Induction of apoptosis by the *Amsacta moorei* entomopoxvirus

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CF-70-B2 cells derived from the spruce budworm (*Choristoneura fumiferana*) undergo apoptosis when infected with *Amsacta moorei* entomopoxvirus (AMEV), as characterized by membrane blebbing, formation of apoptotic bodies, TdT-mediated dUTP nick-end labelling (TUNEL) staining, condensed chromatin and induction of caspase-3/7 activity. The apoptotic response was reduced when cells were infected with UV-inactivated AMEV, but not when infected in the presence of the DNA synthesis inhibitor, cytosine β-d-arabinofuranoside. Hence, only pre-DNA replication events were involved in inducing the antiviral response in CF-70-B2 cells. The virus eventually overcame the host’s antiviral response and replicated to high progeny virus titres accompanied by high levels of caspase-3/7 activity. The CF-70-B2 cells were less productive of progeny virus in comparison to LD-652, a *Lymantria dispar* cell line routinely used for propagation of AMEV. At late stages of infection, LD-652 cells also showed characteristics of apoptosis such as oligosomal DNA fragmentation, TUNEL staining, condensed chromatin and increased caspase-3/7 activity. Induction of apoptosis in LD-652 cells was dependent on viral DNA replication and/or late gene expression. A significantly reduced rate of infection was observed in the presence of general caspase inhibitors Q-VD-OPH and Z-VAD-FMK, indicating caspases may be involved in productive virus infection.

INTRODUCTION

Apoptosis or programmed cell death is an active process characterized by various morphological and biochemical changes eventually leading to cell death. Caspases, a family of cysteine proteases, play a crucial role in the regulation and execution of apoptosis. Caspases are present in cells as inactive zymogens (procaspases) and are activated in response to apoptotic stimuli. There are two types of caspases; initiator caspases (caspases-8, -9 and -10) and effector caspases (caspases-3, -6 and -7). Initiator caspases are activated through death receptor signalling (extrinsic) or mitochondrial (intrinsic) pathways, which result in the activation of effector caspases. The active effector caspases, through cleavage of specific host proteins, are instrumental in directing subsequent morphological and biochemical changes associated with apoptosis. These changes include reduction of cellular volume, chromatin condensation, fragmentation of nucleus, phosphatidylserine exposure on the cell surface, DNA fragmentation, membrane blebbing and formation of apoptotic bodies. Cells undergoing apoptosis may exhibit some or all of these features (Fink & Cookson, 2005; Riedl & Shi, 2004).

Many viruses induce apoptosis, both *in vivo* and in cultured cells. In non-permissive hosts, apoptosis is an effective antiviral mechanism, which can lead directly to premature cell death limiting viral replication and spread. The *Autographa californica* multicapsid nucleopolyhedrosis virus (AcMNPV) induces apoptosis in *Spodoptera littoralis* SL2 cells (Chejanovsky & Gershburg, 1995), *Choristoneura fumiferana* CF-203 cells (Palli et al., 1996) and in various tissues of *Spodoptera litura* larvae (Zhang et al., 2002). The IPLB-LD-652Y cells undergo apoptosis in response to infection by several nucleopolyhedroviruses (NPVs) from *Bombyx mori* (BMNPV), *Hyphantria cunea* (HycuNPV), *Spodoptera exigua* (SeMNPV), *Orgyia pseudotsugata* (OpMNPV) and *S. litura* (SpltMNPV) (Ishikawa et al., 2003). Limited SpltMNPV propagation and infectivity in *S. exigua* larvae was attributed to cell death by apoptosis, which was observed in the haemolymph and fat body tissues (Feng et al., 2007).

To counter the host defence response, many viruses encode anti-apoptotic proteins, which allow the virus to replicate to a high titre and establish a successful infection. Insect viruses inhibit apoptosis by expressing P35 and/or IAP (inhibitor of apoptosis) proteins, which have been found in baculoviruses (Clem et al., 2010), entomopoxviruses (Li et al., 2005a; Means et al., 2007) and iridoviruses (Ińce et al., 2008). P35 and its homologues inhibit apoptosis by...
direct interaction with cellular caspases while viral IAPs appear to act indirectly by inactivating cellular IAP antagonists such as Hid, Reaper and Grim proteins (Clem et al., 2010). Recently, a new gene encoding an apoptosis suppressor was identified in Lymantria dispar MNPV (LdMNPV) (Yamada et al., 2011).

In some cases, apoptosis is related to viral pathogenesis rather than host defence; the virus can replicate and produce infectious progeny despite activation of cellular apoptotic pathways. In these cases, apoptosis is observed at late infection stages concurrently with productive infection (Hinshaw et al., 1994; Ren et al., 2005; Thoulouze et al., 1997). There is emerging evidence that some of these viruses actively promote apoptosis for their own survival and spread (Best & Bloom, 2004; Richard & Tulasne, 2012).

Members of the family Poxviridae contain large, linear dsDNA genomes, and infect both vertebrates (Subfamily: Chordopoxvirinae) and invertebrates (Subfamily: Entomopoxvirinae) (King et al., 2011). The Amsacta moorei entomopoxvirus (AMEV) was first isolated from the lepidopteran red hairy caterpillar (A. moorei) (Roberts & Granados, 1968). The 232 kb genome of AMEV has been completely sequenced (Bawden et al., 2000). Several AMEV genes including FALPE (Alaoui-Ismaili & Richardson, 1996; Alaoui-Ismaili & Richardson, 1998), superoxide dismutase (Becker et al., 2004), inhibitor of apoptosis (Li et al., 2005a; Li et al., 2005b), thymidine kinase (Gruidi et al., 1992; Ltyvyn et al., 1992), spheroidin (Hall & Moyer, 1991) and DNA photolyase (Nalcacioglu et al., 2010) have been well characterized.

AMEV replicates in cultured cells from Estigmene acrea (BTI-EAA, BTI-EAH), L. dispar (SCLD-135, IPLB-652, IPLB-LD-652), Heliothis zea (IPLB-1075), Spodoptera frugiperda (IPLB-21) and B. mori (BM-N) (Goodwin et al., 1990; Granados, 1981; Marlow et al., 1993). Of these, IPLB-LD-652 (commonly referred to as LD-652) is the most widely used cell line for propagation and maintenance of AMEV.

In attempts to find other cell lines permissive to AMEV, we tested several lines present at the Great Lakes Forestry Centre. The cell line CF-70-B2 derived from budworm C. fumiferana (Lepidoptera, Tortricidae) appeared to support the replication of AMEV. We decided to compare the replication of the virus in this cell line with that in the more commonly used LD-652. We observed that AMEV infection triggered an apoptotic response in both CF-70-B2 and LD-652 cells. Since the virus also replicated productively in both lines, we sought to examine this phenomenon further.

RESULTS

AMEV infection in CF-70-B2 and LD-652 cells

CF-70-B2 was found to support parental AMEV replication during a preliminary screening of several insect cell lines. We compared the process of infection between CF-70-B2 cells and the more commonly used LD-652 cells. When infected at an m.o.i. of 10, the LD-652 cells showed cytopathic effects at 1 day post-infection (p.i.) by rounding up and detaching from the surface while the same was not observed until 2 days p.i. in CF-70-B2 cells. At 2 days p.i., all the LD-652 cells were clearly infected and many contained spheroidal occlusion bodies (OBs). Only a few cells with OBs were observed at 2 days p.i. in CF-70-B2. By 3 days p.i., more cells with OBs were observed in both cell lines.

The kinetics of extracellular virus production was monitored over a period of 5 days (Fig. 1a). LD-652 cells were used for assaying virus titres. Extracellular virus production was detected as early as 1 day p.i. in both cell lines when

![Fig. 1.](image-url)
infected at an m.o.i. of 10. The highest virus titre was observed at 2 days p.i. and then declined slightly. The LD-652 cells were more productive in terms of virus replication than CF-70-B2; the extracellular virus titre was 5-10-fold lower in CF-70-B2 than in LD-652 at all time points tested p.i.

AMEV infection in the two cell lines was also tested using a recombinant virus expressing gfp under the control of the spheroidin promoter (Fig. 1b). Since spheroidin is a very late gene, gfp expression from the same locus indicates that the virus has gone through a full cycle of replication. Both cell lines showed low levels of auto-fluorescence at 0 days p.i. GFP was first observed at 2 days p.i. in both LD-652 and CF-70-B2 cells. In LD-652, gfp expression peaked at 3 days p.i. and remained stable throughout the experimental period while it continued to show a slow but gradual increase up to 5 days p.i. in CF-70-B2 cells. GFP levels were considerably higher in LD-652 than in CF-70-B2 from the time GFP was first detected at 2 days p.i., which confirmed that the latter is less amenable to infection by AMEV.

**Antiviral response (early apoptotic response) in CF-70-B2 cells**

AMEV induces an antiviral response in CF-70-B2 cells. We observed that CF-70-B2 cells undergo apoptosis as a result of AMEV infection (Fig. 2). Many cells appear to die of apoptosis before the virus can complete the infection cycle and produce OBs, indicating that this is an antiviral response. The apoptotic response was first evident at 1 day p.i., with a few blebbing cells. Apoptotic bodies as well as many cells with blebbing were visible at 2 days p.i. (Fig. 2a). Apoptosis was also tested by TdT-mediated dUTP nick-end labelling (TUNEL), which is an assay for in situ detection of DNA strand breaks in apoptotic nuclei, in combination with DAPI, which stains both apoptotic and non-apoptotic nuclei. TUNEL-positive (green) nuclei are seen in Fig. 2(b), confirming that AMEV infection leads to apoptosis in CF-70-B2 cells. In addition, DAPI staining indicated that nuclei of infected cells were highly condensed as opposed to those of mock-infected control cells (Fig. 2b). Next, we quantified the apoptotic response by measuring the caspase-3/7 activity over a period of 5 days (Fig. 2c). In accordance with morphological observations, a low level of caspase-3/7 activity was observed at 1 day p.i. when cell blebbing was first evident. Caspase-3/7 continued to increase with the progression of infection leading to high levels of activity at the end of the observation period (5 days p.i., Fig. 2c).

Viral DNA replication and late gene expression are not required for the antiviral response in CF-70-B2 cells. Cytosine β-D-arabinofuranoside (AraC) inhibits AMEV DNA replication (Winter et al., 1995). In order to determine if AMEV DNA replication or events thereafter were needed for the antiviral response in CF-70-B2, we studied the infection in cells treated with AraC (Fig. 3).

Mock-infected cells failed to divide in the presence of AraC indicating that cellular DNA synthesis was suppressed. The lack of OBs in infected cells suggested that viral DNA synthesis was also inhibited by AraC. Some mock-infected cells treated with AraC showed membrane blebbing and formation of apoptotic bodies, suggesting that the inhibitor also induces apoptosis. However, the apoptotic response was more pronounced in AraC-treated infected cells indicating that virus replication is not required for the antiviral response in CF-70-B2 cells (Fig. 3a).

The effect of AraC on apoptosis was confirmed by analysing caspase-3/7 activity in CF-70-B2 cells at 4 days p.i. (Fig. 3b). In comparison to cells infected in the absence of AraC, those infected in the presence of the inhibitor (either 200 or 300 μg ml⁻¹) showed a significant (P<0.01) increase in caspase-3/7 activity. The high levels of caspase-3/7 activity in AraC-treated, infected cells indicated that suppression of viral DNA replication and subsequent events had no effect on induction of apoptosis in CF-70-B2 cells.

The antiviral response is induced by early gene expression. Data obtained from treatment with AraC indicated that pre-DNA replication events are needed to induce apoptosis in CF-70-B2 infected cells. In order to ascertain that early gene expression was needed to induce apoptosis, we used UV-inactivated virus inoculum for infection. Treatment of virus particles with UV radiation damages the DNA genome and restricts mRNA synthesis, without compromising virus attachment and cell entry (Tsung et al., 1996). The virus inoculum was exposed to 5 J cm⁻² UV radiation before inoculating CF-70-B2 cells (Fig. 4). As expected, the untreated virus induced membrane blebbing and activation of caspase-3/7. In contrast, cells infected with the UV-treated virus remained morphologically similar to mock-infected control cells. Caspase-3/7 activity in cells infected with the untreated virus was significantly (P<0.05) higher than that in those infected with the UV-treated virus. These results indicate that viral gene expression is essential for induction of apoptosis. However, as the late events in infection (replication and late gene expression) have no effect on the apoptotic response (Fig. 3), it can be concluded that early genes are involved in inducing apoptosis in CF-70-B2 cells.

**Apoptosis in LD-652 cells**

AMEV induces apoptosis in LD-652 cells. LD-652 has been the line of choice to propagate AMEV because almost every cell becomes infected and the virus replicates to a high titre. The cells exhibit no visual signs of apoptosis (cell blebbing or apoptotic bodies) at any time during infection. As we were conducting a comparative study between CF-70-B2 and LD-652, we decided to test for apoptosis in the latter despite the lack of obvious morphological characteristics. Interestingly, we found compelling evidence of apoptosis induction by AMEV at late stages of infection (Fig. 5).
Shortly after infection, LD-652 cell DNA became fragmented with an apparent DNA ladder beginning at 3 days p.i. that became more distinct thereafter (Fig. 5a). TUNEL staining indicated that a large number of infected cells underwent apoptosis, at 4 days p.i. (Fig. 5b). In addition, nuclei of infected cells showed apoptotic morphology as evidenced by DAPI staining. Control (mock-infected) cells contained large nuclei with diffused chromatin while the nuclear chromatin of infected cells was highly condensed (Fig. 5b). A marked increase in caspase-3/7 activity was also observed from 3 days p.i., which further confirmed AMEV induced late apoptosis in LD-652 cells (Fig. 5c).

Viral DNA replication and/or late gene expression are required to induce apoptosis in LD-652 cells. The DNA synthesis inhibitor AraC prevented cell division of LD-652 (Fig. 6a). No OBs were seen in infected cells in the presence of 300 µg AraC ml⁻¹ (Fig. 6a), whereas a few (one or two) cells containing OBs were present at a reduced concentration of 200 µg ml⁻¹ (not shown). AraC treatment also induced apoptosis in some mock-infected LD-652 cells as seen by occasional cell blebbing (not shown).

Fig. 6(b) shows caspase-3/7 activity in LD-652 cells, in the presence or absence of AraC, at 4 days p.i. Mock-infected cells showed basal levels of caspase-3/7 activity, which increased as a result of AraC treatment (200 or 300 µg ml⁻¹). As expected, AMEV infection also resulted in an increase of caspase-3/7 levels in LD-652 cells in the absence of the inhibitor. In comparison to cells infected in the absence of AraC, those infected in the presence of the
inhibitor (200 or 300 µg ml\(^{-1}\)) showed a significant (\(P<0.05\)) reduction in caspase-3/7 activity. Hence, suppression of DNA synthesis by AraC treatment inhibits caspase-3/7 activity in infected cells. These results suggest that late events in infection (viral DNA synthesis and/or late gene expression) are involved in inducing apoptosis in LD-652 cells.

In the presence of AraC, the cells infected with AMEV showed less caspase-3/7 activity than mock-infected cells (Fig. 6b). The lower caspase-3/7 activity in infected cells could be attributed to the expression of anti-apoptotic proteins P33 and IAP (Li et al., 2005a; Means et al., 2007).

Caspases and productive virus infection

Our results show that replication of the viral genome triggers caspase activation in the highly productive LD-652 cells. To investigate the role of caspases in viral infection, we infected the cells in the presence of broad spectrum caspase inhibitors, Q-VD-OPH and Z-VAD-FMK (Fig. 7).

A recombinant virus expressing \(gfp\) in the spheroidin locus was used for infecting the cells at two different m.o.i. (0.1 and 1), and GFP expression was monitored over a period of 5 days. A detectable increase in GFP was observed at 3 days p.i. and continued to increase up to 5 days p.i. After 3 days p.i., the cells treated with Z-VAD-FMK showed a significant decrease for both 0.1 m.o.i. (\(P<0.001\)) and 1 m.o.i. (3–4 days p.i.: \(P<0.001\); 5 days p.i.: \(P<0.05\)) in GFP expression, compared to control cells (cells infected in the absence of inhibitors). The inhibitor Q-VD-OPH was less effective in suppressing GFP expression than Z-VAD-FMK. Nevertheless, at both 0.1 and 1 m.o.i., GFP expression in cells treated with Q-VD-OPH was significantly (\(P<0.05\)) lower than the control cells at 3–5 days p.i. Taken together, our data indicate that caspases may play a role in productive virus infection.

DISCUSSION

During the course of virus infection, many cells limit infection from spreading to the rest of the organism by undergoing apoptosis. For most RNA viruses, apoptosis does not appear to be a limiting factor for infection; some even utilize it as a means of disseminating progeny virions while evading inflammatory and immune responses of the host (Koyama et al., 1998; Teodoro & Branton, 1997). In many DNA viruses, apoptosis is an antiviral mechanism where the host cell sacrifices itself to restrict the replication and spread of virus to neighbouring cells (reviewed by Clem et al., 2010). There are exceptions, however, where the virus induces apoptosis in host cells with no detectable effect on the replication of its DNA genome or production of infectious progeny. Here, we present evidence to place AMEV in this category. We show that while AMEV can induce an antiviral response in some cell lines, an apoptotic response is initiated late in the replication cycle in permissive cells, without apparent effect on productive virus replication.

The antiviral response

We investigated AMEV-induced apoptosis in two lepidopteran cell lines. Of these two, LD-652 is the most commonly used cell line for propagation of AMEV. The second cell line, CF-70-B2, has not been previously tested for AMEV infection but it clearly supports viral replication, albeit not to the same extent as LD-652 cells. We observed that infection with AMEV induces an early apoptotic response in CF-70-B2 as evidenced by high levels of caspase activity, TUNEL staining, chromatin condensation and
severe blebbing, leading to apoptotic cell death. Oligosomal DNA fragmentation was not observed in CF-70-B2 cells (data not shown). The antiviral response was first evident at early stages of infection. However, a subset of CF-70-B2 cells succumbs to infection and produces extracellular virus and OBs, though at lower levels compared with the more productive LD-652.

Activation of the apoptotic cascade can occur at any stage of the virus life cycle, depending on the specific interactions between the virus and a particular host. Some viruses such as bovine herpesvirus 1 (BHV-1), avian leukosis-sarcoma virus and reovirus induce apoptosis at the time of attachment and some (e.g. Sindbis virus, mouse hepatitis virus) do so during fusion and entry (Brojatsch et al., 2000; Brojatsch et al., 1996; Chitnis et al., 2008; Connolly et al., 2001; Hanon et al., 1998; Jan et al., 2000; Liu & Zhang, 2007; Ramsey-Ewing & Moss, 1998). Yet others require a post-entry step (e.g. frog virus 3) such as uncoating (e.g. avian reovirus S1133) to elicit an apoptotic response (Chinchar et al., 2003; Labrada et al., 2002). In some cases, apoptosis induction is dependent on de novo viral protein synthesis (e.g. vaccinia virus), virus replication and/or post-replication events (e.g. Semliki Forest virus, AcMNPV) of the life cycle (Humlova et al., 2002; Schultz & Friesen, 2009; Urban et al., 2008). In this study, AMEV infection of CF-70-B2 cells resulted in apoptotic cell death; this was suppressed by UV irradiation of the inoculum prior to infection, but not by treatment of cells with AraC, a DNA synthesis inhibitor. These results suggest that the antiviral response triggered by AMEV in CF-70-B2 cells is mediated by early gene products. A similar observation was reported for the chordopoxvirus vaccinia virus (VACV) where apoptotic cell death was dependent on early gene expression in the murine macrophage line J774.G8 (Humlova et al., 2002). However, in Chinese hamster ovary (CHO) cells, a post-binding step associated with cell entry by VACV was sufficient to induce apoptosis (Ramsey-Ewing & Moss, 1998).

**Fig. 4.** The effect of UV irradiation of AMEV inoculum, on infected CF-70-B2 cells, at 3 days p.i. An m.o.i. of 10 was used for infection. (a) Light micrographs, Bars, 20 μm. (b) Caspase-3/7 activity in mock-infected cells and cells infected with AMEV or UV-irradiated (5 J cm⁻²) AMEV. Caspase-3/7 activity (normalized to viability) is expressed as a ratio of the mock-infected control. Results shown are from one of three representative experiments, each containing three replicates. Error bars represent ± SEM.

Apoptosis and productive infection

Many RNA viruses induce apoptosis in permissive cells without detrimental effects on virus replication (Bitzer et al., 1999; Du et al., 2004; Ren et al., 2005). In this study, we observed increased caspase levels, oligosomal DNA fragmentation, condensed chromatin and TUNEL-positive
nuclei in infected LD-652 cells. The apoptotic characteristics in LD-652 were observed at late stages of infection, together with high virus titres, suggesting a process other than an antiviral response. Productive infection associated with apoptosis has also been reported in a few DNA viruses. In the red sea bream iridovirus (RSIV), virus-induced DNA fragmentation was seen in parallel with viral DNA replication (Imajoh et al., 2004). The varicella-zoster virus (VZV) induced apoptosis in human foreskin fibroblasts but not in human sensory neurons, although both were susceptible to VZV infection (Hood et al., 2003).

Mosquito densoviruses usually establish persistent infections in mosquito cells with no obvious cytopathic effects. One exception is the *Hemagogus equinus* densovirus (HeDNV) which induces apoptosis accompanied by vigorous production of progeny in the mosquito cell line C6/36 (Paterson et al., 2005).

Unlike the host defence response in CF-70-B2 where a number of cells die before infection is established, apoptosis in LD-652 cells was exclusively dependent on virus replication and/or late gene expression. Many other

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**Fig. 5.** Apoptosis in LD-652 cells infected (m.o.i. 10) with AMEV. (a) DNA fragmentation in infected cells at 0, 3–6 days p.i. M, molecular mass markers (bp). (b) TUNEL staining (green) and DAPI counterstaining (blue) of mock-infected and infected cells at 4 days p.i. The same field is viewed under normal light; and under UV light with either an FITC or DAPI filter to detect TUNEL- or DAPI-stained nuclei, respectively. Note the TUNEL-positive (green) nuclei in infected cells containing OBs, on the merged image. Bars, 20 μm. (c) Caspase-3/7 activation in infected cells. Caspase-3/7 activity (normalized to viability) is expressed as a ratio of the mock-infected control at day 0. The results shown are from one of two representative experiments, each with three replicates. Error bars represent ± SEM.
viruses exhibit replication-dependent apoptosis (Acrani et al., 2010; Chen et al., 2010; Du et al., 2004; Kassis et al., 2004; Krejbich-Trotot et al., 2011; Ren et al., 2005; Schultz & Friesen, 2009; Urban et al., 2008). In AcMNPV, events associated with viral DNA replication and inhibition of host protein synthesis could trigger the host apoptotic response (Schultz & Friesen, 2009). In another DNA virus, the minute virus of canines (MVC), a moderate level of genome replication in permissive cells induces a cellular DNA damage response, which contributes to enhanced virus replication and cell death, suggesting an intricate relationship between viral replication and host death responses (Luo et al., 2011).

In VACV, the presence of phosphatidylserine on the surface of mature virions was essential for virus entry, which led Mercer & Helenius (2008) to conclude that VACV uses apoptotic mimicry to enter host cells. They also observed that many cells at the edge of viral plaques displayed exposed phosphatidylserine which led them to suggest that VACV-infected cells undergo apoptosis at late stages of infection. However, the significance of late stage apoptosis in chordopoxvirus infection is yet to be investigated.

**Role of caspases in virus infection**

Caspases are essential for propagation of some viruses through their involvement in replication, maturation or virus release from infected cells (reviewed by Best & Bloom, 2004; Richard & Tulasne, 2012). In maedi-visna virus (MVV, a lentivirus), caspase inhibitors delayed MVV-induced cell lysis and subsequent virus release (Duval et al., 2002). In enterovirus 70 (EV70) and human astrovirus (HAstV) caspase inhibitors reduced the release of virus progeny without affecting intracellular virus production.
apoptosis has also been reported for at least two viruses: Stoka (2008; Stoka et al., 2006) and Schotte (2003; Schotte et al., 2005). Downstream activation of caspases (Droga-Mazovec et al., 1999) by degrading anti-apoptotic Bcl-2 proteins, leading to apoptosis by activating the pro-apoptotic protein Bid and contributing to AMEV-induced apoptosis and cell death. (Bideshi et al., 2005; Krebich-Trotot et al., 2011).

In addition to regulating virus release from infected cells, caspases are also involved in virus replication and maturation. In Aleutian mink disease virus (AMDV), caspases play opposing roles in infection. They are involved in cleaving the non-structural protein NS1, resulting in virus replication. In contrast, caspase cleavage of the capsid protein leads to limited production of mature virions. Hence, caspases play a vital role in balancing virus production to ensure persistent infection (Best et al., 2002, 2003; Cheng et al., 2010). In HaAstV, caspases were reported to be involved in virus maturation through their role in proteolytic processing of the structural protein VP90 to the mature capsid protein VP70. In HaAstV, caspases were also involved in virus release through a non-lytic mechanism (Banos-Lara & Mendez, 2010; Mendez et al., 2004). Caspases play an active role in influenza virus propagation by facilitating the export of viral ribonucleoprotein complexes formed in the nucleus to the cytoplasm (Wurzer et al., 2003). Interestingly, in baculoviruses, caspases have a detrimental effect on virus particles. It was shown that activation of caspases during infection of a p35 mutant AcMNPV would lead to the production of progeny virions with defects in stability and infectivity (Bryant & Clem, 2009). Evidently caspases play a number of roles in response to virus infection and are not confined to limiting virus replication and propagation.

In this study, when LD-652 cells were treated with general caspase inhibitors Q-VD-OHP or Z-VAD-FMK, virus infection declined significantly. This suggests that caspases may be involved in productive virus infection. However, the reduction in infection was more pronounced in the presence of Z-VAD-FMK. It has been shown that Z-VAD-FMK is less effective in inhibiting caspase activity than the more potent inhibitor, Q-VD-OHP (Chauvier et al., 2007). Hence, the drastic reduction in infection in the presence of Z-VAD-FMK could not be attributed exclusively to caspase inhibition. Z-VAD-FMK has also been shown to inhibit other cysteine proteases such as cathepsins, papain and legumain (Chauvier et al., 2007; Rozman-Pungercar et al., 2003; Schotte et al., 1999). Therefore, it is possible that in addition to caspases, these other cysteine proteases are also contributing to AMEV-induced apoptosis and cell death. In vitro studies have shown that cathepsins can induce apoptosis by activating the pro-apoptotic protein Bid and by degrading anti-apoptotic Bcl-2 proteins, leading to downstream activation of caspases (Droga-Mazovec et al., 2008; Stoka et al., 2001). Cathepsin activation followed by apoptosis has also been reported for at least two viruses: the herpes simplex virus type 1 and murine norovirus (Furman et al., 2009; Peri et al., 2011). Lyosomal cathepsins are also involved in autophagy, a highly conserved cellular degradation pathway, which plays a major role in innate and adaptive immunity against viral infections (Chiramel et al., 2013; Kudchodkar & Levine, 2009; Muller et al., 2012). The difference between the effect of Q-VD-OHP and Z-VAD-FMK on AMEV infection suggests that autophagy may play a role in AMEV infection. It will be of interest to investigate if caspases are involved in AMEV infection, either through autophagy, or by contributing to caspase activation and apoptosis.

**Concluding remarks**

This is the first study to show that AMEV replicates productively in CF-70-B2 cells and delineates a comprehensive study on the mechanism of cell death by entomopoxvirus infection. We show that early gene products of AMEV can induce an antiviral response in CF-70-B2 cells, which limits virus replication and yield. More importantly, our findings suggest that virus replication/late gene expression triggers apoptosis in permissive LD-652 cells, and that caspase activation does not appear to limit infection. It is tempting to suggest that in the case of AMEV, caspases may play a role in productive infection.

**METHODS**

**Cells and virus.** The LD-652 (IPLB-LD-652) and CF-70-B2 (FPMI-CF-70-B2) cells were maintained at 28 °C. LD-652 cells were propagated in a medium consisting of 45% GM (Sigma-Aldrich), 45% Excell 420 (SAFC Biosciences) and 10% FBS (PAA, The Tissue Culture Company). The CF-70-B2 cell line was maintained in 90% GM supplemented with 10% FBS.

The parental AMEV stock used in this study was kindly provided by Drs Marie Becker and Richard Moyer (University of Florida, Gainesville, FL, USA). The recombinant AMEV expressing gfp was constructed in our laboratory (Perera et al., 2010). The gene encoding GFP was inserted in the sph locus under the sph promoter.

Cells were infected with AMEV at an m.o.i. of 10 p.f.u. per cell, unless otherwise stated. For infection, cells were seeded in tissue culture plates at 1 × 10^5 cells cm^{-2}. After allowing the cells to attach for at least 1 h at room temperature, the culture medium was removed and virus inoculum was added. The plates were incubated at 28 °C with gentle rocking to allow for virus adsorption for 2 h. At the end of the incubation period, the inoculum was replaced with culture medium and the plates were incubated at 28 °C.

**UV irradiation of virus.** An aliquot of AMEV in an uncovered, clear plastic tissue culture plate was exposed to a total dose of 5 J cm^{-2} UV radiation in a Stratalinker UV Cross-linker (Stratagene).

**Assays for AMEV infection**

**TCID<sub>50</sub> assay.** LD-652 cells were seeded in tissue culture plates and infected at an m.o.i. of 10 as described above. To assay extracellular virus production, the cells and media were collected at various times p.i. and centrifuged at 1000 g for 5 min. The virus titres in the supernatant were determined by TCID<sub>50</sub> analysis as described by Reed & Muench (1938).
**GFP expression.** Cells were plated in 96-well tissue culture plates and infected at an m.o.i. of 10, 1 or 0.1 as described above, and infected with a recombinant AMEV expressing gfp in the spheroidin locus. GFP fluorescence in the wells was measured directly in a plate reader equipped with 485nm/520nm filters.

**Assays for apoptosis**

**Caspase and viability assays.** Cells were infected at an m.o.i. of 10 and harvested at desired time points. Caspase-3/7 activity and cell viability were measured using the Caspase-Glo 3/7 Assay (Promega) and Cell Titer-Blue Cell Viability Assay (Promega) according to manufacturer’s instructions. Each assay was done in triplicate. Caspase activity was normalized to cell viability and presented as a ratio of the mock-infected control cells.

**Fragmentation of DNA.** Cells were seeded on 25 cm² flasks and infected as described above. Cells were collected by centrifugation at 1000 g for 5 min and DNA was extracted according to published protocols (Sambrook et al., 1989). Ten micromgrams of DNA was analysed on a 1.5% agarose gel.

**TUNEL assay.** Cells were infected and collected by centrifugation as described above. The TUNEL assay was carried out using the DeadEnd Fluorometric TUNEL system (Promega). Fixing and staining of cells were done according to the Promega technical bulletin (TB235) provided with the TUNEL assay kit. Cells were counterstained with DAPI (VECTASHIELD Hardset Mounting Medium with DAPI) and visualized in a Nikon Eclipse TS100 microscope.

**Inhibitors.** Cytosine β-D-arabinofuranoside (araC, Sigma-Aldrich) at 200 and 300 μg ml⁻¹ was used to suppress AMEV DNA replication. To inhibit caspases, 20 μM of the general caspase inhibitors Z-VAD-FMK (MP Biomedicals) and Q-VD-OPH (MP Biomedicals) were used. The above two caspase inhibitors were used because Q-VD-OPH has a wider spectrum of action than Z-VAD-FMK, the latter being the more commonly used inhibitor (Chauvier et al., 2007). The inhibitors were present in the medium at all times including the initial virus adsorption period.

**Statistical analysis.** Student’s t-test was used for statistical analysis. Values of P<0.05 were considered statistically significant.

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**REFERENCES**


