Clin Exp Immunol 1993; 92:7–13

Characterization of cytokine production in infectious mononucleosis studied at a single-cell level in tonsil and peripheral blood

J. ANDERSSON*† & U. ANDERSSON*‡ *Department of Immunology, Arrhenius Laboratories for Natural Sciences, Stockholm University, Stockholm, †Department of Infectious Diseases, Karolinska Institute, Huddinge Hospital, Huddinge, and ‡Department of Pediatrics, Karolinska Institute, St. Görans Hospital, Stockholm, Sweden

(Accepted for publication 6 January 1993)

SUMMARY

Cytokine profile and production was studied at a single-cell level in cells obtained from 14 patients with acute infectious mononucleosis (IM), with less than 7 days of symptomatic disease, by use of cytokine-specific MoAbs and indirect immunofluorescence technique. In producer cells, all the studied cytokines, except IL-1, accumulated in the Golgi system, which resulted in a characteristic morphology of the staining. Less than one in a thousand mononuclear cells obtained directly from IM blood and stained within 2 h of sampling produced IL-2, interferon-gamma (IFN-γ), IL-4, IL-5, IL-6, IL-10, GM-CSF, tumour necrosis factor-alpha (TNF-α) or TNF-β, spontaneously. However, these cells were induced to cytokine synthesis by T cell receptor ligation *in vitro* using immobilized anti-CD3 MoAbs for 2–3 h restimulation under conditions which did not activate normal cells. By this approach 168 ± 120 cells/10 000 peripheral blood mononuclear cells produced IFN-γ as compared with 10 ± 8 cells/10 000 non-stimulated cultured cells obtained from IM patients ($P < 0{\cdot}001$) and 1/10 000 cells obtained from healthy controls, respectively. No induced production of IL-2, IL-3, IL-4, IL-5, IL-10, GM-CSF or TNF-β was detected in IM cells obtained from peripheral blood by this restimulation. In contrast, a spontaneous cytokine production was evident in tonsil material obtained from four IM patients tonsilectomized because of respiratory obstruction. From this site 160 ± 40 cells/10 000 cells produced IL-2, 40 ± 30 cells IL-6, 30 ± 30 cells TNF-β and 35 ± 25 cells IFN-γ, respectively. No such spontaneous IL-2, IL-6, TNF-β or IFN-γ production was evident in control cells obtained from patients tonsilectomized because of chronic tonsil hyperplasia.

Keywords Epstein–Barr virus infectious mononucleosis cytokines immunopathology

INTRODUCTION

Infectious mononucleosis (IM) is an acute and normally self-limiting lymphoproliferative disease caused by a primary infection of Epstein–Barr virus (EBV) [1]. At the time of clinical onset of acute IM one finds a vast expansion of large lymphoblastoid cells with prominence of basophilic and cytoplasmic azurophilic granules not only in peripheral blood but also invading parenchymal organs [2,3]. However, even though B lymphocytes are target cells for EBV, most expanded lymphocytes are actually T lymphocytes and $CD16^+$ natural killer cells [4].

The T cell population is dominated by the $CD8^+$ subset, expressing HLA-DR, CD45RO and the p70 subunit of the IL-2 receptors on their cell surface [4–6]. This indicates that these cells are activated, memory type T cells with cytotoxic properties and considered to be the result of an intense defence response against EBV-infected target cells.

We have previously shown that the early institution of high doses of the antiviral drug acyclovir significantly inhibited oropharyngeal EBV replication in patients with IM without affecting the clinical symptoms [7]. Thus, the disease is probably a consequence of the immunologic response induced to control the virus rather than a direct effect on the viral replication itself. When we studied serum samples from patients with IM ≤ 7 days of symptoms we found significant raised levels of interferon-gamma (IFN-γ), soluble IL-2 receptors (sIL-2R) and soluble CD8 (sCD8) molecules as well as increased serum neopterin levels [8]. It is thus plausible that the increased production of cytokines by activated cells may cause many of the symptoms characteristic of IM.

This study was initiated to elucidate the cytokine pattern at the cellular level in cells obtained from peripheral blood and from tonsil samples from patients tonsilectomized because of fulminant IM and upper respiratory obstruction. Cytokine production was studied at the single-cell level by u.v.-microscopy of fixed permeabilized cells using cytokine-specific MoAbs for determination of IL-1α, -β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, tumour necrosis factor-alpha (TNF-α), TNF-β, IFN-γ and GM-CSF producing cells [9].

Correspondence: Jan Andersson, MD, Department of Immunology, Stockholm University, 106 91 Stockholm, Sweden.

PATIENTS AND METHODS

Patients

Seventeen patients, 18–26 years old, hospitalized at the Department of Infectious Diseases, Danderyd Hospital, Stockholm, Sweden, with clinical diagnosis of acute infectious mononucleosis, were enrolled. Informed consent was obtained from each patient, their parents or guardians before entering the study. The study was approved by the Ethical Committee at the Karolinska Institute, Stockholm, Sweden. The Ethical Committee, Karolinska Institute, approved both blood analyses as well as tonsilectomy and experimental study in patients with respiratory obstruction and acute IM. All the patients were heterophile antibody-positive and had IgM antibodies to the EBV capsid (VCA) antigen. Four patients were referred to the Department of Otorhinology because of fulminant IM complicated by upper respiratory tract obstruction and were subsequently tonsilectomized.

The control population consisted of 14 normal healthy blood donors (18–40 years old) and the samples were obtained at the same time as the IM samples and handled identically. Control tonsil sections were obtained from four 16–17-year-old individuals, without serological evidence of acute EBV infection, suffering from chronic tonsil hyperplasia, and were also obtained from the Department of Otorhinology. The tonsil material from these patients was handled identically to the tonsils obtained from IM patients.

Isolation of peripheral blood and tonsillar mononuclear cells

Mononuclear cells (MNC) were isolated from buffy coats of healthy blood donors and acute IM blood samples by Lymphoprep centrifugation (Nycomed, AS, Oslo, Norway) and washed three times with sterile PBS (BSS). The harvested cells were immediately cryopreserved, using a programmable freezing unit and then stored at −135°C. Following Lymphoprep centrifugation one part of the cell pool was immediately fixed, permeabilized and stained for spontaneous cytokine-producing cells. The tonsil material was directly placed on ice, the tissue was pressed through a 0·6-mm sterile silver grid in BSS containing 10 mg/ml gentamycin and 100 U/ml of Bencylpenicillin in room temperature followed by Lymphoprep centrifugation and additional three washes in BSS. Approximately $150–500 \times 10^6$ cells were either fixed, permeabilized and stained or cryopreserved in a similar manner to peripheral blood.

Immobilized anti-CD3 for detection of lymphokine-secreting cells

Ninety-six-well microplates (Costar, Cambridge, MA) were coated at 4°C overnight with anti-CD3 antibodies (purified OKT3 antibody) (Ortho Pharmaceutical, Raritan, NJ). Each well was coated with 100 μl of OKT3 in a concentration of 10 μg/ml which was diluted in an acetate coating buffer (Na_2CO_3 1·59 g, $NaHCO_3$ 2·93 g, NaN_3 200 mg, Aq. dest ad 1000 ml, pH 9·6). The wells were subsequently washed in sterile BSS five times (to remove unbound antibody) and stored at 4°C until use. Microplates without immobilized anti-CD3 were used as negative controls in each experiment. Wells with immobilized anti-CD3 were incubated with thawed cells from IM patients or control cells from buffy coats, 1×10^6 cells/ml, which were washed in sterile RPMI and then added in a concentration of 2×10^5 cells/well for indicated periods of time. The cells were cultured for 2 and 3 h at 37°C in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with lipopolysaccharide (LPS)-free 5% fetal calf serum (FCS) in a humidified air atmosphere containing 5% CO_2. The selected culture conditions with immobilized OKT3 did not activate control MNC obtained from healthy blood donors during the 2–3 h of restimulation. However, when we cultured buffy coat cells from healthy blood donors with immobilized OKT3 for 24 h we did see cytokine production in the control cells as well (data not shown). Microplates without immobilized anti-CD3 were used as negative controls in each experiment.

In order to assess the potential for maximal cytokine production we cultured MNC from IM patients or controls with 0·5 mM ionomycin (ATC31005; Calbiochem, La Jolla, CA) in combination with 1 ng/ml phorbol myristate acetate (PMA; Sigma, St Louis, MO). Cells were also cultured in medium alone. In order to reduce contaminating endotoxin, all solutions including RPMI and FCS were run through Affi-Prep polymyxin columns (Bio-Rad, Richmond, CA) according to the manufacturer's instructions, reducing potential endotoxin content by at least 99%.

Cytokine-specific antibodies

The following antibodies were used: a cocktail of three different anti-human IL-1α (1277-89-7, 1277-82-29, 1279-143-4) and anti-human IL-1β (2-D-8), all mouse IgG1 from Dr H. Towbin (Ciba-Geigy, Basel, Switzerland) [10]; a polyclonal anti-IL-1Ra antiserum, raised in New Zealand white rabbits immunized with human IL-1Ra [11], was used at a dilution of 1:500, and a goat-anti-hIL-1Ra, BDA29 (British Biotechnology Ltd., Abingdon, UK) was used at a dilution of 1:60 [11]; IL-2 (17.H.12), IL-3 (3.G.11), IL-4 (25.D.2), IL-5 (39.D.10), IL-10 (19.F.1), TNF-α (20.A.4), GM-CSF (5A2 and 21C11), all rat IgG MoAbs from Dr J. Abrams (DNAX, Palo Alto, CA) [12]; IL-8 (NAP-1) mouse IgG1 MoAb from Dr M. Ceska (Sandoz, Vienna, Austria) [13]; TNF-β (LTX-21) mouse IgG2b MoAb from Dr G. Adolf (Boehringer-Ingelheim, Vienna, Austria) [14]; and IFN-γ (DIK-1) mouse IgG1 MoAb from Dr G. Andersson (Kabi, Stockholm, Sweden) [15] and IL-6 (BSF and IG61), all mouse IgG1 from Dr T. Matsuda and Dr T. Nobuto (Toray Industries, Kamakura, Japan) [16,17]. The cytokine-specific MoAbs were used at a final concentration of 1–5 μg/ml [9,18].

Fixation of the cells and immunofluorescence staining for cytokines

Cells obtained immediately after Lymphoprep centrifugation or thawed cells were cultured and harvested after indicated periods of time and washed in Ca^{2+}- and Mg^{2+}-containing BSS (GIBCO, Paisley, UK) supplemented with 0·01 M HEPES buffer. Cells were transferred to adhesion slides (Bio-Rad Lab. GMBH, Munich, Germany) and were allowed to adhere electrostatically to the slides for 10 min at room temperature. Excess cells were washed away and unbound surface area on the adhesive fields was blocked with 2% fetal bovine serum in BSS by a 10-min incubation. Approximately 4×10^4 cells were fixed on each field with phosphate-buffered 4% paraformaldehyde at pH 7·4 for 30 min. After subsequent washes with BSS we incubated the cells with 10 μl of the cytokine-specific MoAb for 30 min in BSS supplemented with 0·1% saponin as a detergent, which enabled the MoAb to penetrate intracellularly. The cells were washed in BSS-saponin and exposed for another 30 min to 10 μl of the

FITC-coupled second step antibodies. We used FITC-labelled anti-mouse IgG1 or anti-IgG2b from Caltag Lab. (South San Francisco, CA) at a final concentration of 1:300 or FITC-anti-rat IgG from Vector Lab. (Burlingame, CA) at 1:100. For indirect immunofluorescence staining with rabbit sera for presence of cytoplasmic IL-1Ra we used a FITC-coupled goat anti-rabbit H+L (Caltag) diluted 1:300 + 5% AB serum. For indirect staining with the goat sera we used FITC-coupled rabbit anti-goat IgG (Dakopatts A/S, Copenhagen, Denmark) diluted 1:100. After washes in BSS-saponin a final wash was performed with only BSS, which prevented leakage from the cells of stained cytokine, since the permeabilization of the cell membrane by saponin is a reversible process. The cells were left to dry on the slides before they were mounted in buffered glycerol containing 2% diazobicyclooctane to reduce u.v. quenching.

Surface staining of mononuclear cells

The following FITC-coupled antibodies were used to identify cell surface antigens by use of flow cytometric analyses using a FACScan (Becton Dickinson, Mountain View, CA): OKT11 (CD2, sheep erythrocyte receptor), OKT3 (CD3, pan T cell), OKT4 (CD4 helper/inducer T cell) and OKT8 (CD8, cytotoxic T cell), all from Dakopatts; Leu-11a (CD16, LGL/NK cell) from Becton Dickinson, and DAKO-CD19 (CD19 and CD20, B cell) from Dakopatts.

Fluorescence microscopy

The frequency of cytokine-producing cells was determined by u.v. microscopic evaluation of 10000 cells for each cytokine staining. Each cytokine-specific staining was evaluated by two independent persons. The slides were examined with a Reichert-Jung fluorescence microscope equipped with a 200 W mercury lamp and filter sets for FITC, PE and TRITC.

Statistical analysis

Student's *t*-test was used for all statistical analyses.

RESULTS

Spontaneous cytokine production in peripheral blood

Cytokine-specific MoAbs and indirect immunofluorescence have previously enabled us to identify individual IL-1α, IL-1β, IL-1Ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, TNF-β and GM-CSF producing cells obtained from *in vitro* stimulated cultures. All the studied cytokines (apart from IL-1) accumulate in the Golgi system, which results in a characteristic morphology of the staining with or without additional cytoplasmic or cell membrane immunofluorescence (Fig. 1). Only producer cells, but not target cells even if they were exposed to recombinant or natural cytokine, could generate this staining pattern [9]. The incidence of spontaneous cytokine-containing cells in peripheral blood obtained from 150 healthy blood donors was always below one in a thousand for all the above-mentioned cytokines [9]. In this study no increased frequency of cytokine-stained cells was found in MNC from 14 patients with acute IM compared with 14 buffy coat control from healthy blood donors. The MNC were fixed, permeabilized and stained with the cytokine-specific MoAb within 2 h of sampling. The incidence of spontaneous IL-α, IL-1β, IL-1Ra, IL-6, IL-8, IL-10 or TNF-α expressing cells was always below one in a thousand (0·1%) cells, and IL-2, IL-4, IFN-γ, TNF-β or GM-CSF-positive cells were not found above one in ten thousand cells (0·01%) in any of the two groups studied.

Ligation of T cell receptor for detection of lymphokine-producing cells

Since the constitutive lymphokine-producing cells were below detectable levels in blood MNC from IM patients despite numerous activated T cells as judged by surface markers, we speculated that ongoing presumptive cytokine synthesis could be magnified and revealed by *in vitro* cross linkage of T cell receptors (TCR) by immobilized anti-CD3 antibodies. The cells were harvested after 2 and 3 h of restimulation. In pilot experiments, to be explained, we have found that preactivated, but not resting, cells could be induced to cytokine production under these culture conditions with solid-phase bound anti-CD3 MoAb. We titrated the system by activating normal blood MNC with the protein kinase C stimulator PMA and the calcium ionophore ionomycin for 6 h in culture, followed by a 48-h culture period without mitogen. These cells, which at this stage had ceased to produce detectable levels of cytokines, were then restimulated with the immobilized anti-CD3 MoAb and showed production of considerable amounts of many cytokines within 2–3 h. The cytokine profile was identical to that found after primary PMA/ionomycin stimulation. Control cells, which had not been preactivated, did not show any cytokine synthesis under these conditions.

Following TCR ligation, MNC obtained from all 14 IM patients produced IFN-γ (Figs 1b and 2a). An increased number of IFN-γ-expressing cells were found in each individual patient (Fig. 2a). The range of IFN-γ-containing cells varied from 40 to 350 per 10000 cells for each patient. In the anti-CD3-stimulated controls, IFN-γ-synthesizing cells were only evident in 2 of 14 buffy coat samples (Fig. 2a) and they contained a much lower incidence of IFN-γ cytokine-positive cells, 4 and 8 per 10000 counted cells, respectively ($P < 0{\cdot}001$). Unstimulated cultured MNC from buffy coat controls did not produce detectable IFN-γ-synthesizing cells. Because of the low number of IFN-γ-containing cells found even after TCR ligation, samples from 6 of 14 IM patients were repeated blindly. The results from the second experiments did not differ by more than ±15% for any of the donors compared with the primary result (data not shown).

There were no detectable IL-2, IL-3, IL-4, IL-5, IL-10, TNF-β or GM-CSF containing cells in the IM samples after TCR ligation or after culture of unstimulated cells from IM or controls (Fig. 2a). However, this was not due to a general down-regulation of these cytokines, because following *in vitro* stimulation with phorbol ester plus calcium ionophore, both MNC from IM and controls produced a similar number of IFN-γ (Fig. 1a), IL-2, IL-3, IL-4, IL-5, IL-10, TNF-α as well as GM-CSF synthesizing cells (Fig. 2b).

Constitutive lymphokine production in tonsil

A spontaneous production of IL-2, IL-6, TNF-β and IFN-γ was found in MNC obtained from tonsils from four patients with fulminant IM and upper respiratory obstruction, requiring tonsillectomy. The lymphokine-producing cells were evident in all four tonsils, except for TNF-β, which was only found in one tonsil. The frequency of spontaneous IL-2 cytokine-synthesizing cells ranged from 120 to 200 cells (Fig. 3), IL-6 was found in

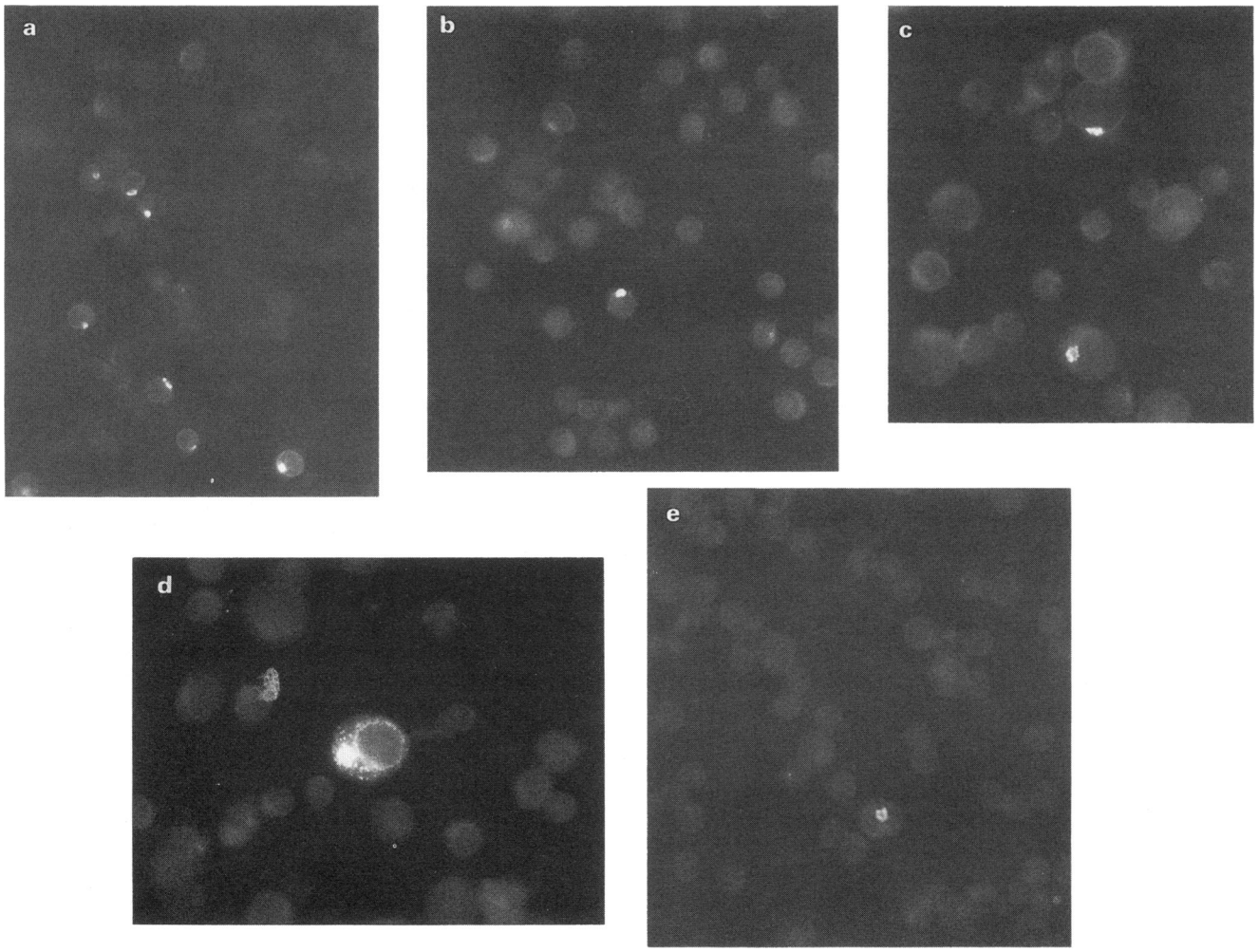

Fig. 1. Different cellular patterns of cytokine staining. Note the localized Golgi-staining, juxtanuclear, in all figures which, in some cells, is also combined with a cytoplasmic and outer membrane cytokine expression. (a) Mononuclear cells obtained from peripheral blood from a patient with acute infectious mononucleosis (IM) and stimulated *in vitro* with phorbol myristate acetate (PMA) which activates protein kinase C, plus the calcium ionophore, ionomycin, for 6 h. Approximately 10% of the cells were stained for IFN-γ, which is the same frequency as found in control samples from healthy blood donors. (b) Cells from the same donor stained for IFN-γ after *in vitro* stimulation for 2 h by cross-linking CD3 receptors with immobilized anti-CD3 MoAb. Approximately 2% of the cells contained IFN-γ. (c) Spontaneous production of IL-2 detected in cells obtained from tonsil from an IM patient. (d) Cells from the same tonsil constitutively producing tumour necrosis factor-beta (TNF-β). Note that all the positive cells are large lymphoblasts. (e) Spontaneous production of IL-6 in mononuclear cells (MNC) from the same tonsil. Original magnification $\times 400$.

10–70 cells (Fig. 3), IFN-γ in 10–60 cells, and TNF-β in 100 cells out of 10000 counted cells (Fig 3), respectively. There was no evidence of increased numbers of IL-1α, IL-1β, TNF-α, IL-3, IL-4, IL-5, IL-8, IL-10 or GM-CSF containing cells. The control samples obtained from patients tonsillectomized because of chronic tonsillar hyperplasia did not contain detectable lymphokine-secreting cells above one in 10000 counted cells (Fig. 3). In contrast to IM samples, the latter group had an increase of IL-1α, IL-1β, IL-8 and TNF-α expressing cells (data not shown).

In vitro, stimulation with phorbol ester (PMA) plus calcium ionophore which bypasses physiologic surface-activation via receptors for induction of IL-2, IL-4, IFN-γ, TNF-α and TNF-β production, resulted in comparable numbers of cells expressing these cytokines in MNC tonsil cells from both IM and controls (data not shown).

Surface staining of mononuclear cells

Consistent with previous reports [19–21], the IM patients examined showed increased percentages of $CD8^+$ T cells compared with control cells obtained from healthy blood donors (Table 1). A much lower incidence of T cells and a significantly higher number of B cells were found in the tonsils. However, the incidence of $CD8^+$ T cells was significantly increased in IM tonsils compared with control tonsils obtained from the patients with hyperplasia without serological evidence for ongoing EBV infection (Table 1).

DISCUSSION

We have previously reported increased serum concentrations of soluble IL-2 receptors, soluble CD8 molecules, neopterin and IFN-γ during acute IM [8]. In this report we have extended our

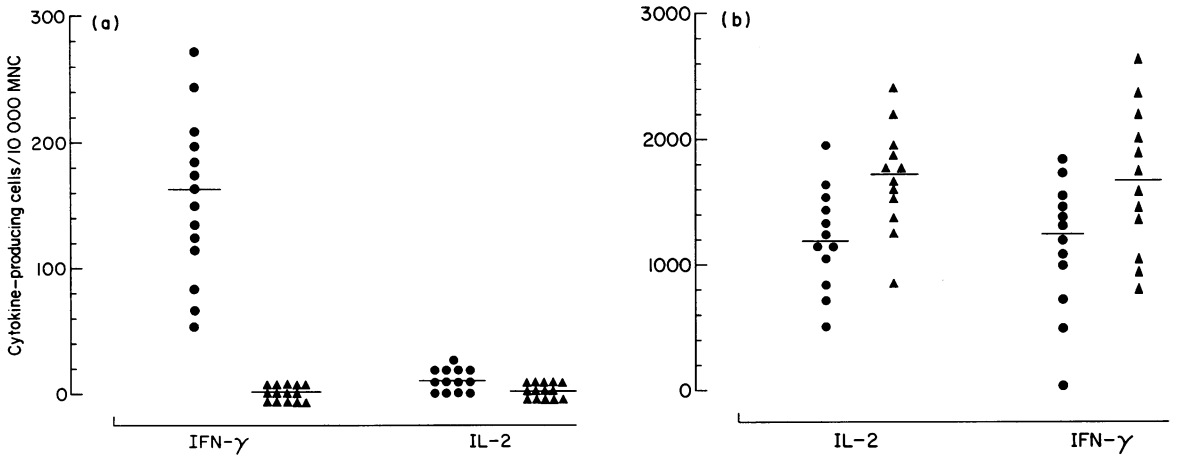

Fig. 2. (a) The frequency of cytokine-producing cells per 10 000 mononuclear cells (MNC) obtained from peripheral blood from 14 patients with acute infectious mononucleosis (IM). The MNC were *in vitro* stimulated for 2–3 h by cross-linking CD3 receptors with immobilized anti-CD3 MoAb. The frequency of IFN-γ and IL-2 producing cells (●) was determined by indirect immunofluorescence using cytokine-specific MoAb and u.v.-microscopy. Cultured non-anti-CD3 MNC cells (▲) from the same donors were assessed concomitantly. Each dot in the figure represents one IM patient. In the IM population, the mean frequency of IFN-γ-producing cells after anti-CD3 stimulation was 168/10 000 MNC compared with 10/10 000 MNC in the non-restimulated cultured cells ($P < 0{\cdot}001$). No IL-2, IL-3, IL-4, IL-5, IL-10, GM-CSF or tumour necrosis factor-beta (TNF-β) containing cells (< 0·01%) were detected constitutively or after OKT3 stimulation. Control cells, from healthy blood donors ($n = 14$), treated by T cell receptor ligation using immobilized anti-CD3 MoAbs, were not induced to any lymphokine synthesis during this short culture period. ●, OKT3 restimulation; ▲, spontaneous production. (b) The frequency of IL-2 and IFN-γ-producing cells after phorbol myristate acetate (PMA)-ionomycin stimulation for 6 h is demonstrated. No significant difference in numbers of IL-2 or IFN-γ-producing cells could be determined between MNC obtained from IM patients or buffy coat control. Cells from the same donors as illustrated in (a) were used. The incidence of IL-3, IL-4, IL-10, TNF-α and CM-CSF synthesizing cells was also similar in the two groups (data not shown). ●, IM patient; ▲, healthy control.

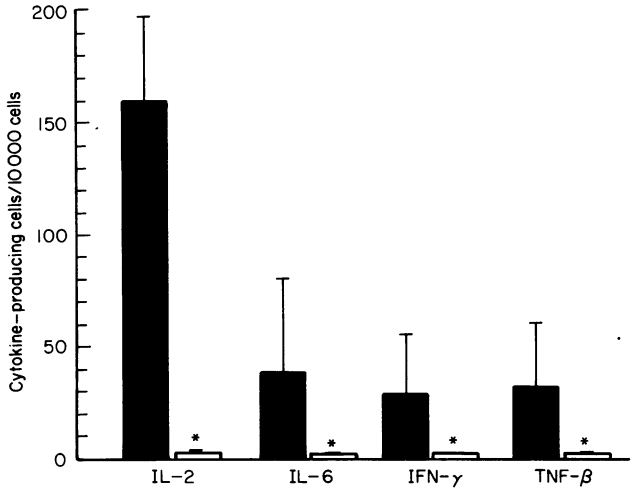

Fig. 3. Constitutive cytokine-producing cell in tonsil obtained from four infectious mononucleosis (IM) patients with respiratory obstruction, and compared with mononuclear cells (MNC) obtained from an age-matched group suffering from chronic tonsilar hyperplasia. ■, The mean ± s.d. of cytokine-producing cells obtained from IM. Cytokine determination was performed by cytokine-specific MoAb and immunofluorescence technique, counting at least 10 000 cells. A spontaneous production of IL-2, IFN-γ, IL-6 was evident in all four IM patients and tumour necrosis factor-beta (TNF-β) in one patient, respectively. The comparative figures for MNC obtained from chronic hyperplasia were 0–4 positive cells/10 000 cells. * A significant difference between the two groups ($P < 0{\cdot}001$). We did not find spontaneous production of IL-3, IL-4, IL-5, IL-10, TNF-α or GM-CSF in the IM samples. However, increased numbers of cells expressing IL-1α, IL-1β, IL-8, and TNF-α were found in the chronic hyperplasia samples (data not shown). □, Control.

Table 1. Characterization of subpopulations of cells obtained from peripheral blood and tonsils from infectious mononucleosis (IM) patients, tonsils and peripheral blood from patients with hyperplasia and healthy controls, respectively

	Percentage (%)*			
	Peripheral blood		Tonsil	
Cells	IM	Control	IM	Control
$CD3^+$	70±4	75±3	23±2	22±1
$CD4^+$	20±4	39±4	13±3	10±1
$CD8^+$	36±6	26±2	18±3	2±2
$CD19^+20^+$	8±2	20±2	65±12	72±3
Mac387†	10±3	9±4	4±2	2±1

* The surface markers were evaluated by flow cytometric analysis.

† Mac387 is a MoAb detecting a cytoplasmic protein evident in monocytes and granulocytes.

studies of pathogenetic mechanisms operating in IM to assess the synthesis of several cytokines at a single-cell level. An undetectable serum level of a cytokine does not rule out its production. Local synthesis of cytokines with para- or autocrine action, with no access to circulation, is one reason for negative serum levels. The recent findings of a receptor-directed focusing of produced cytokines to adjacent cells further explains the difficulties in finding produced interleukins in serum [22].

Previous reports of the occurrence of a prominent polyclonal activation and proliferation of T lymphocytes in the acute

phase of IM cannot be explained by IFN-γ production alone [23]. By the use of cytokine-specific MoAbs and indirect immunofluorescence we aimed in this study to determine the cytokine profile at single-cell level. Even though great caution was exercised and 10 000 cells or more were counted in each cytokine staining, we were not able to detect spontaneous cytokine-producing cells in peripheral blood.

However, very little is known about the frequency of cytokine-producing cells *in vivo* in acute infectious diseases. Similar analyses in mice undergoing lethal acute allogeneic graft-*versus*-host disease have indicated that *in vivo* activated, detectable $CD4^+$ and $CD8^+$ lymphokine-producing spleen or lymph node T cells were below 0·1% [24]. A significant up-regulation was noticed following *in vitro* restimulation with immobilized anti-CD3 causing ligation of TCR. This approach caused lymphokine production in approximately 30% of the T cells. The response appeared to be restricted to activated T cells, since only 0·1% of normal spleen cells responded under the same conditions [24]. It has also previously been shown in another study that when cells which were preactivated by mitogen were restimulated, the lymphokines were produced more rapidly [25] and in greater quantities than in resting cells. The pattern of lymphokine production induced by the primary stimulus was dominant also in the secondary cultures, irrespective of stimulation conditions [26]. In this study a modification of the method for TCR ligation was used. Immobilized anti-CD3 was titrated so that unstimulated cells were not activated when the culture time for restimulation was reduced to 2 and 3 h. By this approach *in vitro*-activated human cells, kept in culture without stimulation for 2 days, were induced to lymphokine synthesis within 2 h with a similar cytokine pattern to that seen in the primary response (data not shown). The detection of IFN-γ-producing cells in peripheral blood in IM in combination with the lack of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10 or TNF synthesizing cells indicates that cytokine production in circulating cells is specifically restricted during acute IM. We believe that the discrepant results with IFN-γ production in uncultured and anti-CD3 MoAb-stimulated blood cells may be explained by quantitative rather than qualitative differences. It is plausible that *in vivo* cytokine production in peripheral blood is below the detection level of our assay and that there is a need to magnify it *in vitro* by cross-linkage of the T cell receptors in order for it to be detected.

In contrast to the negative findings in peripheral blood, spontaneous lymphokine-producing cells were evident in tonsil tissue from IM patients. Not only IFN-γ but also IL-2, IL-6 and TNF-β-producing cells were present at this site. These findings are in agreement with reports of cytokine expression after *in vitro* EBV infection of MNC from seropositive individuals showing synthesis of IL-2, IFN-γ and IL-6 [27,28]. The lack of detection of most monokines (IL-1α, IL-1β, IL-8, IL-10 and TNF-α) in the IM samples is also noteworthy, since EBV have been reported to have down-regulatory properties of monokine production *in vitro*.

The results presented indicate that IM is associated with a constitutive production of IL-2, IFN-γ, IL-6 and TNF-β, all of which are probably needed for the induction of a specific humoral and cellular immune response to the potentially immortalized EBV-infected B cells. The increased production of these cytokines may also be responsible for the clinical symptoms in acute IM. In addition, the release of this specific type of cytokines would provide signals for the generation of a cytotoxic natural killer and T cell response [29,30].

ACKNOWLEDGMENTS

The authors would like to thank Caroline Ekberg and Lena Radler for excellent technical assistance, and acknowledge the gift of cytokine-specific antibodies from Günther Adolf (Vienna), Harry Towbin (Basel), Miroslav Ceska (Vienna), John Abrams (Palo Alto, CA) and Gudrun Andersson (Stockholm). This work was supported by grants awarded by the National Cancer Institute (grant no. 2490-B91-05XAC); the Karolinska Institute; Gustav V:s minnesfond; the Swedish Association against Rheumatism; the Medical Research Council; the Berth von Kantzov's Foundation; the Crafoord Foundation; the Nanna Swartz Foundation; the af Uggla's Foundation; and the Sven Jerring Foundation.

REFERENCES

1 Henle G, Henle W, Deihl V. Relation of Burkitt tumor associated herpes-type virus to infectious mononucleosis. Proc Natl Acad Sci USA 1968; **59**:94–101.

2 Shiftan TA, Mendelson J. The circulating atypical lymphocytes. Hum Pathol 1973; **9**:51–6.

3 Sheldon PJ, Papamichail M, Hemsfed EH, Holborow EJ. Thymic origin of atypical lymphoid cells in infectious mononucleosis. Lancet 1973; **1**:1153–5.

4 Tomkinson BE, Wagner DK, Nelson DL, Sullivan JL. Activated lymphocytes during acute Epstein–Barr virus infection. J Immunol 1987; **139**:3802–7.

5 Miyawaki T, Kasahara Y, Kanegane H, Ohta K, Yokoi T, Yachie, A, Taniguchi N. Expression of CD45RO (UCHL1) by CD4+ and CD8+ T cells as a sign of *in vivo* activation in infectious mononucleosis. Clin Exp Immunol 1991; **83**:447–51.

6 Kamio M, Uchiyama T, Hori T *et al.* Selective expression of the p70 subunit of the interleukin-2 receptor on lymphocytes from patients with infectious mononucleosis. Blood 1990; **75**:415–20.

7 Andersson J, Britton S, Ernberg I, Andersson U, Henle W, Sköldenberg B. Effect of acyclovir on infectious mononucleosis: a double-blind, placebo-controlled study. J Infect Dis 1986; **153**: 283–90.

8 Linde A, Andersson B, Svenson SB *et al.* Serum levels of lymphokines and soluble cellular receptors in primary Epstein–Barr virus infection and in patients with chronic fatigue syndrome. J Infect Dis 1992; **165**:97–105.

9 Sander B, Andersson J, Andersson U. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. Immunol Rev 1991; **119**:65–93.

10 Rordorf-Adam C, Lazdins J, Woods-Cook K *et al.* An assay for the detection of interleukin-1 synthesis inhibitors: effects of antirheumatic drugs. Drugs Exptl Clin Res 1989; **8**:355–62.

11 Andersson J, Björk L, Dinarello CA, Towbin H, Andersson U. Lipopolysaccharide induces human interleukin-1 receptor antagonist and interleukin-1 production in the same cell. Eur J Immunol 1992; **22**:2617–23.

12 Abrams JS, Roncarolo M-G, Yssel H, Andersson U, Gleich GJ, Silver J. Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples. Immunol Rev 1992; **127**:5–24.

13 Martich GD, Danner RL, Ceska M, Suffrendini AF. Detection of interleukin-8 and tumor necrosis factor in normal humans after intravenous endotoxin: the effect of anti-inflammatory agents. J Exp Med 1991; **173**:771–4.

14 Lamche HR, Adolf GR. Highly sensitive enzyme immunoassay for antibodies to human necrosis factor (TNFα) and lymphotoxin (TNF-β). J Immunol Methods 1990; **131**:283–9.

15 Andersson G, Ekre H-P, Alm G, Perlmann P. Monoclonal antibody two-site ELISA for human IFN-γ. J Immunol Methods 1990; **125**:89–94.

16 Matsuda T, Hirano T, Kishimoto T. Establishment of an interleukin 6 (IL-6)/B cell stimulatory factor 2-dependent cell line and preparation of an anti-IL-6 monoclonal antibody. Eur J Immunol 1988; **18**:951–6.

17 Ida N, Sakurai S, Hosaka T *et al.* Establishment of strongly neutralizing monoclonal antibody to human interleukin-6 and its epitope analysis. Biochem Biophys Res Commun 1989; **165**:728–34.

18 Andersson J, Nagy S, Björk L, Abrams J, Holm S, Andersson U. Bacterial toxin induced cytokine production studied at the single cell level. Immunol Rev 1992; **127**:69–96.

19 Reinherz EL, O'Brien C, Rosenthal P, Schlossman SF. The cellular basis for viral-induced immunodeficiency: analysis by monoclonal antibodies. J Immunol 1980; **125**:1269–73.

20 De Waele M, Thielemans C, Van Camp BKG. Characterization of immunoregulatory T cells in EBV-induced infectious mononucleosis by monoclonal antibodies. N Engl J Med 1981; **304**:460–5.

21 Tomkinson BE, Wagner DK, Nelson DL, Sullivan JL. Activated lymphocytes during acute Epstein–Barr virus infection. J Immunol 1987; **139**:3802–7.

22 Poo WJ, Conrad L, Janeway CA. Receptor-directed focusing of lymphokine release by helper T cells. Nature 1988; **332**:378–80.

23 Malkovsky P, Sondel W, Dalgleish A. The interleukins in acquired disease. Immunology 1988; **74**:151–61.

24 Kelso A. Frequency analysis of lymphokine-secreting $CD4^+$ and $CD8^+$ T cells activated in a graft-*versus*-host reaction. J Immunol 1990; **145**:2167–76.

25 Cardell S, Sander B. Interleukin 2, 4 and 5 are sequentially produced in mitogen-stimulated murine spleen cell cultures. Eur J Immunol 1990; **20**:389–95.

26 Sander B, Cardell S, Möller E. Interleukin 4 and interferon gamma production in restimulated $CD4^+$ and $CD8^+$ cells indicates memory type responsiveness. Scand J Immunol 1991; **33**:287–96.

27 Lotz M, Tsoukas CD, Fong S, Dinarello CA, Carson DA, Vaughan JH. Release of lymphokine after Epstein–Barr virus infection *in vitro*. I. Sources of and kinetics of production of interferons and interleukins in normal humans. J Immunol 1986; **136**:3636–42.

28 Gosselin J, Flamand L, D'Addario M, Hiscott J, Menezes J. Infection of peripheral blood mononuclear cells by herpes simplex and Epstein–Barr viruses. J Clin Invest 1992; **89**:1849–56.

29 Maraskovsky E, Chen W-F, Shortman K. IL-2 and IFN-γ are two necessary lymphokines in the development of cytolytic T cells. J Immunol 1989; **143**:1210–4.

30 Borysiewicz LK, Haworth SJ, Cohen J, Murdin J, Rickinson A, Sissons JGP. Epstein–Barr virus-specific immune defects in patients with persistent symptoms following infectious mononucleosis. Q J Med 1986; **58**:111–21.