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Characterization of the expanded T cell population in infectious mononucleosis: apoptosis, expression of apoptosis-related genes, and Epstein–Barr virus (EBV) status

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SUMMARY

Infectious mononucleosis (IM), a manifestation of primary infection with EBV, is characterized by a massive expansion of the T cell population. In this study we examined this expanded T cell population regarding its EBV status, its proliferative and apoptotic activity, and its expression of apoptosis-related genes. Whereas previous studies were performed on *ex vivo* cultures or on peripheral blood, our investigations included *in vivo* analysis of IM tonsillectomy specimens (14 cases) by *in situ* hybridization for viral RNA (EBERs) combined with immunohistochemistry (IHC; CD3, CD45RO, CD20, CD79a, Ki-67, Bcl-2, Bax, Fas, FasL) and the TUNEL method. Of the $EBER^+$ cells 50–70% showed expression of the B cell markers CD20/CD79a. The remainder of the $EBER^+$ cells expressed neither B nor T cell antigens. No co-expression of EBERs and T cell antigens was detected in any of the specimens. In accordance with a high rate of apoptosis (up to 2·37%) within the expanded T cell population, Bcl-2 expression was drastically reduced and FasL expression remarkably increased. The levels of Bax and Fas expression showed no or moderate up-regulation. In conclusion, the massive expansion of IM T cells is not caused by EBV infection of these cells but merely represents an intense immune reaction. Through altered expression of Bcl-2/Bax and Fas/FasL, the activated T cells are subject to enhanced apoptosis while residing within the lymphoid tissue, which eventually allows the efficient silencing of this potentially damaging T cell response.

Keywords infectious mononucleosis Epstein–Barr virus T cells apoptosis

INTRODUCTION

EBV is the causative agent of infectious mononucleosis (IM) and has been strongly linked to the development of endemic Burkitt's lymphoma and other non-Hodgkin's lymphomas, Hodgkin's disease, and several malignant non-lymphoid neoplasias [1].

Since the initial discovery of the virus in Burkitt's lymphoma cell lines, EBV has been known as a B cell lymphotropic virus. To date however controversy exists as to which cellular compartments mediate primary EBV infection. Whereas observations from bone marrow transplant recipients [2] and histopathological analysis of IM tonsils and reactive lymph nodes [3–5] favour primary infection and persistence of EBV in B lymphocytes, recent reports on the association of EBV with T cell proliferation and lymphomas [6–8], together with evidence for *in vitro* infection of thymocytes by EBV [9], raise the question whether T lymphocytes may be a target of EBV infection. Indeed, during IM there is a massive proliferation of highly activated T cells which may either result from viral T cell infection or merely represent a vigorous immune reaction. Furthermore, recent investigations showed that this T cell proliferation is accompanied by increased susceptibility to apoptosis of T cells in the peripheral blood of IM patients [10,11]. Although down-regulation of Bcl-2 in these T cells was revealed by flow cytometric analysis [12], the *in vivo* situation in tonsillar tissue has not been investigated before. Therefore, the present study was undertaken to characterize *in vivo* the T cell population in IM tonsils regarding its EBV status, its proliferative and apoptotic activity, and its expression of apoptosis-related genes (Bcl-2, Bax, Fas, FasL).

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MATERIALS AND METHODS

Materials

Formalin-fixed, paraffin-embedded bilateral tonsillectomy specimens of 14 patients with IM were reviewed. The diagnosis of primary EBV infection had been confirmed serologically in all but four patients. The tonsillectomies were performed at the same

Table 1. Clinical data of infectious mononucleosis patients

Case no.	Age/sex	Serology*
1	17/M	H^+
2	18/M	H^+
3	14/F	VCA^+
4	16/F	ND
5	19/M	VCA^+
6	17/F	ND
7	15/F	VCA^+
8	22/F	VCA^+
9	34/M	ND
10	22/M	H^+
11	10/M	ND
12	13/M	VCA^+
13	16/F	VCA^+
14	2/M	H^+

*H^+, Positive detection of heterophilic antibodies (IgM); VCA^+, positive detection of antibodies (IgM) against viral capsid antigen; ND, not done.

stage of the disease, as all patients had presented similar clinical symptoms (pharyngitis, cervical lymphadenopathy, absent to mild splenomegaly) for approximately 5 days. Clinical data of the IM patients are shown in Table 1. As controls, eight tonsillectomy specimens of chronic tonsillitis with lymphofollicular hyperplasia were used. None of the 22 patients had evidence of immunodeficiency.

In situ *hybridization*

For *in situ* hybridization (ISH) of viral RNA, FITC-conjugated RNA probes complementary to EBER (EBV Early RNAs; Novocastra, Newcastle upon Tyne, UK) were hybridized to deparaffined sections of the tonsillectomy specimens. Detection was performed by alkaline phosphatase-conjugated rabbit anti-FITC antibody using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Novocastra) as the chromogen. The sensitivity of the ISH was tested in two ways: (i) in a case of Hodgkin's disease, known to be EBV^+, virtually all Hodgkin and Reed–Sternberg cells were found to be $EBER^+$, and (ii) comparison of the number of $EBER^+$ cells with the number of cells stained positive by immunohistochemistry (IHC; described below) for the viral proteins LMP-1 (Latent Membrane Protein 1) and EBNA2 (EBV Nuclear Antigen 2) showed equivalent results.

TUNEL

Free DNA-ends yielded by apoptosis were identified by TdT (terminal deoxyribonucleotidyl transferase; Promega, Madison, WI; 0·1 U/μl)-mediated tailing with biotin-labelled nucleotides (biotin-16-dUTP; Boehringer, Mannheim, Germany) and subsequent detection via a streptavidin–alkaline phosphatase complex (Jackson ImmunoResearch, West Grove, PA) and fast red (Boehringer) chromogen. All tissue sections were pretreated with proteinase K.

Immunohistochemistry

IHC was performed on paraffin-embedded tissue sections with an alkaline phosphatase-conjugated streptavidin-biotin detection system (Jackson ImmunoResearch) and fast red. The MoAbs and polyclonal antibodies used included CD20, CD79a, CD45RO, Bcl-2, LMP-1, EBNA2 (MoAbs; Dako, Glostrup, Denmark), CD3, CD4, CD8 (MoAbs; Novocastra), Ki-67 (MoAb; Dianova, Hamburg, Germany), Bax (polyclonal antibody; Dako), Fas and Fas ligand (polyclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA). Antigen retrieval by standard microwaving was performed for all antibodies except anti-CD45RO and anti-LMP-1. For CD45RO detection, tissue sections were pretreated with pronase. For the negative control, the primary antibody was omitted. The specificity of FasL detection had been tested previously on testicular and placental tissue. For combined detection of proteins and EBER or apoptosis, IHC was performed subsequent to ISH and prior to TUNEL using BCIP/NBT as an additional chromogen in the latter. Double labelling was also performed for Ki-67/LMP-1 and Ki-67/CD3, using the Dako Envision TM^+ Doublestain Kit for the latter combination.

Table 2. Results of EBER *in situ* hybridization, TUNEL reaction (AI), and immunophenotyping for Ki-67 (PI), Bcl-2, CD4, and CD8 in infectious mononucleosis (IM) and control tonsillar tissues

Case no.	$EBER^+$ cells*	PI†	AI‡	Bcl-2§	CD8/CD4¶
IM					
1	63	23·20	1·31	38·20	1·29
2	114	51·10	2·01	33·70	1·45
3	145	38·30	1·36	39·50	1·41
4	184	46·20	1·37	18·80	2·01
5	302	42·70	1·45	29·10	2·21
6	22	28·60	1·39	58·40	0·47
7	25	26·90	0·71	39·40	0·54
8	278	43·40	1·84	27·40	1·75
9	191	37·60	1·60	36·20	1·97
10	483	42·00	2·14	18·20	1·65
11	215	39·30	2·08	29·80	2·15
12	32	32·50	1·94	35·60	1·32
13	378	44·00	2·10	24·30	2·16
14	451	41·00	2·37	27·60	1·93
Mean value	206	38·34	1·69	32·58	1·59
Control					
15	–	11·00	0·10	67·70	0·19
16	–	9·70	0·25	54·10	0·21
17	–	9·00	0·15	42·70	0·22
18	–	16·90	0·26	55·30	0·29
19	–	5·40	0·30	35·40	0·28
20	–	9·40	0·34	44·90	0·21
21	–	12·50	0·30	75·50	0·17
22	–	4·90	0·27	59·80	0·18
Mean value	–	9·85	0·25	54·43	0·22

*Number of $EBER^+$ cells per 10 000 lymphocytes.

†Proliferation index, assessed exclusively in interfollicular T cells.

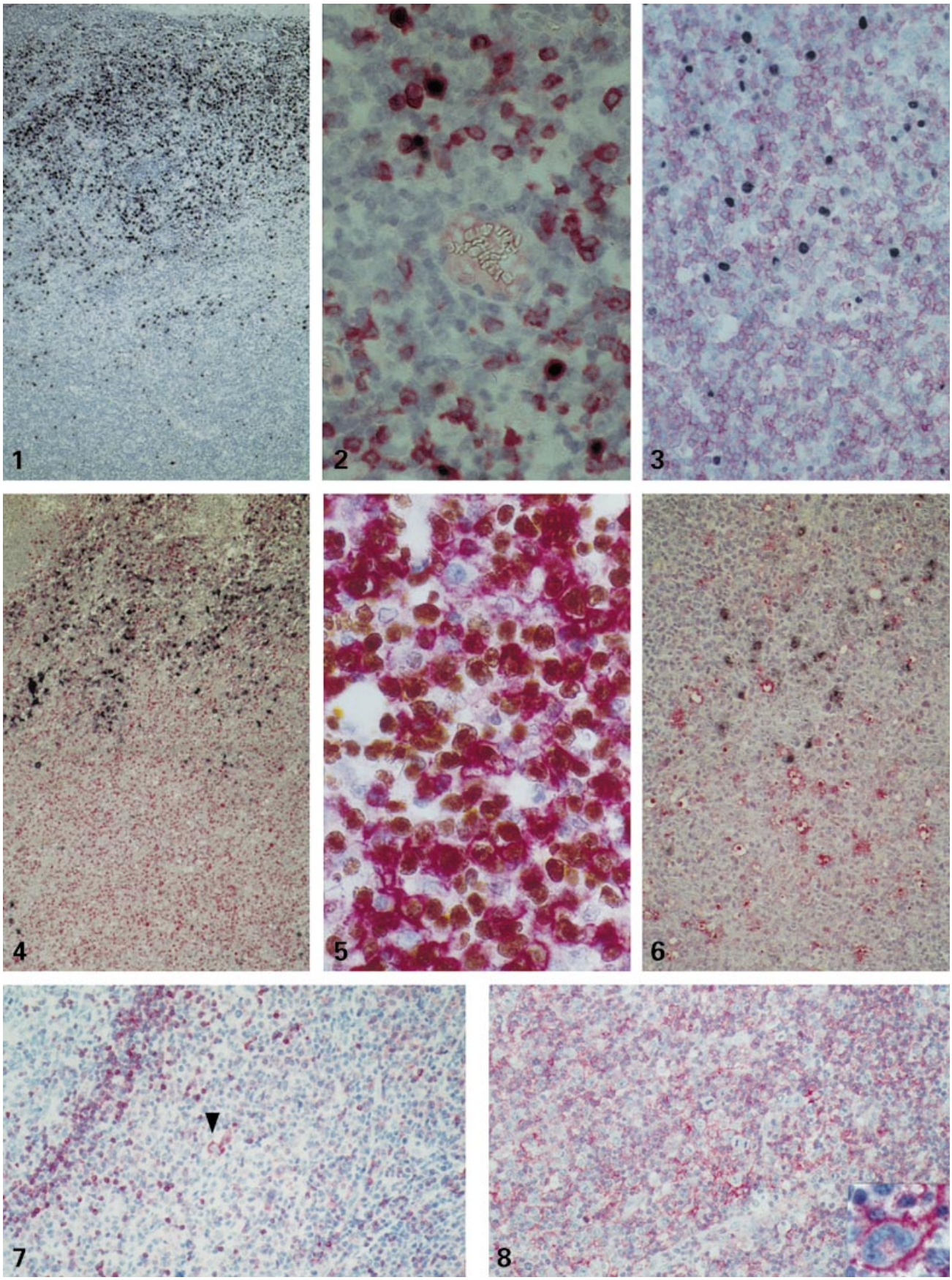
‡Apoptosis index, assessed exclusively in the interfollicular T cell zones.

§Percentage of Bcl-2-expressing cells, counted exclusively in the interfollicular T cell zones.

¶Ratio of CD8- to CD4-expressing cells, counted exclusively in the interfollicular T cell zones.

Semiquantitative assessment and statistical analysis

Semiquantitative assessment of the proliferative and apoptotic activity, as well as the expression of Bcl-2, CD4 and CD8, was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performed by scoring the percentage of labelled cells in at least 5 high-power fields (HPF) (×400). In combined ISH and IHC, the percentage of EBER$^+$ cells co-expressing T or B cell markers was counted throughout the complete section surface.

Statistical correlation of the Bcl-2 score and CD8/CD4 ratio with the number of EBER$^+$ cells was assessed by the Fisher's exact test.

Western blotting

Frozen tissue from three cases (cases 10, 11 and 13) was incubated in lysis buffer (20 mM Tris–HCl buffer pH 7·4; 150 mM NaCl; 1·5 mM EDTA; 3% glycerol; 1 mg/ml bovine serum albumin (BSA); 1% NP40; 1 μg/ml leupeptin; 5 μg/ml aprotinin; 2 μg/ml pepstatin A; 0·5 mM pefabloc) for 30 min at 0°C. SDS–PAGE (12%) and transfer to PVDF membranes (NEN Life Sciences, Boston, MA) was followed by incubation with 250-fold diluted anti-FasL polyclonal antibody. Immunoreaction was visualized by a secondary antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch) and colorimetric detection (NBT/BCIP). Testicular and placental tissue samples were used as positive controls. For the negative control, BSA solution was analysed.

RESULTS

EBER$^+$ cells with the morphology of lymphoid cells of various sizes or of Hodgkin/Reed–Sternberg (H/RS)-like cells, were detected in all cases of IM. All non-lymphoid cells were consistently EBER$^-$. The number of EBER$^+$ cells varied considerably (Table 2). There was no significant correlation between the number of EBER$^+$ cells and the intensity or duration of clinical symptoms.

EBER$^+$ cells were not homogeneously distributed in the tonsillar tissue but were most frequently found adjacent to the crypts: within the crypt epithelium, in the subepithelial lymphoid tissue, and less numerously in the superficial interfollicular zones (Fig. 1). Only few EBER$^+$ cells were found within the deeper interfollicular zones, remaining lymph follicles or peritonsillar soft tissue. In control tonsils (5/8) only very few, solitary EBER$^+$ lymphoid cells of small to moderate size ($<$ 3/section) were observed.

More than 50%, in some cases up to 70%, of all EBER$^+$ cells expressed CD20/CD79a, independent of their morphology and size (Fig. 2). In no case could EBER$^+$ cells co-expressing either CD3 or CD45RO be detected. Of the EBER$^+$ 30% to $<$ 50% cells expressed neither CD20/CD79a nor CD3/CD45RO.

Massive expansion of the interfollicular zones with progressive disappearance of pre-existent lymph follicles was due to dramatic activation and blastic transformation of EBER$^-$or LMP$^-$ interfollicular cells (Fig. 3). Ki-67/CD3 double-labelling showed that most of the highly proliferating interfollicular cells were of a CD3$^+$/CD45RO$^+$ phenotype, corresponding to activated T cells. Their proliferative activity reached a mean proliferation index (PI) of 38·3%, which was significantly higher than that found within the interfollicular zones of control tonsils (PI 9·9%; Table 2; Figs 4 and 5). Immunostaining for CD4 and CD8 revealed a disproportionate T cell expansion as the CD8/CD4 ratio rose more than three-fold, especially in cases with many EBER$^+$ cells (Table 2).

Although the extent of necrosis was variable even among cases with high viral load, the apoptotic activity was constantly very high throughout the expanded interfollicular zones. The apoptosis index (AI) reached values up to 2·37% (mean 1·69%) and was thus comparable to the apoptotic activity in florid germinal centres of control tonsillar tissue (AI in germinal centres 1·8%; in interfollicular zones 0·3%; Table 2; Fig. 6).

Compared with the T cells in control tonsils, the expression of Bcl-2 in the activated T cells of IM was markedly decreased (54·4% *versus* 32·6%). Down-regulation of Bcl-2 expression seemed to be time-dependent, since Bcl-2 expression was significantly lower in cases with abundant EBER$^+$ cells (25·9%) than in tonsils with little viral load (cases 1, 6, 7 and 12: 42·9%; $P < 0{\cdot}015$) (Table 2). In contrast, Bcl-2 expression within remaining lymph follicles in IM was not altered (Fig. 7).

The level of Bax expression was not, or only slightly, increased in the interfollicular T zones of IM compared with control tonsils. H/RS-like cells in the vicinity of the tonsillar crypts showed weak to moderate Bax and strong Bcl-2 expression (not shown).

In accordance with previous reports [13], Fas expression was detected in germinal centre cells but not in mantle cells of control lymphoid tissues. In IM specimens, a moderate increase in staining intensity as well as in the number of interfollicular cells expressing Fas could be observed in all except two cases (nos 7 and 12). H/RS-like cells, representing EBV-infected lymphocytes, exhibited moderate Fas expression (not shown).

In all IM cases except nos 7 and 12, FasL IHC produced a very strong membranous signal in many lymphoid cells of the interfollicular zone (Fig. 8). As we described before [14], pre-existent lymph follicles showed a characteristic meshwork-like expression pattern within their germinal centres. In some H/RS-like cells a very strong membranous signal for FasL was found (Fig. 8, inset). In contrast, no significant positivity was seen in the interfollicular areas of control tonsils.

Western blot analysis of FasL expression in IM tonsils produced in all samples including the positive controls a protein

Fig. 1. Numerous EBER$^+$ cells with large, often bizarre nuclei within and underneath the crypt epithelium as well as in superficial interfollicular zones (case no. 2; ISH for EBER, ×100).

Fig. 2. Expression of CD79a in EBV-infected lymphoid cells (case no. 2; combined ISH for EBER (NBT/BCIP) and IHC for CD79a (fast red); ×400).

Fig. 3. Expanded T zone underneath the EBV-infected cells close to the crypt surface (case no. 6; combined ISH for EBER (NBT/BCIP) and IHC for CD3 (fast red); ×200).

Fig. 4. High proliferative activity in EBV$^-$ cells of the expanded T zone (case no. 2; double labelling for LMP (NBT/BCIP) and Ki-67 (fast red); × 100).

Fig. 5. High proliferative activity in T cells of the expanded interfollicular zones (case no. 2; double labelling for Ki-67 (DAB) and CD3 (fast red); ×400).

Fig. 6. High apoptotic activity of EBV$^-$ interfollicular cells (case no. 6; combined TUNEL (fast red) and IHC for LMP (NBT/BCIP); ×100).

Fig. 7. Down-regulation of Bcl-2 expression in interfollicular cells. Pre-existent small secondary lymph follicle (upper left corner) with strong Bcl-2 expression in the follicle mantle. Strong Bcl-2 expression in a single Hodgkin/Reed–Sternberg (H/RS)-like cell, compatible with an EBV-infected lymphocyte (arrowhead) (case no. 4; IHC for Bcl-2; ×200).

Fig. 8. Strong membranous expression of FasL in activated, partially blast-like transformed interfollicular T cells. Inset: strong FasL expression in EBV-infected cells with Hodgkin/Reed–Sternberg (H/RS)-like morphology (case no. 11; IHC for FasL; ×200, inset ×400).

band of 33 kD, compatible with membrane-bound FasL [15]. In addition, a protein band of 52 kD, probably representing dimeric soluble FasL [15], was detected in testis lysate. Negative control samples showed no protein bands (Fig. 9).

DISCUSSION

IM represents a benign self-limiting lymphoproliferative disorder characterized by primary EBV infection of B lymphocytes and massive proliferation of activated T cells. Whether T cells are also a target of EBV infection or whether their massive expansion solely represents an immune reaction with activation and subsequent rapid elimination of T lymphocytes is controversial.

In our study we found no evidence of EBV infection in T cells. A number, varying from $>50\%$ to 70%, of the EBER$^+$ cells showed expression of the B cell markers CD20/CD79a. The remainder of the EBER$^+$ cells (30% to $<50\%$) expressed neither CD20/CD79a nor CD3/CD45RO. The lineage allocation of these cells could thus not be determined definitively by our investigations. Two observations indicate that these cells may represent B cells, however: (i) former studies were able to label these cells with antibodies detecting B cellular or plasmocytoid differentiation antigens [4,5]; and (ii) a selective down-regulation of several B cellular markers including CD20 by EBV infection of (neoplastic) B cells has been observed *in vitro* and *in vivo* [16,17].

These data are in line with our observation that EBV$^+$ T cells do not occur in IM. Hence, our findings imply that B cells represent the target of primary EBV infection and that EBV infection of T cells is at least an unusual event in IM. Previous studies addressing the possibility of EBV infection of T cells in IM have led to conflicting results, however. Some authors observed expression of T cell antigens in EBV-infected cells [18], the numbers of EBV$^+$ T cells varying from very few in IM tonsils of Caucasians [5] up to 66% in Japanese individuals [19]. These discrepant findings may indicate that geographical and/or racial factors are possibly involved, as has been suggested for the different incidence of EBV association in peripheral (non-nasal) T cell lymphoma in Caucasians and Orientals [20,21]. Very recently however EBV was detected more frequently in reactive B cells within peripheral T cell lymphomas than in the neoplastic T cell population itself [22,23]. This interesting observation casts doubt on primary EBV infection of non-neoplastic T lymphocytes and infers the possibility of a secondary infection of neoplastic T cells.

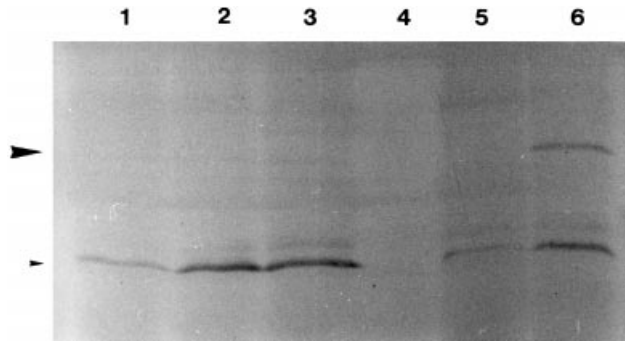

Fig. 9. Western blot analysis of FasL expression: protein bands of 33 kD (small arrowhead) in infectious mononucleosis (IM) tonsils and an additional band of 52 kD (large arrowhead) in control testis (lanes 1–3, IM tonsil (cases 10, 11, 13); lane 4, negative control; lanes 5 and 6, positive control (5, placenta; 6, testis)).

Thus, primary EBV infection targets B cells and is accompanied by a prominent reactive expansion of CD45RO$^+$ T cells, involving the cytotoxic CD8$^+$ subset especially. This intense T cell proliferation is accompanied by a striking increase in apoptotic activity in the expanded T zones, which we found to be comparable to that in germinal centres. This observation implies that primed T cells in IM are eliminated at a high rate by apoptosis while residing within the lymphoid tissue. These findings are in line with experiments which reported on (i) high susceptibility of IM T cells to apoptosis after short-term culture *ex vivo* [24]; and (ii) *in vivo* apoptosis of T cells in the peripheral blood of IM patients [10,11]. The latter reports detected lower apoptotic rates in peripheral blood T cells of IM patients (0·6% [11]) than we observed within the tonsillar lymphoid tissue (up to 2·37%), however, which infers that the major T cell elimination takes place within the lymphoid tissue and that only a smaller proportion of the viable T cells leaving the lymphoid tissue later die through apoptosis while circulating in the peripheral blood. Thus, our observation of apoptosis of T cells residing within the lymphoid tissue is in contrast to the hypothesis that apoptosis arises when T cells leave the local lymphoid tissue, an area which actively produces soluble factors required for their survival [24]. Furthermore, as EBV-infected T cells were not detected, apoptotic T cell death is more likely to result from activation-induced cell death (AICD [25]) than from viral infection.

The large-scale T cell expansion followed by rapid cell death during primary EBV infection is thought to prevent immunopathogenesis caused by the persistence of activated T cells and to ensure the selection of the most appropriate candidates for immunological memory. The mechanisms of regulated death by apoptosis and selective survival of T cells after resolution of acute disease are unknown. In contrast to former studies which used IM T cells from peripheral blood or *ex vivo* cultures [12,26,27], we analysed the *in vivo* expression of apoptosis-related genes in the expanded T cell population *in situ*.

We observed that in conjunction with the progression of the viral infection and concomitant T cell activation, Bcl-2 expression was drastically reduced in the T cell population, as the Bcl-2 level correlated inversely with the number of EBER$^+$ cells and the CD8/CD4 ratio ($P < 0{\cdot}06$, $P < 0{\cdot}01$, respectively). This observation confirms previous reports of Bcl-2 down-regulation in expanded CD45RO$^+$ T cell populations after prolonged *in vitro* stimulation [26] or in peripheral blood T cells of patients with IM [12]. Unlike Bcl-2, expression of Bax was virtually unaltered. As the overall effect on cell survival depends on the Bcl-2/Bax ratio, the remarkable decrease in the level of Bcl-2 expression in combination with the minimal increase in Bax expression in IM T cells implies a switch towards cell death. A similar, quasi unaltered expression of Bax in short-term cultures of peripheral blood T cells during acute infections with other human herpesviruses has been reported before [27]. Among the cytokines playing a major role in preventing activated T cell apoptosis the main candidates are the IL-2 receptor (IL-2R) common γ chain (γc) family [28,29] and type I interferons (IFN) [30,31]. The former regulate T cell survival and apoptosis by co-ordinating the balance between pro-apoptotic (Bax, Bcl-xS) and anti-apoptotic (Bcl-2, Bcl-xL) genes. The decreased Bcl-2 expression and increased apoptosis in the IM T cell population as observed in the current study may result from inhibition of survival signal

transduction through the γc pathway, since down-regulation of IL-2R on activated IM T cells has been described before [32,33].

Experiments with Bcl-2 transgenic mice however revealed unaffected T cell apoptosis during acute viral infections, indicating that other routes of cell death may be operative [34]. In this study we found a moderate increase in Fas expression and a remarkable increase in the expression of the ligand FasL in the T cell zones during IM. The up-regulation of Fas on T cells during acute herpesvirus infection has been documented previously by *ex vivo* analysis [27,35]. To the best of our knowledge however the *in vivo* expression of FasL by T cells during the resolution of primary EBV infection has not been described before. Western blot analysis revealed the expression of membrane-bound and dimeric soluble FasL protein forms which are both biologically active [15]. Thus, Western blot and IHC findings infer that the Fas/FasL system is involved in the extensive T cell apoptosis in IM, as has been suggested before by *in vitro* studies highlighting the critical role of Fas and FasL in T cell AICD [36,37]. Very recently type I IFNs were shown to rescue activated T cells from Fas-induced apoptosis [38,39] by a pathway distinct from that of the γc family [30]. As these cytokines are efficiently produced during a viral infection, they may to some extent counteract Fas-mediated AICD and play an important role in the generation and maintenance of immunological memory.

Taken together, our *in vivo* investigations in IM tonsils support the concept that the massive expansion of T cells is not caused by EBV infection of these cells but rather results from an intense cytotoxic immune reaction. The enhanced apoptosis of activated IM T cells seems to be regulated by multiple mechanisms, including a decrease of the Bcl-2/Bax ratio and an up-regulation of FasL and to a lesser extent of Fas. These apoptosis-inducing mechanisms impose constraints on the survival of activated T cells after IM and may thus act as a safeguard against chronic immune aggression. Further studies are needed to elucidate the triggers of this altered gene expression and to address the potential role of the γc family in the establishment of an effective immune response and specific immunological memory by the regulation of T cell death and survival in IM.

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