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Bcl-2 immunoreactivity in salivary gland neoplasms is unrelated to the expression of mRNA for natural killer cell stimulatory cytokines interleukin (IL)-2 and IL-12

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Abstract Certain cytokines are involved in the generation of natural killer (NK) cells and participate in the regulation of the proto-oncogene *bcl*-2. We aimed to study the mRNA expression of interleukin (IL) -2, IL-4 and IL-5, the composition of the tumour infiltrating lymphocytes (TIL), and the expression of *bcl*-2 in 14 benign and malignant human parotid tumours. T IL were predominantly composed of T lymphocytes and NK cells. We found evidence for the homing of T cells, and for generation of NK cells in the vicinity of the tumours. mRNA for IL-2 and IL-12, were identified but IL-4 mRNA was not found. The cytokine profiles and the composition of TIL of the two tumour categories were indistinguishable, suggesting that these host-response variables do not explain the differences in biological behaviour of these particular tumours. The results support a shift towards Th1 (T helper 1) cells and interferon-γ production, and that IL-12 also in vivo may play an important role in the regulatory interaction between innate resistance and adaptive immunity in tumour diseases. Most infiltrating lymphocytes showed strong expression of *bcl*-2; an interesting observation with regard to lymphocytic apoptosis in neoplastic diseases. The immunoreactivity fot the *bcl*-2 protein varied considerably between and within tumours, and almost all benign tumours showed strong *bcl*-2 positively whereas several of the malignant tumours showed weak or absent staining. The variable expression of *bcl*-2 protein suggests a different susceptibility of tumour cells to apoptosis. The results also indicate that *bcl*-2 cannot play a major role as protective agent in the specific apoptotic pathway induced by NK cells.

Key words Salivary gland neoplasms · Cytokines · *bcl*-2 · Apoptosis · NK cells

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Introduction

Tumour infiltrating lymphocytes (TIL) have been investigated extensively concerning the host-immune response in neoplastic disease [13, 21, 34, 43, 45, 46]. In this infiltrate macrophages and antigen presenting cells (APC), capable of interleukin-12 (IL-12) production and clearance of apoptotic cells, are found. Many of the TIL are cytokine-producing T lymphocytes of both helper and cytotoxic phenotypes [15, 23, 29, 34, 46]. IL-2, a T helper 1 (Th1) associated cytokine, and IL-4 (Th2 associated cytokine), are both of interest due to their cytotoxic properties. IL-2 enhances the cytolytic activity of T lymphocytes and natural killer (NK) cells, and stimulates the proliferation of these cells. IL-2 also synergizes with IL-12 in the generation of lymphokine-activated killer (LAK) cells. IL-4 down-modulates IL-2-induced NK- and LAK-cell activity [41]. IL-12 (NK stimulatory factor; for review see [4]) is a 70 kDa heterodimeric cytokine composed of two covalently linked chains, p40 and p35. The p35 subunit is related to IL-6 whilst p40 has no significant homology to any cytokines but rather is related to the extracellular domain of the IL-6 receptor [4, 14, 28]. The heavy chain p40 has not been shown to be biologically active in any system examined to date [48]. Though macrophages are the major source for IL-12 recent data indicate that IL-12 may be expressed and released by mononuclear cells [8] and monocytes (via CD40/CD40L interaction [39], polymorphous leukocytes

[5], by human keratinocytes and epidermoid carcinoma cell lines [2] and by dendritic cells [26]. The biological active heterodimer IL-12 has multiple immunoregulatory effects on various cell populations [22]. It activates the increase of TIL independently of IL-2 [1], and induces NK cells to produce interferon (IFN)-γ, which promotes the development of naive $CD4^+$ T cells into type 1 effector cells producing high levels of IFN-γ but no IL-4 [39. NK cells, being independent of the classical MHC-restriction [17], inhibit tumour cell growth by irreversibly damaging the target cell DNA by the specific DNA fragmentation characteristic of apoptosis [10, 11, 25].

Apoptosis is partly regulated by the *bcl*-2 proto-oncogene. Dimers of the *bcl*-2 protein family are localized to the same intracellular sites as those of oxygen free radical generation [18, 19]. The *bcl*-2 protein protects cells from apoptosis, hence favouring a prolonged survival of normal as well as neoplastic cells [9, 30, 32, 44]. In the present investigation we have used immunocytochemical methods to study the composition of TIL and the expression of *bcl*-2 protein in human tumour tissue, and reverse transcriptase polymerase chain reaction (RT-PCR) to examine the expression of mRNA for IL-2, IL-4 and IL-12 in situ. The *bcl*-2 protein expression varied between tumours, and within individual tumours. Most benign tumours showed strong *bcl*-2 immunoreactivity whilst many malignant tumours showed weak *bcl*-2 positivity. We found a predominance of T cells among the TIL, many NK cells within and in the vicinity of tumours, and a local production of IL-2 and IL-12 but an apparent lack of IL-4 mRNA. Previous in vitro findings have shown that IL-12 plays a crucial role in the ontogenity of Th1 type immune response. Our demonstration of IL-12 synthesis in situ may indicate an active role of IL-12 in the regulatory interaction between innate resistance and adaptive immunity in human tumour disease.

Materials and methods

Materials

All primary salivary gland tumours were retrieved from the frozen tissue bank (−70° C) at the Pathology Department of Örebro Medical Center Hospital (1988–1993). Frozen tissue samples of 7 benign and 7 malignant tumours were available. All 14 tumours were parotid tumours. Five benign tumours had been diagnosed as pleomorphic adenoma, one as Warthin tumour and one as basal cell adenoma. Three of the malignant tumours had been classified as acinic cell carcinoma, two as poorly differentiated adenocarcinoma NOS, one as adenoid cystic carcinoma and one as oncocytic adenocarcinoma. The RT-PCR for interleukins and the immunocytochemical detection of NK cells and lymphocytes were performed on frozen tissue, whilst the expression of the *bcl*-2 protein and evaluation of the total amount of TIL were investigated on formalin fixed, paraffin embedded material. The different positive and negative control materials are encountered for below.

Polymerase chain reaction

The cytokine (IL-2, IL-4 and IL-12) and control (CD44) specific primers were synthesized using a Applied Biosystem 392 DNA Synthesizer (Foster City, Calif., USA), and the primer sequence specificity and position were checked against updated versions of EMBL Genbank. Phytohaemoagglutinin-stimulated mononuclear cells and tonsil tissue served as positive controls that each primer set yielded a correct sized PCR product. The specificity of cytokine initial PCR amplification was confirmed by a dot blot procedure using as internal probe the same oligonucleotide of the seminested PCR (downstream primer) in every individual case. Digoxygenin labelling of the probe was achieved using a commercially available kit (Boehringer, Mannheim, Germany). Care was taken to avoid cross-contamination between samples and carry-overs of PCR products, as previously recommended [24]. Negative control reactions, using nonretrotranscribed samples, were run for each case.

For RNA extraction ten frozen sections of 10 μm thickness were cut and prepared using a standard procedure [6]. CD44 (the normal epitope variant) was used as positive control for mRNA extraction, as CD44 is present in virtually all lymphocytes except resting peripheral lymphocytes. Briefly, the extraction procedure included a homogenization of the frozen tissue in a 300 μl of 4 M guanidine-thiocyanate (ultrapure GITC, Bethesda Research, USA), 25 mM of a solution of sodium citrate (at pH 7.0), 0.5% sarcosyl (Sigma, St. Louis, USA) and 100 mM of 2-mercaptoethanol (Sigma). Sodium acetate, water-saturated phenol and chloroform-isoamyl alcohol were then sequentially added to the lysate. This final suspension was shaken for 10 sec, chilled at +4° C for 15 min, spun and eventually the aqueous phase was transferred to another tube and the RNA precipitated in an equal volume of isopropanol at −20 °C. Precipitates were later pelleted, washed in RNAse-free ethanol, vacuum-dried and resuspended in RNAse-free water.

Reverse transcription was performed (RNA-PCR kit, Perkin Elmer, Norwalk, Conn., USA) in 20 μl which consisted of 3 μl of the RNA sample, 4 μl of 25 mM magnesium chloride ($MgCl_2$), 2 μl of PCR 10× buffer II, 1 μl of RNAse inhibitor, 1 μl of reverse transcriptase, 2 μl each of the dNTPs and 1 μl of antisense downstream primers (50 pmoles) (Table 1). A Perkin Elmer Thermal cycler was used for the reverse transcription, 42° C for 60 min followed by 99° C for 5 min. The tubes were subsequently stored at +4° C until PCR was done.

For the polymerase chain reaction a mixture of 4 μl of $MgCl_2$, 8 μl of 10× PCR buffer II, 66.5 μl of sterile water, 0.5 μl of *Taq* polymerase (Perkin Elmer) and 50 pmoles sense upstream primer was added to each tube during hot start at 80° C. Thirty-five cycles were conducted with 94° C denaturation (1 min), 40° C and 45° C respectively (1 min; Table 1) and 72° C primer extension (1 min; 10 min last cycle). Amplified products were stored (+4° C) until separated on an ethidium-bromide stained 3% NuSieve agarose gel (FMC Bioproducts, Me., USA), ultraviolet examination, and photography.

For IL-2, IL-4 and IL-12, seminested and nested PCR, were subsequently performed. The amplified products of IL-2, IL-4 and IL-12 were further amplified for 35 cycles in a mixture of 50 μl mixture containing buffer II, 1 μl of each dNTP, 0.25 μl of *Taq* polymerase, sterile water and 50 pmoles of each primers. Five microlitres of $MgCl_2$ was added at hot start at 80° C. The thermal profiles were identical to the initial PCR except for the annealing temperatures (Table 1). Storage was performed as for PCR products.

Immunocytochemistry

Approximately 5 μm thick cryosections were cut, transferred to 3-well slides (ph099 black, Cel-line Associates, Newfield, N.J., USA), air-dried at room temperature for 5 min, fixed in acetone for 10 min at room temperature, dried as above, wrapped in aluminium foil, and stored at −20° C until stained. The immunocytochemical staining was performed according to the alkaline phosphatase-anti alkaline phosphatase (APAAP) technique ([7]; tissue sections were allowed to come to room temperature before unwrapping of the aluminium foil). Primary incubation with the

Table 1 Primer sequences for CD44, interleukin (IL)-2, IL-4 and IL-12 with annealing temperatures and the polymerase chain reaction (PCR) product sizes and their location (*AT* annealing temperature, *bp* base pairs)

Primer Sequence	PCR		PCR (Seminested)		PCR (nested)	
	AT	Product location	AT	Product location	AT	Product location
CD44	40° C	198 bp				
Upstream 5′-ACAGTCCCTGGATCACCGACA-3′		738–758				
Downstream 5′-CCAAGATGATCAGCCATTCTGG-3′		914–935				
IL-2	40° C	300 bp				
Upstream 5′-ACAACTGCAGCATTTACTGCTGGA-3′		203–226				
Downstream 5′-GCTGTCTCATCAGCATATTCACAC-3′		479–502				
Downstream 5′-TAGCACTTCCTCCAGAGGTTTGAG-3′			45° C	175 bp 354–377		
IL-4	40° C	210 bp				
Upstream 5′-ACTGAGAAGGAAACCTTCTGCAGG-3′		253–276				
Downstream 5′-CAACGTACTCTGGTTGGCTTCCTT-3′		439–462				
Downstream 5′-GTTTCAGGAATCGGATCAGCTGCT-3′			45° C	136 bp 365–388		
IL-12	40° C	251 bp				
Upstream 5′-CATCTCCCCTCGTGGCCATATGGG-3′		63–86				
Downstream 5′-CGAATGGCTTAGAACCTCGCCTCC-3′		289–313				
Nested					45° C	169 bp 115–283
Upstream 5′-ATTGGATTGGTATCCGGATGCCCC-3′		115–138				
Downstream 5′-ACAGGTGTACTGGCCAGCATCTCC-3′		259–283				

monoclonal antibodies was performed at room temperature for 30 min, and the solution was then washed in TRIS buffered saline (TBS) and placed in a TBS bath on a shaker for 5 min. The monoclonal antibodies used for T lymphocytes were DAKO-CD3 (pan-T), DAKO-CD4 (helper T subset), DAKO-CD8 (suppressor T subset) and DAKO-CD25 (an early marker of T cell activation, the IL-2 receptor; all mouse, IgGlκ; Dako, Copenhagen, Denmark). Subsets of B lymphocytes were investigated by the monoclonal antibodies DAKO-CD19 (pan-B), -CD20 (pan-B) and -CD23 (marker of B cell activation), all being mouse IgG1κ antibodies (Dako). NK cells were studied by monoclonal antibodies DAKO-CD 16 (mouse IgMκ, Fc γ Receptor III), DAKO-CD56 (mouse, IgG1κ) and CD57 (Immunotech AS, France). Test titrations gave an optimal dilution of 1/100 for all antibodies. The second incubation was carried out in rabbit anti-mouse immunoglobulins (Dako, dilution 1:50) incorporating a 1:25 dilution of normal human serum for 30 min (DAKO P260). After washing in TBS the sections were incubated in APAAP (Dako). Levimasole was used as to minimize endogenous enzyme activity. One repeat step was performed to enhance the staining. Fast red (naphthol AS-MX Phosphate, Sigma) was added for 15 min to give the immunopositive cells a bright red colour, suitable for computerized image analysis of positive cells. A weak counterstaining with Mayer's haematoxylin was applied for 5 s. Tonsillar tissue was used as positive control and omission of the primary antibody served as negative control. The evaluation of the immunostaining was conducted by computer-assisted image analysis using a black and white CCD camera attached to the microscope. The method can give both the number of immunostained objects per mm^2 and the percentage of immunopositive area per examined microscopical field [20]. The programme is not capable of distinguishing between different $CD16^+$ subsets (such as granulocytes and macrophages), and therefore the values for $CD16^+$ cells given in Table 3 cannot be interpreted as absolutely representative for NK cells.

Due to the complex nature of NK cells and their ability to express different CD antigens, double immunostainings were carried out wherever sufficient material was available. However, the shortage of representative material made this part of the investigation incomplete, and the results are only viewed as supportive. NK cells can be defined as CD3–/CD56+/CD16+, CD3–/CD56+/CD16– or CD8+/CD56+ lymphocytes, and we performed double immunostainings for CD3 and CD16, CD56 and CD57, and for CD8 and CD56. The avidin-biotin complex method was used for the first antibody (DAKO-CD3 or DAKO-CD8) which was revealed with a peroxidase reaction and the second antibody (DAKO-CD16, DAKO-CD56 or CD57) was applied according to the APAAP method.

Sections of approximately 5 μm thickness were cut from the routine blocks and one section was stained with haematoxylin and eosin. The other sections were immunostained for *bcl*-2 protein using the streptavidin-biotinylated peroxidase complex. Briefly, the sections were treated with 3% hydrogen peroxide in distilled water to inhibit endogenous peroxidase activity, and then immersed in boiling citrate buffer pH 6, in a microwave oven set at 600 W [38], two changes of 5 min each. After extensive washing in tap and distilled water, the sections were subsequently incubated with 1:20 dilution of normal rabbit serum for 20 min at room temperature, 1:100 dilution of the 124 monoclonal antibody to *bcl*-2 protein overnight at +4° C, 1:200 dilution of a biotinylated rabbit antiserum to mouse immunoglobulins for 30 min at room temperature and 1:100 dilution of the streptavidin-biotinylated peroxidase complex for 30 min, also at room temperature. Peroxi-

dase activity was developed in diaminobenzidine chromogenic substrate. All reagents were purchased from DAKO.

A known positive control (a formalin-fixed paraffin-embedded follicular lymphoma carrying the (t 14; 18) chromosomal translocation) was immunostained with the test samples. The positive control section displayed strong immunoreactivity of the neoplastic cells. Negative control sections (where the specific monoclonal antibody was substituted with the immunoglobulin fraction of nonimmune mouse sera) remained unstained. The immunoreactivity for *bcl*-2 was evaluated both with regard to the staining intensity and the percentage of immunoreactive tumour cells. Neoplastic cells displaying definite cytoplasmic staining of the same intensity as shown by lymphoma cells or higher were graded as 3+. Tumour cells with moderate intensity staining reaction were graded 2+, weakly positive cells 1+ and negative tumour cells were recorded as –. In cases where the intensity of immunoreactivity varied within the tumour, the dominating areas overruled. The extent of immunoreactivity was evaluated as 1+ if less then 25% of tumour cells were positive, 2+ if 25%–75% were positive and 3+ if more than 75% of cells were positive. When possible, this semiquantitative estimation of the *bcl*-2 immunoreactivity also included surrounding normal parotid tissue as well as TIL.

The number of TIL was evaluated on the paraffin sections instead of on the small frozen sections; this was thought to be more representative of the tumour. Furthermore, computer-assisted image analysis was developed for frozen sections only [20]. Numerous lymphocytes were recorded as 3+, a moderate number as 2+ and a sparse amount of lymphocytes as 1+. The gradings were performed on two occasions with identical results.

Statistics

Differences between the numeric values for lymphocytes (Table 3) were analysed using Wilcoxon rank sum test.

Results

The review of the routine sections revealed five cases of pleomorphic adenoma, two of which were very myxoid and hypocellular (cases 1 and 5) whilst the other three showed a more common picture with a mixture of epithelial und myoepithelial cells embedded in a myxoid/chondroid stroma (cases 2, 3 and 4). A Warthin tumour represented the sixth case and its stroma contained sparse lymphoid tissue. A basal cell adenoma was the last of the benign tumours; a mixed trabecular and tubular variant (case 7). There were three cases of acinic cell carcinoma where one case showed areas of widespread necrosis (case 8) and another, apparently also of relatively high grade malignancy, showed widespread invasion into adjacent muscle and massive spread to intraparotid lymph nodes (case 9). The remaining case of acinic cell carcinoma showed usual features and multinodular growth pattern (case 10). One of the two cases initially diagnosed as poorly differentiated adenocarcinoma showed features compatible with salivary duct carcinoma (case 11) whilst the other (case 12) was considered to be a poorly differentiated, sclerotic adenocarcinoma NOS. There was one case of adenoid cystic carcinoma which showed massive intra- and perineural growth, as well as vascular invasion (case 13). The last tumour of the series was an oncocytic carcinoma (case 14) showing local infiltration and areas of clear cell differentiation (Table 2). Normal parotid tissue was present in all sections but one (case 12). Aggregates of lymphoid tissue were present in most cases, and in two cases peri- and intraparotid lymph nodes with germinal centres were seen (cases 6 and 9). Hence the material consisted of seven benign and seven malignant salivary gland neoplasms (Table 2).

Table 2 Histological diagnoses, reverse transcriptase (RT)-PCR of CD44, IL-2, IL-4 and IL-12, and amount of tumour infiltration lymphocytes (TIL) in 14 salivary gland neoplasms (*PA* pleomorphic adenoma, *WA* warthin tumour, *BCA* basal cell adenoma, *ACC* acinic cell carcinoma, *SDC* salivary duct carcinoma, *AC* adenocarcinoma NOS, *ADCC* adenoid cystic carcinoma, *OAC* oncocytic adenocarcinoma, + positive bands, – no bands detected)

Case no.	Diagnosis	CD44	IL-2	IL-4	IL-12	TIL[a]
1	PA	+	+	–	+	+
2	PA	+	+	–	+	+++
3	PA	+	+	–	+	++
4	PA	+	+	–	+	+
5	PA	+	+	–	+	+
6	WA	+	+	–	+	+++
7	BCA	+	+	–	–	++
8	ACC	+	+	–	+	+++
9	ACC	+	+	–	+	+++
10	ACC	+	+	–	–	++
11	SDC	+	+	–	–	+
12	AC	+	+	–	+	+
13	ADCC	+	+	–	+	+
14	OAC	+	+	–	+	+++

[a] TIL grading: + few cells, ++ moderate number, +++ numerous cells

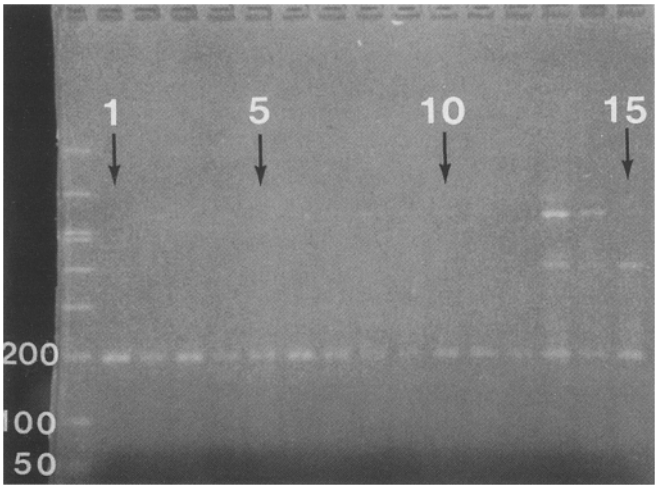

Fig. 1 Polymerase chain reaction (PCR) products of control CD44. *Lane 1* constitutes a positive control. The amplified bands for CD44 (198 bp) in *lanes 2–15* verify the presence of extracted amplifiable RNA. The *lanes 2–15* correspond to cases 6, 5, 3, 4, 9, 2, 14, 7, 1, 10, 11, 13, 8 and 12, respectively. To the left, the molecular-weight markers are of 50 (indicated), 100 (indicated), 200 (indicated), 300, 400, 500, 600 and 700 bp

PCR yielded amplified CD44 bands (198 bp) in all 14 specimens (Fig. 1). PCR for the interleukins did not yield clear bands in all cases, whereby seminested or nested PCR were performed to increase the sensitivity. All 14 tumours expressed mRNA for IL-2 (seminested, 175 bp) but IL-4 (seminested, 136 bp) was not detected in any case (Table 2; Fig. 2).

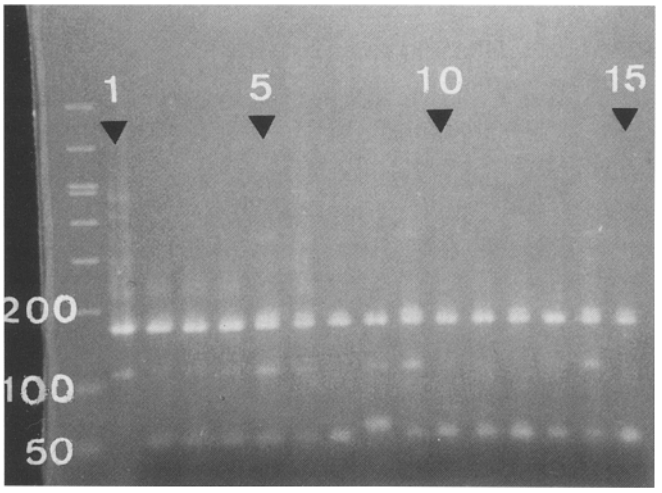

Fig. 2 Fifteen lanes (*1–15*) with positive bands of PCR products of interleukin-2 (IL-2, seminested, 175 bp). Lane 1 constitutes a positive control. The *lanes 2–15* are arranged in the same order as in Fig. 1. Molecular-weight markers to the left are indicated as in Fig. 1

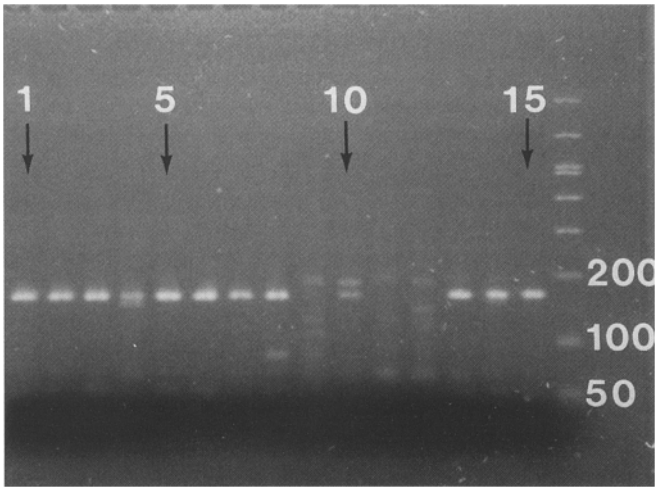

Fig. 3 Twelve lanes with positive bands for IL-12 (nested, 169 bp). *Lane 1* is a positive control, and the *lanes 2–15* are arranged in the same order as in Fig. 1. No positive bands are detectable in lanes 9, 11 and 12 (corresponding to cases 7, 10 and 11, respectively). Molecular-weight markers to the right as indicated as in Fig. 1

Amplified bands of IL-12 (nested, 169 bp) were obtained in all cases but three (cases 7, 10 and 11; Fig. 3). No specific bands were observed in negative control experiments.

The results of the computerized image analysis of lymphocyte subsets and NK cells in frozen sections are shown in Table 3. T lymphocytes greatly outnumbered B lymphocytes. The differences were significant considering benign and malignant tumours; regardless of whether they were analysed as one group together, or separately (CD3 vs CD19 $P<0.01$; CD3 vs CD20 $P<0.01$). In the benign and in the malignant tumours, there were more $CD3^+$ T cells than both $CD19^+$ and $CD20^+$ B cells together ($P<0.05$ in all cases). In the Warthin tumour T lymphocytes greatly outnumbered B cells, but there was no significant difference between the numbers of $CD4^+$ and $CD8^+$ T lymphocytes. The CD16 antigen is expressed on NK cells but also on granulocytes and on a small subset of T lymphocytes, and so the significance of the difference between the number of T cells ($CD3^+$) and $CD16^+$ cells ($P<0.01$) must be interpreted with caution. However, it is more reliable to compare $CD3^+$ T cells with $CD57^+$ NK cells, and there were significantly more T cells then NK cells ($P<0.05$; Table 3). In terms of recruitment of NK cells, or generation on site, it is of interest to investigate the local NK cell ($CD57^+$) percentage of cells and compare it with that of peripheral blood. In peripheral blood nu-

Table 3 Subsets of lymphocytes and natural killer (NK) cells in salivary gland tumours evaluated by computerized image analysis. The figures correspond to the mean number of immunopositive cells per mm^2 (standard deviation within brackets)

Antibody	Benign tumours[a]	Malignant tumours[b]	Warthin tumour
CD3	166 (88)	201 (134)	360
CD4	161 (125)	197 (169)	316
CD8	126 (61)	148 (90)	340
CD25	98 (46)	72 (24)	183
CD19	33 (27)	74 (66)	169
CD20	54 (27)	78 (46)	209
CD23	47 (22)	53 (24)	65
CD16	200 (127)	358 (215)	455
CD57	119 (69)	71 (26)	460

[a] Benign tumours: five cases of pleomorphic adenoma (cases 1–5) and one case of basal cell adenoma (case 6)
[b] Malignant tumours: three cases of acinic cell carcinoma (cases 8–10), one salivary duct carcinoma (case 11), one adenoid cystic carcinoma (case 13), and two cases of adenocarcinoma (cases 12 and 14)

Table 4 Immunoreactivity of *bcl-2* protein in 14 salivary gland tumours

Case no.	Diagnosis	Intensity of immunoreactivity[a]	Extent of immunoreactivity[b]
1	PA	+++	+++
2	PA	+++	+++
3	PA	+++	++
4	PA	++	+++
5	PA	+++	+++
6	WA	+++	+++
7	BCA	+	++
8	ACC	+++	+++
9	ACC		
	Primary	++	+
	Metastasis	+++	+++
10	ACC	++	+++
11	SDC	Negative	
12	AC	+	++
13	ADC	+++	+++
14	AC	++	+

[a] + weak, ++ moderate, +++ equal or stronger than positive control
[b] + <25% of cells, ++ between 25% and 75% of cells, +++ >75% cells

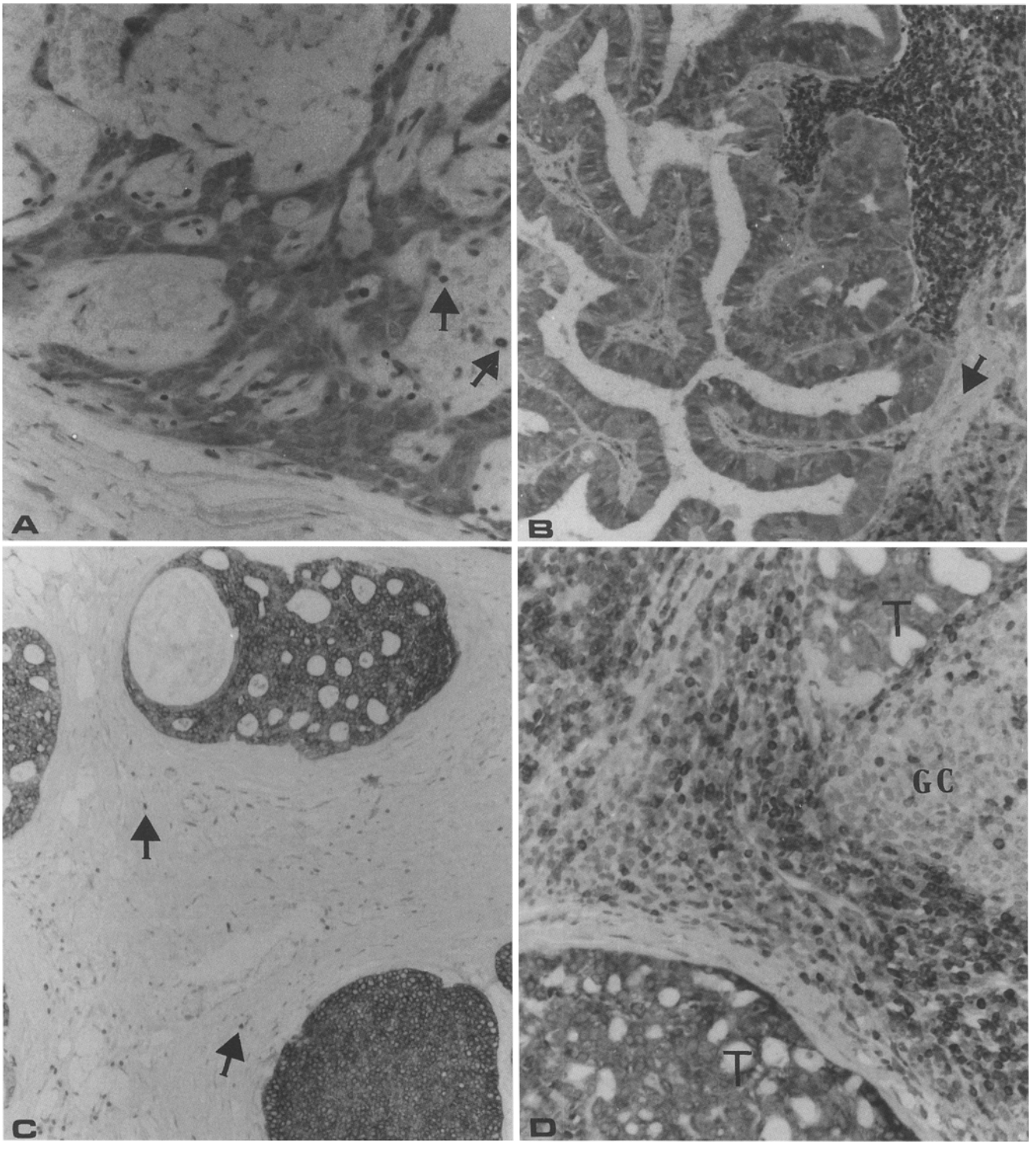

Fig. 4A–D *Bcl*-2 staining (streptavidin-biotinalyted peroxidase complex) on paraffin sections. **A** Strong positivity of myoepithelial tumour cells in an otherwise hypocellular pleomorphic adenoma. Note strongly positive lymphocytes (*arrows*; case 5, *bcl*-2, ×200). **B** Warthin tumour showing a moderate staining intensity. The majority of cells of this tumour did however show a strong reaction (Table 4). Note the negative stroma between strongly positive lymphoid tissue and tumour epithelium (*arrow*; case 6, *bcl*-2, ×100). **C** Adenoid cystic carcinoma showing strong staining reaction for *bcl*-2. Note scattered positive lymphocytes (*arrows*; case 13, *bcl*-2, ×100). **D** Metastatic acinic cell carcinoma with strongly positive tumour cells (*T*). Note negative lymphocytes in the germinal centre (*GC*) and positive lymphocytes around it (case 9, *bcl*-2, ×200)

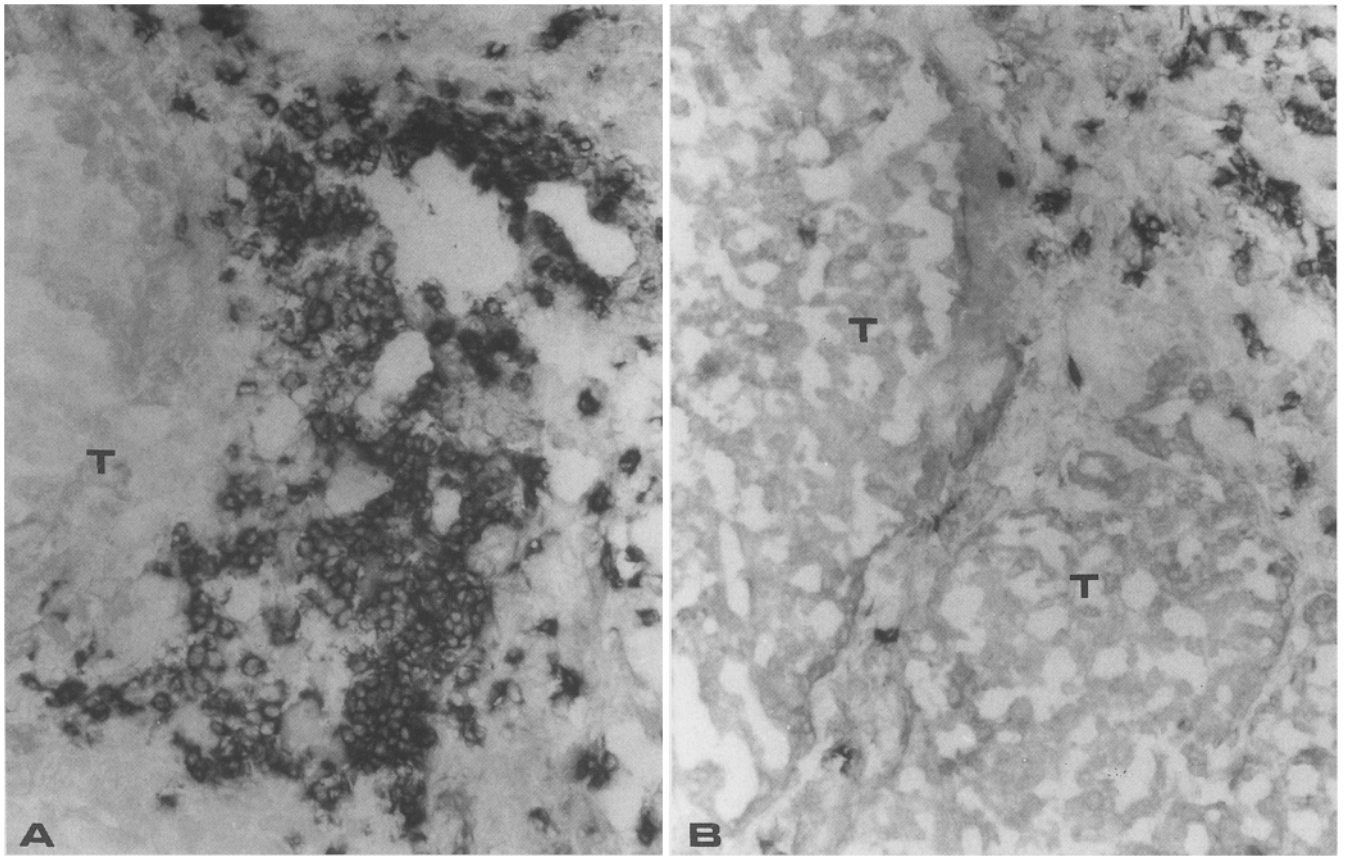

Fig. 5 **A** Photomicrograph illustrating a tumour with numerous tumour infiltrating lymphocytes (TIL). The picture is taken from a frozen section and the lymphocytes are stained with CD3 [*T* tumour; case 8, alkaline-phosphatase anti-alkaline phosphatase (APAAP)], ×200). **B** A moderate number of TIL surrounding the tumour (case 10, APAAP, CD3, ×200)

merical values for NK cells have been estimated at a mean of 14% standard deviation (SD)=6.1 [35]. In our study the percentage of tissue bound $CD57^+$ NK cells (malignant and benign tumours together) was 33% (SD=18) of the total ($CD3^+$, $CD19^+$, $CD57^+$) which represents a significantly higher percentage than in peripheral blood ($P<0.05$; Table 3).

Double-staining of NK cells was performed on few cases only due to lack of material. However, in those cases investigated, several $CD3^-/CD57^+$ cells were seen, as were $CD3^-/CD56^+$ cells. The small subset of T lymphocytes that may also be $CD16^+$ was not present in our cases of double-stained sections ($CD16^+/CD3^-$) and importantly, other double stainings showed most $CD56^+$ cells to express CD8, thus compatible with a NK cell phenotype. The double-stainings support the idea that the majority of the cells detected by the image analysis as immunoreactive for CD57 represented NK cells. This applies to a lesser extent to $CD16^+$ cells as these cells are a mixture of NK cells, activated T cells, macrophages, granulocytes and other cells expressing the Fc-γIIIR. Other evidence favouring the accumulation in the tumours of NK cells ($CD8^+$ cells which are not $CD3^+$) is provided by the number of $CD4^+$ and $CD8^+$ cells, the sum of which vastly outnumbers $CD3^+$ cells (Table 3).

Immunostaining for *bcl*-2 showed a wide variation between tumours both concerning staining intensity and the number of positive cells within a tumour (Table 4). All benign tumours (except the basal cell adenoma) showed strong staining intensity in more than 75% of tumour cells. All malignant tumours (except the salivary duct carcinoma) also expressed the *bcl*-2 protein, but the intensity was in general not as strong, and only in three cases were more than 75% of tumour cells positive. In the one case of acinic cell carcinoma with peri- and intraparotid lymph node metastases (case 9), the primary tumour showed scattered positive cells only, whilst most of the metastatic tumour cells were strongly *bcl*-2 positive (Table 4; Fig. 4). In most tumours the majority of TIL showed strong positive *bcl*-2 immunoreactivity.

The semiquantitative evaluation of the number of TIL revealed a great variation between tumours. TIL were sparse in two benign and three malignant tumours, moderate in two benign and one malignant, whilst there were numerous lymphocytes in one benign (plus the case of Warthin tumour) and in three malignant tumours (Table 2; Fig. 5).

Discussion

The two main mechanisms of cell loss from growing tumours are apoptosis and necrosis [47]. Recent data sug-

gest that oncogene expression (*myc*, *ras*) determines intrinsic apoptotic rates [3]. However, cytolytic T lymphocytes (CTL) and related NK, large granular lymphoid and LAK cells, are all important effector agents relevant to tumour apoptosis. Cell death apoptosis has been demonstrated by cytolytic T cell mediated killing of target cells [10], and NK cells can act in a similar way. The presence of NK cells, transcription of IL-2 and IL-12 in situ, and that Th1 cells prevail over Th2 cells in TIL (as evaluated by mRNA for IL-2 and IL-4), are some of the important findings in the present study.

Messenger RNA for IL-2 was found in all 14 tumours, indicating in situ synthesis of this cytokine. IL-2 synthesis is mainly performed by activated Th1 cells (most TIL expressed CD25; Table 3), and to a lesser extent by a subset of activated B cells [40] which could be of importance as IL-2 is a growth-promoting signal during the development of CTL [33]. No mRNA for IL-4 could be detected. The absence of IL-4 transcription may be explained by inhibition of the development of IL-4 producing Th2 cells by IL-12 [27]. Furthermore, a Th2 to Th1 switch due to the IL-12-dependent induction of IL-2 producing Th1 cells would decrease IL-4 production [3, 27, 42]. IL-12 mRNA was demonstrated in all tumours but three. The latter cases represented a basal cell adenoma, an acinic cell carcinoma and a salivary duct carcinoma. Notably, all these three cases had a sparse inflammatory cell infiltrate (Table 2), which, again, supports the idea that IL-12 mRNA is produced by "professional" APC [48] rather than by tumours cells. IL-2 and IL-12 apparently occur both in benign and malignant tumours, and therefore they would not seem to serve as good discriminating prognostic factors. We did not use competitive PCR and any possible difference in the amount of the cytokines produced can therefore not be discussed.

The in situ production of NK cell stimulatory cytokines IL-2 and IL-12 is important as picomolar concentrations of IL-12 are as effective as nanomolar concentrations of IL-2 in enhancing the cytolytic activity of NK cells expanded in vivo by IL-2 [36]. It in not certain what is the proportion of tissue-bound NK cells recruited into the tumour tissue from the blood as null cells, and converted to NK cells on site, or recruited as NK cells. We found NK cells in the TIL population in all tumours approximately equaling the number of T cells, and exceeding the number of B cells (Table 3). IL-2 and IL-12 both act in an auto- or paracrine fashion and it is therefore reasonable to conclude that the majority of NK cells were generated on site. The present study thus supports the homing of T cells and in situ generation of NK cells within the tumours.

IL-4 down-modulates the IL-2-induced NK cell activity. Our data (no IL-4 mRNA) can be interpreted as suggesting that the immune system actively takes part in tumour cell killing in situ via the cytokine network (interaction between IL-2 and IL-12). Furthermore, the Th2 synthesis of IL-4 in vitro is inhibited by IL-12, both on transcriptional and translational level [48]. Local production of IL-4 would diminish the effect of IL-2-generated NK cells. Therefore, the presence of both IL-2 and IL-12 but absence of IL-4 supports local NK cell activity. Tumour cell apoptosis is one effect of the NK cells.

The TIL and cytokine profiles were similar in benign and malignant tumours. There were no significant differences between the amount of TIL and the *bcl*-2 immunoreactivity (Tables 3 and 4) but the expression of *bcl*-2 varied between tumours. A high cellular expression of the *bcl*-2 protein is potentially indicative of a reduced susceptibility to apoptosis; the actual size of *bcl*-2 immunoreactive tumours does not necessarily depend on high proliferative rate. In other solid tumours, *bcl*-2 positivity correlates, with lower malignant potential and better differentiation [9, 31, 32]. In contrast tumours with little or no *bcl*-2 staining can be regarded as being more prone to apoptosis, and their growth or size is likely sustained by a higher proliferative rate. This is consistent with their more aggressive clinical behaviour. It is of interest that the salivary duct carcinoma was the only *bcl*-2-negative tumour. Salivary duct carcinoma has one of the poorest prognoses of all salivary gland neoplasms, and probably the most dismal prognosis [12, 16, 37]. If factors released from NK cells influence apoptosis via a pathway involving the *bcl*-2 protein, many tumour cells should lack staining, or show weak staining, for *bcl*-2. In the present investigation weak or absent staining for *bcl*-2 was not found in any of the seven benign tumours. Thus *bcl*-2 may not be involved in the NK-cell induced type of apoptotic pathway. Only one benign tumour showed a combination of weak staining intensity and moderate staining (25%–75% of cells). In contrast, two of the malignant tumours showed weak or absent staining, and another two cases showed only scattered positive cells (less than 25%). NK cell activity may hence differ between tumours and/or individuals, and other components of the immune system may differ between benign and malignant tumours in terms of possible interference with apoptotic pathways.

The present demonstration in salivary tumours of mRNA for IL-2 and IL-12, and the diversity in *bcl*-2 staining patterns, calls for carefully designed prospective studies. As IL-12 responsiveness, unlike IL-2, is limited to those T and NK cells that have been pre- or coactivated, IL-12 may have a great potential as an immunotherapeutic agent.

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