitopes occurs together with the influx of bystander lymphocytes, creating a population of polyclonal T cells. There may nevertheless be evidence of overexpression at later times. This may explain the contradictory results in various studies, which are most probably due to samples analysed at different stages of the disease.

This study extends previous reports on cytokine gene expression in SS. Earlier studies did not detect IL-4 expression in SS tissue samples [18], thus arguing against a predominant Th2 response in this disease. Two recent studies have shown IL-4 expression in some and IL-13 expression in the majority of SS salivary gland biopsies, casting some doubts on the predominance of a Th1 response in SS [34, 35]. In the present study, neither IL-4 nor IL-13 were detected in any of the samples analysed, which indicates a Th1 response in at least some patients with SS, or at least at some stage during the disease process. These contradictory results are possibly due to biopsy samples taken during different stages of the inflammatory process. On the other hand, IL-10 was expressed in the majority of the samples, which is consistent with previous studies showing its expression in tissue samples and CD4+ T-cell clones derived from SS labial biopsies [18, 21].

IL-10 is produced by both Th1 and Th2 cells in man, and activates B cells [36], suggesting a role for this cytokine in the autoantibody production frequently seen in SS. The strong expression of IL-10 in two patients correlated with high serum antinuclear antibody levels and in one of these patients with elevated anti-Ro/anti-La antibody levels.

In agreement with previous studies [18, 37], TNF-α was detected in nearly all the biopsy samples. TNF-α is an important mediator of immunological and inflammatory phenomena, and its local production has been implicated in tissue destruction [38]. Furthermore, as a potent inducer of fibroblast proliferation [39], TNF-α may be involved in salivary gland fibrosis, an occasional feature of SS. Similarly to previous reports [18], IL-1α was expressed in the majority of SS but none of the CS samples. However, this is in contrast to other studies where no differences could be found between SS and CS in regard to IL-1α.
expression [20], and this could be related to different methodological techniques used in the latter study. IFN-γ was found in the majority of SS but not in CS samples, with intense bands detected in patients with a high labial gland focus score. Using similar techniques, two studies have demonstrated IFN-γ mRNA in SS tissue samples [34, 40]. However, its expression was not related to the degree of lymphocytic infiltration. SS-derived lymphocytes have the ability to produce IFN-γ in vitro [41], and this cytokine induces HLA class II expression on salivary gland epithelial cells [42], and stimulates these cells to produce pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α [18]. Taken together, the above findings suggest a role for IFN-γ in the pathogenesis of the disease.

One drawback of the RT-PCR technique is that mRNA expression does not necessarily correlate with protein production, and ideally studies on mRNA expression should be complemented with protein detection. However, current immunohistological methods are difficult to apply to small tissue samples, and their sensitivity is far less than that of RT-PCR, which may also result in misleading interpretations.

In summary, the cytokine profile was similar in both the SS and the CS groups, apart from IL-1α and IFN-γ which were exclusively expressed by SS samples. The study of TCR usage showed limited heterogeneity rather than clear restriction in the majority of SS samples. However, one SS sample showed restricted Vα gene usage, but also a restricted cytokine profile, raising the possibility of an artificial restriction that should be taken into account when applying such methodologies. There was no apparent association between the repertoire of the TCR-α genes expressed and the cytokine profile observed, suggesting a general diversity of T-cell phenotype within the salivary gland infiltrate, at least by the time the disease is clinically apparent.

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