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Inhibition of CD95 (Fas/Apo1)-mediated apoptosis by vaccinia virus WR

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SUMMARY

Stimulation of the CD95 (Apo-1/Fas) molecule either by the CD95 ligand or by monoclonal antibodies induces programmed cell death by apoptosis in a variety of cell lines and primary cells. In this study we observed that infection of B lymphoblast and T lymphoblast cell lines with vaccinia virus strain WR and recombinant vaccinia WR constructs, but not strain Copenhagen, rendered cells refractory to CD95-mediated apoptosis. In particular, vaccinia virus infection suppressed anti-CD95 antibody-induced membrane disintegration, apoptotic nuclear morphology of cells, and DNA fragmentation. Inhibition of apoptosis was not mediated by CD95 down-regulation or reduced binding of anti-CD95 antibody to infected cells, and occurred at a time point when cellular metabolism was not yet affected by the lytic vaccinia virus infection. Vaccinia virus (WR)-infected cells were resistant to CD95 ligand–CD95-mediated lysis by $CD4^+$ and $CD8^+$ T lymphocytes. Because cytolysis mediated by CD95 is one of two major mechanisms used by cytotoxic T lymphocytes to kill target cells, inhibition of CD95-mediated apoptosis may constitute a novel immune escape mechanism for this virus. Additionally, this mechanism may contribute to the higher pathogenicity of vaccinia virus strain WR compared with strain Copenhagen.

Keywords CD95 vaccinia apoptosis Apo-1 Fas

INTRODUCTION

The CD95 (Fas/Apo1) molecule, a member of the tumour necrosis factor (TNF) receptor family, mediates cell death by apoptosis [1,2] and is constitutively or inducibly expressed by a variety of cells. Cross-linking of CD95 by MoAbs causes apoptosis of the cell reflected by chromatin condensation, nuclear and DNA fragmentation and blebbing of the cellular membrane which ultimately results in rupture of the membrane [1]. The ligand of CD95 has recently been identified and is expressed on the surface of a variety of cells, including stimulated lymphocytes [3]. It was recently demonstrated that the CD95 molecule is involved in cytolysis mediated by T lymphocytes [4,5] and activation-induced cell death [6]. In addition, it was suggested that CD95-mediated cell killing may be crucial in regulating the T cell life cycle [7].

Vaccinia virus is a complex double-stranded DNA virus of the poxvirus family. Although closely related to each other by genetic restriction and sequence analysis, numerous vaccinia virus strains exist which exhibit broad heterogeneity in regard to their pathogenicity in the animal model [8,9]. The genome of vaccinia contains more than 200 open reading frames and has been sequenced in its entirety [10]. In addition to genes that are essential to viral replication, several gene products of vaccinia or related pox viruses have been identified that may enhance viral resistance against the host immune response. The mechanisms by which these viruses evade the immune system may include defective presentation of antigens by the MHC class I molecules [11] and blockade of complement activity [12,13]. In addition, pox virus proteins may interfere with the release and action of anti-inflammatory cytokines including IL-1 [14–16], interferon [17–19] and TNF-α [20,21].

Because cytolysis mediated by the CD95 molecule constitutes a major lytic mechanism used by T lymphocytes and may be involved in cytotoxic cellular immune responses [4,5], the aim of this study was to investigate the effect of vaccinia virus infection on CD95-mediated apoptosis in B and T lymphoblasts.

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MATERIALS AND METHODS

Vaccinia infection

Epstein–Barr virus (EBV)-transformed B lymphoblasts (B-LCL) and the T lymphoblast cell lines Jurkat E6-1 [22] and A3.01 [23] were infected with wild-type vaccinia virus strain WR (Western Reserve) or Copenhagen, or recombinant vaccinia virus constructs expressing a truncated HIV-1 IIIB envelope glycoprotein (WR, vPE17), HIV-1 polymerase (WR, vCF21) or measles virus haemagglutinin (Copenhagen, vvH) [24] at a multiplicity of infection of 1–10 plaque forming units (PFU)/cell and incubated for 12–16 h at 37°C. To determine the extent of vaccinia virus expression, a

polyclonal rabbit anti-vaccinia antiserum (a kind gift of Dr H. Wege, Federal Research Institute for Viral Diseases of Animals, Insel Riems, Germany) or the anti-HIV-1 envelope MoAb 902 [25] recognizing the expression of the recombinant vaccinia virus vPE17, were used for flow cytometric analyses.

Preparation of $CD4^+$ and $CD8^+$ T lymphocytes

$CD4^+$ and $CD8^+$ T lymphocytes were prepared from peripheral blood mononuclear cells (PBMC) by positive selection using anti-CD4 and anti-CD8 immunomagnetic beads (Dynal, Hamburg, Germany). The cells were stimulated *in vitro* with the anti-CD3 MoAb 12F6 at 0·1 μg/ml, 100 U/ml recombinant IL-2 (rIL-2) and allogeneic feeder cells for 10–14 days. The resulting polyclonal cell lines contained 90–95% $CD4^+$ or $CD8^+$ T cells, respectively, at the time of the experiments.

Chromium release assay

Standard chromium release assays were performed as described previously [26]. Briefly, cells were labelled with 80–100 μCi $Na_2{}^{51}Cr\ O_4$ for 60 min. For determination of anti-CD95 MoAb-mediated lysis, 1×10^4 ^{51}Cr-labelled cells were incubated for an additional 3 h with 200 ng/ml of the anti-CD95 MoAb CH-11 (Dianova, Hamburg, Germany). For determination of CD95 ligand-mediated lysis by T lymphocytes, 1×10^4 ^{51}Cr-labelled uninfected and vaccinia-infected B-LCL were incubated for 8 h with $CD4^+$ or $CD8^+$ effector T lymphocytes in the presence of 10 μg/ml concanavalin A (Con A) and 1·5 mM EDTA. After incubation, supernatants were harvested and counted in a gamma counter. Per cent specific lysis was calculated by the formula: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Maximum chromium release was determined by lysis of targets in 1·5% Triton X-100. Spontaneous chromium release was always < 30%.

Determination of intracellular DNA fragmentation

For agarose gel separation of low molecular weight DNA, 1·5–2×10^6 cells were incubated for 3 h with 200 ng/ml anti-CD95 MoAb. Cells were pelleted, lysed for 10 min on ice in a 10 mM Tris/EDTA solution containing 1% Triton X-100. High molecular weight DNA was pelleted by ultracentrifugation and discarded. Low molecular weight DNA in the supernatant was obtained after phenol/chloroform extraction and ethanol precipitation as described [27]. DNA gel electrophoresis was performed using 1·8% agarose gels. Gels were run in Tris-acetate EDTA buffer.

Fluorescence staining

For flow cytometric analysis 5×10^4 cells were incubated for 3 h with 400 ng/ml anti-CD95 MoAb. After incubation, cells were washed and stained with a secondary FITC-conjugated goat anti-mouse MoAb (Dako, Hamburg, Germany). For examination of apoptotic nuclear morphology, 5×10^4 cells were incubated for 5 h with 200 ng/ml anti-CD95 MoAb. Cells were stained by addition of the nuclear dye H33342 to the culture medium at a final concentration of 1 μg/ml.

MTT assay

Cells (5×10^4) were incubated with or without 200 ng/ml anti-CD95 MoAb for 3 h in the presence of 0·5 mg/ml MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue; Sigma, Munich, Germany) in 96-well flat-bottomed plates. After incubation, three quarters of the supernatant were removed and the same amount of 99% isopropanol/0·1N HCl added. Absorbance was measured at 550 nm *versus* 630 nm [28]. Relative metabolic activity was calculated as follows: relative metabolic activity of vaccinia virus-infected cells (%): absorbance of infected cells/absorbance of uninfected cells × 100; relative metabolic activity of anti-CD95 MoAb-treated cells (%): absorbance of anti-CD95-treated cells/absorbance of untreated cells × 100.

RESULTS

CD95-mediated cytolysis is inhibited by vaccinia virus strain WR but not Copenhagen

Activation of the CD95 molecule on the surface of permissive cells causes death by apoptosis. To examine the effect of vaccinia virus infection on CD95-mediated apoptotic cell death, EBV-transformed human B-LCL were infected with wild-type and recombinant vaccinia viruses strain WR and Copenhagen and treated with the anti-CD95 IgM MoAb CH-11. Membrane disintegration representing cell death was measured by a standard chromium release assay. Figure 1a demonstrates that a fraction of uninfected B-LCL was lysed when incubated for 3 h with the anti-CD95 MoAb. In contrast, infection with both wild-type and recombinant vaccinia virus strain WR suppressed CD95-mediated cell killing. However, CD95-mediated cell lysis was unaffected by infection with wild type and recombinant vaccinia virus strain Copenhagen.

Having demonstrated inhibition of CD95-mediated cytotoxicity by vaccinia virus WR using the B cell line 010-035i, additional B-LCL and Jurkat and A3.01 T cells were infected with wild-type and recombinant vaccinia viruses WR, and cell death was induced by addition of the anti-CD95 MoAb. Figure 1b shows that vaccinia WR infection similarly rescued all cell lines tested from cell death caused by the apoptosis-inducing MoAb CH-11.

Since vaccinia virus infection of cells may vary depending upon the cell line and the vaccinia preparation used, it was important to regularly monitor the extent of vaccinia virus expression in parallel to the apoptosis experiments. A polyclonal rabbit anti-vaccinia antiserum was used in the experiments shown in Fig. 1 to measure the level of vaccinia expression by flow cytometric analysis. Because an HIV-1 envelope-specific MoAb was available in this laboratory and lysis of cells was inhibited by both wild-type vaccinia virus WR and recombinant vaccinia WR constructs, a recombinant vaccinia virus WR encoding a truncated HIV-1 envelope glycoprotein (vPE17) under control of an early promotor was used for most of the experiments presented, and the extent of infection was regularly monitored by measuring the expression of HIV envelope glycoprotein on the surface of infected cells. At least 50%, although in most instances more than 85%, of the cells expressed the vaccinia construct at the time the cytotoxicity experiments were performed.

Suppression of CD95-induced DNA fragmentation

A hallmark of apoptosis is the fragmentation of cellular DNA into multiples of 180 base pairs. In order to examine DNA fragmentation in cells treated with the anti-CD95 MoAb, agarose gel electrophoresis of nuclear DNA was performed. Incubation of uninfected B-LCL with anti-CD95 MoAb resulted in fragmentation of cellular DNA, as indicated by the typical DNA ladder. In contrast, the amount of fragmented DNA obtained from vaccinia WR-infected cells was markedly reduced (Fig. 2).

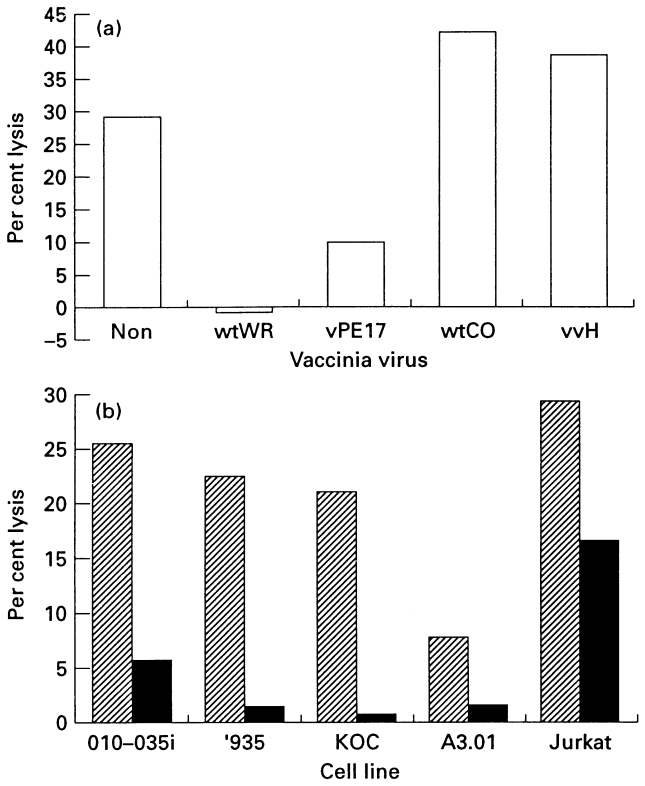

Fig. 1. Inhibition of CD95-mediated cytolysis by vaccinia virus WR but not Copenhagen. B and T lymphoblast cell lines were infected with vaccinia virus for 16 h. Cells were labelled with ^{51}Cr and incubated for 3 h with 200 ng/ml anti-CD95 MoAb CH-11. ^{51}Cr release into the supernatant was measured and percentage specific lysis was calculated. Representative results of three independent experiments are depicted. (a) Uninfected Epstein–Barr virus-transformed B lymphoblasts (B-LCL) 010-035i (non) or B-LCL 010-035i infected with wild-type vaccinia virus WR (wtWR), recombinant vaccinia WR construct vPE17, wild-type Copenhagen (wtCo) and the recombinant Copenhagen construct vvH were incubated with the anti-CD95 MoAb CH-11. (b) B-LCL 010-035i, '935 and KOC were infected with the vPE17 vaccinia construct. The T cell lines A3.01 and Jurkat-E6 were infected with the vaccinia WR construct vCF21. ▨, Uninfected; ■, infected.

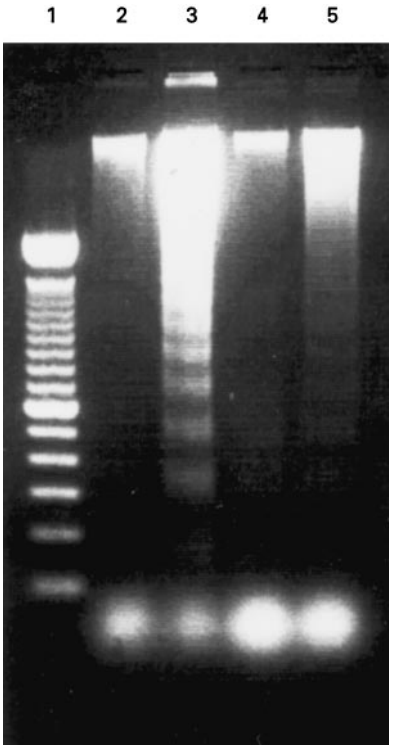

Fig. 2. Suppression of CD95-mediated DNA fragmentation. Epstein–Barr virus-transformed B lymphoblasts (B-LCL) 010-035i were infected with the vaccinia virus WR construct vPE17 for 16 h. After incubation of $1{\cdot}5 \times 10^6$ cells with and without 200 ng/ml anti-CD95 MoAb for 3 h, low molecular weight DNA was prepared and separated by agarose gel electrophoresis. Lane 1, 100 base pair marker; lane 2, uninfected cells without anti-CD95 MoAb; lane 3, uninfected cells with anti-CD95 MoAb; lane 4, infected cells without anti-CD95 MoAb; lane 5, infected cells with anti-CD95 MoAb.

Inhibition of apoptotic nuclear morphology

Apoptotic cells undergo characteristic cellular and nuclear changes, including membrane blebbing, chromatin condensation and nuclear fragmentation [1]. The nuclei of untreated and anti-CD95-treated cells were stained with the dye Hoechst 33342 and examined by fluorescence microscopy. Nuclei of uninfected cells treated with anti-CD95 MoAb demonstrated condensed chromatin and fragmented nuclei. In contrast, no CD95-induced changes of the nuclear morphology were detected in vaccinia WR-infected cells (Fig. 3).

Inhibition of apoptosis is not mediated by CD95 down-regulation or a secreted antibody binding protein

It was previously demonstrated that pox viruses inhibit the activity of the inflammatory cytokines TNF and IL-1 by secreting cytokine-binding proteins that interfere with the interaction of the cytokines with their receptors [14,16,20,21]. To exclude the possibility that inhibition of apoptosis is mediated by CD95 down-regulation or by soluble factors secreted by vaccinia virus-infected cells which interfere with the binding of anti-CD95 MoAb to the CD95 molecule, binding of the antibody to the cells was investigated by flow cytometric analysis. After 3 h of incubation, the amount of anti-CD95 MoAb bound to the cells was measured by addition of a FITC-conjugated anti-mouse IgM antiserum. Figure 4 depicts the cell size and anti-CD95 MoAb binding to uninfected (b) and infected treated (d) cells in comparison with untreated cells (a,c). The reduced forward scatter height of a large fraction of uninfected cells incubated with anti-CD95 MoAb (Fig. 4b, region 3) reflects apoptosis of these cells [29]. These cells also have reduced amounts of antibody bound to their surface, indicating fewer CD95 molecules on the surface of dying, apoptotic cells. In contrast, the vaccinia WR-infected cell population incubated with the anti-CD95 antibody contained only few apoptotic cells in region 3 (Fig. 4d). The almost identical mean fluorescence values of viable uninfected and infected cells (Fig. 4b,d region 2) indicate equal amounts of anti-CD95 MoAb bound to the surface of each cell population (y mean) and argue against the release of a soluble anti-CD95 MoAb binding factor or down-regulation of CD95 expression following vaccinia WR infection.

Suppression of apoptosis at a time point when cellular metabolism is still unaffected

Infection with vaccinia virus ultimately leads to destruction of the cellular metabolism and cell death. Since induction of apoptotic

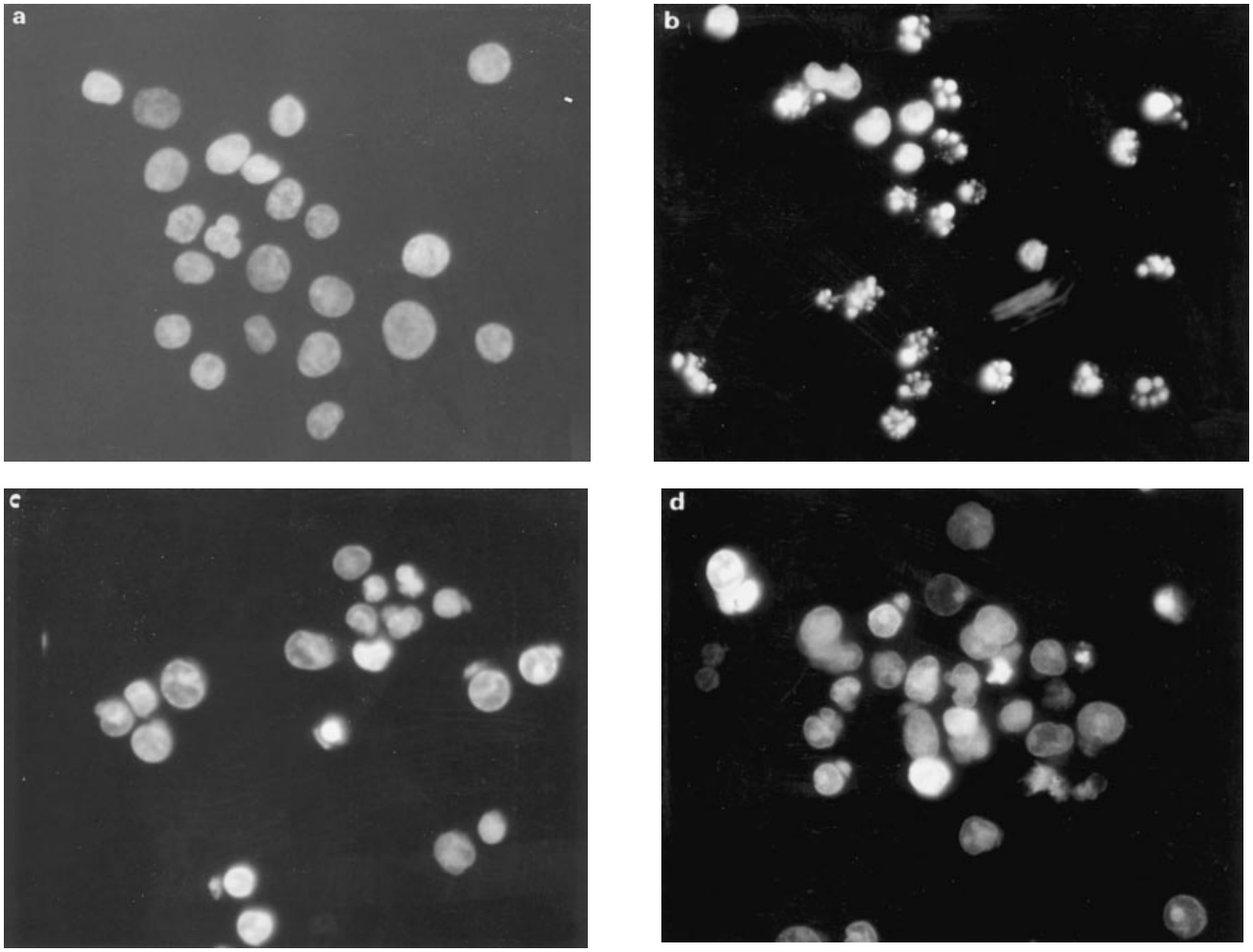

Fig. 3. Inhibition of apoptotic nuclear morphology. Epstein–Barr virus-transformed B lymphoblasts (B-LCL) 010-035i were infected with vaccinia virus WR construct vPE17 for 16 h. Cells were incubated with 200 ng/ml anti-CD95 MoAb for 5 h, stained with the nuclear dye H33342, and analysed by fluorescence microscopy. (a) Uninfected cells. (b) Uninfected cells after anti-CD95 MoAb treatment. (c) Vaccinia-infected cells. (d) Vaccinia-infected cells treated with the anti-CD95 MoAb.

cell death may require an active cell function in certain instances, the metabolic activity of infected and uninfected cells was quantified using the tetrazolium salt MTT [28]. Figure 5a shows the metabolic activity of vaccinia virus-infected cells in relation to uninfected cells, and demonstrates that 12–18 h after infection with vaccinia virus the cellular metabolism decreased due to the lytic effect of this viral infection. In parallel, infected and uninfected cells were incubated for 6, 12 and 18 h post-infection and for an additional 3 h with and without anti-CD95 MoAb in the presence of MTT. Figure 5b shows that whereas the metabolic activity of uninfected MoAb-treated cells was markedly reduced compared with the activity of untreated cells at all time points tested, the relative metabolic activity of vaccinia virus-infected MoAb-treated cells compared with untreated cells increased. At 18 h post-infection the metabolism of infected anti-CD95 MoAb-treated cells almost reached the activity of untreated cells, indicating complete inhibition of apoptosis at this time point. Incomplete inhibition of apoptosis at 6 h and 12 h and complete inhibition at 18 h post-infection probably reflect expression of the relevant vaccinia virus gene in an increasing fraction of the cell population. Additional statistical analysis was performed with data obtained from three independent experiments. The analysis confirmed that inhibition of CD95-mediated cell death was significant even at 12 h post-infection when cellular metabolism was not yet affected by vaccinia virus. These data demonstrated that inhibition of the CD95-mediated cell killing by vaccinia virus WR infection occurred before damage of the overall cellular metabolism by vaccinia infection (data not shown).

Vaccinia virus WR confers resistance to CD95-mediated cytolysis by $CD4^+$ and $CD8^+$ T cells

Incubation of stimulated T lymphocytes with allogeneic target cells in the presence of Con A results in a non-MHC-restricted killing of target cells. It has recently been demonstrated that a portion of this cytolysis is independent of extracellular calcium and mediated by the CD95/CD95 ligand pathway [30]. Experiments were thus designed to allow cell lysis only by the second lytic pathway, CD95. Uninfected and vaccinia virus-infected B lymphoblasts were incubated together with stimulated $CD4^+$ and $CD8^+$ T cells in the presence of Con A and EDTA. As shown in

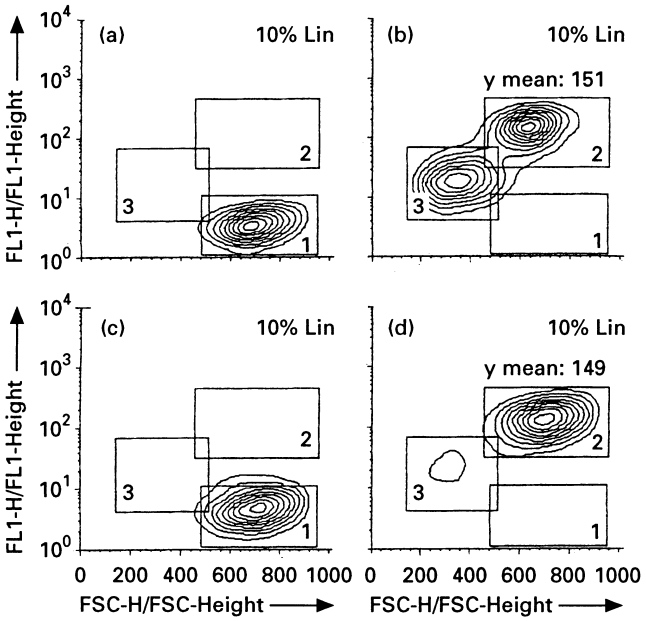

Fig. 4. Inhibition of CD95-mediated apoptosis is not caused by down-regulation of CD95 or reduced binding of the antibody to the cells. Vaccinia (WR) vPE17-infected Epstein–Barr virus-transformed B lymphoblasts (B-LCL) 010-035i were incubated with and without 200 ng/ml anti-CD95 MoAb for 3 h. Cells were stained with a secondary FITC-conjugated goat anti-mouse MoAb and analysed by flow cytometric analysis. In this contour dot plot unstained cells appeared in region 1, stained viable cells in region 2, and stained apoptotic cells in region 3. (a) Uninfected cells without anti-CD95 MoAb. (b) Uninfected cells incubated with anti-CD95 MoAb. (c) Infected cells without anti-CD95 MoAb. (d) Infected cells incubated with anti-CD95 MoAb.

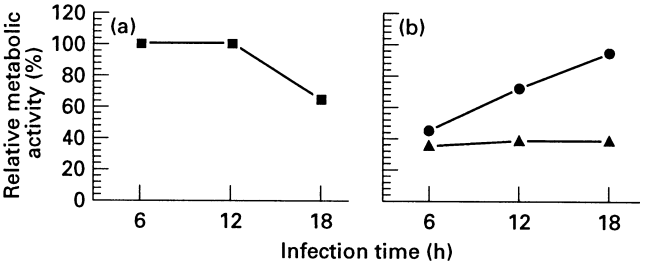

Fig. 5. Inhibition of CD95-mediated apoptosis in relation to the time point after vaccinia virus infection. Epstein–Barr virus-transformed B lymphoblasts (B-LCL) 010-035i were infected with vaccinia virus WR construct vPE17 for 6–18 h. Cells were incubated with and without 200 ng/ml anti-CD95 MoAb CH-11 for an additional 3 h in the presence of MTT dye as described in Materials and Methods. (a) Metabolic activity of vaccinia virus-infected cells in percentage of metabolic activity of uninfected cells. (b) Metabolic activity of anti-CD95 MoAb-treated vaccinia virus-infected (●) and uninfected cells (▲) in percentage of metabolic activity of untreated cells.

Fig. 6, uninfected B cells were readily killed by the T cells, while vaccinia WR-infected cells escaped cytolysis.

DISCUSSION

This study demonstrates that apoptosis mediated by anti-CD95 MoAb is suppressed in cells infected with vaccinia virus strain WR and recombinant vaccinia WR constructs, an observation that was reproducible. Inhibition of apoptosis occurred before a decrease of the cellular metabolism caused by the lytic vaccinia infection. Vaccinia-induced suppression of CD95-mediated apoptosis was not due to down-regulation of the CD95 antigen or to decreased binding of the anti-CD95 MoAb to the surface of infected cells.

Inhibition of CD95-mediated apoptosis by vaccinia virus has not been described previously and suppression of other apoptotic pathways by vaccinia virus, in addition to the effect described here, has not yet been investigated. However, inhibition of apoptotic pathways by the closely related cowpox virus has been reported previously. This virus contains a host range gene, CHOhr, which prevented virus-induced apoptosis in Chinese hamster ovary cells [31]. In addition, cowpox virus encodes a gene product, dubbed crmA, which inhibited apoptosis caused by over-expression of the murine IL-1 β-converting enzyme in rat fibroblasts and prevented apoptotic cell death after depletion of nerve growth factor in rat neuron cells [32,33]. In addition, it was recently reported that this gene also inhibits CD95-mediated apoptosis [34,35]. Interestingly, vaccinia virus strain WR contains a homologue of the cowpox crmA gene called B13R or SPI-2 [36], the open reading frame of which is disrupted and therefore non-functional in vaccinia virus strain Copenhagen [10]. It remains to be determined whether this gene may also be involved in the inhibition of CD95-mediated apoptosis by vaccinia virus WR.

Apoptotic cell death mediated by CD95 and the release of perforin and granzyme from cytoplasmic vesicles constitute the two major cytotoxic pathways used by $CD4^+$ and $CD8^+$ T lymphocytes [4,5]. Although the role of CD95-mediated cytolysis in the defence against pathogens including pox viruses has not yet been determined, protection of infected cells from CD95-mediated lysis by $CD4^+$ and $CD8^+$ T lymphocytes by vaccinia virus WR may indicate a novel immune escape mechanism for this virus.

Suppression of apoptosis induced by anti-CD95 MoAbs has been observed in infection with adenovirus and the human T cell leukaemia virus (HTLV)-I. In adenovirus infection, the E1B oncogene product suppressed CD95-mediated apoptosis [37] by binding to a cellular apoptosis-inducing protein called bak [38]. This molecule may also inhibit additional pathways leading to apoptosis, including TNF-mediated cytotoxicity and apoptosis induced by cisplatin or the adenovirus E1A protein [39–41]. Similarly, the HTLV-I tax protein inhibited anti-CD95 MoAb-mediated apoptosis in HTLV-I-infected T lymphocytes [42].

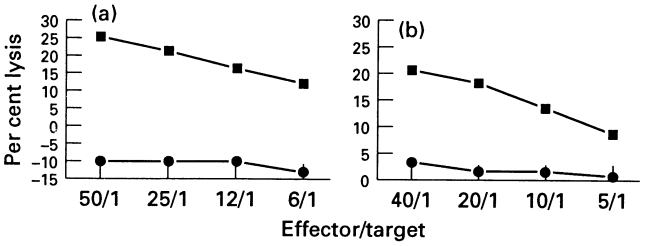

Fig. 6. Vaccinia virus WR renders infected cells resistant to CD95-mediated lysis by T lymphocytes. $CD4^+$ (a) and $CD8^+$ (b) T lymphocytes were incubated for 8 h with ^{51}Cr-labelled uninfected (■) and vaccinia WR (vCV21)-infected (●) Epstein–Barr virus-transformed B lymphoblasts (B-LCL) at the indicated effector-to-target cell ratios in the presence of 10 μg/ml concanavalin A and 1·5 mM EDTA.

However, this molecule may also be involved in the enhancement of apoptosis induced by serum deprivation [43].

Apoptosis is an important final effector mechanism to resist viral infection. Conversely, viruses may have evolved ways to antagonize apoptotic cell death. It was postulated that inhibition of apoptosis by viral gene products may prevent death of the host cell during viral replication either by inhibition of cell suicide induced by virus infection, or by blocking apoptosis triggered by cells of the immune system [42,44]. The capacity of vaccinia virus WR, but not Copenhagen, to inhibit apoptosis may therefore ensure survival and enhance proliferation of the virus in infected cells and organisms, and may contribute to the highly more vigorous pathogenicity of the WR compared with the Copenhagen strain in the animal model [9].

Finally, recombinant vaccinia virus constructs of both WR and Copenhagen origin are frequently used to measure the antigen-specific immune response mediated by cytotoxic T lymphocytes [45]. The observation that recombinant vaccinia virus WR constructs infer resistance to CD95-mediated cytolysis therefore indicates that the use of these constructs may preclude detection of cytotoxicity mediated by this molecule.

The characterization of the vaccinia gene product responsible for the inhibition of apoptosis might give new insight into the yet unknown pathway of CD95-mediated apoptosis.

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REFERENCES

1 Itoh N, Yonehara S, Ishii A *et al.* The polypeptide encoded by the cDNA for the human cell surface antigen Fas can mediate apoptosis. Cell 1991; **66**:233–43.

2 Oehm A, Behrmann I, Falk W *et al.* Purification and molecular cloning of the Apo-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. J Biol Chem 1992; **267**:10709–15.

3 Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993; **75**:1169–78.

4 Kägi D, Vignaux F, Ledermann B *et al.* Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Nature 1994; **265**:528–30.

5 Lowin B, Hahne M, Mattmann Ch, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and the Fas lytic pathway. Nature 1994; **370**:650–2.

6 Ju S-T, Panka DJ, Cui H *et al.* Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 1995; **373**:444–8.

7 Krammer PH, Behrmann I, Daniel P, Dhein J, Debatin K-M. Regulation of apoptosis in the immune system. Curr Op Immunol 1994; **6**:279–89.

8 Fenner F. The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 1958; **5**:502–29.

9 Tartaglia J, Perkus ME, Taylor J *et al.* NYVAC: a highly attenuated strain of vaccinia virus. Virology 1992; **188**:217–32.

10 Goebel SJ, Johnson GP, Perkus ME *et al.* The complete DNA sequence of vaccinia virus. Virology 1990; **179**:247–66.

11 Townsend A, Bastin J, Gould K *et al.* Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. J Exp Med 1988; **168**:1211–24.

12 Kotwal GJ, Isaacs SN, McKenzie R, Frank MM, Moss B. Inhibition of the complement cascade by the major secretory protein of vaccinia virus. Science 1990; **250**:827–30.

13 Isaacs SN, Kotwal GJ, Moss B. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. Proc Natl Acad Sci USA 1992; **89**:628–32.

14 Alcami A, Smith GL. A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host immune response. Cell 1992; **71**:153–67.

15 Ray CA, Black RA, Kronheim SR *et al.* Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell 1992; **69**:597–604.

16 Spriggs MK, Hruby DE, Maliszewski ChR *et al.* Vaccinia and cowpox viruses encode a novel secreted interleukin-1-binding protein. Cell 1992; **71**:145–52.

17 Chang H-W, Watson JC, Jacobs BL. The E3 4 gene of vaccinia virus encodes an inhbitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proc Natl Acad Sci USA 1992; **89**:4825–9.

18 Upton C, Mossman K, McFadden G. Encoding of a homolog of the IFN-γ receptor by myxoma virus. Science 1992; **258**:1369–72.

19 Carroll K, Elroy-Stein O, Moss B, Jagus R. Recombinant vaccinia virus K34 gene product prevents activation of double-stranded RNA-dependent, initiation factor 2α-specific protein kinase. J Biol Chem 1993; **268**:12837–43.

20 Palumbo GJ, Buller RM, Glasgow WC. Multigenic evasion of inflammation by poxviruses. J Virol 1994; **68**:1737–49.

21 Schreiber M, McFadden G. The myxoma virus TNF-receptor homologue (T2) inhibits tumor necrosis factor-α in a species-specific fashion. Virology 1994; **204**:692–705.

22 Weiss A, Wiskocil RL, Stobo JD. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL2 production reflects events occuring at a pre-translational level. J Immunol 1984; **133**:123–8.

23 Folks T, Benn S, Rabson A *et al.* Characterization of a continous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc Natl Acad Sci USA 1985; **82**:4539–43.

24 Drillien R, Spehner D, Kirn A *et al.* Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or fusion protein. Proc Natl Acad Sci USA 1988; **85**:1252–6.

25 Chesebro B, Wehrly K. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J Virol 1988; **62**:3779–88.

26 Jassoy C, Johnson RP, Navia BA, Worth J, Walker BD. Detection of a vigorous HIV-1-specific cytotoxic T-lymphocyte response in cerebrospinal fluid from infected persons with AIDS dementia complex. J Immunol 1992; **149**:3113–9.

27 McConkey DJ, Aguilar-Santelises M, Hartzell P *et al.* Induction of DNA fragmentation in chronic B lymphocytic leukemia cells. J Immunol 1991; **146**:1072–6.

28 Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. J Immunol Methods 1986; **89**:271–7.

29 Darzinkiewicz Z, Bruno S, Bino G Del *et al.* Features of apoptotic cells measured by flow cytometry. Cytometry 1992; **13**:795–808.

30 Ju S-T, Cui H, Panka DJ, Ettinger R, Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. Proc Natl Acad Sci USA 1994; **91**:4185–9.
31 Ink BS, Gilbert CS, Evan GI. Delay of vaccinia virus-induced apoptosis in nonpermissive chinese hamster ovary cells by the cowpox virus CHOhr and adenovirus E1B 19K genes. J Virol 1995; **69**:661–8.
32 Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J. Induction of apoptosis in fibroplasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced*–3. Cell 1993; **75**:653–60.
33 Gagliardini V, Fernandez P-A, Lee RKK *et al.* Prevention of vertebrate neuronal death by the crmA gene. Science 1994; **263**:826–8.
34 Enari M, Hug H, Nagata S. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature 1995; **375**:78–81.
35 Los M, Craen M van der, Penning LC *et al.* Requirement of an ICE/CED-3 protease for Fas/Apo-1-mediated apoptosis. Nature 1995; **375**:81–83.
36 Kotwal GJ, Moss B. Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. J Virol 1989; **63**:600–6.
37 Hashimoto S, Ishii A, Yonehara S. The E1b oncogene of adenovirus confers cellular resistance to cytotoxicity of tumor necrosis factor and monoclonal anti-Fas antibody. Int Immunology 1991; **3**:343–51.
38 Chittenden T, Harrington EA, O'Conner R *et al.* Induction of apoptosis by the Bcl-2 homologue Bak. Nature 1995; **374**:733–6.
39 Debbas M, White E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev 1993; **7**:546–54.
40 Subramian T, Tarodi B, Govindarajan R *et al.* Mutational analysis of the transforming and apoptosis suppression activities of the adenovirus E1B 175R protein. Gene 1993; **124**:173–81.
41 Wold WS. Adenovirus genes that modulate the sensitivity of the virus-infected cells to lysis by TNF. J Cell Biochem 1993; **53**:329–35.
42 Copeland K, Haaksma AGM, Goudsmit J, Krammer PH, Heeney JL. Inhibition of apoptosis in T cells expressing human T cell leukemia virus type I tax. AIDS Res Hum Retrovir 1994; **10**:1259–67.
43 Yamada T, Yamaoka S, Goto T *et al.* The human T-cell leukemia virus type I tax protein induces apoptosis which is blocked by the bcl-2 protein. J Virol 1994; **68**:3374–9.
44 Vaux DL, Haecker G, Strasser A. An evolutionary aspect on apoptosis. Cell 1994; **76**:777–9.
45 Moss B. Vaccinia virus: a tool for research and vaccine development. Science 1991; **252**:1662–7.