EFFECT OF POKEWEED ANTIVIRAL PROTEIN ON THE INDUCTION OF APOPTOSIS IN LARGEMOUTH BASS VIRUS INFECTED CELLS

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ABSTRACT

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Largemouth Bass Virus (LMBV), a member of the family *Iridoviridae*, infects warm freshwater fish, Asian ornamental fish, and is responsible for the killing of Largemouth Bass (*Micropterus salmoides*). This study demonstrates that ultraviolet light-inactivated LMBV induces apoptosis in Bluegill fry-2 (BF-2) infected cell monolayers via the extrinsic (caspase-8) pathway. Addition of pokeweed antiviral protein (PAP) to virus infected cells at a concentration sufficient to inhibit viral replication failed to inhibit LMBV-induced apoptosis. The results demonstrate LMBV-induced apoptosis is not dependent on viral protein synthesis and LMBV induced apoptosis occurs early in the infection cycle prior to host cell shut down and viral early gene expression. Apoptosis was observed 6h p.i. (post infection) following the addition of UV inactivated virus and

12h p.i. following the addition of infectious virus. The data suggest LMBV infection inhibits the extrinsic apoptotic pathway. Viral protein synthesis is necessary to re-initiate apoptosis via the intrinsic (caspase-9) pathway which occurs at 18h p.i. Activation of the caspase-9 pathway may be a response to viral induced cell damage.

INTRODUCTION

Largemouth Bass Virus (LMBV) is a member of the family Iridoviridae, which contain four genera: Iridovirus, Ranavirus, Lymphocystivirus, and Chloriridovirus. The viruses are tropic for fish, amphibians and arthropods (Williams 1994; Williams and Cory 1994; Whittington, Kearns et al. 1997; Chen, Zheng et al. 1999; Mao, Wang et al. 1999; Yu, Bearzotti et al. 1999; Paperna, Vilenkin et al. 2001; Betts AM 2003; Qin, Chang et al. 2003). Representative species within each genera include serologically distinct Invertebrate Iridescent virus 1 (Iridovirus), Frog Virus 3 (Ranavirus), Lymphocystis Disease Virus 1 (Lymphocystis virus), and Invertebrate Iridescent virus 3 (Chloridovirus) (Bahr U 1997; Tidona and Darai 1997; van Regenmortel 2000; D'Costa, Yao et al. 2001; Essbauer, Bremont et al. 2001; Essbauer S 2002; Qin, Chang et al. 2003). Sequence variation studies revealed LMBV to be a member of genus Ranavirus (Williams and Cory et al. 1994). Several members of Iridoviridae have been characterized by DNA sequencing and phylogenetic trees for Iridoviridae have been constructed (Goldberg, Coleman et al. 2003). Iridoviridae have a worldwide distribution and pathogenicity on several different hosts has been studied (Miocevic, Smith et al. 1993; Bollinger, Mao et al. 1999; Nakajima, Maeno et al. 1999; Yu, Bearzotti et al. 1999; Betts et al. 2003).

Structurally, Iridoviridae are composed of three domains: an outer protein capsid, a middle phospholipid membrane, and an inner DNA genome (van Regenmortel et al.

2000). Some members of the Iridoviridae family have protein spikes protruding from their capsid. Iridoviridae form large crystalline lattices, when visualized via transmission electron microscopy (van Regenmortel et al. 2000).

Vertebrate Iridoviridae possess an envelope obtained from host membranes. Capsids vary in size from 120-200 nm in diameter and are comprised of identical capsomeres which vary in number, and determine particle size (van Regenmortel et al. 2000). Iridoviridae encode 25 to 75 different proteins that range in molecular weight from 12,000 to 150,000 kDa, however, all possess a common 50 kDa capsid protein (Webby et al. 1998). Ranavirus encodes for 29 structural proteins, which do not undergo extensive posttranslational processing (van Regenmortel et al. 2000).

Ranavirus virions contain a short double stranded DNA genome that is circularly permuted and terminally redundant (Yan, Olson et al. 2000; Jakob, Muller et al. 2001). The virion contains a single dsDNA molecule, 150-170 kbp, with a GC content of 53% (van Regenmortel et al. 2000). All genomic cytosines are methylated by a viral-encoded DNA methyltransferase, which protects from viral endonucleases (van Regenmortel et al. 2000).

Iridoviridae genomes replicate both in the cytoplasm and the nucleus of the host cell. The first step in viral replication is attachment and entry into the host cell, which occurs by pinocytosis, followed by viral uncoating. The parental viral genome is transported to the nucleus of the host cell followed by host macromolecular synthesis shut down and transcription using a virus modified host RNA polymerase II. Progeny genomes are transported to the cytoplasm where concatamers are formed by recombination and late transcription occurs. The concatamers are resolved into packaged

lengths and loaded into newly formed capsids, possibly by the "headful" approach (Webby 1998; van Regenmortel et al. 2000). Virions exit the host cell by either budding or lysis (Webby 1998; van Regenmortel et al. 2000).

Transcription of viral proteins take place in three major stages: immediate-early (α proteins), delayed-early (β proteins), and late (γ proteins) (Webby et al. 1998). During both immediate-early and delayed-early transcription, enzymes for genome synthesis and host inhibition are synthesized (Webby et al. 1998). During the late phase, structural proteins (γ) are primarily transcribed. Proteins produced during each transcription phase provide negative feedback for viral protein synthesis (Martin, Aubertin et al. 1982). This mechanism allows for efficient transcription of proteins prior to virion assembly and budding from the host cell.

Iridoviridae were first characterized in 1954 by Claude Rivers, following his discovery of a large blue iridescent patch on the hind gut of a grub larvae (Webby et al. 1998). The Ranavirus genus is primarily tropic for warm freshwater fish and ornamental Asian fish (Mao, Wang et al. 1999; Paperna, Vilenkin et al. 2001; Goldberg, Coleman et al. 2003). Largemouth bass virus was first observed in 1995 following a major fish kill in the Santee-Cooper reservoir (Grizzle, Altinok et al. 2002). LMBV has a significant impact on the killing of sport-fish, either in fisheries or following fishing tournaments (Grizzle, Altinok et al. 2002).

Little is known of the epidemiology of LMBV (van Regenmortel et al. 2000). The major reservoir of this virus has not been determined nor is it known how the virus progresses among freshwater bodies. LMBV exerts a great economic effect by the killing of sport-fish, primarily Largemouth bass *Micropterus salmoides* (Scott et al. 2003).

Iridoviridae are poiklothermic and pathogenicity is temperature dependant (Webby et al. 1998). Fish kills usually follow fishing tournaments or a rapid increase in water temperature that results in stress. It is unknown whether LMBV is endemic in Largemouth Bass populations, due to lack of major fish kills witnessed since 2002 (Fries et al. 2006). Fish infected with Largemouth Bass Virus exhibit lesions on the swimbladder and a loss in equilibrium, characterized by corkscrew swimming. Pathogenic effects include darkening of pigmentation on the skin, inflammation and necrosis at the original site of infection, distended abdomen, lateral recumbency, and destruction of gill tissue which leads to decreased respiratory function. Histologically, exterior portions of the internal organs became fibrous in nature, however, the interior appear normal except for necrosis of the gastrointestinal mucosa (Zilberg, Grizzle et al. 2000).

The effects of temperature, pH and other environmental factors contribute greatly to the pathogenicity of Iridoviruses (Webby 1998; van Regenmortel 2000; Rojas, Richards et al. 2005). The pathogenicity of these viruses has been examined. High titers of this family of viruses yield relatively low mortality rates (Hedrick and McDowell et al. 1995).

RT-PCR methods of identification and quantification were developed specifically for LMBV and a PCR-based technique has been advanced as a method for the rapid detection of LMBV (Grizzle, Altinok et al. 2003). Additional methods for rapid detection of LMBV include the use of loop-mediated isothermal amplification (Caipang, Haraguchi et al. 2004). Serological assays are also available to identify the virus *in vivo* (Bollinger,

Mao et al. 1999). An ELISA assay is currently being developed to determine LMBV antibody titers in hatchery fish (Fries et al. 2006).

Inhibition of LMBV replication has been studied following treatment of LMBV infected cells the with antiviral agents ribivarin, guanidine hydrochloride and pokeweed antiviral protein (PAP) (Scott et al. 2003). At low multiplicities of infection, PAP was shown to provide 100% protection to cells from viral replication (Scott 2003). Ribivarin and guanidine hydrochloride were also effective in inhibiting the replication of LMBV, 47% and 34% respectively (Scott et al. 2003).

Inactivation of LMBV by chemical disinfectants including bleach, ethanol, and formalin has been determined (Scott 2003). Ethanol (70%), formalin (2%) and, bleach (0.07%) all reduced viral infectivity by 99.9% (Scott et al. 2003). The effects of UV radiation on LMBV infected cells was also determined. Virions exposed to UV radiation ($2x10^3$ $ergs \cdot s^{-1} \cdot cm^{-2}$) for 60 seconds resulted in a 50% decrease in infectivity (Scott et al. 2003). Temperature also had an effect on the infectivity of the virus. The infectivity of LMBV decreased greater than 50%, at 37°C for 5 days (Scott et al. 2003).

APOPTOSIS

Eukaryotic cells exhibit programmed cell death (apoptosis) following cellular damage which results in a loss of cell function. In higher organisms, apoptosis is beneficial for multiple reasons. Apoptosis causes destruction of cells which do not code for functional proteins or cells that have unchecked proliferation. During T and B-cell development apoptosis is used to eliminate cells which produce self-reactive proteins (Holm et al. 2004).

Apoptosis can be initiated by either external or internal cellular signals. External signals include the binding of fas ligand or tumor necrosis factor alpha (TNF-α) to their respective receptors. Internal signals such as the inhibition or destruction of p53, inhibition of protein kinase C, and genome destruction also trigger apoptosis. Other internal initiators of apoptosis include the destruction of mitochondria (Strauss et al. 2002).

CD8 T-cells release perforins and granzymes which cause target cells to undergo apoptotis. This results in destruction of organelles, DNA fragmentation, and death of mitochondria in the target cell. Cellular remains localize and form vesicles called apoptotic bodies. Lysosomes within the cell lyse causing the contents of apoptotic bodies to be degraded. Any remaining cellular portions are digested by macrophages (Strauss et al. 2002).

Ultraviolet radiation can cause the formation of thymine dimers in cell DNA which leads to p53 gene activation and apoptosis (Potratz, Mlody et al. 2005). Other external signals that induce apoptosis include cell membrane destruction or exposure to chemicals such as staurosporine. Apoptosis may also be induced through the lack of binding of an apoptosis inhibitor i.e., failure of leptin to bind to neutrophils (Bruno, Conus et al. 2005).

Annexins are a group of proteins that bind phospholipids in the presence of calcium. During the early stages of apoptosis, apoptotic cells lose membrane phospholipid asymmetry. In living cells phosphotidylserine (PS) is transported to the inner plasma membrane leaflet by the Mg-ATP dependant enzyme aminophospholipid

translocase (Koopman, Reutelingsperger et al. 1994; Pigault, Follenius-Wund et al. 1994; Dachary-Prigent, Pasquet et al. 1995; Kuypers, Lewis et al. 1996).

Both external and internal intiators of apoptosis converge on and activate the caspase cascade (De Falco, Penta et al. 2005). The caspase cascade includes seven enzymes activated by the fasL pathway, the TNF pathway, Lck pathway or internal signals such as DNA degradation, that lead to apoptosis. FasL binds and activates fas which activates of FADD and procaspase-8. Following procaspase-8 initiation, initator caspases such as caspase-8 are activated which act upon mitochondria and, in turn, activate effector caspases which result in enhanced membrane permeability (Castedo M 2003; Lay S 2003; Misumi S 2004; Mori N 2004; Shih WL 2004; Ozegbe, Chernajovsky et al. 2005). Internal signals such as destruction of mitochondria activate caspase-9 which converges on caspase 3, 6, and 7, which initiate apoptosis (Chen, Texada et al. 2006).

VIRUS INDUCED APOPTOSIS

Viral infections may induce apoptosis in order to circumvent the host's immune response. Apoptotic bodies do not elicit inflammation which allows progeny virions time to mature (Strauss et al. 2002). Several viruses, including human immunodeficiency virus 1 (HIV-1), cowpox virus, and rabies virus have been shown to induce apoptosis (Moyer 1998; Lay 2003; Holm et al. 2004). Some viruses use apoptosis as a strategy to bud progeny virus without eliciting a host immune response (Strauss et al. 2002). HIV-1 circumvents the host immune response, by coding for a protein, released from infected cells, which induces apoptosis in T-cells (Holm et al. 2004). HIV-1 employs apoptosis to increase infectivity in mucosal surfaces. Viral proteins nef, tat and gp120 to induce

apoptosis in mucosa (Acheampong, Parveen et al. 2005). In some cases viral pathogenicity is increased as a result of apoptosis. Apoptosis during Coronavirus infection aids in the destruction of host cells (Yan et al. 2004). Epstein Barr virus induces apoptosis re-establishing infection in B-cells prior to the end of its latent cycle, which allows the virus to re-establish infection without interference from the host immune system (Fukuda and Longnecker et al. 2005).

Hepatitis B virus and herpesviruses, induce apoptosis through *de novo* synthesis of proteins, which activate fas ligand (Hasham 2004; Yoo et al. 2004). Herpesvirus saimiri Tip protein initiates the Lck cascade (Hasham et al. 2004). Hepatitis B virus protein X binds fas (Hasham 2004; Yoo et al. 2004). HIV-1 tat and gp120 proteins induce apoptosis in oral keratinocytes by activating the fas pathways (Acheampong, Parveen et al. 2005).

Some viruses induce apoptosis in the absence of *de* novo protein synthesis. Influenza A and B structural proteins NS1 present in parental virions induce host cells to enter apoptosis (Schultz-Cherry et al. 1998).

The induction of apoptosis is influenced by the presence of Zn^{2+} and Cd^{2+} ions in human T-cell leukemia virus (HTLV) infected cells (Kasai et al. 2004). In the presence of Zn^{2+}, HTLV Tax and Jun proteins induce apoptosis (Kasai et al. 2004). In the presence of Cd^{2+} ions, although Tax and Jun proteins are present apoptosis does not occur (Kasai et al. 2004).

TT virus (TTV) synthesizes a protein specific the for induction of apoptosis in hepatocellular carcinoma cells (HCC) (Kooistra et al. 2004). TTV-derived apoptosis-inducing protein (TAIP) induces apoptosis in HCC cells but not in other cell lines

(Kooistra et al. 2004). Thus, TAIP aids in the pathogenicity of TTV infection associated with hepatitis (Kooistra et al 2004). Other cellular proteins have been shown to aid in the efficiency by which viruses induce apoptosis in host cells. Heat shock protein (Hsp 60) has been shown to increase the efficiency of Hepatitis B virus X protein and cause an increase in cells that enter apoptosis (Tanaka et al 2004). Hsp 60 is thought to act as a chaperon for HBV X protein which increases both the efficiency of apoptotic induction and the infectivity of HBV (Tanaka et al 2004).

Some members of the family Iridoviridae have the ability to inhibit apoptosis, such as Frog Virus-3 (FV-3) and Invertebrate Iridescent Virus-6 (IIV-6). FV-3 encodes a caspase recruitment domain CARD containing protein (vCOP), which may modulate the activation of caspases and thereby control apoptosis and the ensuing inflammatory response (Williams et al 2005). IIV-6 encodes for a protein that is an inhibitor of apoptosis protein (IAP), which delays apoptosis and allows the virus to replicate (Williams et al 2005). Inhibitors of apoptosis proteins (IAP), are characterized by a Zinc/RING finger and BIR (baculovirus inhibitor repeat) domains (Williams et al 2005). There are three different genes of the IAP protein in the IIV-6 genome and all differ greatly with that of other ranaviruses (Williams et al 2005).

Ranaviruses encode for other proteins that allow them the ability to evade or control the host immune system, such as a TNF receptor-like homolog (Williams et al 2005). This protein has the ability to block the external signal for apoptosis that is initiated by TNF binding (Williams et al 2005). Additional proteins that are encoded for immune evasion purposes include Bak-like, TRAF2-like proteins, which inhibit

mitochondrial apoptotic effects and expression of TNFά respectively (Oakes 2004; Kanazawa and Kudo 2005; Williams 2005).

ULTRAVIOLET RADIATION

Ultraviolet radiation is a portion of the electromagnetic spectrum within the wavelengths: (UVA) 400-320nm, (UVB) 320-290nm, and (UVC) 290-200nm. The spectrum between UVA and UVB (320-340nm) is the most photobiologically active (Diffey et al 1991).

Purines and pyrimidines absorb UV radiation at wavelengths 260-265nm (UVC). The major products of UVC radiation on DNA are pyrimidine derivatives. These products are cyclo-butyl type dimers (pyrimidine dimers), pyrimidine adducts, "spore photoproducts", pyrimidine hydrates and DNA-protein crosslinks (Diffey et al 1991). Of these products thymine-thymine dimers are the most commonly known, and lead to errors in DNA replication and frameshift mutations.

Since the 1920's research has been conducted demonstrating the ability of UV radiation to destroy microorganisms. UV radiation acts upon bacteria by accumulating pyrimidine dimers that bacteria cannot repair, which in turn encode for non-functional proteins. UVC has the same inhibitory effect upon viral infectivity (Diffey et al 1991). UVC radiation has been shown to inactivate LMBV (Scott et al2003).

VIROPEXIS

Viropexis is a pinocytotic process by which cells take up extracellular virus. Viropexis allows virions to pass through the cell membrane. Frog virus 3, has been shown to enter susceptible host cells by viropexis (Webby 1998; van Regenmortel 2000). During viropexis: clathrin binds to a portion of the cellular membrane which is in contact with the virion. Envagination occurs forming a pocket which contains the virions. The pocket then becomes encircled with clathrin and forms a small vesicle. Clathrin then dissociates with the vesicle and resets for new vesicle formation (Webby 1998; van Regenmortel 2000).

Viropexis can be subdivided into macropinocytosis (0.5-5 micron) and micropinocytosis, (<0.1 micron) based upon the size of the vesicle produced (Kruth et al 2005). Micropinocytosis may be inhibited by the use of chemicals such as nystatin or filipin (Kruth et al 2005). LY 294,002, a phosphatidylinositol 3-kinase inhibitor, has been shown to inhibit macropinocytosis without inhibiting micropinocytosis (Lee, Liao et al. 2005). LY 294,002 may prevent viropexis of LMBV.

POKEWEED ANTIVIRAL PROTEIN

Pokeweed antiviral protein (PAP) is derived from cell walls of the pokeweed plant *Phytolacca americana* (Bonness et al 1992). The antiviral activity of pokeweed was first observed in 1925 which culminated in the discovery of a specific antiviral agent (Bonness et al 1992). PAP has a broad spectrum of antiviral activity, and is effective against both DNA and RNA containing viruses such as human immunodeficiency virus-

1, herpes simplex virus, and poliovirus (Aron and Irvin 1980; Lee, Crowell et al. 1990; Zarling, Moran et al. 1990).

PAP was identified as a ribosome-inactivating protein (RIP) in 1973 (Obrig et al 1973). RIPs are a group of proteins derived from a wide variety of plants that have the ability to inactivate ribosomes through an enzymatic mechanism (Endo et al 1988). RIPs inhibit ribosomal function by cleaving adenine or guanine at A4234 or G4323 in 28S rRNA (Endo et al 1987). PAP enters cells made permeable following viral infection and inhibits viral protein synthesis. At high concentration PAP is cytotoxic, however, at lower concentration PAP is not cytotoxic and has antiviral properties (Aron et al 1988). At 2 μM PAP has been shown to inhibit poliovirus and LMBV replication (Lee, Crowell et al. 1990).

XTT CELL VIABILITY ASSAY

XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) is a tetrazolium salt that is used for quantifying viable cells (Scott et al 2003). Upon addition to metabolically active cells, XTT molecules are converted to an orange water-soluble formazan salt by cellular mitochondrial dehydrogenase activity (Scott et al 2003). Formazan and XTT have different UV absorbance properties, which allow them to be quantified spectrophotometrically. Since metabolically active cells are essential for the conversion of XTT to formazan, the amount of formazan present is directly related to the amount of viable cells (Scott et al 2003). Cell viability assays using XTT are quick, safe, accurate, and sensitive to low numbers of viable cells. The XTT assay has many uses, including assessing the effect of cytotoxic agents on cell viability studying the affect of various

cellular growth factors on cells, and determining the infectivity of virus particles on cell monolayers (Scott et al 2003). Similar assays employ the tetrazolium salt MTT (3-(4,5-dimethyl-thiazol-2-yl0-2,5-diphenyltetrazolium bromide), which is also converted to a formazan salt in the presence of metabolically active cells (Scott et al 2003). However, MTT cell viability assays require the solublization of the formazan salt, since it is non-water-soluble molecule. The XTT cell viability assay was used to determine whether LMBV reinitiates apoptosis via destruction of the host cell mitochondria (Scott et al 2003).

The objective of this research is to investigate LMBV induced apoptosis in Bluegill Fry cells. The event and time post infection in the viral replication cycle during which apoptosis is initiated was determined. Infection of the cells with UV-inactivated virus, and Pokeweed Antiviral Protein will determine if attachment and viral protein synthesis is required for initiation of apoptosis. Determination of activation of caspases-8 and -9 was used to confirm virus induced external and internal induced apoptotic signals.

MATERIALS AND METHODS

Cell Culture. – Bluegill Fry cells (BF-2) were supplied by A.E. Woods State Fish Hatchery, San Marcos, TX. Cells were grown in Eagle Minimum Essential Medium (E-MEM) containing 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins CO), 1% non-essential amino acids, 0.24% $NaHCO_3$, 2mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO). Cells in 75-cm^2 tissue culture flasks (Sarstedt Inc., Newton, NC) were incubated in zip seal plastic bags at 30°C in a water-jacketed incubator (NuAire Inc., Plymouth, MN). Upon monolayer formation, cells were grown at room temperature and maintained in E-MEM. The BF-2 cells were passaged by enzymatic and physical methods as follows: monolayers were rinsed Earle's Balanced Salt Solution (EBSS; Sigma-Aldrich Chemical Co., St. Louis, MO) followed by the addition of 1% trypsin (Sigma-Aldrich Chemical Co., St. Louis, MO) to place the cells in suspension. Cell scrapers (VWR International Inc., Aurora, CO) were used to dissociate the cell monolayer. Fresh E-MEM was added to each flask at a volume of 50 ml, and the resulting cell solution split 1:2. Cells were incubated at 30°C in a water-jacketed incubator for 24 h or until monolayer formation. To maintain cell monolayers, growth medium was changed 24 h following cell passage and at least once every three to four weeks.

Cell Storage. –BF-2 monolayers (24 h) were harvested using a modified procedure for cell passage, as follows. After the cell monolayer was disassociated from the flask, 10 ml of fresh E-MEM was added and the cell suspension centrifuged at 200 x g for 10 min at 4°C. The supernatant was discarded and the cells resuspended in 2 ml of E-MEM containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific Co., Fair Lawn, NJ). Cell aliquots which contained 1 ml in cryogenic vials (Nalge Co., Rochester, NY) were stored directly in liquid nitrogen (-196°C; Locator 8, Barnstead/Thermolyne Corp., Dubuque, IA).

Virus. – Largemouth Bass Virus (LMBV) was obtained from A. E. Woods State Fish Hatchery, San Marcos, TX. LMBV stocks were prepared by the addition of virus to BF-2 cell monolayers in 75-cm^2 tissue culture flasks. Growth medium was removed from 24 h monolayers, and monolayers rinsed with 10 ml EBSS. LMBV at a titer of 10^6 TCID50/ml was used to infect the monolayers at an approximate multiplicity of infection (MOI) of 1-2. Infected cells were incubated for 90 min at 30°C for 48 h. Following incubation, infected cells were freeze-thawed 3x at -80°C. Cell lysates were removed from the flasks and centrifuged at 2,300 x g for 20 min at 4°C. The supernatant was sterile filtered (0.45 μm) and dispensed in 1.5 ml aliquots. The virus samples, which contained approximately 10^8 TCID50/ml, were stored at -80°C and rapidly thawed in a 35°C water bath just prior to use.

Tissue Culture Infectious Dose 50% Assay. – Virus was rapidly thawed at 35°C, and serially diluted 1:10 in 96-well plates in sextuplet, at a final volume of 90 μl. Plates, which contained diluted virus samples, were placed at 4°C during preparation of the cell suspension. A BF-2 cell suspension was prepared as previously described and

cell concentration was determined using a hemacytometer (Fisher Scientific Co., Fair Lawn, NJ) following a 1:5 dilution of the cell suspension in trypan blue (1:6 solution of 0.4% trypan blue in 0.85% saline; Allied Chemical, Morristown, NJ). The cell suspension was adjusted to 5.5 x 10^5 cells/ml in E-MEM and added (90 µl) to the diluted virus samples. Tissue culture plates, which contained viral dilutions and cell solution, were agitated to ensure proper distribution of cells in suspension, and incubated at 30°C for 5 d. After incubation, the monolayers were observed microscopically for cytopathic effect. The virus titer was determined using the Spearman-Kärber method (Mahy and Kangro 1996).

Apoptosis. - BF-2 cells at 1x10^5 cells/ml were seeded in a 24 well tissue culture plate and grown to a confluent monolayer. BF-2 monolayers were infected with LMBV with an MOI of 15, in duplicate assayed for apoptosis at 12 h intervals. Positive controls include untreated BF-2 cells and, BF-2 cells that were induced into apoptosis with staurosporine (1mg/ml cells) for 2 h. Negative controls include addition of NaOH to increase the pH to 10 and induce necrosis. Samples were stained using an Annexin V-Cy3 Apoptosis Detection Kit (Sigma-Aldrich Chemical Co., St. Louis, MO), visualized and recorded with an Olympus CKX41 epifluorescent microscope and digital camera.

Determination of Activation of Caspases. - BF-2 cells were grown as monolayers in 96-well plates and infected with LMBV at a MOI of 15. Infected cell monolayers were incubated for 24 h at 30°C. Controls included untreated and apoptotically induced monolayers with 1µg/ml staurosporine. Monolayers were tested using an FLICA Poly-Caspase detection kit (Immunochemistry, Bloomington, MN) and visualized using epifluorescent microscopy.

Time of Apoptosis. – BF-2 monolayers were grown in 24 well tissue culture plates. BF-2 cells were infected with both infectious and UV inactivated LMBV at an MOI of 15. Aliquots of infected BF-2 cells were tested for apoptosis and quantified using the FLICA Poly-Caspase detection kit (Immunochemistry, Bloomington, MN) every 6 h up to 24 h p.i. Infected cells were visualized using epifluorescent microscopy.

Effect of UV-inactivated Virus. - LMBV (TCID $10^{6.5}$) were exposed to UV (253.7nm), ($2x10^3$ ergs·s^{-1}·cm^{-2}) light for 60 s and inactivated viral samples stored at 4°C. Aliquots of UV inactivated virus were used to infect BF-2 monolayers at MOI 15. Infected BF-2 monolayers were incubated for 24 h at 30°C and assayed for apoptosis using a FLICA Poly-Caspase detection kit (Immunochemistry, Bloomington, MN) and visualized using epifluorescent microscopy.

Effect of Inhibition of Viropexis. - BF-2 monolayers were treated with 50 μM LY 294,002 for 1 h prior to infection with a 1 ml aliquot of LMBV ($TCID_{50}$ 10^9). Monolayers were incubated for 5 days at 30°C and observed for CPE. BF-2 monolayers (1×10^5 cells/ml) were treated with 50 μM LY 294,002 in 24-well tissue culture plate. The plate including controls were incubated for 1 h at 30°C, following infection with LMBV at an MOI of 15. Following infection monolayers were incubated at 30°C for 24 h. After incubation for 6, 12, 18, and 24 h the samples were assayed for apoptosis using the Annexin V kit (Sigma-Aldrich Chemical Co., St. Louis, MO) and FLICA polycaspase detection kit (Immunochemistry, Bloomington, MN).

Confirmation of Penetration. - LMBV was used to infect cells and 50 μM LY 294,002 was added. The BF-2 cells were incubated for 24 h at 30°C. After incubation the monolayers were tested for the presence of apoptosis with the Annexin V kit (Sigma-

Aldrich Chemical Co., St. Louis, MO) and FLICA polycaspase detection kit (Immunochemistry, Bloomington, MN).

Effect of Inhibition of Viral Protein Synthesis. - BF-2 cell monolayers were infected with LMBV (MOI 15), allowed to attach for 1 h at 30°C. Following attachment, 2.5μM PAP was added to infected cell monolayers and incubated at 30°C for 24 h. Infected cell monolayers were harvested and assayed for apoptosic cells with a FLICA Polycaspase detection kit (Immunochemistry, Bloomington, MN) and visualized using epifluorescent microscopy.

Time of Activation of Intrinsic and Extrinsic Caspases. – Cell monolayers were infected with LMBV (MOI 15), cells were incubated at 30°C and harvested at 6, 12 and 18 h post infection. Cells were assayed using FLICA Caspase-8 (Immunochemistry, Bloomington, MN) and FLICA Caspase-9 detection kits (Immunochemistry, Bloomington, MN) and visualized using epifluorescent microscopy.

Determination of Mitochondrial Destruction. – BF-2 cells were seeded into a 96-well tissue culture at a concentration of $1x10^6$ cells/ml and incubated overnight. Cells were infected with LMBV (MOI 15) for 1 h. 50μL of XTT labeling mixture (Roche, Indianapolis, IN) was added to the monolayers, and the cells were incubated at 30°C for 6, 12, and 18 h. Cells were harvested and absorbance measured at 490nm using a Bio-Tek automated microplate reader (Bio-Tek, Winooski, VT).

RESULTS

Induction of Apoptosis by Largemouth Bass Virus. –BF-2 cell monolayers were infected at a MOI of 15 TCID50/ cell and apoptosis determined at 24 h p.i., following staining with Annexin V-Cy3. Cells were visualized with either epifluorescent microscopy (Fig 1 A, B & E) or scanning laser confocal microscopy (Fig 1 F). Cells that have intact membranes appear green, and cells with permeable membranes stain red. Confocal images (Fig 1 F) were stacked, overlaying green and red images. The results demonstrate apoptosis occurs in Largemouth bass virus (LMBV) infected BF-2 cells.

To determine if LMBV infection induces apoptosis via the activation of cellular caspase cascades, BF-2 cell monolayers were infected with LMBV and monolayers assayed following staining with SR-VAD-FMK (FLICA) which detects activation of caspases (Fig 1C & D). FLICA binds to a highly conserved portion of all activated caspases and following attachment SR-VAD-FMK cleaves sulforhodamine which yields red fluorescence. The data show LMBV infected cell monolayers were positive for active caspases at 24 h p.i., and indicate LMBV induces apoptosis by the activation of cellular caspases.

Time of Apoptosis. – To determine the time apoptosis is induced p.i., cell monolayers were infected with LMBV at a MOI of 15 TCID50/cell, harvested at 6, 12, 18, and 24 h p.i., and assayed for apoptosis following staining with Annexin V-Cy3 (Fig

5). Apoptosis was observed at 12 h p.i. but not at 6 h p.i. The data indicate LMBV induced apoptosis occurs between 6 and 12 h p.i.

Effect of Inhibition of Viral Protein Synthesis. – To determine the effect of inhibition of viral protein synthesis on apoptosis in LMBV infected cells, monolayers were infected with LMBV at a MOI of 15 TCID50 /cell for 24 h in the presence of pokeweed antiviral protein and assayed for apoptosis with Annexin V-Cy3 (Fig 2 A, B & E) and FLICA (Fig 2 C & D). Cells were visualized with either epifluorescent microscopy (Fig 2 A, B & E) or scanning laser confocal microscopy (Fig 2 F). The results demonstrate at 24 h p.i. infected monolayers were both apoptotic, and positive for active caspases. The data suggest that viral protein synthesis is not required for either the induction of apoptosis or activation of cellular caspase cascades.

Effect of Infection with UV-inactivated Virus on Apoptosis. – Cells were infected with UV-inactivated LMBV at a MOI of 15 TCID50/cell and harvested at 6, 12, 18, and 24 h p.i. Infected cells were assayed using Annexin V-Cy3 (Fig 3). The data show that infected BF-2 monolayers were apoptotic at 6 h p.i., and indicate UV-inactivated virus can initiate apoptosis early in the infection cycle. The results suggest an early viral event prior to viral protein synthesis such as: viral attachment, entry or uncoating triggers cellular apoptotic pathways.

Effect of Inhibition of Phosphatidylinositol 3-Kinase. - To determine the effect of the inhibition of cell signaling on the induction of apoptosis, BF-2 monolayers were

infected with LMBV in the presence of 50 μM LY 294,002 an inhibitor of phosphatidylinositol 3-kinase (PI3K). Infected cell monolayers were harvested at 6, 12, 18, and 24 h p.i., and stained with either Annexin V-Cy3 (Fig 4 A, B & E) or FLICA (Fig 4 C, D & F). In the presence of LY 294,002, apoptosis in LMBV infected BF-2 cells was not observed and activation of caspase cascades was not detected. The data indicate that cell signal transduction is necessary for LMBV induced apoptosis and suggest the virus activates cell caspase cascades through the activation of an external signal.

Activation of Caspase-8 and Caspase-9. –To determine if either caspase-8 or -9 is activated following LMBV infection, and if viral protein synthesis is required to induce apoptosis, cells were infected with LMBV at a MOI of 15 TCID50/cell, and harvested at 6, 12, 18, and 24 h, p.i. both in the presence and absence of PAP. Cells were assayed for caspase-8 and -9 with caspase-8 and -9 specific FLICA (Fig 7 & 8). Infected cells were positive for caspase-8 activation both in the presence and absence of PAP at 6 h p.i., which indicates LMBV infection initiates caspase-8 activation early in the viral infection cycle, and that viral protein synthesis is not required to activate caspase-8. The data suggest that virus attachment initiates caspase-8 via the extrinsic pathway. LMBV infected monolayers were positive for active caspase-9 (Fig 8) at 18 h p.i., however, in the absence of protein synthesis, activation of caspase-9 was not detected. The data suggest that capase-8 and -9 are activated early and late, respectively, following infection and that viral protein synthesis is necessary to initiate caspase-9 but not caspase-8.

XTT Assay. –To determine the effect of LMBV infection on mitochondrial activity, infected cells were assayed for formazan dye cleavage by mitochondrial dehydrogenases at 6, 12, 18, and 24 h p.i. (Fig 9). The results demonstrate mitochondrial activity was decreased 75% at 12 h p.i.

DISCUSSION

All cells, vertebrate, invertebrate, eukaryote and prokaryote alike have the ability to self-destruct or become apoptotic. Apoptosis in higher organisms is a highly regulated process that involves the activation of at least 14 enzymes (Santic 2006). Viruses take over and control apoptosis as means to modulate host immune responses, and increase pathogenic effects that occur following infection (Holm GH 2004; Acheampong, Parveen et al. 2005). Iridoviridae such as Frog Virus-3 (FV-3), Invertebrate Iridescent Virus-6 (IIV-6), Chilo Iridescent Virus (CIV), and Lymphocystis Disease Virus (LDV) have been shown to induce apoptosis (D'Costa, Yao 2001; Williams et al. 2005). IIV-6 and FV-3 induce apoptosis via the activation and control of cellular caspases.

Largemouth Bass Virus (LMBV), an Iridoviridae, causes significant economic loss due to fish kills in hatcheries (Paperna, Vilenkin et al. 2001). The study of apoptosis in LMBV infected cells may result in the therapeutic treatment of infected fish. This study describes the effect pokeweed antiviral protein (PAP) on the activation of caspases 8 and 9 in LMBV infected Bluegill Fry (BF-2) cells.

The induction of apoptosis and caspase-8 by UV-inactivated LMBV suggests virus attachment triggers apoptosis via an external pathway and cellular apoptotic receptors (i.e. FasL, FADD or TNF-α) may act as receptors for the virus. If viropexis or uncoating of a capsid protein signaled apoptosis, one would expect activation of caspase 9 (internal pathway) to occur early in the infection cycle. Other viruses have been shown

to induce cellular apoptosis via external signals. UV-inactivated FV-3 and Enterovirus 70 induced apoptosis in fathead minnow cells and Human Chang's Conjuntival cells, respectively (Martin, Aubertin 1982; Chen, Texada et al. 2006). In addition, Enterovirus 71 has been reported to use the external Fas ligand receptors to initiate apoptosis (Chen, Shyu et al. 2006). Influenza virus capsid protein NS1 induces apoptosis in chicken embryo cells (Stacey Schultz-Cherry 1998; Lowy et al. 2003). Recently, Chilo Iridescent Virus (CIV) capsid proteins have been shown induce apoptosis in cotton bowl weevil cells (Bilimoria et al. 2006).

Pokeweed antiviral protein (PAP) has been shown to inhibit viral protein synthesis in poliovirus, herpes simplex virus and LMBV infected cells (Aron 1980; Scott et al. 2003). The activation of caspase-8 early in the infection cycle and in the absence of viral protein synthesis (Fig 7) supports the notion that virus attachment is sufficient to trigger apoptosis. The induction of the external apoptotic pathway was not dependant upon *de novo* viral protein synthesis and indicated apoptosis is triggered by an event prior to virus induced host macromolecular shutdown and virus protein synthesis. LY 294,002, a phosphotidylinositol-3 kinase (PK3) inhibitor, blocks the phosphorylation of caspases and inhibits apoptosis caused by activation of an external signal (Lee, Liao et al. 2005). Enfuvirtide, a PK3 inhibitor prevents Human Immunodeficiency Virus (HIV) replication by blocking viral fusion and entry through the inhibition of signal transduction (Lazzarin et al. 2005). LY 294,002, blocked LMBV induced apoptosis and caspase 8 activation which supports the notion virus attachment induces apoptosis via an external pathway (Fig 5).

Infectious LMBV was observed to delayed caspase 8 induced apoptosis which suggests the virus may synthesize a protein similar in function to the Inhibitor of Apoptosis Protein (IAP) expressed by IIV-6 and the FV-3 caspase recruitment protein (Weikert, Schrader 2005; Williams et al. 2005). This is an effective strategy which allows the virus sufficient time to complete a productive replication cycle.

LMBV infected cells were found to reinitiate apoptosis via caspase-9 late in the infection cycle and was dependant on viral protein synthesis. In addition, LMBV inhibited mitochondrial activity by 75% at 12 h p.i. The destruction of mitochondria and the cell genome has been reported to initiate the caspase-9 intrinsic pathway (Weikert, Schrader et al. 2005; Santic 2006). It has been postulated the continued synthesis of a cell protein may be required for the inhibition of the intrinsic apoptotic pathway (Santic 2006). One would expect caspase-9 to be induced early following infection in the presence of PAP if BF-2 cells constitutively synthesize an apoptotic inhibitor.

This is the first report of virus induced apoptosis in LMBV infected cells. The treatment of infected fish with PAP could be an effective means of controlling virus transmission.

APPENDIX

Figure 1: Effect of LMBV infection on BF-2 cells. BF-2 monolayers were infected with LMBV at a TCID50 of 15/cell for 24 h. Cells were stained with an Annexin V-Cy3 and FLICA. Cells were visualized with epifluorescent microscopy and scanning laser confocal microscopy at 600X. (A) Infected BF-2 cells, Annvexin V-Cy3, FITC filter. (B) Infected BF-2 cells, Annexin V-Cy3, Cy3 filter. (C) Infected cells, FLICA, DAPI filter. (D) Infected cells, FLICA, Cy3 filter. (E) Uninfected (control), Annexin V-Cy3 FITC filter. (F) Infected cells, Annexin V-Cy3, confocal microscopy.

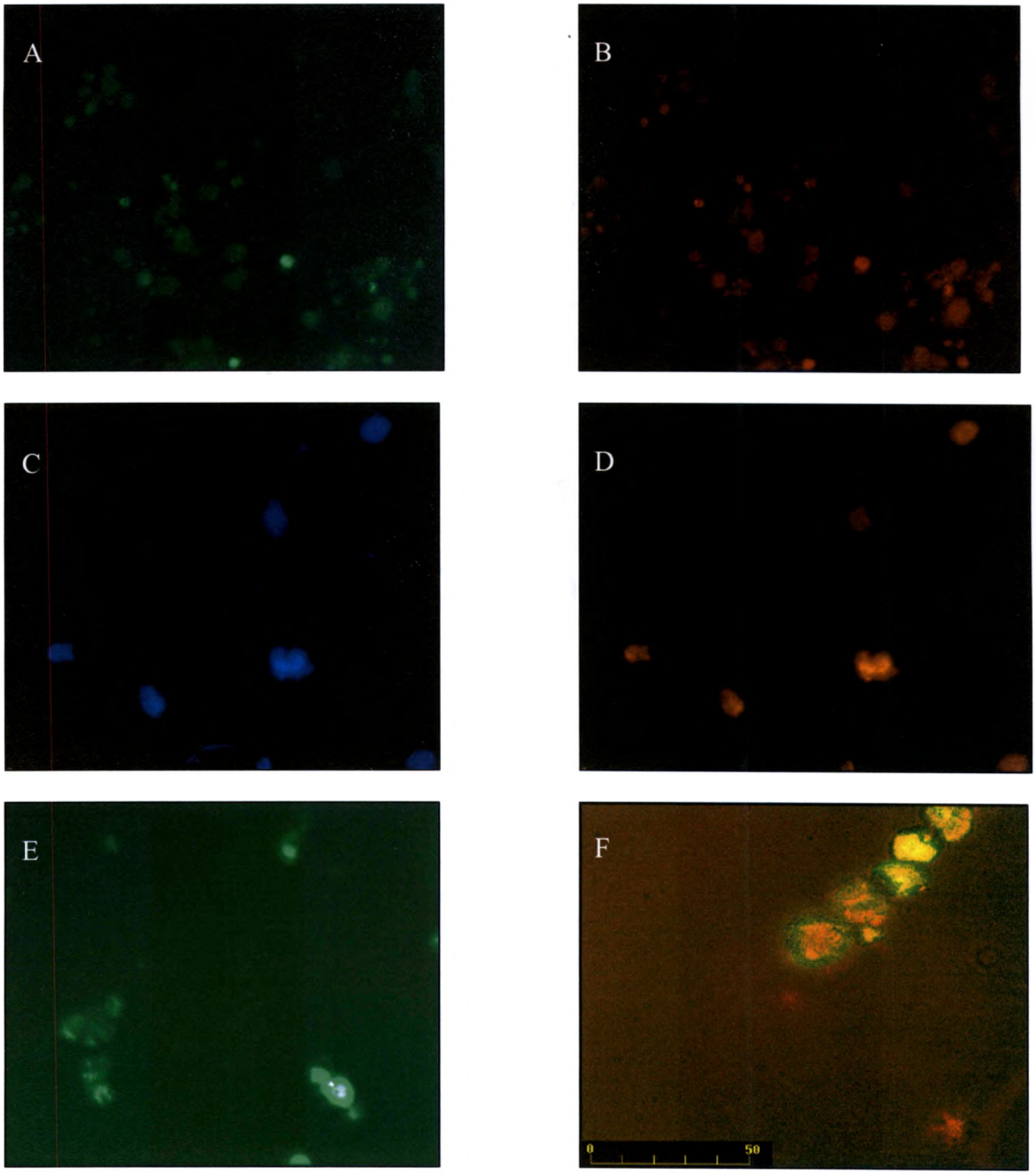
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Figure 2: Time of apoptosis in LMBV infected cells. BF-2 monolayers were infected with LMBV at a TCID50 of 15/cell for 6, 12, and 18 h. Cells were stained with Annexin V-Cy3 and viewed at 600X magnification. (A) Infected cells 6 h p.i., FITC filter. (B) Infected cells 6 h p.i., Cy3 filter. (C) Infected cells 12 h p.i., FITC filter. (D) Infected cells 12 h p.i., Cy3 filter. (E) Uninfected (control) cells 6 h, FITC filter. (F) Uninfected (control) cells 12 h, FITC filter.

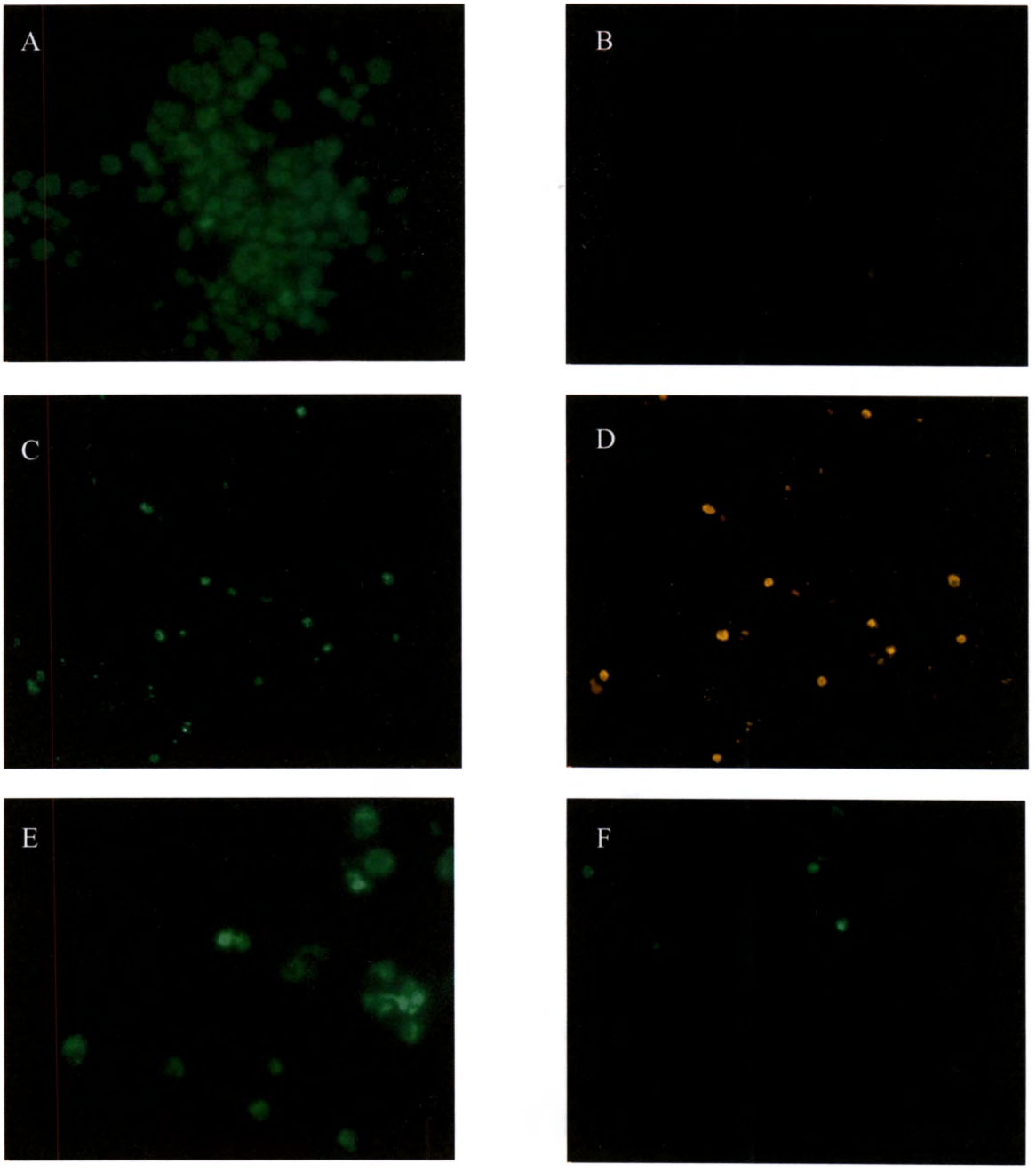
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Figure 3: Effect of inhibition of viral protein synthesis on apoptosis. BF-2 monolayers were infected with LMBV at a TCID50 of 15/cell in the presence of 2.5μM PAP, after 24 h. Monolayers were stained with an Annexin V-Cy3 and FLICA. Cells were visualized using epifluorescent microscopy and scanning laser confocal microscopy at 600x magnification. (A) Infected cells 24 h p.i., Annexin V-Cy3, FITC. (B) Infected cells 24 h p.i., Annexin V-Cy3, Cy3. (C) Infected cells 24 h p.i., FLICA, DAPI. (D) Infected cells 24 h p.i., FLICA, Cy3. (E) Uninfected (control) cells 24 h, Annexin V-Cy3, FITC filter. (F) Infected cells 24 h p.i., Annexin V-Cy3, confocal microscopy.

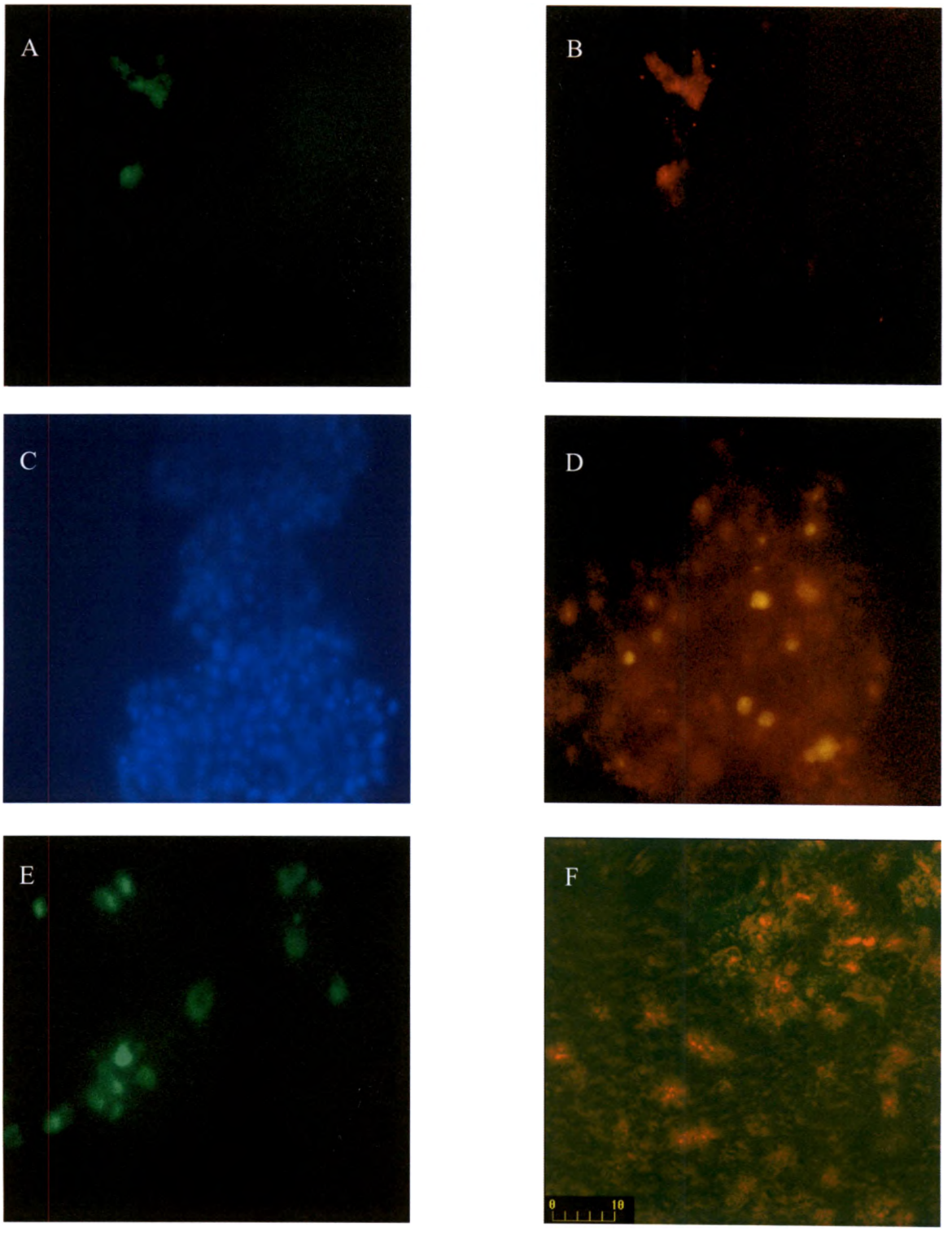
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Figure 4: Effect of UV-inactivated virus on apoptosis. BF-2 monolayers were infected with UV-inactivated LMBV with a TCID 50 of 15/cell for 6, 12,and 18 and 24 h. Cells were stained with an Annexin V-Cy3 and viewed at 600X magnification. (A) Infected cells 6 h p.i., FITC. (B) Infected cells 6 h p.i., Cy3 filter. (C) Infected cells 12 h p.i., FITC. (D) Infected cells 12 h p.i., Cy3. (E) Uninfected (control) cells at 6 h, FITC. (F) Uninfected (control) cells at 12 h, FITC. (G) Infected cells 24 h p.i., FITC. (H) Infected cells 24 h p.i., Cy3. (I) Uninfected (control) cells 24 h, FITC.

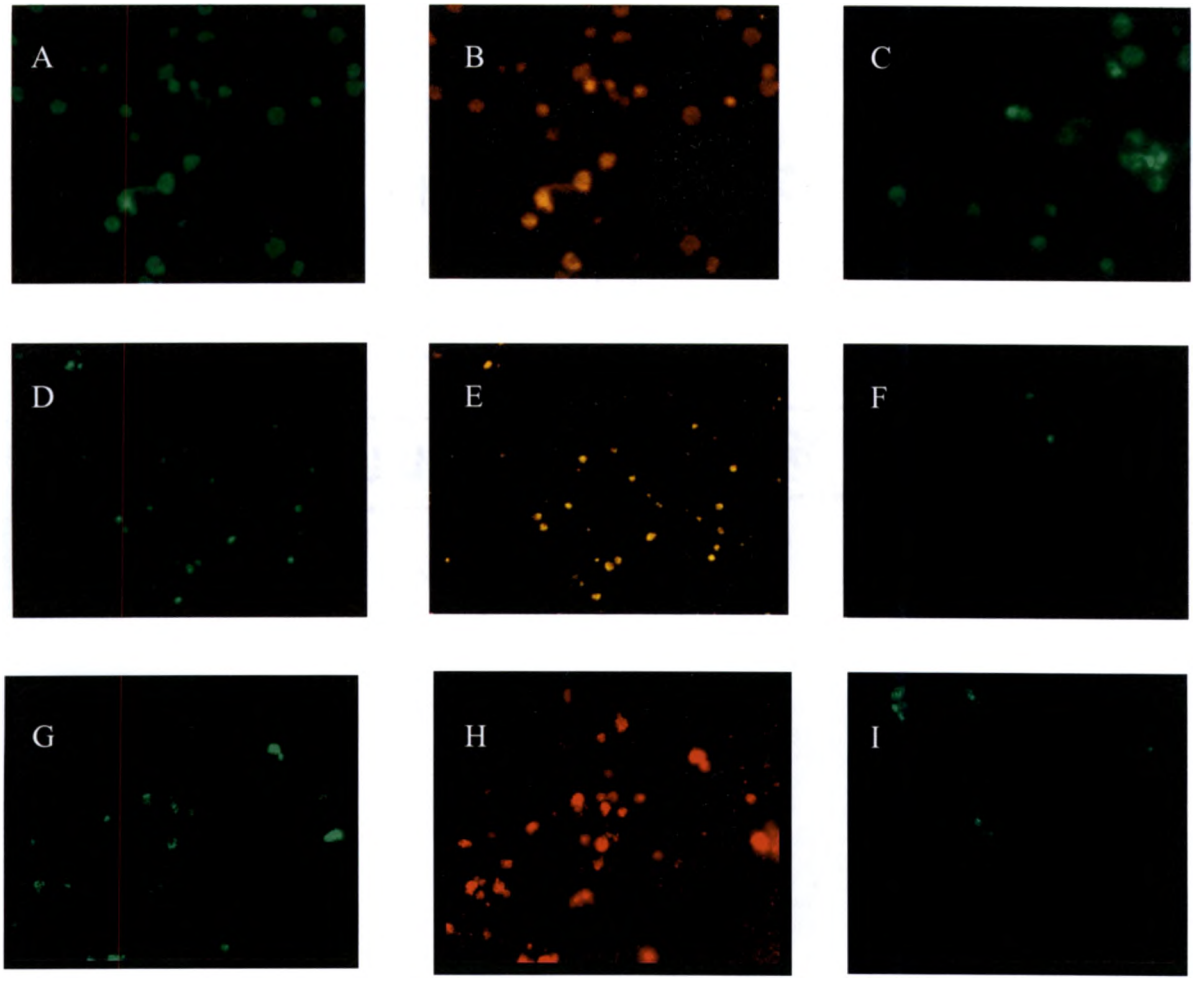
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Figure 5: Effect of inhibition of phosphatidylinositol 3-kinase on apoptosis. BF-2 cell monolayers were infected with LMBV TCID50 of 15/cell for 24 h in the presence of 50μM LY 294,002. Cells were stained Annexin V-Cy3 or FLICA, and viewed with epifluorescent microscopy at 600X magnification. (A) Infected cells, Annexin V-Cy3, FITC. (B) Infected cells, Annexin V-Cy3, Cy3. (C) Infected cells, FLICA, DAPI (D) Infected cells, FLICA, Cy3. (E) Uninfected cells 24 h, Annexin V-Cy3, FITC. (F) Uninfected cells 24 h, FLICA, DAPI.

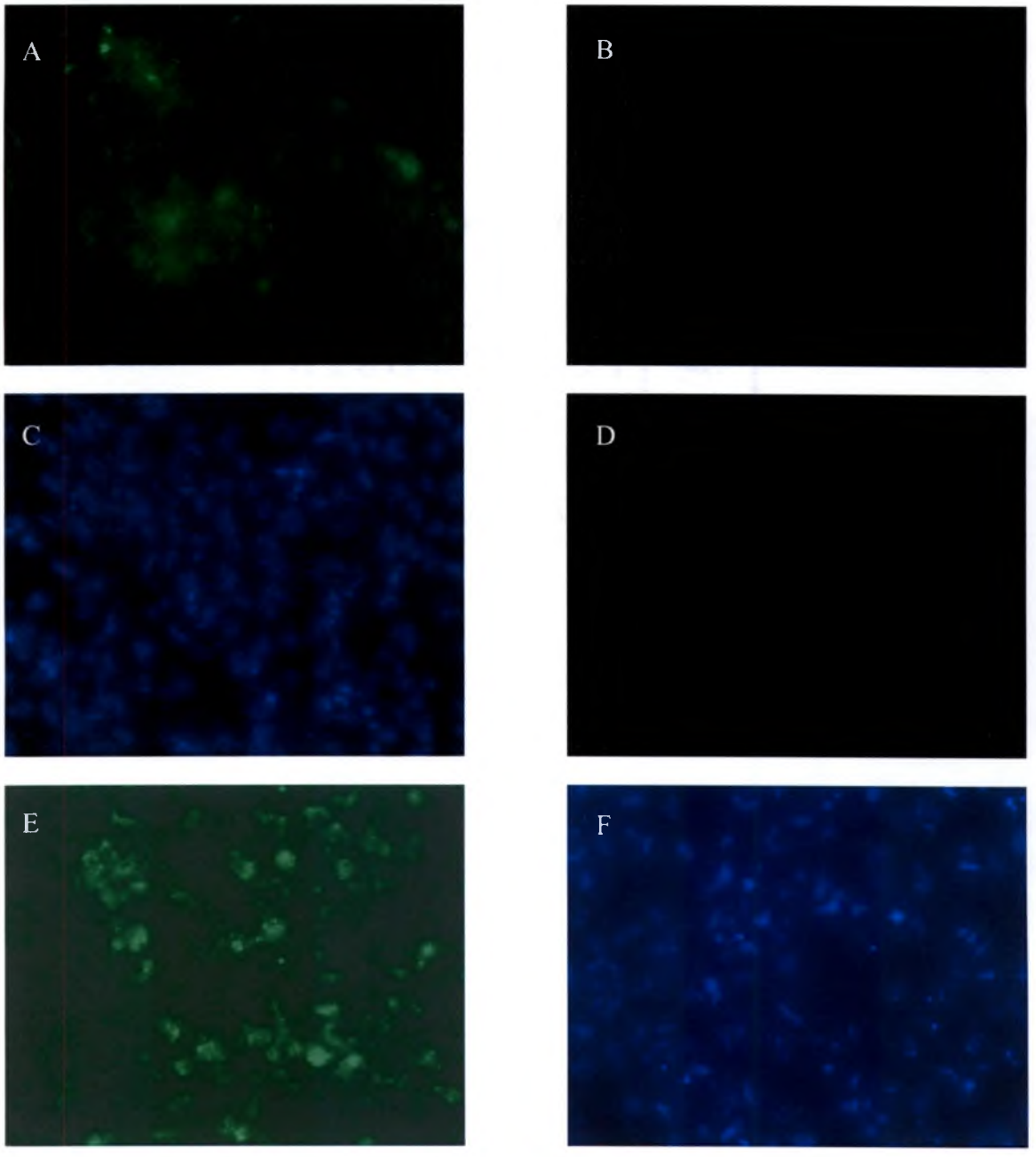
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Figure 6: Effect of LMBV infection on caspase-8 activation. BF-2 cell monolayers were with LMBV (TCID50 of 15/cell) in the presence and absence of 2.5μM PAP. Cells were assayed at 6, 12, 18, and 24 h p.i. for the activation of caspase-8, using FLICA caspase-8 detection and visualized with epifluorescent microscopy at 600X magnification. (A) Infected cells 6 h p.i., DAPI. (B) Infected cells 6 h p.i., FITC. (C) Infected cells in the presence of 2.5μM PAP, 6 h p.i. DAPI. (D) LMBV infected cells in the presence of 2.5μM PAP, 6 h p.i. FITC. (E) and (F) Uninfected (control) cells 6 h, DAPI.

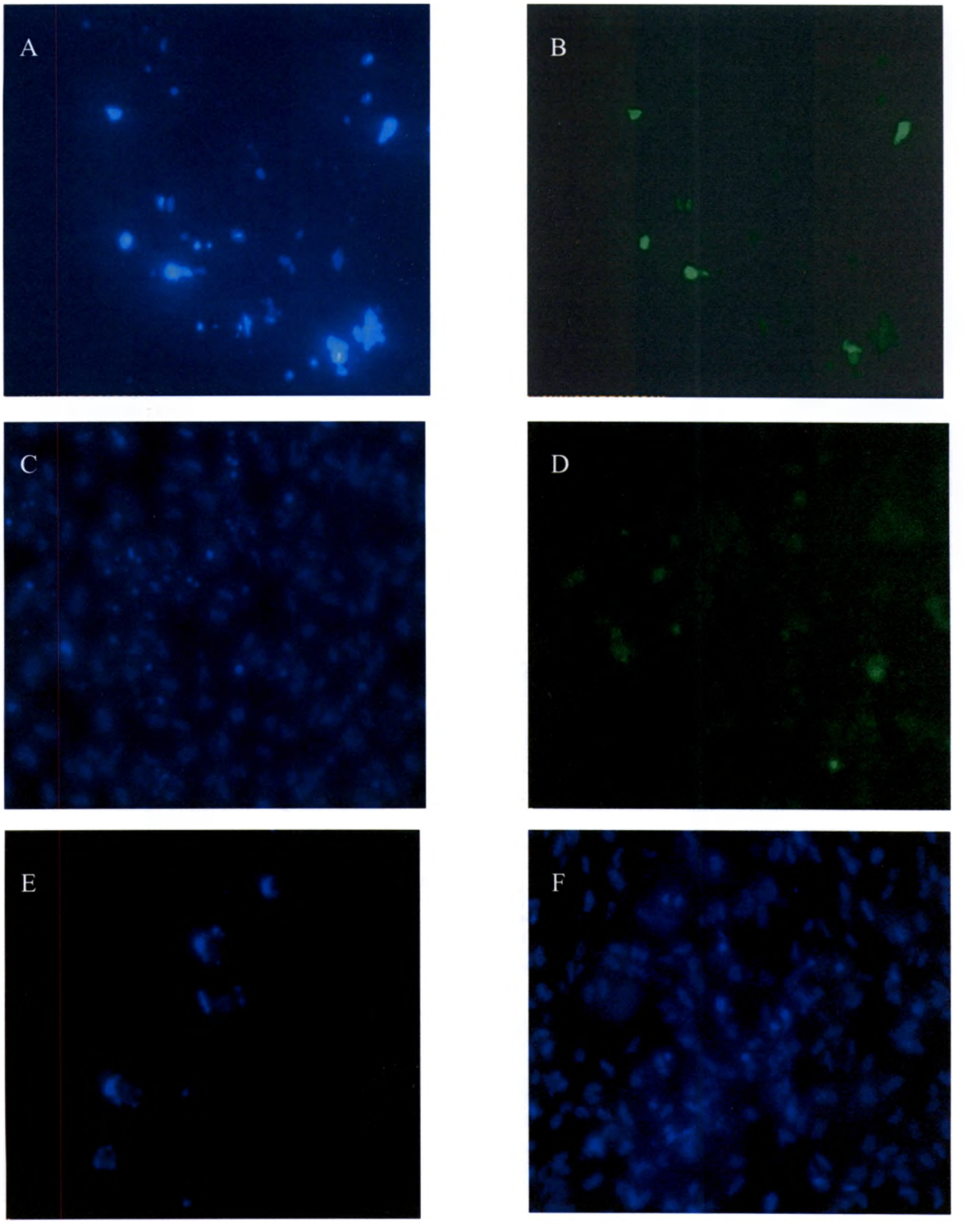
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Figure 7: Effect of LMBV infection on caspase-9 activation. BF-2 cell monolayers were with LMBV (TCID50 of 15/cell) in the presence and absence of 2.5μM PAP. Cells were assayed at 6, 12, 18, and 24 h p.i. for the activation of caspase-9, using FLICA caspase-9 detection and visualized with epifluorescent microscopy at 600X magnification. (A) Infected cells 6 h p.i., DAPI. (B) Infected cells 6 h p.i., FITC. (C) Infected cells in the presence of 2.5μM PAP, 6 h p.i. DAPI. (D) LMBV infected cells in the presence of 2.5μM PAP, 6 h p.i. FITC. (E) and (F) Uninfected (control) cells 6 h, DAPI.

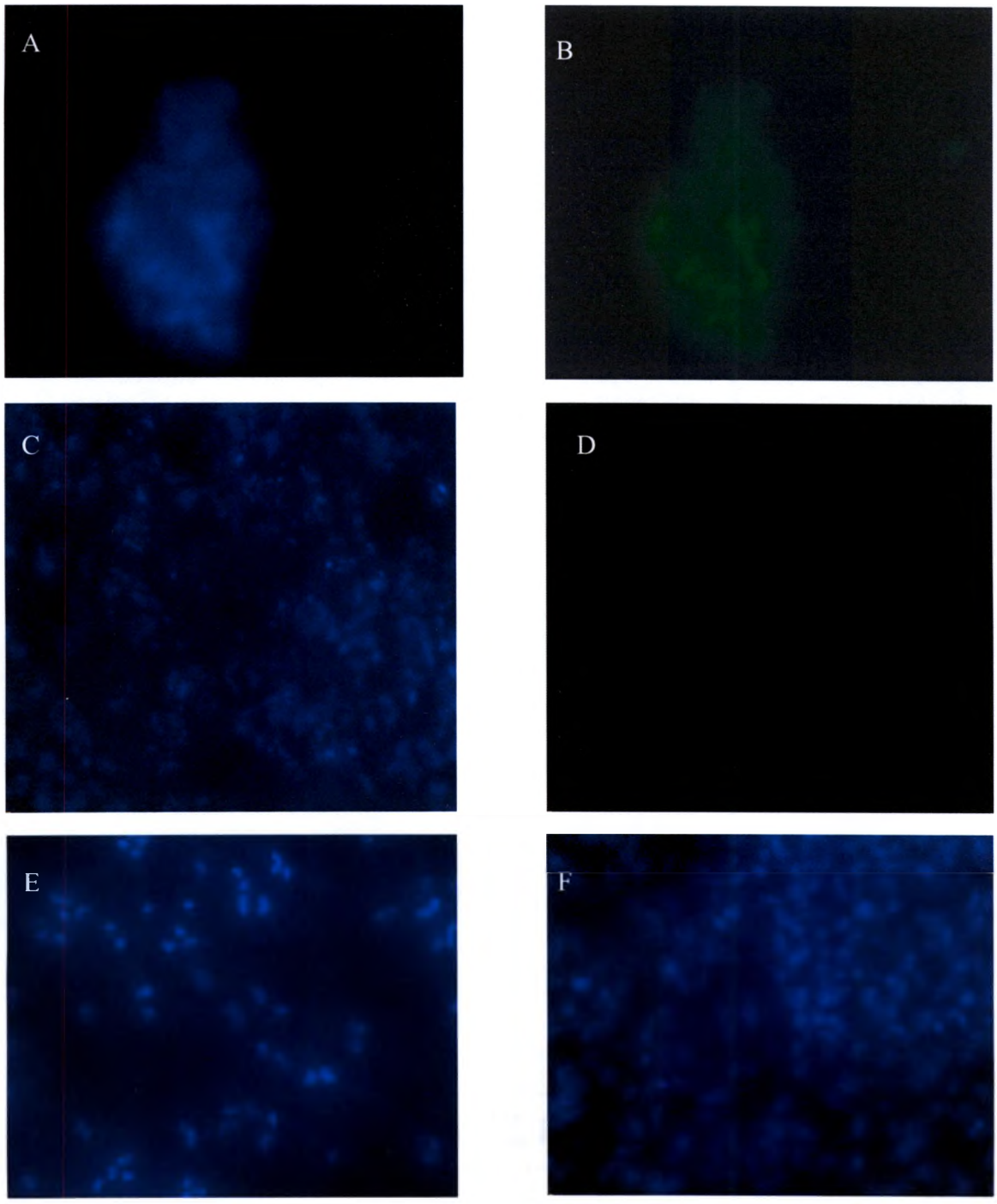
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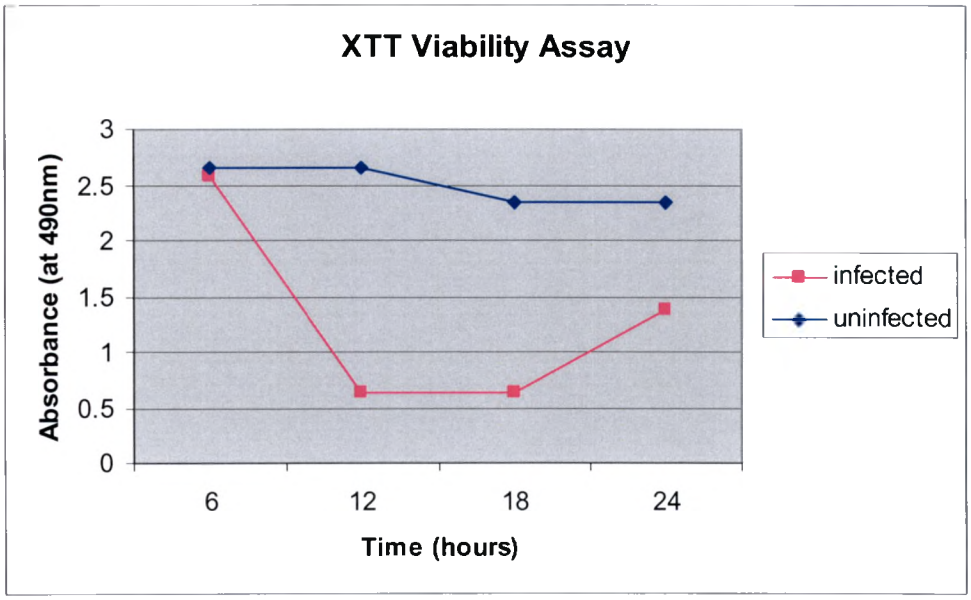
Figure 8: Effect of LMBV infection on mitochondrial activity. LMBV infected BF-2 cells were assayed at 6, 12, 18, and 24 h p.i., for mitochondrial dehydrogenase activity using an XTT cell viability assay. Absorbance was measured at 490 nm.


XTT Viability Assay
Absorbance (at 490nm)
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2.5
2
1.5
1
0.5
0
6
12
18
24
Time (hours)
infected
uninfected

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