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T cell clones from a Sjögren's syndrome salivary gland biopsy produce high levels of IL-10

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SUMMARY

Sjögren's syndrome (SS) is characterized by a focal periductal salivary gland infiltrate consisting mainly of T and B lymphocytes. Most of the T cells bear the memory of $CD4^+$ Th-1-like phenotype and express high levels of class II, though $CD8^+$ cells are also present. We have studied 17 labial salivary gland and 15 peripheral blood T cell clones from a patient with primary SS. The tissue clones were 71% $CD8^+$ and 29% $CD4^+$, and the peripheral blood-derived clones were 60% $CD8^+$ and 40% $CD4^+$. The $CD4^+$ T cell clones from both the salivary gland and autologous peripheral blood were of the Th1 phenotype, in that they produced interferon-gamma (IFN-γ) and IL-2 but very little IL-4 after 24 h stimulation with phorbol myristate acetate and anti-CD3 antibody. The salivary gland-derived $CD4^+$ clones produced 15 times more IL-10 (7·92 ng/ml) than peripheral blood-derived $CD4^+$ clones (0·52 ng/ml, $P \leqslant 0{\cdot}02$). The tissue $CD8^+$ clones produced 1·2 times ($P < 0{\cdot}04$) more IFN-γ and $CD4^+$ clones produced 3·5 times less IL-2 ($P < 0{\cdot}02$) than the respective PBM-derived clones. The accumulation of Th1-type cells producing high levels of IL-10 in the salivary gland suggests a specific immunoregulatory function at the site of inflammation in SS.

Keywords IL-10 Th1 Sjögren's syndrome

INTRODUCTION

Sjögren's syndrome (SS) is an autoimmune exocrinopathy characterized by lymphocytic infiltration of lachrymal and salivary glands. The infiltrate is composed of T and B lymphocytes, plasma cells and a small number of macrophages [1,2]. Most of the T cells are $CD4^+$ [3–5] bearing the phenotype of memory cells [6,7] with 15–20% being $CD8^+$ cells [3,4]. A large proportion of the T cells express class II MHC antigens [8–10], suggesting activation, though few (4%) express the T cell activation antigen. TAC (the IL-2α chain) or IL-2 receptor (IL-2R) [11,12].

Thus within the SS salivary gland there is in infiltration of partially activated $CD4^+$ and $CD8^+$ cells. The duct epithelial cells express class II antigens both in the tissue [8,9] and for prolonged periods in culture [13], and it has been suggested that they act as antigen-presenting cells (APC) (14) leading to the recruitment of T cells to the salivary gland, though the function of these T cells remains unknown.

Some insight into T cell function can be determined by the cytokine profile of T cell clones. Naive (CD45RA) cells tend to have an undifferential phenotype, whereas primed cells (CD45RO) can be split into Th1 or Th2 according to their cytokine profile. In the mouse, Th1 cells classically produce IL-2, interferon-gamma (IFN-γ) and lymphotoxin, but not IL-4, IL-5, IL-6 or IL-10, whereas Th2 cells have a reciprocal cytokine profile. The Th1 cells are responsible for DTH responses, and the Th2 cells for B cell help. In the human the cytokine profiles are not so clear cut, and distinction between Th1- and Th2-type cells relies on IFN-γ/IL-4 ratios.

Immunohistochemistry and mRNA *in situ* hybridization have shown the presence of IFN-γ, IL-2 and transforming growth factor-beta (TGF-β). IL-1, Il-6 and tumour necrosis factor (TNF) have also been detected in salivary gland epithelial cells and saliva [15,16]. IFN-γ has been detected in the lymphocytic infiltrate and on ductal epithelial cells, and may be responsible for class II expression in the salivary gland [9,11,13,17]. We have recently shown that the addition, and more significantly the depletion (by neutralizing MoAb), of IFN-γ down-regulates class II expression on epithelial cells in labial salivary gland primary cultures [14]. These studies suggest that two products of Th1 cells, IFN-γ and IL-2, are reproducibly detected by functional assays, antibodies and mRNA at the site of the lesion in SS, whereas the Th2 cytokines, IL-4 and IL-5, are rarely detected.

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The examination of mixed cell cultures or tissue sections in SS patients has limitations in defining cytokine profiles for differentiating between Th1 and Th2 cells. It is particularly difficult to study cytokines which are produced by a variety of cell types. For example, IL-10 which has been detected in salivary glands [18] is produced by both Th1- and Th2-type cells [19,20], $CD8^+$ cells [20] and macrophages. Its effect can be either inhibitory [20], or stimulatory [21–24] on macrophages and T cells, and it has variable effects on B cells, sustaining resting B cells and stimulating immunoglobulin production from activated B cells [21].

In this study we have determined the cytokine profiles of T cell clones from a salivary gland biopsy and compared them with matching peripheral blood from a newly diagnosed patient with active SS. By this means we were able to circumvent the problems of mixed cell populations and provide evidence of a $CD4^+$, Th1 cell within the gland, which also produced high levels of IL-10. The accumulation of this cell type within the gland suggests it may contribute to the local pathology at the site of inflammation.

MATERIALS AND METHODS

The patient

The patient, aged 48 years, female, presented with keratoconjunctivitis sicca and xerostomia. Relevant blood analyses included an elevated erythrocyte sedimentation rate (ESR; 40 mm/h), low leucocyte count $3{\cdot}2 \times 10^9/l$), positive tests for anti-Ro and anti-La by immunodiffusion, and elevated IgG ($21{\cdot}3\,g/l$). The HLA issue type was DR 3/4, DRw 52/53, DQw2. Schirmer's and salivary flow rate tests were reduced. The histology scores for inflammatory infiltrate using a haematoxylin and eosin-stained paraffin section were grade 4 and focus 6 as defined by Chisholm & Mason [26]. The patient fulfilled six out of six of the Vitali *et al*. [27] criteria for primary SS. The total lymphocyte count was reduced at $900/mm^3$ comprising 10% B cells, 40% CD4 and 31% CD8 cells. The total CD4 count was $360/mm^3$, indicating a CD4 lymphopenia. Serological testing for HTLV-1 and hepatitis C were negative. Although there were no risk factors for HIV infection, the patient was tested at her request, and found to be negative.

Salivary gland biopsy and culture

Six labial salivary gland lobules were removed under local anaesthetic from the lip margin. Two salivary gland lobules were fixed in formaldehyde for histology and grading, two embedded in OCT and cryofixed for imunohistochemistry [9]. The remainder of the biopsy was placed into transport medium (RPMI; GIBCO, Paisley, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS; GIBCO), and 10 U/ml penicillin and 10 ng/ml streptomycin (ICN Biomedical Inc., Flow, UK). Within 60–90 min of excision the explant was placed into culture [13] in the presence of 10 ng/ml or rIL-2 (Hoffman-La Roche, Nutley, NJ). After 7 days the culture contained the biopsy tissue, epithelial cells, a few fibroblasts and numerous mononuclear cells. Fresh medium was added, without any removals, maintaining the final concentration of rIL-2 at 10 ng/ml for a further 7 days.

T cell cloning

Non-adherent cells from the primary culture, containing the mononuclear cells ($1{\cdot}2 \times 10^6$), were pelleted and resuspended into 2 ml of RPMI in the presence of irradiated (40 Gy) allogeneic feeder peripheral blood mononuclear cells (PBMC; 2×10^6/ml; North London Blood Transfusion Centre (NLBTC), London, UK), rIL-2 (20 ng/ml) and phytiohaemagglutinin (PHA; Sigma, Poole, UK) plus 10% heat-inactivated AB positive human serum (NLBTC) with penicillin 10 U/ml and streptomycin 10 μg/ml (complete medium). Cells were incubated for 7 days in a humidified atmosphere at 37°C with 4% CO_2. Cultures were then supplemented with IL-2 for a further 7 days before cloning and characterization. The expanded cell lines were cloned by limiting dilution (0·3 cells/well) in Terasaki plates (20 μl/well; Becton Dickinson, NJ) using irradiated allogeneic PBMC (5×10^5/ml), rIL-2 (20 ng/ml) and mouse anti-human CD3 MoAb (OKT3, 35 ng/ml; ATCC, Rockville, MD) as previously described [28].

PBMC from the same patient were cloned out 1 week later in an identical fashion, except both IL-2 and PHA were added during the initial isolation of T cells. All cells were maintained on a 14 day cycle [22] and were analysed four cycles after cloning and day 14 after anti-CD3 stimulation.

Flow cytometry analysis

Cells (10^5 cells/tube) were washed twice in PBS containing 0·2% sodium azide (BDH Chemicals, Merk, Lutterworth, UK) and 2% FCS (FACS buffer) and the cell pellets were resuspended in 30 μl FACS buffer containing the appropriate mixture of conjugated antibodies. T cell lines derived from the PBL or salivary gland were incubated with CD3 conjugated to Texas red coupled to PE (Coulter Electronics, Luton, UK) plus FITC-conjugated anti-CD4 (Becton Dickinson) plus PE-conjugated anti-CD8 antibody (Becton Dickinson) or CD3 conjugated to Texas red coupled to PE plus a B cell marker (CD19) coupled to FITC (Becton Dickinson). T cell clones were incubated with only the anti-CD4 and anti-CD8 conjugated antibodies. Cells were incubated in the dark, on ice, for 20 min, washed in FACS buffer, then fixed with FACS buffer plus 3% paraformaldehyde (Sigma). Cells were stored in the dark until analysis by a FACStar Plus flow cytometer (Becton Dickinson). The percentage of $CD4^+$ and $CD8^+$ T cells in the lines was determined by the percentage of CD4 and CD8 staining of the $CD3^+$ cells.

Cytokine profiles of tissue and PBMC clones

The salivary gland and PBMC-derived T cell clones (1×10^6/ml) were cultured for 24 h in complete medium in the presence of 10 ng/ml phorbol myristate acetate (PMA; Sigma) and 1 μg/ml soluble anti-CD3 (OKT3) [25]. The cells were pelleted and the supernatants collected and stored at −20°C until use. IFN-γ, IL-4 and IL-10 levels in the supernatants were detected by ELISA. The ELISA reagents were kindly donated by Dr D. Novick (Weizmann Institute, Rehovot, Israel), Dr F. de Padova (Basel, Switzerland), and Drs K. Moore and J. Abrams (Palo Alto, CA). IL-2 was detected by the standard CTLL assay. The criteria for defining the Th subtypes, Th1, Th2, Th0, were the detection limits of the IFN-γ and IL-4 ELISAs as previously described [22,23]: Th1 $>$120 pg/ml IFN-γ and $<$40 pg/ml IL-4, Th2 $>$40 pg/ml IL-4 and $<$120 pg/ml IFN-γ,. and Th0 $>$120 pg/ml IFN-γ and $>$40 pg/ml IL-4.

Statistical analysis

All data were analysed using the Mann–Whitney test.

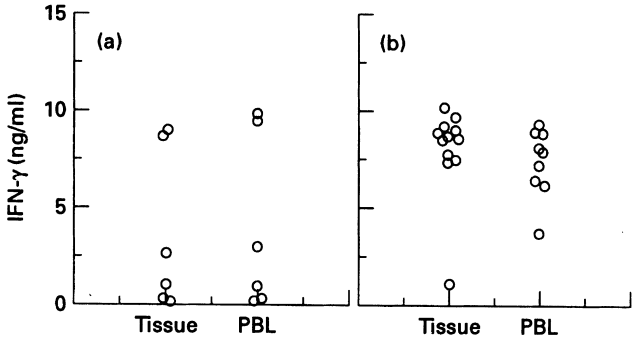

Fig. 1. IFN-γ production (ng/ml) by individual $CD4^+$ (a) and $CD8^+$ (b) T cell clones derived from the salivary gland and autologous peripheralblood. Cytokine production was analysed in T cell clone supernatants after 24 h stimulation with phorbol ester plus anti-CD3 antibody.

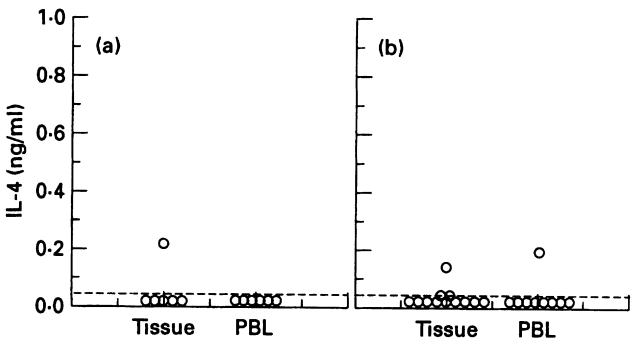

Fig. 3. IL-4 production (ng/ml) by individual $CD4^+$ (a) and $CD8^+$ (b) T cell clones derived from the salivary gland and peripheral blood. ----, Detection limit of assay.

RESULTS

Phenotype of cultured T cells

The PBMC line contained 83% $CD8^+$ and 12% $CD4^+$ T cells. From the PBMC line, 24 PBMC clones were established, and 15 of these were randomly selected and assessed for cytokine production. The tissue cell derived from the salivary gland biopsy contained 52% $CD8^+$ and 42% $CD4^+$ T cells. Thirty-four salivary gland clones were established from the line, and of these 17 were assessed. Contamination of both lines by B cells was <1%. Of the tissue clones, 12 (70%) were $CD8^+$ and five (29%) were $CD4^+$, whilst nine (60%) of the PBMC clones were $CD8^+$ and six (40%) were $CD4^+$. The proportion of $CD8^+$ and $CD4^+$ cells found in the biopsy by immunohistochemistry was 64% $CD8^+$ and 36% $CD4^+$, and in the blood was 50% $CD4^+$and 38% $CD8^+$ cells. This suggested that the high proportion of $CD8^+$ clones recovered mirrored the findings in the biopsy and the blood, with a tendency to select for $CD8^+$ cells.

Cytokine production

The level of cytokines produced by the salivary gland and PBMC-derived clones is shown in Figs 1–4. The tissue $CD8^+$ clones produced significantly more IFN-γ (media = 8·40 ng/ml) than the tissue $CD4^+$ clones (3·64 ng/ml, $P < 0{\cdot}02$) and the PBMC $CD8^+$ clones (7·29 ng/ml, $P < 0{\cdot}04$) (Fig. 1). Salivary gland $CD4^+$ clones produced significantly less IL-2 (0·28 ng/ml) than PBMC $CD4^+$ clones (0·85 ng/ml, $P < 0{\cdot}02$) (Fig. 2). There were no significant differences in IL-4 production; only three clones, two from the salivary gland and one from the peripheral blood, produced IL-4 above the detection limit of the ELISA (Fig. 3). These three were designated Th0 cells, the remainder being Th1.

The most significant finding in terms of differences between the target tissue and the peripheral T cells was the production of IL-10 (Fig. 4). Salivary gland $CD4^+$ clones produced 15 times more IL-10 (7·92 ng/ml) than did the PBMC $CD4^+$ clones (0·52 ng/ml, $P < 0{\cdot}02$), 23 times more than the PBMC $CD8^+$ clones (0·34 ng/ml, $P < 0{\cdot}01$), and 65 times more than the tissue $CD8^+$ clones ($< 0{\cdot}12$ ng/ml, $P < 0{\cdot}002$).

DISCUSSION

Using *in situ* stimulation with IL-2 alone we have been able to generate T cell clones from a salivary gland biopsy. The CD4 clones from the tissue produced high levels of IL-10, suggesting that these cells had a specific function at the site of inflammation in SS. An unexpected finding was that $CD4^+$ clones were in a minority compared with CD8 (ratio 0·5:1 from the tissue and 0·7:1 from the blood). This is partly explained by the fact that the patient also had a $CD4^+$ lymphopenia and a low percentage (36%) of $CD4^+$ cells in the salivary gland, though there was also a selection bias toward CD8 cells during the establishment of the T-cell lines. This bias towards CD8 cells is unlikely to be due to technical factors peculiar to our laboratory, since other studies from our unit have always yielded the expected excess of $CD4^+$ clones [25,30]. The cloning strategy will have selected the

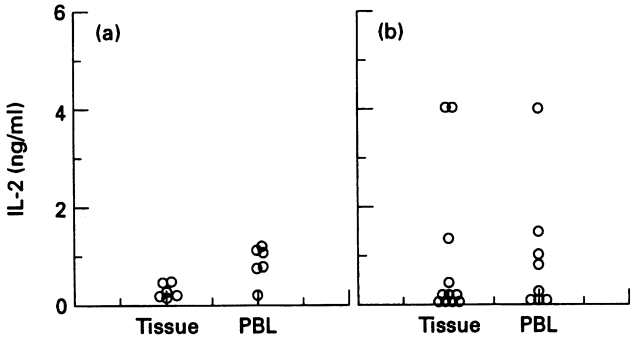

Fig. 2. IL-2 production (ng/ml) by individual $CD4^+$ (a) and $CD8^+$ (b) T cell clones derived from the salivary gland and peripheral blood.

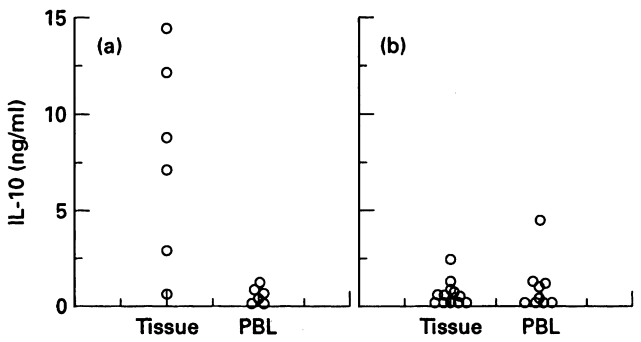

Fig. 4. IL-10 production (ng/ml) by individual $CD4^+$ (a) and $CD8^+$ (b) T cell clones derived from the salivary gland and peripheral blood.

activated cells within the biopsy, which in this case comprised mainly CD8 cells.

The low $CD4^+$ count in the gland, peripheral blood, T cell lines and T cell clones suggests that the patient described here is not typical of SS. Previous studies have reported a predominance of $CD4^+$ cells, with mean ratios of 3:1 in the blood and 5:1 in the gland being reported [3]. However, these general figures conceal the enormous scatter which occurs in SS. In the study of Fox *et al*. of 15 patients [3], four had CD4/CD8 ratios of between 1·0 and 1·3 in the blood, eight had ratios of between 2·2 and 3·9, and three had ratios of >6. Our patient, who had a ratio of 1·29, might be part of a subset of SS similar to that of the group of four in Fox's study. We could hypothesize that this subset could be caused by a particular virus infection. The negative serology for HTLV-1, HIV and hepatitis C excludes three viruses which have been linked to SS (reviewed in [31]), though another lymphotropic virus which can cause a CD4 lymphopenia could well be a candidate.

We have confirmed the observation that in SS the majority of $CD4^+$ cells were of the Th1 type, capable of producing IFN-γ and IL-2, but not IL-4. In addition, we showed that our salivary gland-derived Th1-like $CD4^+$ clones were capable of producing high levels of IL-10. Since IL-10 directly inhibits IL-2 production [21], this would explain our observation that the clones which produced high levels of IL-10 produced little IL-2. Fox *et al*. [18] reported similar findings for $CD4^+$ cells from SS salivary glands at the mRNA level, and proposed three possible explanations: (i) the cells were contaminated with IL-10 producing cells such as macrophages; (ii) the cells were Th2-like but had suppressed ability to produce IL-4 and IL-5; or (iii) the Th1-like cells in salivary glands from patients with SS produce IL-2, IFN-γ and IL-10. Our data, using individual salivary gland clones, confirm the third suggestion.

High levels of IL-10 production by $CD4^+$ clones derived from the autoimmune target have also been found in rheumatoid arthritis (RA) [25]. The implication of high levels of IL-10 produced by T cells in SS, however, is quite different to that for RA. In the presence of an increased number of macrophages, which occurs in the synovial membranes of RA patients, IL-10 is likely to be involved in immunosuppression, as IL-10 inhibits T cell function indirectly by inhibiting antigen presentation by macrophages [21]. The effects of IL-10 in the absence of macrophages, as found in SS salivary gland lesions, may be immunostimulatory. This is particularly relevant in terms of sustaining resting B cells and stimulating immunoglobulin production by activated B cells. The B cell stimulation may result in the hypergammaglobulinaemia particularly characteristic of SS [29]. In addition, IL-10 in the absence of accessory cells can enhance T cell proliferation when in the presence of other T cell cytokines such as IL-2 [30] and IL-3 [23].

This study of one patient with primary SS can be used as a guide for future studies to examine the role of infiltrating cells in the salivary gland. The increased production of IFN-γ and IL-10 suggests that the infiltration is not a random event and that the cells have accumulated to perform a specific function. The production of high levels of IFN-γ supports our previous hypothesis [13] that this cytokine is important in maintaining class II expression in the salivary gland. At present it is not known whether the function of IL-10 in SS is proinflammatory or protective. We cannot state with certainty that the pattern we have observed is common to SS as a whole, or peculiar to a subset of the disease represented by our patient. However, we have demonstrated conclusively that the cloning strategy enables a specific dissection of the T cell subsets and the cytokines which they produce. Future studies will address the question of which of the pleiotropic effects of IL-10 are dominant in Sjögren's syndrome.

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