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High frequency of spontaneous interferon-gamma-producing cells in human tonsils: role of local accessory cells and soluble factors

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SUMMARY

The frequency of mononuclear cells (MNC) spontaneously secreting interferon-gamma (IFN-γ) has been examined in freshly isolated cell suspensions from human palatine tonsils. Two-site reverse enzyme-linked immunospot (ELISPOT) analyses, involving short term (20 h) incubation of MNC in the absence of any added exogenous stimulus, revealed that tonsillar MNC suspensions contain exceptionally large numbers of cells secreting IFN-γ. No significant differences were observed when comparing the frequency of IFN-γ-producing cells between cell suspensions obtained from hyperplastic and tonsillitis specimens. Cell-sorting experiments disclosed that spontaneous tonsillar IFN-γ production was essentially contributed by $CD4^+$ T cells, and required the presence of accessory cells and/or soluble factors to be detected. Thus, depletion of plastic adherent cells or monocytes from the tonsillar MNC suspensions resulted in reduced numbers of detectable IFN-γ-secreting cells. Addition of very small numbers of autologous monocytes restored spontaneous IFN-γ production in tonsillar MNC cultures depleted of monocytes. Neutralization of endogenous IL-1β and IL-2, as well as blocking of the IL-2 receptor, also decreased IFN-γ production from unfractionated tonsillar cells. Addition of exogenous IL-1β restored IFN-γ production in cultures of tonsillar MNC depleted of plastic adherent cells. Furthermore, IL-1β synergized with IL-2 by increasing intracellular as well as cell-free levels of IFN-γ in cultures of unfractionated tonsillar MNC. This study further establishes that the tonsils are highly active immunological organs containing large numbers of T cells spontaneously producing IFN-γ whose detection is contingent upon the presence of functional accessory cells. It also demonstrates that concomitant production of IL-1β and IL-2 occurs in tonsils and is necessary to maintain ongoing synthesis and extracellular accumulation of IFN-γ in these organs.

Keywords tonsils IFN-γ IL-1 IL-2

INTRODUCTION

Mucosal surfaces of the upper aerodigestive tract constitute a major portal of entry in the body of ingested and inhaled antigens. Strategically located at the entrance of this tract, the tonsils are important masses of organized lymphoid tissue that contain all cell types necessary for induction and expression of humoral and cell-mediated immune responses [1–4]. Equipped with a specialized epithelium containing antigen-transporting cells similar to intestinal M cells [5,6] and with lymphoid follicules containing germinal centres [1], tonsils might be regarded as the bronchus-associated lymphoid tissue counterparts of intestinal Peyer's patches. As such, these tissues may play an important part in antibody-mediated protection of the upper aerodigestive tract [1]. Furthermore, the tonsils appear to be continuously engaged in immune responses to antigens encountered not only in the upper respiratory mucosa but also, and at variance with intestinal Peyer's patches, to antigens encountered in extramucosal tissues [7,8]. In fact, recent studies involving engraftment of human tonsillar cells into severe combined immunodeficiency mice indicate that the tonsils contribute an important fraction of B cell precursors destined for the lungs and other lymphoid tissues but not for the gut [9,10].

In contrast to the large body of information accumulated on the functional and migratory behaviour of tonsillar B cells, studies regarding the properties of tonsillar T cells remain scarce. Among major products of activated T cells, interferon-gamma (IFN-γ) is of central importance not only in the regulation of inate and adaptive immune responses [11,12], but also in the control of basic physiological functions of mucosal

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epitheliae. These include barrier function [13], secretion and uptake of electrolytes [14] and transport of potentially protective secretory immunoglobulins [15]. We therefore examined the frequency of cells spontaneously secreting IFN-γ in freshly isolated mononuclear (MNC) suspensions from healthy and recurrently inflamed tonsils.

In this study we demonstrate that spontaneous IFN-γ production occurs in human tonsils but requires accessory cells and/or factors whose presence is mandatory to allow its extracellular secretion. This led us to investigate a possible role for IL-1 and IL-2, which are known co-inducers of IFN-γ synthesis, in this secretory process.

MATERIALS AND METHODS

Preparation of MNC suspensions

With due approval from the Research Ethical Committee of the Medical Faculty, University of Göteborg, surgically resected palatine tonsils were collected from 25 individuals undergoing tonsillectomy indicated by recurrent tonsillitis (five males and seven females, aged 6–38 years) or by severe 'snoring' problems or tonsillar hypertrophy, i.e. individuals with clinically healthy tonsils (10 males and three females, aged 4–61 years). The tonsillar material was kept at 4°C in PBS (0·01 M phosphates, 0·15 M NaCl, pH 7·4) until being processed for isolation of MNC within a few hours later. Venous blood samples were also collected from seven of the patients at the time of tonsillectomy.

Parts of the tonsils macroscopically judged as inflamed were discarded and the remaining tissue was cut into 1×1 mm fragments with a semi-automated tissue chopper (McIlwain, Guilford, UK). The tissue fragments were then disrupted by pressing through a 150 μm nylon mesh and the resulting single-cell suspension was washed three times by centrifugation (530 *g*, 4°C, 5 min) in cold Ca^{++}- and Mg^{++}-free PBS (0·01 M phosphates, 0·15 M NaCl, pH 7·4, GIBCO Europe, Edinburgh, UK). Tonsillar and peripheral blood MNC were isolated by gradient centrifugation (940 *g*, 20°C, 15 min) on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). Interface MNC were collected, washed three times with Ca^{++}- and Mg^{++}-free PBS, and then resuspended in Iscove's medium supplemented with 5% fetal calf serum (FCS) and 100 μg/ml gentamicin (Sigma, St Louis, MO) (complete medium). The medium used contained less than 20 pg/ml of endotoxin as assessed by the limulus lysate test. Cell suspensions were kept on ice before being assayed.

When indicated, tonsillar MNC were fixed with 4% paraformaldehyde in PBS for 10 min at 20°C and thereafter washed three times before use.

Fractionation of MNC

Tonsillar MNC were fractionated by rosetting with magnetic beads coated with relevant antibodies, rosetting with 2-aminoethylisothiouronium bromide (AET) (Sigma) treated sheep erythrocytes and by depletion of plastic adherent cells. MNC suspensions were stained with fluorescein- and/or PE-labelled antibodies specifying the CD2, CD3, CD4, CD8, CD14, CD16 and CD25 (IL-2R) cell surface markers (Becton Dickinson, San Jose, CA). Tonsillar dendritic cells were demonstrated by stepwise exposure of MNC suspensions to a MoAb to human follicular dendritic cells (DRC-1, Dakopatts AS, Glostrup, Denmark) [16] and B cells by exposure to a MoAb reacting with CD20 (Immunotech, Marseille, France), followed by fluorescein-labelled $F(ab')_2$ fragments of rabbit antibodies to mouse immunoglobulin (Dakopatts).

Flow cytometry analyses revealed that tonsillar MNC suspensions contained $27 \pm 6\%$ $CD2^+$ cells and $28 \pm 10\%$ $CD3^+$ cells ($n = 8$ individuals). Among tonsillar T cells, $CD4^+$ cells dominated over $CD8^+$ cells ($19 \pm 8\%$ and $5 \pm 4\%$, respectively) and $2 \pm 4\%$ of tonsillar MNC expressed the IL-2R. $CD16^+$ cells, putative natural killer (NK) cells, accounted for $0{\cdot}2 \pm 0{\cdot}1\%$ of tonsillar MNC and $61 \pm 4\%$ expressed the B cell marker CD20. In addition, $0{\cdot}4 \pm 0{\cdot}2\%$ of tonsillar MNC expressed the monocyte marker CD14, and $17 \pm 10\%$ carried the marker for dendritic cells, DRC-1. It should be noted that DRC-1 antibody has also been shown to stain germinal centre B cells in tonsils [17].

To deplete the tonsillar MNC suspensions of cell subsets, monodisperse magnetic microspheres directly coated with antibodies to either CD2, CD4, CD8 or CD19 (Dynal AS, Oslo, Norway) were used. Beads indirectly coated with antibodies to CD3, CD14 and CD16 were prepared by incubating overnight at 4°C 1 μg of mouse MoAb to human CD3 (T3, Dakopatts), CD14, or CD16 per mg of beads previously coated with sheep antibodies to mouse immunoglobulin (Dynal). Tonsillar MNC were incubated at 37°C for 5 min with the various antibody-coated bead preparations at a bead-to-target cell ratio of 10:1 in 0·5 ml of complete medium, and pelleted by centrifugation (115 *g*, 4°C, 5 min). After a further incubation at 4°C for 1 h, cells and beads were gently resuspended and maintained in suspension for 15 min at 4°C. Cells attached to beads and free beads were then retained by applying a magnetic field. Unbound cells were washed twice and resuspended in complete medium. These magnetic depletion procedures yielded MNC suspensions that contained less than 5% $CD2^+$, $CD3^+$, $CD4^+$ and $CD20^+$ cells, less than 0·5% $CD8^+$ cells, and less than 0·1% $CD14^+$ and $CD16^+$ cells.

$CD2^+$ tonsillar cells were also isolated by rosetting with AET-treated sheep erythrocytes, followed by gradient centrifugation on Ficoll–Hypaque [18]. This procedure routinely yielded cell suspensions containing >95% of $CD2^+$ cells.

Tonsillar cell suspensions were depleted of plastic adherent cells by incubating $1{\cdot}5 \times 10^7$ MNC in 10 ml of complete medium in 9 cm diameter plastic Petri dishes for 2·5 h at 37°C. Non-adherent cells were transferred to a second Petri dish and incubated for 2·5 h at 37°C. The resulting non-adherent cells were washed twice before being assayed. This procedure yielded MNC suspensions containing less than 0·1% of $CD14^+$ cells.

Ficoll–Hypaque fractionated peripheral blood MNC were enriched for monocytes by counter-current centrifugal elutriation at 588 *g* and at a flow rate of 18 ml/min in a centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-6B rotor. This procedure routinely yields cell suspensions containing 50–90% of $CD14^+$ monocytes [19].

Enumeration of IFN-γ-secreting cells

Cell suspensions were assayed for numbers of cells spontaneously secreting IFN-γ by a two-site reverse enzyme-linked immunospot (ELISPOT) technique using epitope-specific mouse anti-human IFN-γ MoAbs as capture and developing reagents [20]. Briefly, various numbers of MNC were incubated for 20 h at 37°C in 100 μl of complete medium in nitrocellulose-bottomed 96-well plates (Millipore, Bedford, MA) coated with

anti-IFN-γ MoAb. Zones of solid phase bound IFN-γ secreted by individual cells were revealed as spots by stepwise addition of biotinylated anti-IFN-γ MoAb, horseradish peroxidase-conjugated avidin, and suitable chromogen substrate.

In some experiments, various amounts of affinity-purified human IL-1α, IL-1β (Glaxo, Geneva, Switzerland) and/or human recombinant IL-2 (Genzyme, Boston, MA), IL-6 (Biosource International, Camarillo, CA), or tumour necrosis factor-alpha (TNF-α; Pharmingen, San Diego, CA) were added during the cell incubation period.

In other experiments, neutralizing Fab fragments of sheep antibodies to human IL-1β [21] and/or Fab fragments of rabbit antibodies to human IL-2 [22], or rabbit IgG antibodies to human TNF-α, were added to the ELISPOT wells at the time of cell plating. IgG and Fab fragments thereof from normal rabbit or sheep serum were used for control purposes.

Quantification of secreted and intracellular IFN-γ

Aliquots of MNC suspensions were incubated at 37°C in flat-bottomed 96-well culture plates (Nunc, Roskilde, Denmark) under conditions (cell density, incubation time) similar to those of the ELISPOT assay. Thereafter, cell suspensions were centrifuged (260 ***g***, 20°C, 5 min), and the culture supernatants were harvested and kept frozen (−20°C) before being assayed. After three washings by centrifugation, intracellular IFN-γ was extracted by exposure of the cells for 1 h at 37°C to 0·01% (w/v) digitonin (Sigma) in complete medium. After centrifugation the cell-free extract was harvested and kept frozen at −20°C before being assayed. The IFN-γ contents of cell culture supernatants and digitonin extracts were measured by an ELISA [23]. The assay can detect as little as 0·01 U of purified lectin-induced human IFN-γ per millilitre.

Assessment for de novo *synthesis of IFN-γ*

Tonsillar MNC were incubated for 3 h at 37°C in complete medium containing 2 mM of cycloheximide (Sigma). Thereafter, cells were washed three times and plated in ELISPOT assay dishes or flat-bottomed 96-well culture plates in complete medium with cycloheximide for 20 h. Numbers of IFN-γ-secreting cells and amounts of secreted IFN-γ were detected respectively by the ELISPOT and ELISA assays described above.

Proliferative activity of peripheral blood and tonsillar MNC

Peripheral blood and tonsillar MNC suspensions were incubated in 96-well flat-bottomed culture plates under conditions identical to those of the cell incubation step of the ELISPOT assay. Tritiated thymidine (1 μCi; Amersham International, Amersham, UK) was added to the cultures at the start of the incubation period. After 20 h, cells were harvested on a glass fibre filter (Inotech, Wohlen, Switzerland) and the amount of incorporated radioactivity was determined in an automated argon-activated β-scintillation counter (Inotech).

RESULTS

Baseline frequencies of tonsillar IFN-γ-secreting cells

Freshly isolated tonsillar MNC suspensions were assayed for numbers of IFN-γ-secreting cells by a two-site reverse ELISPOT technique [20]. High numbers of cells spontaneously secreting IFN-γ were detected in tonsillar MNC suspensions plated for 20 h at 10^6 MNC/assay well (geometric mean 108 IFN-γ-secreting cells/10^6 MNC, range 27–360, $n=25$ donors). This contrasted with peripheral blood MNC suspensions obtained from the same individuals which contained a few spontaneous IFN-γ-secreting cells (geometric mean 6 IFN-γ-secreting cells/10^6 MNC, range 0–32, $n=7$ donors). There was no significant difference in frequencies of tonsillar IFN-γ spot-forming cells (SFC) between patients with recurrent tonsillitis (geometric

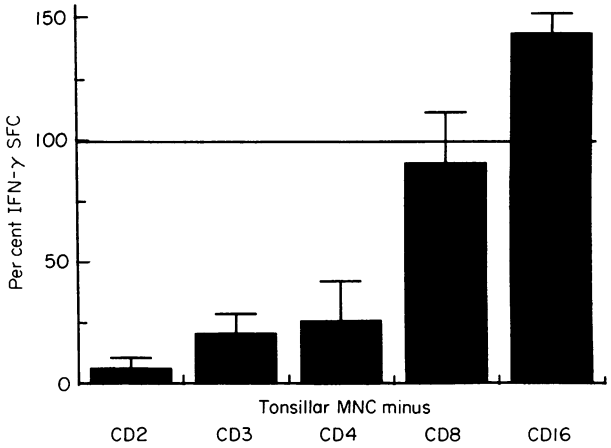

Fig. 1. Phenotypic characteristics of spontaneous IFN-γ-secreting cells in human tonsils. Bars indicate the frequency of cells spontaneously secreting IFN-γ, detected by ELISPOT assay, after depletion of cells with magnetic beads coated with antibodies to the indicated cell surface markers. All cell suspensions were adjusted to 10^6 mononuclear cells per assay well and cultured for 20 h. Control cell suspensions were treated with magnetic beads coated with antibodies to a streptococcal protein antigen. The frequencies of IFN-γ-secreting cells are expressed as per cent of spot-forming cell (SFC) numbers determined by comparison with control cells. Data are pooled and expressed as mean + s.d. of three-to-five tonsil donors. MNC, Mononuclear cells.

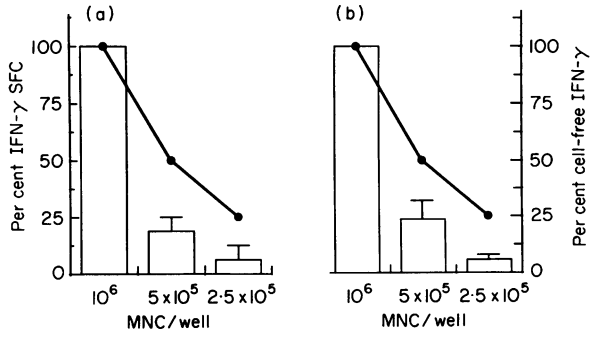

Fig. 2. Spontaneous secretion of IFN-γ by human tonsillar mononuclear cells is dependent on cell density. Tonsillar mononuclear cells (MNC) were incubated for 20 h in ELISPOT assay dishes or in flat-bottomed microculture plates. Open bars indicate the number of cells spontaneously secreting IFN-γ (a) or the amounts of secreted IFN-γ (b) per assay well at the indicated cell numbers. Results are expressed respectively as mean % + s.d. of spot-forming cell (SFC) numbers and amounts of secreted IFN-γ determined in MNC cultures plated at 10^6 cells per assay well and are compared with the predicted values assuming a constant frequency of IFN-γ-secreting cells (line). Data are pooled from 25 (ELISPOT assay) and five (ELISA assay) tonsil donors.

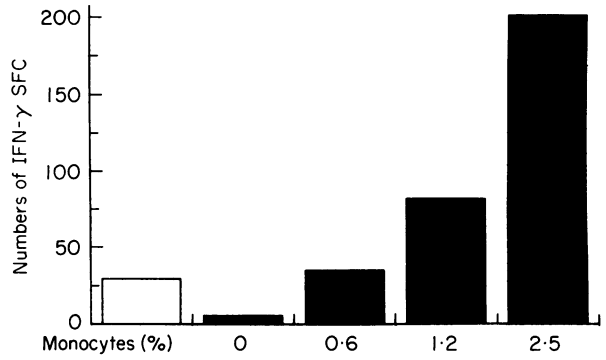

Fig. 3. Tonsillar IFN-γ secretion is dependent on monocytes. Bars indicate the frequency of IFN-γ-secreting cells in one unfractionated tonsillar mononuclear cell (MNC) suspension (□) and in corresponding suspensions depleted of $CD14^+$ cells via magnetic bead rosetting (■) and plated at 10^6 cells per ELISPOT assay well. Peripheral blood monocytes enriched by counter-current centrifugal elutriation were added at the frequencies indicated at the time of plating to MNC cultures depleted of $CD14^+$ cells. Data from a representative experiment are shown. SFC, Spot-forming cells.

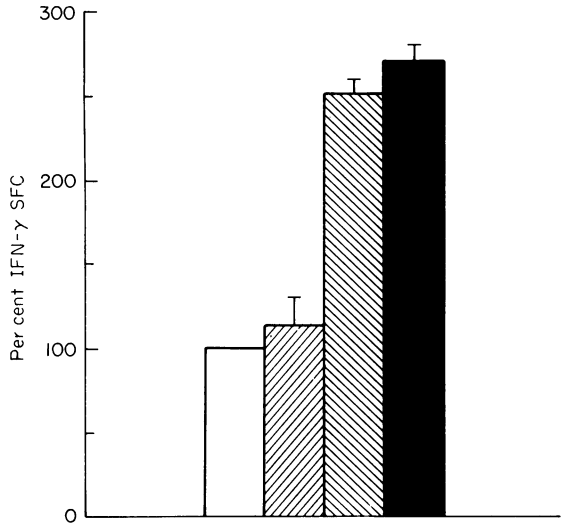

Fig. 4. Exogenous IL-2 increases spontaneous IFN-γ production by tonsillar mononuclear cells (MNC). Tonsillar MNC were incubated for 20 h with exogenous IL-1β (▩), IL-2 (▧), or IL-1β together with IL-2 (■) in ELISPOT assay dishes. Numbers of IFN-γ-secreting cells were determined by ELISPOT assays in cultures of 10^6 MNC per assay well. Data are expressed as mean % + s.d. of spot-forming cell (SFC) numbers determined by comparison with untreated MNC cultures (□) and are pooled from five tonsil donors.

mean 90 IFN-γ-secreting cells/10^6 MNC, s.d. 79, $n = 12$ donors) and patients with 'snoring' problems or tonsillar hypertrophy (geometric mean 131 IFN-γ-secreting cells/10^6 MNC, s.d. 144, $n = 13$ donors).

A good correlation ($r = 0{\cdot}83$, $P < 0{\cdot}001$, Pearson's correlation test) was obtained between numbers of detected IFN-γ-secreting cells and amounts of secreted IFN-γ detected by ELISA, indicating an average secretion rate of approximately 100 μU of IFN-γ per tonsillar IFN-γ SFC per 20 h.

Requirement for *de novo* protein synthesis in the assays was documented by the effects of exposure of MNC suspensions to cycloheximide during the cell incubation period. Such treatment virtually abrogated IFN-γ secretion as detected by ELISPOT and ELISA. In addition, cell-free IFN-γ could not be detected in supernatants from MNC cultured at 4°C for 20 h.

Tonsillar $CD4^+$ T cells are the major secretors of IFN-γ

Depletion of $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$ or $CD16^+$ cells from tonsillar MNC suspensions demonstrated that the large majority of tonsillar MNC spontaneously secreting IFN-γ resided within the T cell population ($CD2^+$, $CD3^+$), and belonged mainly to the $CD4^+$ helper/inducer T cell subset (Fig. 1). Tonsillar NK cells ($CD2^+$, $CD16^+$, $CD3^-$), detected in small numbers by flow cytometry analyses, did not appear to contribute to any significant extent to IFN-γ production (Fig. 1). Depletion of $CD19^+$ B cells from tonsillar MNC suspensions increased rather than decreased the frequencies of IFN-γ-secreting cells (data not shown). Since tonsillar MNC suspensions comprise large numbers of B cells (approximately 60%), and since unfractionated and MNC suspensions depleted of various subpopulations were assayed at the same cell densities (10^6 MNC/well) this was largely due to corresponding increases in the relative proportion of T cells.

Spontaneous secretion of IFN-γ by tonsillar T cells is dependent on cell density

Unexpectedly, the relation between numbers of MNC assayed and numbers of detectable IFN-γ-secreting cells was not linear, but decreased dramatically below a threshold cell density (Fig. 2a). This observation was confirmed by ELISA measurements of IFN-γ levels accumulated in supernatants from tonsillar MNC cultures performed under conditions identical to those of the reverse ELISPOT assay (Fig. 2b).

The importance of cell–cell interactions in promoting spontaneous IFN-γ secretion by tonsillar MNC was assessed by adding paraformaldehyde-fixed tonsillar MNC to suboptimal numbers of non-fixed, fresh MNC so as to reconstitute a cell density optimal for IFN-γ secretion. In these experiments, fixed tonsillar MNC could not substitute for metabolically active cells in promoting IFN-γ secretion, indicating that spontaneous IFN-γ secretion by tonsillar MNC is not only dependent on direct cell–cell contact.

Based on the above observations, absolute frequencies of tonsillar IFN-γ-secreting MNC reported in this study are related to cell densities falling within the linear portion of dose-response curves (cell input/numbers of detectable IFN-γ-secreting cells).

Spontaneous IFN-γ secretion by tonsillar T cells is dependent on plastic adherent cells

Among highly purified (>95%) $CD2^+$ tonsillar MNC the frequency of detectable IFN-γ-secreting cells was substantially lower (mean reduction $61 \pm 12\%$ of untreated controls, $n = 3$ experiments) than in corresponding unfractionated MNC suspensions, albeit the latter suspensions contained less than half the number of $CD2^+$ cells.

Depletion of plastic adherent cells (presumably dendritic cells, monocytes and macrophages) from the tonsillar MNC suspensions also resulted in a marked reduction in the frequency of detectable IFN-γ-secreting cells at all cell densities examined (mean $47 \pm 22\%$ reduction at 10^6 MNC per ELISPOT well,

Table 1. Effects of IL-1 and IL-2 on spontaneous intracellular accumulation and secretion of interferon-gamma (IFN-γ) by human tonsillar lymphocytes

Cytokine added (experiment no.)	IFN-γ (mU) per 10^7 tonsillar MNC							
	Intracellular				Secreted			
	Nil	IL-1β	IL-2	IL-1β+IL-2	Nil	IL-1β	IL-2	IL-1β+IL-2
1	<10	<10	<10	30	26	75	88	384
2	27	<10	48	81	1060	776	3640	4160
3	<10	<10	17	29	116	154	662	898
4	ND	ND	ND	ND	168	284	992	1984

ND, Not determined.

$n=10$ experiments) compared with corresponding unfractionated MNC suspensions. Parallel ELISA analyses of supernatants from tonsillar MNC suspensions cultured under identical conditions confirmed the latter observation.

Depletion of $CD14^+$ monocytes from tonsillar MNC suspensions also resulted in a large decrease ($72\pm25\%$, $n=5$ experiments) in the frequency of IFN-γ SFC. Addition of as few as 6×10^3 autologous peripheral blood monocytes to 10^6 tonsillar MNC suspensions depleted of $CD14^+$ cells restored the frequency of IFN-γ-secreting cells to a level comparable to that seen in corresponding unfractionated MNC suspensions (Fig. 3). Addition of larger numbers of monocytes led to even higher frequencies of IFN-γ-secreting cells.

IL-1 and IL-2 regulate both the synthesis and secretion of IFN-γ by tonsillar MNC

Addition of neutralizing anti-IL-1β or anti-IL-2 antibodies during the cell incubation stage in ELISPOT assays reduced the frequency of detectable IFN-γ-secreting cells by $53\pm11\%$ and $44\pm17\%$ ($n=3$ experiments), respectively. The combination of both antibodies led to a further decrease ($69\pm9\%$, $n=3$ experiments) of the frequency of detectable IFN-γ-secreting cells. In contrast, anti-TNF-α antibody did not alter the frequency of tonsillar IFN-γ-secreting cells.

Addition of affinity-purified human IL-1β did not significantly increase the frequency of detectable IFN-γ-secreting cells in cultures containing 10^6 unfractionated tonsillar MNC per assay well (Fig. 4). However, addition of exogenous IL-1β (0·01 U/well) could restore the number of IFN-γ-secreting cells in cultures of plastic adherent cell-depleted MNC suspensions (mean $109\pm1\%$ of the frequency observed in unfractionated MNC suspensions, $n=2$ experiments). Furthermore, addition of IL-1β to unfractionated MNC assayed at suboptimal densities ($\leqslant5\times10^5$ MNC/well) partially restored the predicted but failing linearity between numbers of ELISPOT-detectable IFN-γ-secreting cells and numbers of plated cells. Although IL-1α and IL-6 had similar effects (data not shown), exogenous TNF-α (0·1–100 U/well) did not affect IFN-γ secretion by tonsillar MNC (data not shown).

Addition of recombinant human IL-2 (1 U/well) to tonsillar MNC suspensions increased the frequency of ELISPOT-detectable IFN-γ-secreting cells by about 2·5-fold, as compared with untreated MNC cultures (Fig. 4). Even though incubation of cell cultures with IL-2 induced high absolute numbers of IFN-γ-secreting cells at all cell concentrations assayed, this treatment could not restore a linear relation between numbers of MNC plated and numbers of detectable IFN-γ-secreting cells. The amounts of secreted IFN-γ in cell cultures exposed to IL-2 were on average 4·5 times higher than those recorded in untreated cultures (Table 1), indicating that not only the frequency of IFN-γ-secreting cells increased after exposure to IL-2, but also the average amount of IFN-γ secreted per cell.

In most experiments treatment of tonsillar MNC with both IL-1β and IL-2 resulted in even larger amounts of secreted IFN-γ than treatment with IL-2 alone (Table 1). No corresponding increase could be detected when comparing the frequency of IFN-γ-secreting cells after incubation with IL-2 together with IL-1β with that after incubation with only IL-2 (Fig. 4).

That untreated tonsillar MNC cultures and cultures exposed for 20 h to IL-2, with or without IL-1β, incorporated thymidine to a comparable extent (albeit 6–10 times higher than corresponding blood MNC cultures; data not shown) indicates that treatment of MNC with these cytokines increased the secretion rate of IFN-γ-producing cells rather than their proliferation.

In one additional experiment, antibody to IL-2R completely inhibited secretion of IFN-γ in untreated as well as in IL-1β-treated MNC cultures when added during the cell incubation step. Anti-IL-2R antibody also decreased the total amount of secreted IFN-γ in MNC cultures incubated with IL-2 by 85%.

Intracellular IFN-γ could usually not be detected in untreated or IL-1β-treated cultures, whereas IL-2 induced ELISA-detectable amounts of intracellular IFN-γ in two out of five tonsillar MNC suspensions. The combination of IL-1β and IL-2 during the cell incubation step further increased intracellular IFN-γ content as compared with MNC treated with IL-2 alone, in three out of the five experiments (Table 1).

DISCUSSION

This study establishes that the human tonsils are the site of pronounced T cell activation, as demonstrated by the high frequency of T cells spontaneously producing IFN-γ in this organ, irrespective of the clinical status of the donors. It may reflect constant stimulation of tonsillar lymphocytes by environmental antigens encountered in the upper alimentary tract, rather than overt local inflammation. This situation resembles that of the intestinal mucosa where large numbers of spontaneous IFN-γ-producing cells have been detected in healthy

human volunteers and were even further enlarged after introduction of enteric immunogens [24]. The virtual absence of spontaneous IFN-γ-secreting cells in blood is consistent with the above interpretation.

Previous studies have indicated that IFN-γ production is contributed by $CD4^+$ T cells and $CD8^+$ T cells, as well as by NK cells [11]. In the present study, experiments involving short-term (20 h) assay of tonsillar MNC depleted of various lymphocyte subpopulations indicate that, in the absence of any added stimulus, $CD3^+CD4^+$ T cells are the main cells spontaneously producing IFN-γ in human tonsils. A recent study has demonstrated that among $CD4^+$ T cells, only memory cells have the capacity to produce IFN-γ upon mitogen stimulation [25], indicating that the cells we were detecting may represent memory T cells that had been activated *in vivo*.

In contrast to human intestinal [24] and mitogen-stimulated peripheral blood MNC [20,26] analysed by the same techniques, the relation between numbers of tonsillar MNC assayed and numbers of detectable IFN-γ-secreting cells or amounts of secreted IFN-γ was not linear. This observation suggested that physical cell–cell contacts and/or accessory cells or factors present in limiting numbers or amounts were required to allow secretion of IFN-γ by tonsillar T cells. The fact that addition of fixed tonsillar MNC to suboptimal numbers of fresh autologous tonsillar MNC failed to restore spontaneous IFN-γ secretion argues against the former possibility. The second interpretation is consistent with (i) the paucity of conventional accessory cells such as monocytes and dendritic cells in freshly isolated tonsillar MNC, and (ii) the fact that MNC suspensions depleted of plastic adherent cells or monocytes had markedly reduced capacities to secrete IFN-γ spontaneously. Moreover, addition of small numbers of autologous blood monocytes restored the frequency of IFN-γ-secreting cells in tonsillar MNC suspensions depleted of monocytes. Taken together, these observations demonstrate that monocytes play a mandatory role in spontaneous IFN-γ production by tonsillar cells.

Recent studies have demonstrated that lymphoblastoid B cells can produce a heterodimeric lymphokine capable of inducing IFN-γ production from both resting and activated human peripheral blood lymphocytes [27,28]. In the present study, removal of B cells from tonsillar MNC suspensions did not result in decreased IFN-γ production, indicating that B cells and factors therefrom had no direct role in promoting spontaneous IFN-γ production by tonsillar $CD4^+$ MNC. The possibility that $CD4^+$ cells comprise a subpopulation of T cells providing the critical accessory signal(s) to other $CD4^+$ T cells, without being IFN-γ secretors *per se*, was excluded since highly enriched tonsillar T cell suspensions failed to produce IFN-γ. However, we cannot rule out the possibility that $CD4^+$ tonsillar MNC comprise a subpopulation of macrophages that acted as accessory cells. Nor can we rule out the possibility that dendritic cells were also promoting spontaneous IFN-γ secretion by tonsillar $CD4^+$ T cells, since we were not able to obtain suspensions sufficiently depleted or enriched in dendritic cells.

Earlier studies have demonstrated that IFN-γ production by mitogen and/or IL-2-stimulated T cells requires accessory cells [29–31]. Our study demonstrates for the first time that, in the absence of exogenous mitogen or IL-2, spontaneous IFN-γ secretion by freshly isolated tonsillar T cells also requires accessory cells. A well established function of accessory cells is related to production of IL-1, IL-6 and TNF-α [32–34]. In this respect, neutralization of endogenous IL-1β activity but not of TNF-α in unfractionated tonsillar MNC cultures decreased the frequency of detectable IFN-γ-secreting cells. Furthermore, exogenous IL-1β could fully reconstitute IFN-γ secretion when added to cultures of tonsillar MNC that had been depleted of accessory cells. These observations underline the importance of this cytokine in maintaining spontaneous IFN-γ secretion by tonsillar T cells.

One of the major effects that IL-1 exerts on T cells is the concomitant induction of IL-2 production and of IL-2 receptor(s) expression [32]. Since IL-2 is a known inducer of IFN-γ synthesis [29–31,35–37] and given the fact that IL-1β synergized with IL-2 by increasing both intracellular accumulation and secretion of IFN-γ from tonsillar MNC cultures, we reasoned that IL-1 produced by tonsillar MNC might influence IFN-γ production indirectly by inducing expression of IL-2R and thus uptake of IL-2. Indeed, blocking of IL-2R abrogated spontaneous IFN-γ secretion in cultures of unfractionated tonsillar MNC. Furthermore, addition of IL-2 alone, but not of IL-1β, increased the secretion of IFN-γ in cultures of unfractionated tonsillar MNC, indicating that IL-2, rather than IL-1β, was the limiting factor in such short-term cultures. The synergistic effect of the two cytokines could then result from increased IL-2R expression induced by IL-1 when supplies of IL-2 are no longer limited. The increased frequencies of IFN-γ-secreting cells observed after exposure of tonsillar MNC to IL-1β and/or IL-2 did not appear to result merely from increased cell proliferation, but rather from increased secretion of IFN-γ per cell (resulting in larger numbers of ELISPOT-detectable IFN-γ-secreting cells). This was indicated by the fact that IL-1β and IL-2 increased intracellular as well as cell-free levels of IFN-γ but had no effect on proliferation of tonsillar MNC.

The fact that IL-1β alone, in contrast to IL-2, did not affect secretion or intracellular accumulation of IFN-γ but, together with IL-2, increased intracellular as well as cell-free levels of IFN-γ also indicates that these cytokines have distinct regulatory effects on IFN-γ-producing cells. This also demonstrates that IL-1β and IL-2 can act on cells already synthesizing IFN-γ. Taken together, these observations can be integrated in a model where newly synthesized IFN-γ molecules accumulate into the cytosol and their export in the extracellular milieu requires a signal that appears to be transduced by the binding of IL-2 to its membrane receptor(s), the expression of the latter being maintained by locally produced IL-1. This model is consistent with recent studies [28,38] showing that synthesis of IFN-γ and its secretion appear to be differentially regulated.

Tonsillar IFN-γ secretion is probably an important component in immunological defence of the upper aerodigestive tract with likely effects on both lymphoid [11,12] and epithelial cell functions. Thus, epithelial permeability may be increased by locally produced IFN-γ [13], resulting in transport of potentially protective antibodies across the tonsillar epithelium and uptake of antigens encountered in the upper aerodigestive tract.

In conclusion, this study demonstrates that the tonsils are highly active immunological organs, harbouring large numbers of IFN-γ-secreting T cells. It also infers that production of IL-1 and IL-2 occurs in the tonsils and is required to promote both synthesis and extracellular secretion of IFN-γ. The usefulness of an IFN-γ-specific reverse ELISPOT assay to document in a simple, direct, and sensitive manner the short-term effects of potential immunomodulating agents is thus illustrated.

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