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Concomitant *in vivo* production of 19 different cytokines in human tonsils

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SUMMARY

Accumulating data indicate that cytokines, peptides involved in regulation of both physiological and pathological immune responses, are produced predominantly at the site of local antigen stimulation. Cytokine-producing cells were detected at the protein level in human tonsil tissue obtained from children with recurrent tonsillitis or infectious mononucleosis (IM). Concomitant production of 19 different human cytokines, interleukin-1α (IL-1α), IL-1β, IL-1 receptor antagonist (ra), IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF, tumour necrosis factor-α (TNF-α), TNF-β, interferon-γ (IFN-γ) and transforming growth factor-β1–3 (TGF-β1–3), was identified at a single-cell level by indirect immunohistochemical staining procedures and use of carefully selected cytokine-specific antibodies (Ab). Fresh frozen sections were fixed with 4% paraformaldehyde and permeabilized by 0·1% saponin treatment, eluting cholesterol from the cell-surface membrane and the Golgi complex. The intracellular localization of all cytokines, except IL-1 and IL-1ra, was demonstrated by a characteristic local cytoplasmic perinuclear configuration in producer cells. In addition, the immunoreactivity for certain cytokines (IL-2, IL-4, IL-5, G-CSF and GM-CSF) was expressed on the cell membranes and extended over a large extracellular area encompassing the producer cell. Localization of the cytokine to the Golgi organelle was established by co-staining with a monoclonal antibody (mAb) specific to the Golgi complex. Both the extra- and intracellular cytokine staining reactions could be blocked by preincubation of the cytokine-specific Ab with the corresponding purified natural or recombinant cytokine. A complex cytokine pattern was established in both groups studied, where most T-helper type 1 (Th1) and Th2 lymphokines were expressed in the tonsils but at different frequencies and localizations. Cells expressing IL-4, IL-5, IL-10 and IL-13, (Th2 response) were evident at higher frequencies in recurrent tonsillitis compared to sections from IM, which were associated with a more pronounced IL-2, IFN-γ and TNF-β expression.

INTRODUCTION

The palatine tonsils, a major component of lymphoid tissue in Waldeyer's ring, were chosen for characterization of cytokine production *in vivo*. Immunohistological and lymphocyte phenotyping studies show that this tissue belongs to the so-called mucosa-associated lymphoid tissue (MALT) and appears to function as a first line of defence against micro-organisms.[1,2] Engraftment of human tonsillar cells to combined immunodeficiency mice indicates that the tonsils contribute an important fraction of B-cell precursors designated for the lungs and other lymphoid tissues but not for the gastrointestinal system.[3,4]

The information regarding the functional capacity of tonsillar B cells is substantial. However, the functional properties of tonsillar T cells remain relatively unknown. Analysis of cytokine-producing cells within the microenvironment of this lymphoid tissue may reveal T-cell responses required for primal immunological control. A spontaneous production of interleukin-6 (IL-6) and interferon-γ (IFN-γ) has been previously reported in tonsils obtained from children with tonsillar hypertrophy.[5,6] A significant production of IL-2, IFN-γ, tumour necrosis factor-β (TNF-β) and IL-6 has also been found at this site in children with fulminant infectious mononucleosis (IM).[7] However, these studies were performed using cell suspensions from the tissue. This requires considerable cell handling, which reduces the possibilities of detecting ongoing *in vivo* production of cytokines. The shortcomings of these approaches are obvious considering that receptor-mediated

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Abbreviations: GC, germinal centre; IM, infectious mononucleosis.

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focusing of cell-to-cell release of cytokines and a short half-life have been demonstrated for most cytokines.[8–11] Therefore one can understand that these approaches are of limited clinical value. A new method has been adapted, preserving cellular morphology and protein antigenicity. The permeabilization includes the Golgi organelle, which enables detection of individual cytokine-producing cells in human tonsillar tissue at the protein level. Single-cell cytokine production was identified, through the characteristic accumulation of staining to the Golgi complex in the producer cells, by indirect immunohistochemical staining procedures using cytokine-specific monoclonal antibody (mAb) or affinity-purified polyclonal antibodies. The possible presence of production of 19 human cytokines, IL-1α, IL-1β, IL-1 receptor antagonist (ra), IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF, TNF-α, TNF-β, IFN-γ and transforming growth factor-β1–3 (TGF-β1–3), was studied in fresh frozen tonsil tissues obtained from children with recurrent tonsillitis or acute IM.

MATERIALS AND METHODS

Materials

Fresh surgical tonsil specimens obtained from children with recurrent tonsillitis or acute IM were immediately snap frozen in isopenthan in dry ice. Informed consent was obtained from the parents before the tonsil studies were instituted. The Ethical Committee, Karolinska Institute, Sweden, approved the analysis as well as tonsillectomy in children with recurrent tonsillitis or IM.

Immunohistochemistry

The tonsils were embedded in OCT compound (Tissue-TEK, Mites, Elkhart, IN). Cryostat sections, 8–10 μm, were mounted on HTC glass slides (Novakemi, Stockholm, Sweden) and fixed in paraformaldehyde (PFA; 4%, pH 7·4; Sigma Chemicals, St Louis, MO) for 15 min. Permeabilization was performed by use of 0·1% saponin (Riedel de Haen, AG Seelze, Germany). Endogenous peroxidase was blocked by 1% H_2O_2/0·02% NaN_3 in balanced salt solution (BSS) (Gibco Ltd, Paisley, U.K.) supplemented with 0·1% saponin for 30 min. The slides were washed three times in BSS containing Ca^{2+} and Mg^{2+} supplemented with 0·01 M HEPES buffer. Endogenous biotin was quenched by pretreatment of the sections with an avidin–biotin blocking kit (SP-2001; Vector Lab., Burlingame, CA) for 20 min at room temperature. Following three additional washes in BSS/saponin, the slides were incubated over night at 4° with 10–30 μl of a panel of cytokine-specific mAb (Table 1), or affinity-purified polyclonal antibodies, at a concentration of 2–5 μg/ml. The slides were washed an additional three times in BSS/saponin and incubated with 1% normal goat serum for 15 min, in order to reduce background due to goat IgG Fc interactions. Biotin-labelled secondary antibodies, absorbed against human Ig (biotin-goat anti-mouse IgG1 and IgG2b; Caltag Lab, South San Francisco, CA; biotin-goat anti-rat IgG; Vector Lab.) were used, diluted 1 : 300 and 1 : 100, respectively, during a 30-min incubation at room temperature. Following subsequent washes in BSS/saponin, the slides were incubated either with avidin–biotin–horseradish peroxidase (Vectastain, ABC-HP-kit; Vector Lab.) or with avidin–biotin–alkaline phosphatase (Vectastain, ABC-AP-kit; Vector Lab.). Colour reaction was developed

Table 1. Cytokine-specific antibodies

Cytokine	Antibody	Isotype	Producer	References
IL-1α	1277-89-7, 1277-82-29	Mouse IgG1	H. Towbin (Ciba-Geigy, Basel, Switzerland)	12
	1279-143-4	Mouse IgG1		
IL-1β	2-D-8	Mouse IgG1	H. Towbin (Ciba-Geigy)	12
IL-1ra	1384-92-17-19	Mouse IgG1	H. Towbin (Ciba-Geigy)	12
IL-2	MQ1-17H12	Rat IgG2a	J. Abrams (DNAX, Palo Alto, CA)	13
IL-3	BVD8-6G8	Rat IgG	J. Abrams (DNAX)	13
	BVD3-IF9	Rat IgG1		
IL-4	MP4-25D2	Rat IgG1	J. Abrams (DNAX)	13
IL-5	JES-39D10	Rat IgG2a	J. Abrams (DNAX)	13
IL-6	MQ2-6A3	Rat IgG	J. Abrams (DNAX)	13
IL-8	NAP-1	Mouse IgG1	M. Ceska (Sandoz, Vienna, Austria)	14
IL-10	JES3-19F1	Rat IgG2a	J. Abrams (DNAX)	13
	JES3-12G8	Rat IgG2a	J. Abrams (DNAX)	
IL-13	JES8-5A2	Rat IgG2a	J. Abrams (DNAX)	15
	JES8-30F11	Rat IgG2a	J. Abrams (DNAX)	
GM-CSF	BVD2-21C11	Rat IgG2a	J. Abrams (DNAX)	13
	BVD2-5A2	Rat IgG2a	J. Abrams (DNAX)	
G-CSF	BVD13-3A5	Rat IgG	J. Abrams (DNAX)	13
	BVD11-37G10	Rat IgG	J. Abrams (DNAX)	
TNF-α	MP9-20A4	Rat IgG	J. Abrams (DNAX)	13
TNF-β	LTX 21	Mouse IgG2b	G. Adolf (Boehringer-Ingelheim, Vienna, Austria)	16
	LTX 22	Mouse IgG1		
IFN-γ	DIK1	Mouse IgG1	G. Andersson (KABI, Stockholm, Sweden)	17
TGF-β1	96	Polyclonal rabbit IgG	K. Miyazono (Ludwig Inst., Uppsala, Sweden)	18
TGF-β2	94	Polyclonal rabbit IgG	K. Miyazono (Ludwig Inst.)	18
TGF-β3	95	Polyclonal rabbit IgG	K. Miyazono (Ludwig Inst.)	18

by diaminobenzidine (DAB) 0·5 mg/ml, or an alkaline phosphate substrate kit including levamisole-blocking endogenous alkaline phosphatase (Vector Lab.). The reaction field was blocked after 5–30 minutes by three washes in BSS and dehydrated by 2-min treatment in 50% ethanol + 50% H_2O followed by 100% ethanol. Slides were counterstained with haematoxylin or methylgreen and mounted in a glycerin buffer.

A sequential staining technique was used for two-colour immunohistochemical analysis. The single-colour stain was initially performed as described above for visualization of cytokine-producing cells using the APAAP technique. The complex of alkaline phosphatase and mouse/rat mAb anti-alkaline phosphatase was detected by alkaline phosphatase substrate (red). The sections were incubated with a second biotinylated mAb (for phenotyping of cells or Ki67 a marker for proliferation) in BSS/0·1% saponin, which was developed by avidin–biotin–horseradish peroxidase and DAB for brown peroxidase staining.

All the anti-cytokine mAb used had the capacity to neutralize the biological activity of each cytokine in its pure form. The staining for each mAb could only be blocked with its corresponding cytokine, not with any of the other 18 cytokines investigated in this study. Irrelevant isotype-matched mAb, rabbit anti-mouse IgG subclass-specific and rabbit anti-rat IgG (Dakopatts, Glostrup, Denmark) were used in controls for non-specific staining reactions. Intracellular localization of the cytokine-specific staining was performed by use of mAb specific for the human Golgi complex (1271, mouse IgG1; Chemicon Inc., El Segundo, CA).

Phenotypic characterization and detection of cell proliferation

Biotin-labelled mAb were used to identify cell-surface antigens: OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), DAKO-CD19 (CD19-CD20) and DAKO-Mac 387 (IgG1; Dakopatts), reacting with a cytoplasmic antigen expressed in cells of the myelomonocytic lineage. DAKO-Ki67 (IgG1; Dakopatts) was used to identify a nuclear protein expressed in proliferating cells.

Recombinant and natural cytokines

Highly purified natural or recombinant-produced cytokines were used to block specific cytokine staining. The appropriate cytokine was added in excess, at a concentration of 20–50 μg/ml, to its corresponding cytokine-specific antibody (2–5 μg/ml) at +4° overnight. Staining with the complex was performed as described previously and compared to results obtained by combining anti-cytokine mAb preincubated with other cytokines as a control.

The following cytokines were used: natural IL-1α (Dr C. Heusser, Basel, Switzerland) and IL-1β (Dr C. Dinarello, Boston, MA), recombinant IL-1ra (Dr H. Towbin, Ciba-Geigy, Basel, Switzerland), recombinant IL-2, recombinant IL-3, recombinant IL-4, recombinant IL-5, recombinant IL-6, recombinant IL-8 (Genzyme Corp., Boston, MA), recombinant IL-8 (Dr M. Ceska, Sandoz, Vienna, Austria), natural IL-10 (Dr J. Abrams, DNAX, Palo Alto, CA) recombinant IL-2 (R&D Systems, Abingdon, U.K.) recombinant TNF-α and recombinant TNF-β (Bayer Inc., Hannover, Germany), recombinant IFN-γ (Boehringer-Ingelheim Inc., Vienna, Austria), recombinant GM-CSF (Sandoz Inc., Basel, Switzerland), recombinant G-CSF (Hoffman La-Roche, Basel, Switzerland) bovine TGF-β1 (Dr C. Snapper, Bethesda, MD) and recombinant TGF-β2 (Genzyme Corp.)

Quantification of stained cells

The localization and identification of cytokine-producing areas were visualized in a Reichert-Jung microscope (Polyvar 2; Reichert-Jung, Vienna, Austria) under ×100 magnification. The entire tissue section was examined. Positive stained cells were counted under ×400 magnification.

RESULTS

The morphology of cytokine-staining in tonsil tissue

The intracellular cytokine-staining pattern for most interleukins, as revealed by immunohistochemical staining of fresh frozen tonsil sections, was observed to be cytoplasmic. In certain cells this staining was localized, with a juxtanuclear position in the cytoplasm. The same morphological pattern was also established when staining was performed on *in vitro*-activated cytokine-producing cells in suspension (Fig. 1a). Indeed, the prior demonstration in cell suspensions of a uniform, perinuclear staining pattern greatly facilitated the evaluation (Fig. 1a).[19] This pattern also seemed to reflect the cytokine-accumulation in the Golgi complex of the cytokine-producing cells in the cryopreserved tissue (Fig. 1b). The appearance of this cytoplasmic staining signal in the tonsil sections coincided with the staining appearance obtained with Golgi organelle-specific mAb (Fig. 1c). It has been reported previously that all the cytokines we studied, except the IL-1 family, have hydrophobic amino acid-binding sequences directing secretion through the Golgi–endoplasmatic route.[19]

It was also observed that, in addition to cytoplasmically stained cells, the immunoreactivity extended over a large extracellular area encompassing many producer cells. It is conceivable that these pools of extracellular cytokines may be due to binding to low affinity receptors in the local areas or to extracellular matrix components following cytokine secretion (Figs 1b and 2a–c). This extracellular depot may have a functional role, by prolonging the local bioavailabity of the cytokines. The specificity of the extracellular as well as the intracellular immunoreactivity was verified by a complete abrogation caused by preincubation of the cytokine-specific mAb with the relevant corresponding human cytokines (Fig. 2d).

In contrast, staining of mononuclear cells in suspension for IL-1α, IL-1β and IL-1ra showed a different staining pattern not restricted to the local juxtanuclear area but rather expressed as a diffuse perinuclear or granular appearance in the cytoplasm and on the cell surface membrane (Fig. 3a).[20] Immunohistochemical staining in tissue sections of these three cytokines also revealed a diffuse nuclear and cytosolic compartmentalization without a local intracellular accumulation (Fig. 3b and c). It has recently been established that the intracellular transport of IL-1α, IL-1β and part of IL-1ra follows a different pathway than the other cytokines studied.[19,20]

Constitutive cytokine-producing cells in tonsil tissue

Cryopreserved tonsil sections were examined for the presence of 19 different human cytokines. The occurrence of cytokine-

Table 2. Characterization of cytokine production profile in tonsil tissue by immunohistochemical technique

Cytokine	Frequency of cytokine-producing cells*		Localization of cytokine†
	IM	RT	
IL-1α	+ + +	+ + +	Surface epithelium
IL-1β	—	+	Surface epithelium
IL-1ra	+ + + +	+ + +	Surface epithelium
IL-2	+ +	+	Extrafollicular, T-cell rich area
IL-3	+ +	+ + +	Extrafollicular, T-cell rich area
IL-4	+	+ + + +	Extrafollicular, $CD4^+$ T cells
IL-5	+ +	+ +	Extrafollicular, $CD4^+$ T cells, germinal centre
IL-6	+	+ + +	Extrafollicular, crypt area
IL-8	+ +	+ + +	Epithelium and monocytes in crypts
IL-10	+	+ + +	Extrafollicular, T-cell rich area
IL-13	+	+ +	Extrafollicular, T-cell rich area
TNF-α	+	+	Close to crypts, mononuclear cells
TNF-β	+	—	Extrafollicular, T-cell rich area
IFN-γ	+ +	+	Extrafollicular, T-cell rich area
GM-CSF	+ + +	+ +	Extrafollicular, T-cell rich area
G-CSF	+ + +	+ + + +	Extrafollicular, T-cell rich area
TGF-β1	+ + + +	+ + +	Reticular crypt epithelium, germinal centre
TGF-β2	+ + + +	+ + +	Reticular crypt epithelium, germinal centre
TGF-β3	+ + + +	+ + +	Reticular crypt epithelium, germinal centre, surface epithelium

The cytokine pattern detected in cryopreserved tissue of tonsillitis caused by IM was compared to that of chronic recurrent tonsillitis (RT).

*The frequency of cytokine-producing cells/cryocut section: + 1–5 cells; + + 6–25 cells; + + + 26–75 cells; + + + + >75 cells.

†Each cytokine staining was blocked by preincubating the cytokine-specific mAb with its relevant human recombinant or natural interleukin.

synthesizing cells could be demonstrated in most sections (Figs 1b and 2a–c). A concomitant *in vivo* production of IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF, G-CSF, TNF-α, TNF-β, IFN-γ and TGF-β1–3 was established in the tonsil tissue obtained from children with recurrent tonsillitis or IM (Table 2). The frequency of individual cytokine-producing cells varied for each of the cytokines between the two groups studied (Table 2). In both groups cytokine-producing cells tended to be present in clusters in the tissue. The cells producing different cytokines were detected consistently in the same anatomical location of the tonsils (Table 2). In addition, there were no large variations in occurrence of cytokine-synthesizing cells when serial sections from a given tonsil were repeatedly stained.

Localization of cytokine-producing cells

The aim of this study was to characterize the cytokine production *in vivo* and to localize the specific sites for synthesis. There were in general more cells that contained IL-1α than IL-1β (Fig. 3b and c). The sections from recurrent tonsillitis, however, had a higher incidence of IL-1β-containing cells than those of IM (Table 2). The IL-1α-synthesizing cells were found in particular in the surface epithelial layers (Fig. 3b and c), but also in the endothelial cells of small vessels and occasionally in the extrafollicular area as well as the germinal centres. This immunoreactivity appeared diffusely around the nucleus or throughout the cytosol of the cells, in parallel to earlier findings *in vitro* in lipopolysaccharide (LPS) stimulated mononuclear cells in suspension (Fig. 3a). IL-1ra was more pronounced in the IM sections, and was localized to the same areas as those of the IL-1-producing cells. In addition, this cytokine was found over larger extracellular areas than IL-1, suggesting an increased cytokine secretion or binding to extracellular matrix (Table 2). However, no IL-1ra activity was detected in the endothelial cells of small vessels.

The chemoattractant IL-8 was a more prominent monokine in recurrent tonsillitis than in IM (Table 2). The area of production was confined to the reticular crypt epithelium. The IL-8-synthesizing cells were predominantly found in the crypt epithelium. While IL-6 and IL-10 production had a scattered distribution in the extrafollicular sites (Table 2), both IL-6- and IL-10-producing cells were more prevalent in recurrent tonsillitis than in sections obtained from IM. TNF-α was also gathered in the extrafollicular area, many of the cells being $CD4^+$ as judged by two-colour staining.

Production of lymphokines

In the samples from recurrent tonsillitis, a high number of IL-4-producing cells was found in the T-cell rich extrafollicular area surrounding the germinal centre (Fig. 2a). The frequency of IL-4 synthesizing cells exceeded that of IFN-γ, IL-2 and TNF-β (Table 2). IL-4-producing cells could also occasionally be found in the germinal centre in some of the follicles (Fig. 2a). Many of the IL-4-positive cells were $CD4^+$. A

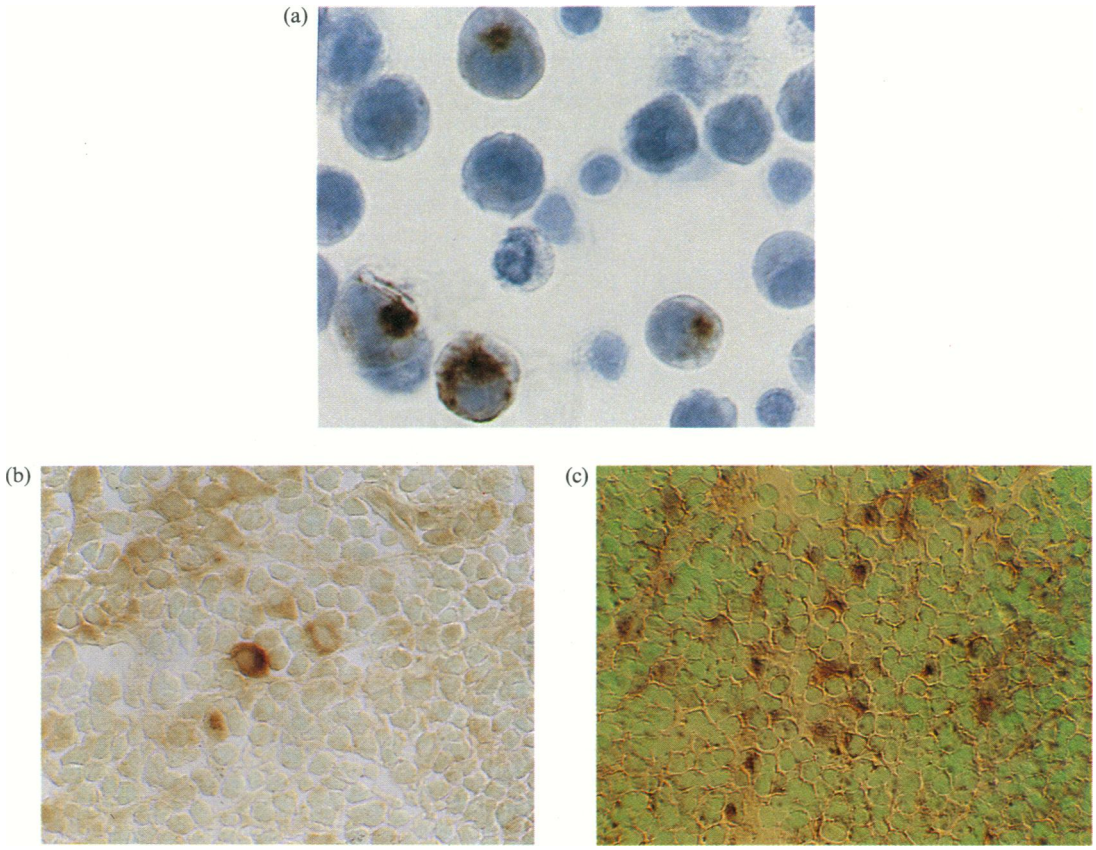

Figure 1.

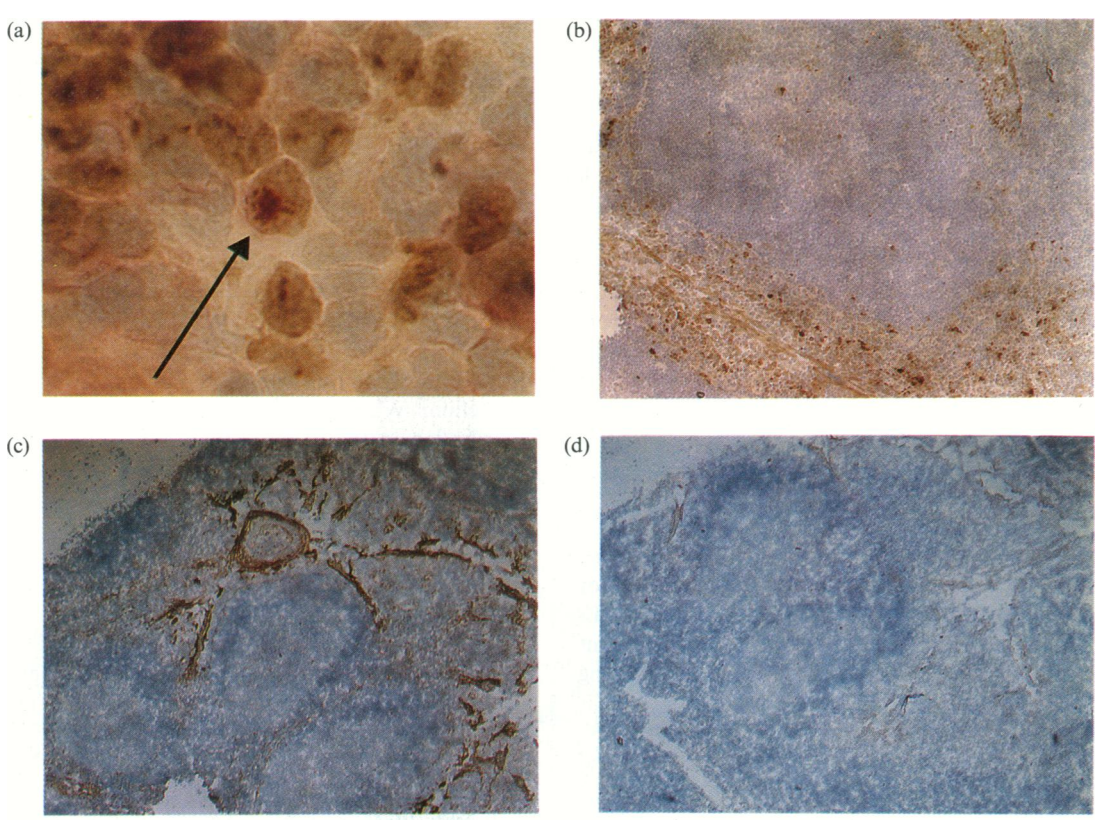

Figure 2.

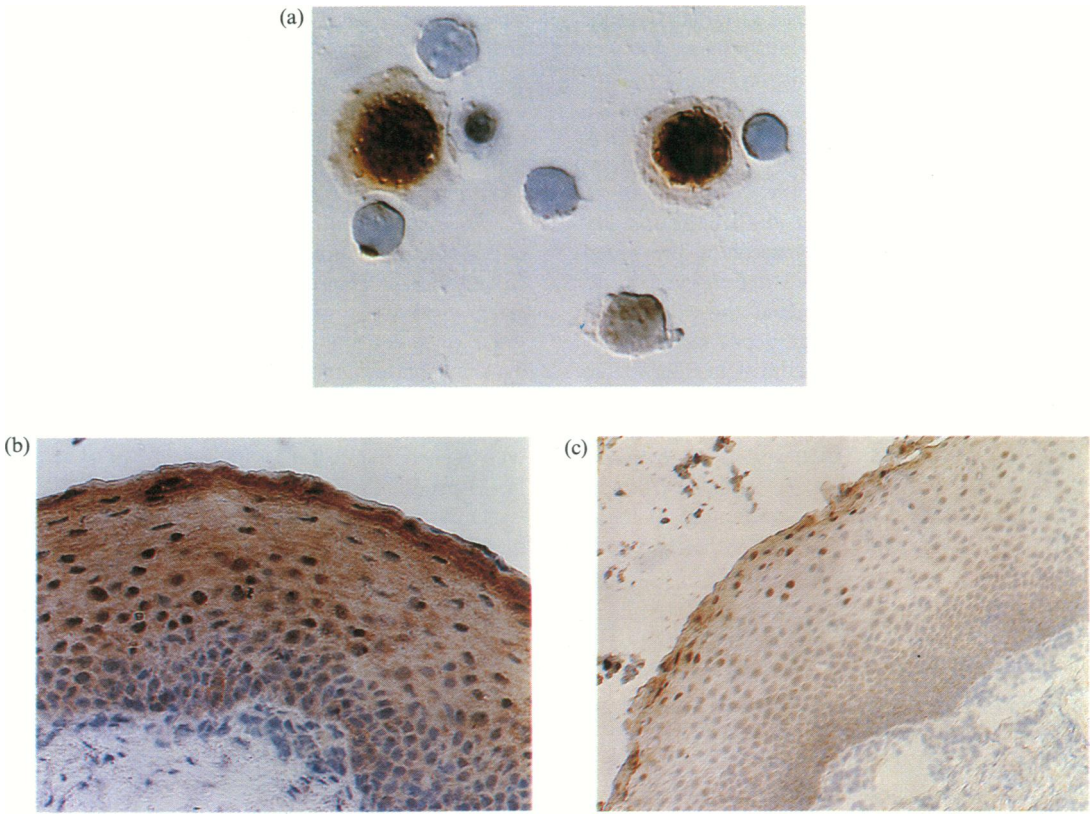

Figure 3. Colour video photographs demonstrating IL-1-producing cells. (a) Illustrates IL-1α-producing cultured monocytes stained in suspension by immunocytochemical peroxidase technique after 3 hr of LPS stimulation. A diffuse nuclear and faint cytoplasmic accumulation of the cytokine was evident. The cells were counterstained with haematoxylin. Original magnification × 800. (b) IL-1α-containing cells detected in the surface epithelium of a fresh frozen tonsil section from recurrent tonsillitis. The cells were counterstained with haematoxylin. IL-1α-containing cells with predominant nuclear staining combined with a diffuse cytoplasmic cytokine accumulation (brown peroxidase), were in vast excess compared to IL-1β. Original magnification × 400. (c) A consecutive section stained for IL-1β, indicating a much lower content in the tissue of this cytokine. The small amount expressed had a nuclear and a diffuse cytoplasmic localization. Original magnification × 320.

Figure 1. (*opposite*) (a) Videoprint photograph illustrating IFN-γ-producing cells in suspension stained by immunoperoxidase after *in vitro* streptococcal enterotoxin-A stimulation for 72 hr. Note the local juxtanuclear deposition of IFN-γ in producer cells caused by the accumulation of IFN-γ in the Golgi organelle. The cells were counterstained with haematoxylin. Original magnification × 800. (b) The photograph illustrates immunohistochemical staining of GM-CSF-producing cells, in a cryopreserved tonsil section (8 μm). Note the similar staining morphology evident in (a), with the local deposition close to the nucleus of the cytokine stained by the immunoperoxidase DAB technique. The nuclei of the cells were counterstained with methylgreen. In addition, puddles of brownish stain in the extracellular matrix close to the synthesizing cells indicate local trapping of the cytokine. Original magnification × 400. (c) A consecutive section to that shown in (b), illustrating the Golgi organelle stained brown with a mAb specific for human Golgi complex, by peroxidase technique. The nuclei of the cells were counterstained with methylgreen. A similar staining pattern to that shown in cytokine-producing cells was evident. Original magnification × 400.

Figure 2. (*opposite*) Colour video photographs illustrating cytokine-producing cells, in cryopreserved tonsil tissue, by immunohistochemical technique. (a) Two-colour staining of proliferating IL-5 synthesizing cells. The local juxtanuclear accumulation of cytokine, stained red by alkaline phosphatase technique, and a diffuse nuclear antigen, stained brown by peroxidase, indicate the intracellular presence of the proliferative antigen, Ki67, in the IL-5-producing cells. The nuclei of the cells were counterstained with haematoxylin. The cryopreserved tonsil tissue was obtained from a patient with IM. Original magnification × 500. (b) Brown peroxidase staining of numerous IL-4-synthesizing cells in recurrent tonsillitis, localized to the extrafollicular area encompassing the germinal centre. A few cells were also localized to the central part of the germinal centre. Accumulation of the cytokine in the extracellular area was also evident around producer cells. Original magnification × 125. (c) A section obtained from a patient with recurrent tonsillitis showing TGF-β2-containing cells localized to the crypts and to the germinal centres without activity in the mantel zones. The cells were counterstained with haematoxylin. Original magnification × 80. (d) Staining of a consecutive section following preincubation of the anti-TGF-β2 Ab with recombinant human TGF-β2, which ablated the cytokine staining in the TGF-β2-producing cells. Original magnification × 80.

much lower incidence of IL-5-producing cells was registered (Table 2 and Fig. 2b). Most of the IL-5-synthesizing cells were proliferating, Ki 67 antigen-positive cells, as judged by two-colour staining. The localization of these cells was both in the germinal centres and in the extrafollicular area. There were more IL-13- than IL-2-positive cells, again with an extrafollicular distribution. In contrast, the TGF-β1–3 family was concentrated to the reticular crypt epithelium and also to the central zone of the germinal centres of the tonsils (Fig. 2c). The TGF-β staining was impressive in intensity for all the three subtypes studied. The persistent and extracellular localization indicated a localized storage depot in all stained specimens. There was no immunoreactivity in the surface epithelium except for TGF-β3. The sections obtained from patients with recurrent tonsillitis consistently contained increased numbers of IL-4, IL-5, IL-10 and IL-13 cytokine-producing cells, compared to snap-frozen sections from IM (Table 2). In contrast, IM sections contained a higher incidence of IL-2-, IFN-γ- and TNF-β-synthesizing cells (Table 2).

Colony-stimulating growth factors

Significant numbers of cells expressing G-CSF, GM-CSF and IL-3 were regularly found in the extrafollicular parts of the tonsils (Fig. 2a and Table 2). The production was restricted to non-epithelial cells. The biological function of this production in the tonsils still has to be elucidated. In particular, GM-CSF was associated with a pronounced extracellular distribution. The frequency of IL-3- and G-CSF-synthesizing cells was, in parallel to the general lymphokine production profile of Th2, also consistently higher in the recurrent tonsillitis group (Table 2).

DISCUSSION

The combination of PFA and saponin for fixation and permeabilization preserved cellular ultrastructure and protein antigenicity. Permeabilization with saponin made it possible to stain for several cytokines, of epitopes recognized by carefully selected cytokine-specific mAb. The use of saponin was superior to treatment with other detergents such as Triton-X, n-octyl-beta-D-glucopyranoside, Tween-20, or organic solvents such as acetone, ethanol and methanol, or without using any detergent at all.[19,21]

The important question as to whether the specific staining pattern encountered in the tissue was generated by intracellular cytokine production or by uptake of cytokines was addressed by incubating cells in culture with purified natural or recombinant cytokines before fixation. This procedure only generated a diffuse increase in background staining, clearly different from the staining pattern outlined in Fig. 1a and b.[19] Thus, the appearance of a perinuclear accumulation of a cytokine reflects intracellularly produced rather than absorbed cytokine. The gradual occurrence of this specific staining pattern in cultured cells correlated to increasing extracellular accumulation of the cytokine, measured by ELISA, and also by the appearance of cytokine-specific mRNA identified by reverse transcriptase-polymerase chain reaction (RT-PCR).[22] The co-localization of the cytokine to the Golgi organelle was also verified by staining with a mAb specific to the Golgi complex (Fig. 1c).

The specificity of the cytokine-detecting antibodies was established by bioassays, by staining permeabilized T-cell clones and cytokine gene-transfected cells, and by ablating the staining by prior incubation of the antibodies with human natural or recombinant cytokines.

This study indicates that human tonsils are sites of continuous lymphoid activation, as demonstrated by high frequencies of spontaneous cytokine-producing cells. It may reflect constant stimulation by environmental antigens encountered in the oropharyngeal cavity. The complexity of the cytokine pattern indicates that both Th1 and Th2 types of cytokine responses were expressed in tonsil tissue. However, for most sections and, in particular, in sections obtained from cases with recurrent tonsillitis, the number of IL-4-, IL-5-, IL-10-, IL-13-producing cells exceeded that of IFN-γ, IL-2 and TNF-β.

Differential production of Th cells during an immune reaction has important regulatory effects on the nature of the immune activation.[23] Responses in mice that are characterized by substantial IFN-γ, TNF-β and IL-12, but little or no IL-4, IL-5 and IL-13 production, that is Th1 responses, result in activation of macrophages to kill intracellular micro-organisms, cytotoxicity, delayed-type hypersensitivity (DTH) and in IgG2a synthesis.[24–26] In contrast, the Th2 responses, in which the production of IL-4, IL-5, IL-10 and IL-13 predominates, generate IgG1 and IgE secreting cells preferentially and may induce eosinophilia.[27,28] Human $CD4^+$ T-cell clones, specific for bacterial antigens on the one hand and allergens on the other, exhibit Th1- or Th2-like cytokine production profiles in line with responses described for mouse Th cells.[29] However, limited information is available about the functional roles of different Th cells *in vivo* in human tissue. The results of this study do not support the completely restricted scenario described above, but favour a more complex pattern where all Th1 and Th2 cytokines are constitutively expressed, but at different frequencies and localizations (Table 2).

On the other hand, the children with recurrent tonsillitis were tonsillectomized when healthy, and had not received antibiotics during the preceding 4 weeks. Thus, the cytokine pattern found in the tissue most likely reflects a homeostatic balance between different arms in the immune response. One may speculate that a more polarized cytokine production profile would have been detected if the tonsil tissue was obtained during exacerbation. A spontaneous production of IL-4, IL-5 and IL-6 has been described recently in normal nasal mucosa.[28] Constitutive production of IFN-γ has been detected in intestinal mucosa, in healthy individuals, and in tonsils.[6,30] Thus, health may require a persistent production of cytokines and immunoactive lymphocytes in the mucosa.

The pro-inflammatory cytokines IL-1β and TNF-α were rarely found in the tonsil tissue (Table 2). IL-1α- and IL-1ra-containing cells were much more prominent, indicating that the tissue was kept under close immunoregulation.[31] The TGF-β1–3 family content in all tissues was substantial. Recently it has been shown that these cytokines are involved in the regulation of IL-1 production, the Ig switch to all IgG subclasses, IgA production, and also in induction of the Bcl-2 gene, thus preventing apoptosis of immune-activated B lymphocytes.[32–34] These biological properties can explain the localization of these cytokines to the crypts and the middle zone

of the follicles (Fig. 2c). The follicular TGF-β-producing cells were probably dentritic cells, as judged by morphology.

In total, these results indicate that the interleukin-synthesizing dominating sites of the tonsil are located to the epithelial areas of the crypts as well as the extrafollicular T-cell rich area, where $CD4^+$ T cells seem to be the dominant phenotype of producer cells. In the tonsil site the lymphokine pattern in recurrent tonsillitis was dominated by IL-1α, IL-1ra, TGF-β1–3, IL-4, IL-5, IL-10 and IL-13, which would indicate that the effector cells should be induced to a humoral immune response, while a more pronounced IL-2, IFN-γ and TNF-β response was verified in the IM case.

Thus, it is possible, by immunohistochemical techniques, to identify human cytokines in frozen fixed tissue. Distinct cytokine patterns may be useful for differential diagnosis. The morphology of cytokine staining in tissue often appears to be a combination of intracellular Golgi staining in producer cells and extracellular deposition in surrounding tissue.

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