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Factors that influence activated CD8$^+$ T-cell apoptosis in patients with acute herpesvirus infections: loss of costimulatory molecules CD28, CD5 and CD6 but relative maintenance of Bax and Bcl-X expression

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SUMMARY

The expanded CD8$^+$ T-lymphocyte population arising in response to viral infection controls the virus but could also prove damaging to the host unless safely removed at the end of the immune response. Apoptosis provides a mechanism whereby this can be achieved, as apoptotic cells are recognized and engulfed by macrophages. Peripheral blood CD8$^+$ T lymphocytes from individuals with acute viral infections were highly susceptible to apoptosis after short-term culture *in vitro*. This spontaneous cell death could be prevented by interleukin-2 (IL-2) and was related to a decreased expression of Bcl-2 but not Bax or Bcl-X_L, additional molecules that promote or prevent apoptosis, respectively, as well as an increase in CD95. After stimulation with anti-CD3 antibody, T cells from these patients also underwent an activation-induced cell death (AICD) that could not be prevented by IL-2. Interestingly, CD8$^+$ T cells from this patient group expressed lower than normal levels of three costimulatory molecules, CD28, CD5 and CD6, suggesting that stimulation in the absence of a second signal is a possible mechanism for the defective reactivation of these cells. Thus multiple mechanisms, including loss of Bcl-2, increased CD95 and loss of costimulatory molecules, place constraints on the survival and reactivation of activated CD8$^+$ T cells after viral infections. This enables immune activation to be controlled and cellular homeostasis to be re-established during resolution of viral diseases *in vivo*.

INTRODUCTION

The immune response elicited by acute infection with the herpesviruses, Epstein–Barr virus (EBV), varicella zoster virus (VZV) and cytomegalovirus (CMV), results in a massive expansion of T lymphocytes, mainly of the CD8$^+$ subset.[1–3] Peripheral blood T cells from these patients show evidence of *in vivo* stimulation, as they express high levels of HLA class II and CD45RO.[4] In addition, the CD8$^+$ subset is highly cytolytic when assayed *in vitro* against infected target cells.[5] This suggests that the expanded lymphocytes are an activated effector population that arises in response to, and is directed against, the invading pathogen. However, such effector cells are potentially hazardous to the host and must be safely removed after virus clearance.[6]

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Abbreviations: AHVI, acute herpesvirus infections; AICD, activation-induced cell death.

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Apoptosis is a mechanism of cell death that, when it occurs *in vivo*, results in the phagocytosis of the dying cell before it can release its cell contents.[7] This process is controlled by a number of genes whose products act to induce or prevent cell death.[8] The best described of these is *bcl-2*, which inhibits cell death.[8] There are a number of other apoptosis-regulating genes that share sequence homology with *bcl-2*. These include *bax*, which promotes apoptosis,[9] and *bcl-X*, whose large (*bcl-x$_L$*) and small (*bcl-X$_S$*) forms prevent or induce apoptosis,[10] respectively. In mature lymphocytes apoptosis can result passively when cells are deprived of factors essential for cell survival. For example, cytokine-dependent cell lines die rapidly by apoptosis upon cytokine withdrawal.[11] This passively induced apoptosis involves the down-regulation of *bcl-2* and *bcl-X$_L$*.[12] Alternatively, apoptosis can be induced by the ligation of surface molecules such as CD95 (Fas/APO-1) by its ligand CD95-L or anti-CD95 antibody.[13,14] In addition, inappropriate activation through the T-cell receptor (TCR) can also lead to apoptosis, a phenomenon known as activation-induced cell death (AICD).[15] This can occur when mature T cells are activated in the absence of costimulatory signals normally provided by antigen-presenting cells (APC),[16] and involves the CD95 pathway.[17–19] There are a number of molecules found on T lymphocytes that are costimulatory for T-cell activation after interaction with their

specific ligands on APC.[20] Key amongst these is CD28, although other molecules including CD5 and CD6 have also been shown to have costimulatory activity.[21–23]

T lymphocytes from patients with acute herpesvirus infections (AHVI) are highly susceptible to apoptosis after short-term culture *in vitro*[24,25] and this is associated with a decrease in Bcl-2 protein expression in freshly isolated T cells.[24,25] Because Bcl-2 can be up-regulated and apoptosis prevented when interleukin-2 (IL-2) is added to these cells,[24,25] it is likely that this spontaneous death *in vitro* is due to cytokine withdrawal from the *in vivo*-activated T cells. However, the expression of other apoptosis-related proteins, such as Bax and Bcl-X, is unknown. In addition, a previous report has shown that after reactivation *in vitro*, T lymphocytes from mice with acute lymphocytic choriomeningitis virus (LCMV) do not proliferate but die rapidly by apoptosis, suggesting that AICD can also occur in T cells during acute viral infections.[26]

In this study we have extended our investigations of factors that may influence apoptosis in AHVI patients. In particular, we show that as well as spontaneous apoptosis, T cells from these patients also undergo an AICD. We have also investigated the expression of other *bcl-2*-related gene products by the apoptosis-prone T cells and have examined changes in expression of costimulatory molecules that could account for the AICD after reactivation *in vitro*.

MATERIALS AND METHODS

Patients and controls

Peripheral blood was obtained from patients at the infectious diseases unit, Coppetts Wood Hospital (London, UK). Patients were confirmed acute EBV by the presence of characteriztic clinical features, supported by atypical lymphocytosis and heterophile antibodies in the Monospot test. Acute VZV diagnosis was based on clinical history and a characteriztic pruritic vesicular skin rash. A total of 34 patients was investigated (EBV 13, VZV 21). No differences were found between the patient groups and therefore results for both have been pooled. Controls were healthy laboratory personnel known to be free of any upper respiratory tract or viral infection for at least 2 weeks.

Lymphocyte purification and activation

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, and T-enriched populations were prepared by rosetting with neuraminidase-treated sheep erythrocytes. Natural killer (NK) cells were removed by complement lysis using anti-CD16 (Leu-11b; IgM; Professor J. R. Thompson, University of Kentucky, Lexington, KY) and complement (Serological Reagents Ltd, East Grinstead, UK). Cells were cultured in RPMI-1640 (Gibco Ltd, Paisley, UK) containing 10% fetal calf serum (FCS; M.B. Meldrum Ltd, Bourne End, UK), antibiotics (Gibco) and supplemented with 2 mM L-glutamine (ICN Biomedicals Ltd, High Wycombe, UK). T cells were activated with anti-CD3 (OKT3; American Tissue Typing Collection, ATTC, Rockville, MD) in the presence or absence of IL-2 (2–5 ng/ml; R&D Systems, Abingdon, UK).

Enumeration of viable cells and cells in apoptosis

Viable cells were distinguished by their forward angle scatter (FSC) and 90° side scatter (SSC) profiles and were counted using a Cytoron*Absolute* flow cytometer (Ortho Diagnostics Ltd, High Wycombe, UK). Cells in apoptosis were identified morphologically in cytospin preparations stained with May–Grünwald–Giemsa (MGG, Merk Ltd, Poole, UK). A total of at least five fields per sample (> 500 cells) were counted by two independent investigators. In some cases, apoptosis was confirmed by electron microscopy and DNA electrophoresis, as described elsewhere.[24]

Analysis of intracellular Bcl-2, Bcl-X and Bax expression

For flow cytometric studies, PBMC or purified subsets were permeabilized using Permeafix (Ortho Diagnostics Ltd) prior to immunophenotyping. Bcl-2 expression was measured using direct staining with a fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (mAb) (Dako Ltd, High Wycombe, UK). Bcl-X and Bax expression was measured using purified rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) together with a species-specific FITC-conjugated second layer [Southern Biotechnology Associates (SBA), Birmingham, AL]. Expression of intracellular antigens was confirmed on cytospin preparations stained indirectly with antibody plus goat anti-mouse (GAM)–FITC (SBA). Cytospin preparations were also dual stained with anti-Bcl-2 plus GAM–IgG–TRITC (SBA) and either anti-Bax or anti-Bcl-X plus goat anti-rabbit–FITC (SBA).

Analysis of cell-surface phenotype by flow cytometry

The following mAb were used in double and triple combinations for FACScan analysis of T-cell phenotype. CD4 (RFT4; IgG1) and CD8 (RFT8; IgG1 and IgM) were used to identify helper and suppressor/cytotoxic cells, respectively. The antibodies CD45RA (SNI30; IgG1), CD45RO (UCHL1; IgG2a; provided by Professor P. C. L. Beverley, University College and Middlesex School of Medicine, London, UK) and CD45RB (PD7/26; IgG1; Dako Ltd) recognize isoforms of the leucocyte common antigen. Fas (CD95) expression was measured using a FITC-conjugated reagent (UB2; IgG1; Immunotech, Marseille, France). HLA-DR (RFD2; IgG1) and CD38 (RFT10; IgM) were used to identify activated T cells. T lymphocytes were also investigated for their expression of CD28 (L293; IgG1; Becton Dickinson, Oxford, UK), CD5 (RFT1; IgG1) and CD6 (3F7B5; IgG1; Ancell Corporation, Bayport, MN). Biotinylated reagents were visualized with a streptavidin–tricolour conjugate (Dako Ltd). Cell-surface expression of CD28, CD5 and CD6 was quantified using Quantum Simply Cellular microbeads (Flow Cytometry Standards Corporation, Leiden, the Netherlands).

Immunoblotting

The expression of Bcl-X in T cells was also investigated by immunoblotting.[27] Briefly, protein was extracted from 5×10^6 T lymphocytes after lysis and removal of nuclei. Protein concentration was determined using a modified Lowry assay[27] and 20 μg loaded onto each lane. After electrophoresis, the protein was transferred onto nitrocellulose paper (Hybaid Ltd, Teddington, UK) and stained with either anti-Bcl-X (Santa Cruz Biotechnology Inc.) or anti-actin (Oncogene Science Inc., Cambridge, MA) followed by a peroxidase-conjugated second layer (SBA). Staining was visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham Int. plc, Amersham, UK).

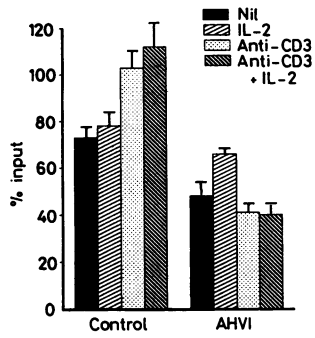

Figure 1. Lymphocyte survival after culture *in vitro*. PBMC from five healthy controls and five patients with acute VZV infection were cultured for 3 days in medium containing anti-CD3 or IL-2 as indicated. The number of viable cells remaining after the culture period was determined based on FSC and SSC profiles on the Cytoron*Absolute*. The data are expressed as a percentage of the original cell input; mean ± SEM.

RESULTS

Death and/or proliferation of lymphocytes from AHVI patients after short-term culture *in vitro*

Experiments were carried out to determine the effect of IL-2 on the survival of resting or anti-CD3-activated T lymphocytes from control and AHVI patients. Initial studies using [^{3}H]thymidine ([^{3}H]TdR) incorporation to measure proliferation showed that IL-2 enhanced the proliferation of anti-CD3-stimulated cells from normal individuals, and on its own induced low levels of [^{3}H]TdR uptake over a 3-day culture period. PBMC from AHVI patients, however, showed reduced [^{3}H]TdR uptake under all conditions (data not shown). When cell recovery was investigated (Fig. 1) it was clear that culture in medium alone caused a significant reduction in recovery of viable cells in the AHVI patient group (recovery 48 ± 5·4%) compared to controls (recovery 73·3 ± 2·2%; $P < 0.001$). This spontaneous death occurred by apoptosis as described elsewhere.[24] The addition of IL-2 to the lymphocytes at the start of culture had little effect on cell recovery from control individuals. However, it did significantly increase viable cell recovery from the AHVI patients (recovery 66 ± 3·1%; $P < 0.001$).

Stimulation of lymphocytes from control individuals with anti-CD3 or anti-CD3 plus IL-2 resulted in a increase in cell recovery compared to control cells cultured alone for 3 days (recovery control 73·3 ± 2·2%; anti-CD3 103 ± 9·8%; anti-CD3 + IL-2 112 ± 10·7%; $P < 0.001$). Minimum levels of apoptosis were detected in these cultures. In AHVI patients, stimulation through the TCR did not result in an increase in cell recovery and there was evidence of extensive apoptosis. However, small numbers of lymphoblasts indicative of proliferating cells were detected. Thus the net effect on TCR stimulation of AHVI patients was a balance between considerable apoptosis and minimal proliferation. Nevertheless, the numbers of TCR-activated lymphocytes recovered was not maintained or expanded even in the presence of IL-2, indicating that this cytokine could not rescue the cells from activation-induced death. Thus T cells from AHVI patients undergo spontaneous death due to lack of cytokines such as IL-2, and also exhibit an activation-induced death after stimulation through the TCR that occurs independently of this cytokine.

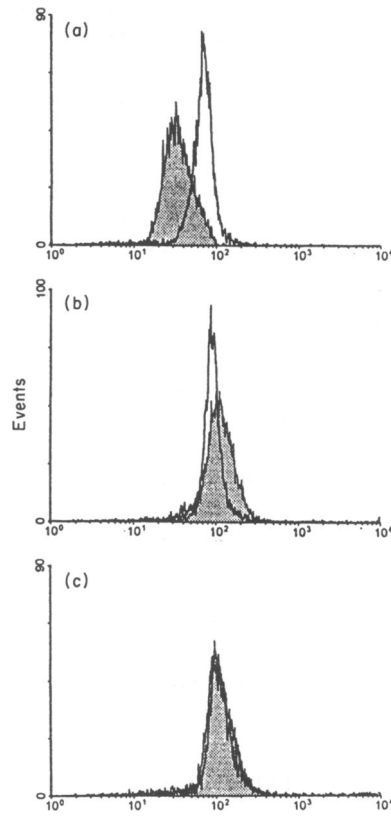

Figure 2. The expression of Bcl-2, Bax or Bcl-X on CD8$^+$ T lymphocytes. Purified T lymphocytes, depleted of NK cells, were investigated for (a) Bcl-2, (b) Bax or (c) Bcl-X expression within the CD8$^+$ T lymphocyte subset by two-colour immunofluorescence. During data acquisition a gate was set up around the CD8$^+$ T cells and 5000 events collected. The results from one representative healthy control (open histogram) and one EBV patient (filled histogram) are shown. In each case cells stained with irrelevant control antibodies fell within the first log decade.

Changes in Bcl-2, Bax and Bcl-X in AHVI patients

Our previous studies showed that spontaneous death in AHVI patients occurs in CD8$^+$ T cells[28] and is associated with a decrease in Bcl-2, but the expression of other apoptosis-related gene products by these cells was not known. A flow cytometric study of the expression of these death-related molecules showed that levels of Bcl-2 were significantly reduced, especially in the expanded CD8$^+$ subset (Fig. 2a). This study did not reveal a totally Bcl-2-negative population but rather a shift in intensity of staining. In contrast, using fluorescence microscopy clearly positive and negative populations could be detected (Fig. 3),[24] indicating that there may be some non-specific binding of anti-Bcl-2–FITC after permeabilization for FACScan analysis in this system. Unlike Bcl-2, CD8$^+$ T lymphocytes from both control and AHVI patients expressed high levels of Bax (>98%; Fig. 2b) and there was no indication of a loss of Bax expression. The anti-Bcl-X reagent reacts with both the large and small forms of this molecule. However, Western blot

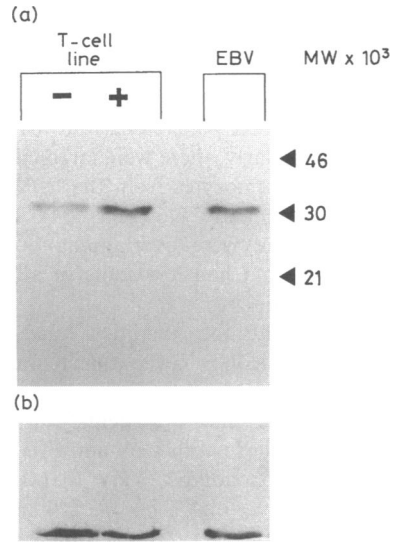

Figure 3. Immunoblot of Bcl-X staining in T lymphocytes. (a) The expression of Bcl-X was investigated in an IL-2-dependent T-cell line cultured for 48 hr in the presence (+) or absence (−) of IL-2, and also in T lymphocytes from a patient with acute EBV infection. Note that although the antibody used detects both large and small forms of Bcl-X, only Bcl-X_L (30 000 MW) but not Bcl-X_S (21 000 MW) was detected. Note also that removal of IL-2 from an IL-2-dependent line led to a reduction in Bcl-X_L. (b) The expression of actin in the same samples.

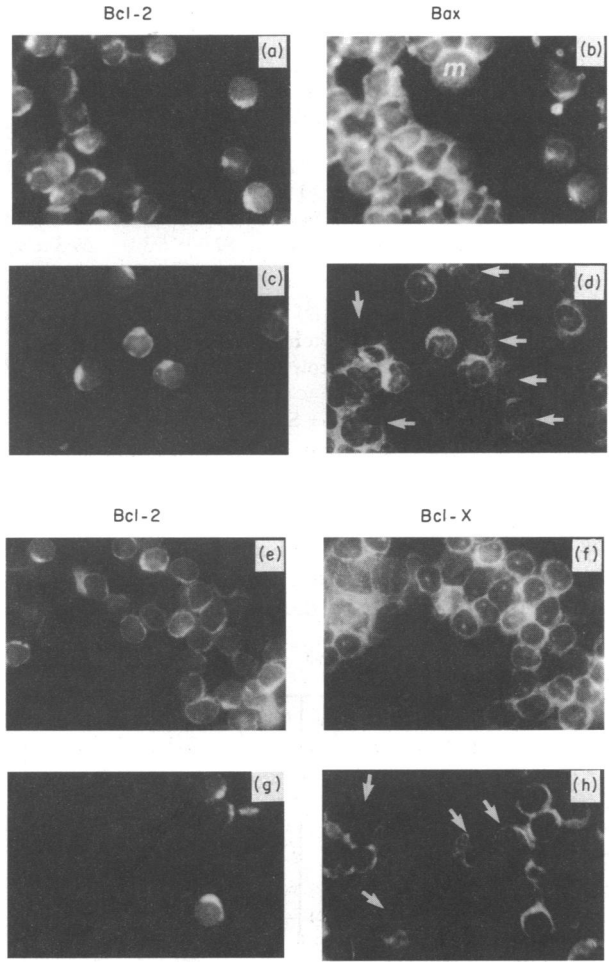

Figure 4. The dual expression of Bcl-2 and either Bax or Bcl-X on lymphocytes. Cytospin preparations of peripheral blood lymphocytes were dual stained with anti-Bcl-2 (a,c,e,g) and either anti-Bax (b,d) or anti-Bcl-X (f,h). The same fields from one healthy individual (a,b and e,f) and one EBV patient (c,d and g,h) are shown. The arrows indicate the presence of some Bcl-2-negative cells that retained expression of Bax or Bcl-X. m, contaminating monocytes.

analyses showed that in both healthy and virally infected individuals the large form, which prevents apoptosis, predominated[29,30] and Bcl-X_S was not seen (Fig. 3). Interestingly, Bcl-X, which was highly expressed in the healthy control group, was not decreased in freshly isolated T cells from the herpesvirus-infected patients (Fig. 2c). Culture of these cells for 24 hr in the absence of exogenous stimuli, however, resulted in a decrease in Bcl-X_L (data not shown). In healthy individuals, the majority of lymphocytes showed dual positivity for both Bcl-2/Bax (Fig. 4a,b) and Bcl-2/Bcl-X (Fig. 4e,f). However, purified T lymphocytes from individuals with acute EBV infection showed large numbers of lymphoblasts that had lost Bcl-2 while retaining both Bax (Fig. 4c,d) and Bcl-X (Fig. 4g,h).

As previously reported, we found that CD95 expression was increased in AHVI patients. In healthy individuals higher expression of CD95 was found on $CD4^+$ $CD45RO^+$ T cells compared to $CD8^+$ lymphocytes. The expanded $CD8^+$ population from herpesvirus-infected patients showed an increase in the percentage of cells expressing CD95 (control 12·6 ± 5·4, AHVI 57·6 ± 4·5; $n = 12$; $P < 0{\cdot}001$) although the intensity of the staining was not altered when compared to normal.

Decreased expression of costimulatory molecules in AVI patients

Lymphocyte activation in the absence of costimulatory signals results in a defective proliferative response.[16] One possibility is that the poor response of lymphocytes from AHVI patients is due to defective costimulatory signals. In patients with either human immunodeficiency virus type-1 (HIV-1) infection or with common variable immunodeficiency, CD28 expression was decreased on $CD8^+$ T cells.[31,32] We therefore investigated the expression of three molecules previously shown to have costimulatory activity, namely CD28, CD5 and CD6, in $CD4^+$ and $CD8^+$ T lymphocytes from AHVI patients, to determine if down-regulation of these molecules could account for abnormal proliferative responses. NK cells which can express low levels of CD8 but lack CD28[31] were removed prior to the phenotypic analyses.

The vast majority of $CD4^+$ T lymphocytes from all individuals expressed high levels of all three costimulatory molecules (Table 1). The $CD8^+$ subset from both control and AHVI patients was found to have a slightly lower expression of these molecules in terms of percentage positivity. A quantification of antigen density showed that $CD4^+$ T cells from both healthy controls and virally infected patients expressed $4{\cdot}2 \times 10^3$ molecules/cell (m.p.c.) of CD28 compared to $2{\cdot}4 \times 10^3$ m.p.c. on $CD8^+$ T cells. This was also observed with CD5, reduced from $22{\cdot}8 \times 10^3$ m.p.c. in $CD4^+$ to $13{\cdot}2 \times 10^3$ m.p.c. in $CD8^+$, but was less marked with CD6, reduced from

Table 1. Decreased expression of costimulatory molecules on $CD8^+$ T cells during acute viral infections*

		CD28	CD5	CD6
$CD4^+$	Control†	98·6 ± 0·3‡	99·6 ± 0·2	95·8 ± 0·8
	AVI	95·4 ± 3·1	98·6 ± 0·7	93·8 ± 1·4
$CD8^+$	Control	77·6 ± 5·4	91·4 ± 4·8	84·5 ± 5·1
	AVI	55·6 ± 7·7§	82·3 ± 6·3	59·3 ± 9·7§

* The expression of CD28, CD5 and CD6 on T-cell subsets was determined by two-colour flow cytometry on NK-depleted PBMC.

† A total of seven healthy controls and seven AHVI patients (4 VZV, 3 EBV) were investigated.

‡ Data are expressed as mean ± SEM.

§ $P < 0.05$.

85×10^3 m.p.c. to 75×10^3 m.p.c. Although the majority of $CD8^+$ T cells from healthy individuals expressed the three costimulatory molecules, albeit at a lower density, these percentages were significantly decreased compared to controls in the AHVI patient group (Table 1). This is also illustrated in Fig. 5, which shows representative results from a healthy individual and a patient with acute EBV infection. Whereas in the healthy individual only 17·5% of the $CD8^+$ T cells were $CD28^-$, this was dramatically increased to 67·0% in the EBV patient (Fig. 5a,b). Similarly, there were large increases in the number of $CD8^+$ T lymphocytes from the EBV patient that had lost CD5 (Fig. 5c,d) and CD6 (Fig. 5e,f). These percentages indicate that there were overlapping populations of $CD8^+$ T lymphocytes that had lost some or all of the three costimulatory molecules.

During acute viral infections there is a huge expansion of $CD8^+$ T cells. In the cohort of patients studied, $CD8^+$ lymphocytes increased from 581 ± 65 cells/mm^3 in the control group to 4018 ± 1720 cells/mm^3 in patients. Furthermore, the majority of the $CD8^+$ cells in both VZV and EBV patients had a primed, $CD45RO^+$ phenotype (VZV 61·1 ± 9·2%; EBV 70·1 ± 7·4%) compared to only 27·1 ± 3·2% in the healthy controls ($P < 0.001$). In fact, the absolute number of $CD8^+$ $CD45RO^+$ T cells increased over 19-fold from 169 ± 24 cells/mm^3 in the control group to 3237 ± 1418 cells/mm^3 in the AHVI patients as a whole. In contrast, more modest increases were seen in the $CD8^+$ $CD45RA^+$ subset (control 412 ± 57 cells/mm^3; AHVI 851 ± 396 cells/mm^3). This expanded $CD8^+$ $CD45RO^+$ population in AHVI patients contained a high proportion of $CD28^-$ cells (64·4 ± 10·0; $n = 6$). Thus a substantial proportion of the increased pool of $CD8^+$ T cells in AHVI patients lacked costimulatory molecules. Our results suggest that this may be one mechanism that leads to the

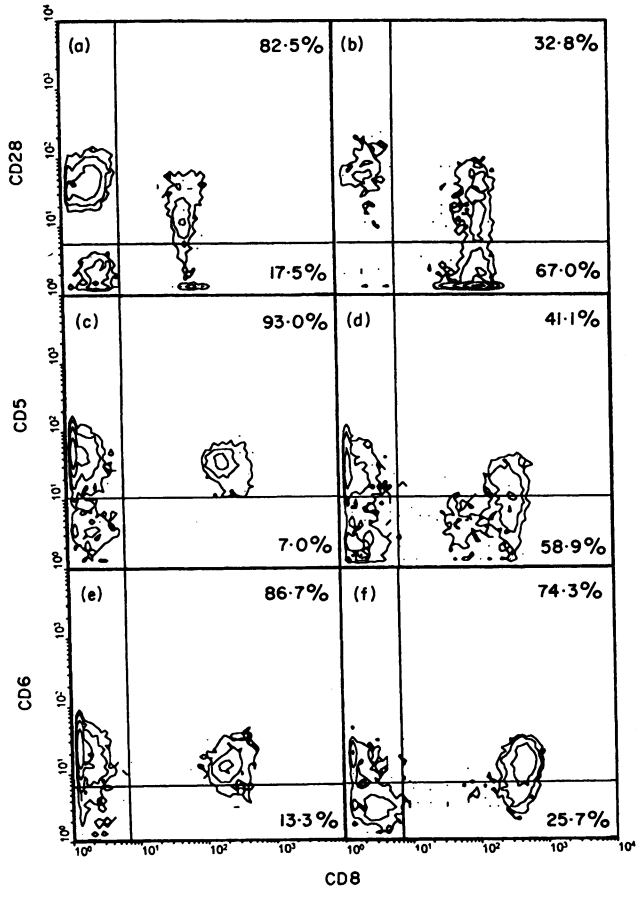

Figure 5. The expression of costimulatory molecules on $CD8^+$ T cells. Purified peripheral blood T lymphocytes from one healthy control (a,c,e) and one EBV patient (b,d,f) were investigated for the expression of CD28 (a,b), CD5 (c,d) and CD6 (e,f) by two-colour immunofluorescence. Quadrants were set using irrelevant antibody conjugate controls. The numbers show the percentages of $CD8^+$ T cells that express or lack either CD28, CD5 or CD6.

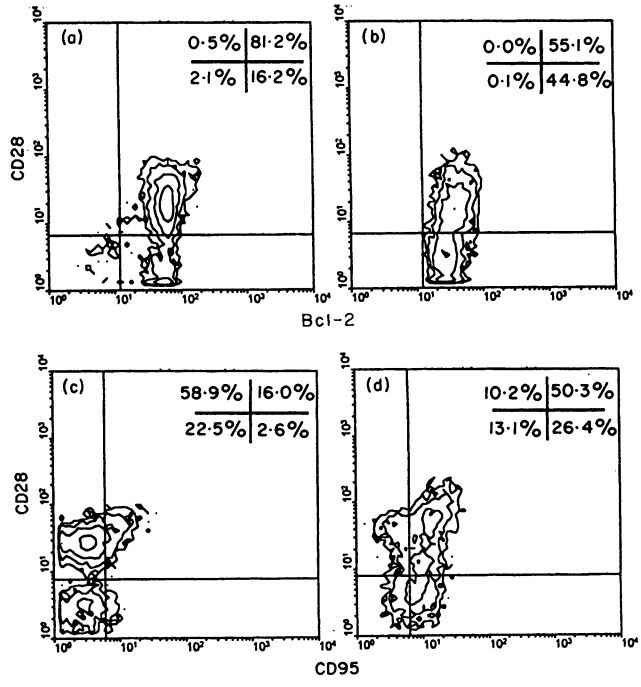

Figure 6. Comparison of Bcl-2, CD95 and CD28 expression. Purified peripheral blood T lymphocytes from one healthy individual (a,c) and one patient with acute EBV infection (b,d) were dual stained with either CD28 and Bcl-2 (a,b) or CD28 and CD95 (c,d). The numbers indicate the percentages of lymphocytes within the quadrants set using control antibodies.

diminished capacity of these cells to proliferate in response to antigen.

Concomitant loss of Bcl-2 and costimulatory molecules

To determine if the same cells that had lost Bcl-2 protein expression also had decreased levels of costimulatory molecules, purified T lymphocytes were dual stained with either CD28, CD5 or CD6 and cytoplasmic Bcl-2. There was no indication in healthy individuals of decreased expression of Bcl-2 within the $CD28^-$ T-cell subset (Fig. 6a). In contrast, although the acute EBV patient illustrated had an overall loss of Bcl-2 protein expression, as indicated by the decreased mean fluorescence intensity (MFI), this was more pronounced in the $CD28^-$ population (MFI: $CD28^+$ control 64·1, EBV 43·3; $CD28^-$ control 60·7, EBV 31·9; Fig. 6a,b). A similar pattern was also observed when CD5 and Bcl-2 were compared. However, no such correlation was seen with CD6 (data not shown). Additionally, when CD28 and CD95 were investigated, CD95 was found to be more brightly expressed on the $CD28^+$ T cells from healthy individuals, while $CD8^+$ T cells from patients with acute viral infection showed increased CD95 expression on both the $CD28^+$ and $CD28^-$ subsets (Fig. 6c,d).

Thus, virus-induced lymphocyte proliferation *in vivo* results in the expansion of large numbers of $CD8^+$ lymphocytes that have lost expression of costimulatory molecules and Bcl-2, while retaining Bax and Bcl-X_L. Nevertheless, despite the presence of Bcl-X_L, this population is highly susceptible to apoptosis and fails to proliferate when stimulated with anti-CD3.

DISCUSSION

Peripheral blood lymphocytes from individuals with AHVI are highly susceptible to spontaneous apoptosis when cultured *in vitro*, and this is related to a decreased expression of Bcl-2.[24,25] We have investigated two other apoptosis-related proteins, Bax and Bcl-X, and showed that expression of these was unaltered in freshly isolated T lymphocytes from AHVI patients. Thus Bcl-2 seems to play a crucial role in the control of apoptosis of mature T cells stimulated *in vivo*. Because spontaneous death could be partially prevented by IL-2, this form of cell death results from cytokine deprivation *in vitro*.[12] The inability of IL-2 to restore proliferation and prevent apoptosis in anti-CD3-stimulated cultures indicates that these T cells are also susceptible to AICD.

Several studies have documented AICD in mature T lymphocytes and have shown that death occurs only in cells that have entered the cell cycle.[16,33] Thus death can be triggered in T lymphocytes that are reactivated in a non-resting state. The T lymphocytes expanded during an acute viral infection may be an *in vivo* example of this phenomenon, where subsequent *in vitro* stimulation of *in vivo*-activated lymphocytes leads to cell death by apoptosis.

Recent blocking studies in T-cell clones, activated peripheral T cells and T-cell hybridomas have highlighted the critical role of CD95 in AICD.[17–19] This was supported by the lack of TCR-induced apoptosis in lymphoproliferative (*lpr*) mutant mice that have a defect in CD95.[17] Indeed it is recognized that activation of these cells induces the expression of CD95 and the expression and secretion of its ligand, CD95-L. The interaction between CD95/CD95-L results in apoptosis even in single-cell cultures, indicating that it can occur in an autocrine manner.[17–19] The increased expression of CD95 on T lymphocytes during acute viral infections and the observed AICD suggests that CD95 may also be involved in TCR-induced apoptosis of this *in vivo*-activated T-cell population.

The two-signal model of lymphocyte activation first proposed by Bretscher & Cohn[34] predicts that occupancy of the TCR alone is insufficient for optimal stimulation and that costimulatory signals provided by accessory molecule/ligand interactions are necessary for optimal proliferation.[20,35] Stimulation of T lymphocytes in the absence of such signals leads to anergy and cell death.[16,36,37] To date, the most powerful costimulatory signal in T-cell activation is provided through CD28 on the T-cell combining with one of its ligands, CD80 or CD86.[38,39] Thus, the observation of decreased CD28 expression in the AHVI patient group suggests that this population is functionally deficient and unlikely to respond normally to activation signals through the TCR. A similar situation is seen in patients with chronic HIV-1 infection, who also show decreased CD28 expression[31] and evidence of AICD, particularly within the $CD28^-$ population.[31]

CD28 signalling has recently been shown to prevent AICD both in the murine thymus *in vivo*[40] and in the anti-CD3-induced apoptosis of activated human T cells.[41] In addition, costimulation with anti-CD28 can prevent HIV-1 gp120-mediated apoptosis in $CD4^+$ T cells.[42] These data indicate that CD28 is able to modify apoptotic signals and prevent cell death. Anti-CD28 costimulation of activated T cells has been shown to lead to an increase in Bcl-X_L to levels that can prevent apoptosis induced either by TCR cross-linking, anti-CD95 antibodies or IL-2 withdrawal.[41] Other investigators have shown that either Bcl-2 or Bcl-X_L transfectants are able to resist CD95-induced apoptosis.[43] Thus, high levels of endogenous Bcl-2 or Bcl-X_L, which can be induced by costimulation through CD28, can alter susceptibility to CD95-induced death. This implies that CD28 costimulation may prevent CD95-induced death.

It is interesting to note that the *in vitro* expansion of T-cell lines with IL-2 also results in an accumulation of $CD8^+$ cells that express CD95 but lack CD28.[44,45] Thus the loss of CD28 expression and acquisition of CD95 might result normally after prolonged stimulation, providing a mechanism to prevent the reactivation of effector T-cell populations. The observation that $CD8^+$ T lymphocytes from AHVI patients also showed lower expression of CD5 and CD6, both of which have costimulatory activity, suggests there may be a general down-regulation of costimulatory molecules during the antiviral immune responses. This may act as a natural safeguard against chronic immune activation.

We have previously shown that cell death in AHVI patients occurs in cells that have lost expression of Bcl-2 and, significantly, that levels of Bcl-2 decrease in normal T cells after prolonged activation *in vitro*.[24] The *in vivo* patterns of Bcl-2 protein expression suggest that it plays an important role in controlling lymphocyte survival. For example, Bcl-2 is absent from short-lived germinal centre B cells but is strongly expressed by the long-lived memory B cells of the mantle zone.[46] Also, cortical thymocytes undergoing selection in the thymus lack Bcl-2 while medullary thymocytes retain expression.[46] In addition, studies on lymph nodes from

HIV-1-infected patients showed that Bcl-2 is absent from the majority of $CD8^+$ T cells.[47] These results suggest that the expression of Bcl-2 by peripheral blood T-cell populations is mirrored by cells of the lymphoid tissues.

It is perhaps surprising that Bcl-X_L, as a molecule that prevents apoptosis, is not also down-regulated during acute viral infections. However, a number of recent studies have shown that the tissue distribution of Bcl-X_L is very different from that of Bcl-2 and both molecules show reciprocal patterns of expression. For instance, Bcl-X_L is highly expressed in immature thymocytes but decreases with maturation.[29] Also, Bcl-X_L unlike Bcl-2, is present at high levels in germinal centre B cells.[29] The observations that Bcl-X-deficient mice die early *in utero*, showing extensive apoptosis in maturing neurones and haematopoietic cells, has led to the hypothesis that Bcl-X and Bcl-2 play complementary roles in controlling apoptosis in immature and mature cells, respectively.[29] The demonstration of high levels of Bcl-X_L but an absence of Bcl-2 in acutely infected patients underlies the central importance of Bcl-2 in the maintenance of survival of mature T lymphocytes. However, it should be noted that levels of Bcl-X_L decreased in T cells from AHVI patients after culture overnight in the absence of exogenous stimuli, when substantial apoptosis was also observed (N. J. Borthwick *et al.*, unpublished observation).

The large numbers of effector $CD8^+$ lymphocytes that develop in response to viral infections are a potent force for the elimination of the pathogen. However, they also form a potential hazard if uncontrolled. Apoptosis provides a method for the safe removal of these cells once the infection has resolved.[6] Our study shows that this apoptosis could result from restimulation of $CD8^+$ T cells in the absence of T-cell costimulatory signals. Alternatively, these $CD8^+$ T cells can also be eliminated by apoptosis related to cytokine withdrawal, related to the loss of Bcl-2 protein expression. These observations provide a rationale for the maintenance of T-cell homeostasis after viral infections and highlight the importance of factors that prevent apoptosis of T cells, thus enabling immune memory to persist.[6]

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REFERENCES

1. Reinherz E.L., O'Brien C., Rosenthal P. & Schlossman S.F. (1980) The cellular basis for viral-induced immunodeficiency: analysis by monoclonal antibodies. *J Immunol* **125,** 1269.
2. Crawford D.H., Brickell P., Tidman N., McConnell I., Hoffbrand A.V. & Janossyg. (1981) Increased numbers of cells with suppressor T cell phenotype in the peripheral blood of patients with infectious mononucleosis. *Clin Exp Immunol* **43,** 291.
3. Maher P., O'Toole C.M., Wreghitt T.G., Spiegelhalter D.J. & English T.A. (1985) Cytomegalovirus infection in cardiac transplant recipients associated with chronic T cell subset ratio inversion with expansion of a Leu-7^+ TS-C^+ subset. *Clin Exp Immunol* **62,** 515.
4. Miyawaki T., Kasahara Y., Kanegane H., Ohta K., Yokoi T. & Yachie A. (1991) Expression of CD45R0 (UCHL1) by $CD4^+$ and $CD8^+$ T cells as a sign of *in vivo* activation in infectious mononucleosis. *Clin Exp Immunol* **83,** 447.
5. Enssle K.H. & Fleischer B. (1990) Absence of Epstein–Barr virus-specific, HLA class II-restricted $CD4^+$ cytotoxic T lymphocytes in infectious mononucleosis. *Clin Exp Immunol* **79,** 409.
6. Akbar A.N., Salmon M., Savill J. & Janossy G. (1993) A possible role for bcl-2 in regulating T-cell memory—a 'balancing act' between cell death and survival. *Immunol Today* **14,** 526.
7. Duvall E., Wyllie A.H. & Morris R.G. (1985) Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology* **56,** 351.
8. Korsmeyer S.J. (1992) Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* **80,** 879.
9. Oltvai Z.N., Milliman C.L. & Korsmeyer S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74,** 609.
10. Boise L.H., Gonzalez-Garcia M., Postema C.E. *et al.* (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74,** 597.
11. Cohen J.J. (1991) Programmed cell death in the immune system. *Adv Immunol* **50,** 55.
12. Akbar A.N., Borthwick N.J., Wickremasinghe R.G. *et al.* (1996) Interleukin-2 receptor common γ-chain signalling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (*bcl-2, bcl-x_S*) but not pro-apoptotic (*bax, bcl-x_L*) gene expression. *Eur J Immunol* **26,** 294.
13. Itoh N., Yonehara S., Ishii A. *et al.* (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66,** 233.
14. Suda T. & Nagata S. (1994) Purification and characterization of the Fas-ligand that induces apoptosis. *J Exp Med* **179,** 873.
15. Kabelitz D., Pohl T. & Pechhold K. (1993) Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol Today* **14,** 338.
16. Liu Y. & Janeway C.A. (1990) Interferon gamma plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J Exp Med* **172,** 1735.
17. Dhein J., Walczak H., Baumler C., Debatin K.M. & Krammer P.H. (1995) Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* **373,** 438.
18. Brunner T., Mogil R.J., LaFace D. *et al.* (1995) Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* **373,** 441.
19. Ju S.T., Panka D.J., Cui H. *et al.* (1995) Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* **373,** 444.
20. Liu Y. & Linsley P.S. (1992) Costimulation of T-cell growth. *Curr Opin Immunol* **4,** 265.
21. Jenkins M.K., Taylor P.S., Norton S.D. & Urdahl K.B. (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol* **147,** 2461.
22. Ceuppens J.L. & Baroja M.L. (1986) Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-phase-bound OKT3. *J Immunol* **137,** 1816.
23. Swack J.A., Mier J.W., Romain P.L., Hull S.R. & Rudd C.E. (1991) Biosynthesis and post-translational modification of CD6, a T cell signal-transducing molecule. *J Biochem* **266,** 7137.
24. Akbar A.N., Borthwick N., Salmon M. *et al.* (1993) The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *J Exp Med* **178,** 427.
25. Uehara T., Miyawaki T., Ohta K., Tamaru Y., Yokoi T. & Nakamura S. (1992) Apoptotic cell death of primed $CD45RO^+$ T lymphocytes in Epstein–Barr virus-induced infectious mononucleosis. *Blood* **80,** 452.
26. Razvi E.S. & Welsh R.M. (1993) Programmed cell death of T lymphocytes during acute viral infections: a mechanism for virus-induced immune deficiency. *J Virol* **67,** 5754.

27. Panayiotidis P., Ganeshaguru K., Jabbar S.A. & Hoffbrand A.V. (1994) Alpha-interferon (α-IFN) protects B-chronic lymphocytic leukaemia cells from apoptotic cell death *in vitro*. *Br J Haematol* **86,** 169.
28. Akbar A.N., Savill J., Gombert W. *et al.* (1994) The specific recognition by macrophages of CD8$^+$,CD45RO$^+$ T cells undergoing apoptosis: a mechanism for T cell clearance during resolution of viral infections. *J Exp Med* **180,** 1943.
29. Ohta K., Iwai K., Kasahara Y. *et al.* (1995) Immunoblot analysis of cellular expression of Bcl-2 family proteins, Bcl-2, Bax, Bcl-X and Mcl-1, in human peripheral blood and lymphoid tissues. *Int Immunol* **7,** 1817.
30. Krajewski S., Krajewska M., Shabaik A. *et al.* (1994) Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. *Cancer Res.* **54,** 5501.
31. Borthwick N.J., Bofill M., Gombert W.M. *et al.* (1994) Lymphocyte activation in HIV-1 infection. II. Functional defects of CD28$^-$ T cells. *AIDS* **8,** 431.
32. North M.E., Akbar A.N., Borthwick N., Sagawa K., Funauchi M. & Webster A.D. (1994) Co-stimulation with anti-CD28 (Kolt-2) enhances DNA synthesis by defective T cells in common variable immunodeficiency. *Clin Exp Immunol* **95,** 204.
33. Russell J.H., White C.L., Loh D.Y. & Meleedy-Rey P. (1991) Receptor-stimulated death pathway is opened by antigen in mature T cells. *Proc Natl Acad Sci USA* **88,** 2151.
34. Bretscher P. & Cohn M. (1970) A theory of self-nonself discrimination. *Science* **169,** 1042.
35. Mueller D.L., Jenkins M.K. & Schwartz R.H. (1989) Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* **7,** 445.
36. Harding F.A., McArthur J.G., Gross J.A., Raulet D.H. & Allison J.P. (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356,** 607.
37. Tan P., Anasetti C., Hansen J.A. *et al.* (1993) Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J Exp Med* **177,** 165.
38. Linsley P.S., Brady W., Grosmaire L., Aruffo A., Damle N.K. & Ledbetter J.A. (1991) Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* **173,** 721.
39. Caux C., Vanbervliet B., Massacrier C., Azuma M., Okumura K. & Lanier L.L. (1994) B70/B7–2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med* **180,** 1841.
40. Shi Y., Radvanyi L.G., Sharma A. *et al.* (1995) CD28-mediated signaling *in vivo* prevents activation-induced apoptosis in the thymus and alters peripheral lymphocyte homeostasis. *J Immunol* **155,** 1829.
41. Boise L.H., Minn A.J., Noel P.J., June C.H., Accavitti M.A. & Lindsten T. (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* **3,** 87.
42. Tuosto L., Piazza C., Moretti S. *et al.* (1995) Ligation of either CD2 or CD28 rescues CD4$^+$ T cells from HIV-gp120-induced apoptosis. *Eur J Immunol* **25,** 2917.
43. Itoh N., Tsujimoto Y. & Nagata S. (1993) Effect of bcl-2 on Fas antigen-mediated cell death. *J Immunol* **151,** 621.
44. Salmon M., Pilling D., Borthwick N.J. *et al.* (1994) The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis. *Eur J Immunol* **24,** 892.
45. Testi R. & Lanier L.L. (1989) Functional expression of CD28 on T cell antigen receptor γ/δ-bearing T lymphocytes. *Eur J Immunol* **19,** 185.
46. Hockenbery D.M., Zutter M., Hickey W., Nahm M. & Korsmeyer S.J. (1991) BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* **88,** 6961.
47. Bofill M., Gombert W., Borthwick N.J. *et al.* (1995) Presence of CD3+CD8$^+$Bcl-2(low) lymphocytes undergoing apoptosis and activated macrophages in lymph nodes of HIV-1+ patients. *Am J Pathol* **146,** 1542.