Mechanisms of Induced Cell Death in Bluetongue Virus Challenged Human Cell Lines

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MECHANISMS OF INDUCED CELL DEATH IN BLUETONGUE VIRUS CHALLENGED HUMAN CELL LINES

by

Justin Darrel Hoopes

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biology

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2009
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ABSTRACT

Mechanisms of Induced Cell Death in Bluetongue Virus
Challenged Human Cell Lines

by

Justin Darrel Hoopes, Doctor of Philosophy
Utah State University, 2009

Major Professor: Dr. Joseph K.-K. Li
Department: Biology

Bluetongue virus (BTV) is a pathogenic member of the Reoviridae family. BTV does not cause disease in humans, but is capable of selectively infecting and killing certain transformed human cell lines. Understanding BTV’s oncotrophism may lead to new therapeutics for treating cancer. This study focused on the underlying mechanisms of BTV-induced cell death in carcinoma cell lines. It was our hypothesis that BTV infects human carcinoma transformed cells, produces mRNA and protein, induces a strong inflammatory response, induces mitogen activated protein kinase (MAPK)-based pro-apoptotic signaling, inhibits PKB-based signaling, and eventually kills the cell by inducing apoptosis.

Three carcinoma cell lines (A498, HEP-G2, and A549) were independently infected with BTV. In each cell line we determined: (1) cell viability over the course of infection; (2) BTV induced cytokine expression profile and magnitude of expression; (3) BTV viral RNA expression profile and magnitude of expression;
(4) BTV viral protein expression profile and magnitude of expression; (5) changes in BTV induced cell death and cytokine expression in cells with protein kinase B (PKB), p38-MAPK, extracellular receptor kinase (ERK-1/2), stress-activated protein kinase (SAPK-JNK), Src kinase, platelet-derived growth factor receptor (PDGFR) kinase, epidermal growth factor receptor (EDGFR) kinase, or Janus kinase (JAK) activity inhibited; (6) intracellular changes in PKB, p38-MAPK, ERK-1/2, and SAPK-JNK phosphorylation as a result of BTV infection; and (7) BTV-induced changes in tyrosine phosphorylation.

We determined that BTV infects and kills all three cell lines in a cell line dependent manner. Relative cell death between cell lines was proportional to cytokine expression, but inversely proportional to viral protein expression. Only tyrosine kinase inhibitors influenced BTV-induced cell death and cytokine expression. Both A498 and A549 cells constitutively expressed phosphorylated PKB and p38 MAPK, of which both were de-phosphorylated during BTV infection. Tyrosine phosphorylation remained active, with elevated tyrosine phosphorylation exclusively in infected cells.

We conclude that BTV-induced cell death and cytokine expression are a function of the cell’s response to infection and are directly related through intracellular signaling. These pathways are only partially poly I:C inducible, but include PKB and tyrosine kinase signaling.
ACKNOWLEDGMENTS

I would like to thank Dr. Joseph K.-K. Li for his guidance and support over the years both academically and personally. I also thankfully acknowledge my committee members, Dr. Gregory J. Podgorski, Dr. Dennis L. Welker, Dr. Scott A. Ensign, and Dr. Jeffery R. Broadbent, for their patience, guidance, and academic support. I would also like to thank Quansys Biosciences for their contribution of resources, equipment, and expertise in cytokine testing as well as Ryan Jackson and Luck Peterson for their assistance in process and analyzing the RT-PCR data.

I would like to particularly express gratitude to my wife, Kari Hoopes, for her continual patience and support through the years. I would not be the person I am without her.

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Justin Darrel Hoopes
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

_BTV in the News_

On 18 August, 2006, an outbreak of bluetongue virus (BTV) was reported in southern Netherlands; the first reported incidence of BTV anywhere in northern Europe (IAHD, 2006). Following confirmation in the Netherlands and the UK, an immediate ban on live exports of sheep, cattle and goats and "living products" including sperm, cells and embryos was established. In addition, transportation was restricted for all ruminants within 100 kilometers of the incident case (BBC News, 2006). Europe's International Animal Health Division, a division of the Department of Environment Food and Rural Affairs, categorizes BTV among its list of high impact diseases of major concern. Since the initial report on 18 August, outbreaks of BTV have been reported in France, Luxemborg, and Germany. The causative agent was determined to be serotype 8, the first incidence of this serotype anywhere in Europe (BBC News, 2006).

This report from the Netherlands is an example of the epizootic nature of the BTV and the economic impact this virus can have. Historically, the United States has experienced multiple outbreaks of BTV (Ackerman and Giroux, 2006). The economic impact of these outbreaks on international trade poses a greater threat to the livestock industry than the disease itself (McDill, 2002). Because of this, BTV was listed among the registered agents of the Agricultural Bioterrorism
Protection Act (2002). Economically, BTV is estimated to cost the United States $125 million annually in lost trade (Bram et al., 2002). Because of the recent epidemic, and resultant economic impact, BTV is generating greater interest by governments, the veterinary industry, and by researchers.

**Viral Phylogeny and Epidemiology**

BTV is classified in the *Reoviridae* family among the *Orbivirus* genus and is the most studied virus within this genus. The *Reoviridae* family encompasses nine distinct genera of segmented double-stranded RNA (dsRNA) viruses (Joklik, 1983). Members of the *Reoviridae* family are extremely diverse in tropism as well as pathology (Urbano and Urbano, 1994). Generally, members of the *Reoviridae* share a non-enveloped icosahedral capsid approximately 70 nm in diameter, a dsRNA genome composed of 10 to 12 segments, incomplete virion uncoating, non-polyadenylated 5' capped mRNA, and viral packaging of all proteins necessary for replication (Nibert et al., 1996). The *Orbivirus* genus includes 14 serogroups based on complement fixation (Borden et al., 1971), including viruses such as African horse sickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV), and BTV (Verwoerd et al., 1979). There is no common antigen to all serogroups though BTV identification is often hindered by cross-reactions with many of these antigenically related viruses (McDill, 2002). BTV is the prototype species and the most studied member of this genus (Verwoerd et al., 1979).

Five serotypes of BTV circulate within the United States, while there are 24 known serotypes worldwide. The 24 serotypes are classified into three major
groupings: North American, Australian, and African (Gould, 1988b). The endemic U.S. serotypes include 2, 10, 11, 13, and 17, though serotype 1 was isolated from a white-tail deer in Louisiana in Nov 2004 (Johnson et al., 2006). Gene homology studies suggest serotype 10 is the principle ancestor to the U.S. serotypes, while serotype 2 is the most distantly related (Hwang et al., 1993). Historically, natural genetic reassortment has been observed only within specific groups (Gould, 1988a).

BTV causes bluetongue disease in both domestic and wild ruminants. BTV primarily affects sheep and is most severe in European breeds (Gorman, 1990). White-tailed deer and antelope are also very susceptible. Cattle, goats, North American elk, elephants, buffalo, and dromedary camels may also be infected by BTV but usually display subclinical symptoms, potentially serving more as a reservoir for the virus (American Veterinary Medical Association, 2006). BTV is not contagious. It is only transmissible through an arthropod vector, most commonly biting midges in the genus Culicoides (Erasmus, 1975). As a result, disease outbreak and transmission are associated with Culicoides habitat and not animal habitat or behavior. While habitat and behavior have little impact, bluetongue disease severity is heavily dependent on the species, breed, age, health status, multiplicity of infection (MOI) and infecting viral serotype (Roy, 1992).

Disease Pathology

The hallmarks of bluetongue disease include the production of primary lesions (inflammation and ulceration of mucosal surfaces) around the mouth 7-10
days post infection (P.I.) and cyanosis of the tongue (Spruell, 1905). Discoloring of the tongue is due to oxygen deprivation caused both by inflammatory capillary constriction as well as disseminated intravascular coagulation caused by viral-induced endothelial damage (Howerth and Tyler, 1988). In sheep it causes soremuzzle, characterized by fever (up to 107.6 F) as well as depression, emaciation, and swelling of the muzzle, eyelids, and ears (Hardy and Price, 1952). A sheep displaying the symptoms of bluetongue disease is shown in Fig. 1-1. Severe inflammation can also compromise mobility and respiration. Fetal abortion usually occurs if ewes are infected during the first trimester of pregnancy. Observed mortality rates have been as high as 75% in some outbreaks (Manso-Ribeiro et al., 1957). If death does occur it is usually within 8 to 10 days as a result of pharyngeal lesions and/or heart failure (Mahrt and
Osburn, 1986). Surviving animals can often experience hair loss, sterility, cracked hooves and muzzle, as well as delayed growth (Jeggo et al., 1983).

**BTV Virion Structure**

BTV is a nonenveloped icosahedral virus with two capsid layers, arranged in a T3 structural morphology around the inner core (Els and Verwoerd, 1969). The total virus particle is around 68-70 nm wide (Martin and Zweerink, 1972). The genome of BTV consists of 10 dsRNA segments (Verwoerd, 1970; Verwoerd et al., 1970), ranging from 822 to 3954 base pairs in size, for a total genome of $1.9 \times 10^4$ base pairs (Verwoerd et al., 1970). The genome segment and size of each protein can be seen in Table 1-1. The names of each gene are derived from their electrophoretic motility, classified into large (L), medium (M), and small (S). Each gene encodes a specific protein, except for the smallest gene segment (S4), which encodes two proteins via an overlapping second reading frame (Mertens and Sangar, 1985). Of the 11 proteins, 7 are found within the structure of the virus, while 4 facilitate the replication cycle, but are not included within the viral particle itself. The exact functions of these non-structural proteins are unknown. A schematic of the virus can be seen in Fig. 1-2. The inner core is composed of viral proteins (VP)1, VP4, and VP6 closely associated with viral dsRNA. Surrounding this is the inner capsid composed of VP3 and VP7. The outer capsid is composed of VP2 and VP5 non-covalently associated with the inner capsid (Verwoerd et al., 1972; Huismans et al., 1987b).
### TABLE 1-1

Bluetongue Virus Gene Segments, Encoded Proteins, Location Within Virion, and Putative Function.

<table>
<thead>
<tr>
<th>Genome Segment</th>
<th>Base Pairs</th>
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<th>Function</th>
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<tr>
<td>L1</td>
<td>3954</td>
<td>VP1</td>
<td>Inner core</td>
<td>RNA polymerase/transcriptase</td>
</tr>
<tr>
<td>L2</td>
<td>2926</td>
<td>VP2</td>
<td>Outer shell</td>
<td>Structural – Adhesion</td>
</tr>
<tr>
<td>L3</td>
<td>2772</td>
<td>VP3</td>
<td>Core</td>
<td>Structural</td>
</tr>
<tr>
<td>M1</td>
<td>2011</td>
<td>VP4</td>
<td>Inner core</td>
<td>5'- mRNA capping enzyme</td>
</tr>
<tr>
<td>M2</td>
<td>1639</td>
<td>VP5</td>
<td>Outer shell</td>
<td>Structural – hemeagglutination</td>
</tr>
<tr>
<td>M3</td>
<td>1770</td>
<td>NS1</td>
<td>Non-structural</td>
<td>Virion transport</td>
</tr>
<tr>
<td>S1</td>
<td>1156</td>
<td>VP7</td>
<td>Core</td>
<td>Structural</td>
</tr>
<tr>
<td>S2</td>
<td>1123</td>
<td>NS2</td>
<td>Non-structural</td>
<td>mRNA binding</td>
</tr>
<tr>
<td>S3</td>
<td>1046</td>
<td>VP6</td>
<td>Inner core</td>
<td>RNA helicase</td>
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<td>S4</td>
<td>822</td>
<td>NS3/3A</td>
<td>Non-structural</td>
<td>Viral progeny release</td>
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FIG. 1-2. BTV virion structure. The virion is composed of 10 dsRNA segments packaged along with VP1, VP4, and VP6 within a VP3/VP7 capsid. The inner VP3/VP7 capsid is subsequently covered in a VP2/VP5 outer capsid. The virus is not enveloped. Each viral protein is indicated on the diagram by their respective number.
The largest viral protein is VP1 (Huang et al., 1995). Derived from the L1 gene, VP1 is a subcore component which functions as an RNA-dependent RNA polymerase, serving dual roles as transcriptase and replicase (Roy, 1992; Boyce et al., 2004). *In vitro* replicase activity shows little specificity for BTV genes, suggesting specificity is derived from its association with VP4 and VP6 (Boyce et al., 2004). After endocytosis and upon removal of the outer capsid layer, VP1 begins production of mRNA from within the core particle (Watanabe et al., 1968; Grimes et al., 1998). Following mRNA production, VP1 replicates new genomic dsRNA from previously synthesized mRNA by strand displacement (Roy, 1992).

VP2 and VP5, derived from the L2 and M2 genes respectively, constitute the outer capsid layer. VP2 represents the major variable epitope which defines serotype (Kahlon et al., 1983) and is the recognition marker responsible for endocytosis (Huijsmans and Erasmus, 1981). Purified VP2 displays hemagglutinin activity through binding to glycophorin A, a sialoglycoprotein component of erythrocytes (Hassan and Roy, 1999) suggesting VP2 may be responsible for transmission by Culicoides. At present, the cellular recognition region on VP2 has not been identified, though Hassan and Roy (1999) suggest the receptor is likely a glycoprotein. Gene sequences show that there are 16 conserved cysteine residues present (Huijsmans and Van Dijk, 1990), though VP2 does not appear to be covalently linked to VP5 (Verwoerd et al., 1972).

VP5 is more structurally conserved than VP2 (Mecham et al., 1986) likely due to VP5’s association with the core proteins (Verwoerd et al., 1972). VP5 is
sometimes referred to as GP5 because it is glycosylated with short unbranched carbohydrates (Yang and Li, 1993). VP5 can bind to different agglutinating lectins, probably contributing to the hemeagglutination properties of BTV. Its main function appears to be membrane permeabilization of the endosomal compartment facilitating release of uncoated virus into the cytoplasm (Hassan et al., 2001). VP5 shares structural features with class I fusion proteins of enveloped viruses (Forzan et al., 2004), which may give BTV its ability to associate with and permeabilize membranes. Extracellular treatment of mammalian cells with recombinant VP2 and VP5 together can result in apoptosis (Mortola et al., 2004), though the mechanism or function of this is currently unknown.

VP3 and VP7, derived from L3 and S1 genes respectively, together make up the inner capsid layer. The VP3 and VP7 sequences are highly conserved among all serotypes (Huismans and Erasmus, 1981). Both are very hydrophobic (Yu et al., 1988; Huismans and Van Dijk, 1990) and can assemble in an alternate host without other viral proteins present (French and Roy 1990; Kar et al., 2005). VP7 exists as a trimer which is inserted into an organized VP3 dimer matrix (Burroughs et al., 1995), as shown in Fig. 1-2. VP7 is the most abundant of BTV proteins found within the viral particle (Huismans et al., 1987b). The N-terminal region of VP7 has been shown to protrude to the surface of the outer capsid (Hyatt and Eaton, 1988), conveying the serogroup-specificity of BTV (Wang et al., 1996). Because of this, VP7 has generated great interest as a target for a universal BTV vaccine. It is the only protein for which the crystal structure is
known (Grimes et al., 1995). BTV can infect insect cells once the inner capsid is assembled (through VP7’s RGD tripeptide motif) but cannot infect mammalian cells until the outer capsid is assembled (Tan et al., 2001).

VP4 (M1 gene product) is a highly conserved inner core protein present at 5-10 copies per virion (Huismans and Van Dijk, 1990). VP4 together with VP1 attach to the underside of VP3 within the viral core in (Nason et al., 2004). VP4 has been shown to nonspecifically bind single stranded RNA (ssRNA) (Huang et al., 1993) via an essential leucine-zipper motif near the c-terminus of the protein (Ramadevi et al., 1998b). VP4 is also an NTP hydrolase (Ramadevi and Roy, 1998), RNA 5’-triphosphatase (Martinez-Costas et al., 1998), inorganic pyrophosphatase (Martinez-Costas et al., 1998), a type 1 and type 2 methylase (Ramadevi et al. 1998a), as well as a guanylyltransferase (Huang et al., 1993). In short, VP4 performs all of the reactions necessary for complete 5’ guanine capping of viral encoded messenger RNA (mRNA), prior to translation.

Gene S3 encodes VP6, the last of the three minor inner core proteins. Each virion carries approximately 36 copies of this protein (Huismans and VanDijk, 1990). VP6 has nucleoside triphosphatase, RNA binding, and helicase activity (Stauber et al., 1997). VP6 is highly conserved among serotypes and is the most polar of all the BTV proteins (Fukusho et al., 1989). ATP binding has been linked to the conserved AXXGXGK(110)V motif (Kar and Roy, 2003). VP6 contains multiple nucleic acid binding domains (Hayama and Li, 1994) and probably functions within the core virion as a dsRNA stabilizer (Roy, 1989; Roy et al., 1990) and during transcription as an RNA-helicase (Roy, 1996).
Gene M3 encodes non-structural protein, NS1. It contains 552 amino acids and is predicted to be 64 kDa in size (Urakawa and Roy, 1988). The M3 gene was originally designated the M2 gene. M2 is now designated to encode VP5. NS1 is the most abundantly produced viral protein (Huismans and Van Dijk, 1990). It is highly conserved among BTV serotypes, though it shows only around 50% sequence conservation with other Orbiviruses (Huismans and Cloete, 1987). Its function within the cell is unknown; however, within the viral inclusion body (VIB) it has been suggested to act as a molecular chaperone to prevent core particle assembly before VP1, 4, 6 and the genome are incorporated (Hewat et al., 1992). Upon entry into the cytoplasm, the NS1 protein will spontaneously polymerize into tubules (Huismans, 1979; Huismans and Els, 1979) between 50 and 68 nm in diameter with a 9 nm banding (Huismans and Els, 1979; Hewat et al., 1992). The tubules associate with the intermediate filaments of the cytoskeleton (Eaton et al., 1988). The presence of NS1 tubules is one of the most notable pathological conditions of BTV infection observed under a microscope (Lecatsas, 1968). The function of these tubules has not been established though they are probably involved in viral transport to and from VIBs (Eaton et al., 1988).

Encoded by the S2 gene, NS2 is produced abundantly within the cell. NS2 alone forms inclusion bodies in transfected insect cells (Thomas et al., 1990). Within inclusion bodies, NS2 binds ssRNA non-specifically, but does not bind to dsRNA (Huismans et al., 1987a; Uitenweerde et al., 1995). Three RNA binding domains have been identified in this protein (Fillmore et al., 2002). NS2’s
proposed role is to associate with newly formed mRNA to protect it from destruction prior to its encapsidation. NS2 may also be responsible for ensuring that only the 10 distinct mRNAs are condensed prior to dsRNA synthesis during genome replication and viral morphogenesis (Eaton et al., 1988; Lymperopoulos et al., 2006).

NS2 is the only known phosphorylated BTV protein (Huismans et al., 1987a). Phosphorylation occurs on two serine residues at positions 249 and 259 (Modrof et al., 2005). NS2 phosphorylation can occur in vitro (Huismans et al., 1987a) but unlike rotavirus NSP2, a functional homolog, NS2 does not autophosphorylate (Taraporewala et al., 2001). Instead, cellular kinases are necessary for phosphorylation. In addition, recombinant NS2 displays nucleotidyl phosphatase activity (Horscroft and Roy, 2000) as opposed to NSP2's NTPase only activity (Taraporewala et al., 2001). Phosphorylation of NS2 somewhat reduces affinity for ssRNA, but appears essential for the formation of VIBs (Modrof et al., 2005).

S4, the smallest gene encodes the NS3 and NS3a proteins. The gene contains two in phase open reading frames (ORFs); the primary ORF starting at base position 20 and the second ORF starting at base position 59 (Van Dijk and Huismans, 1988). The exact functions of either protein is unknown, but they have been shown to be associated with cellular membranes and hydropathy plots show two distinct hydrophobic membrane spanning domains at amino acids 118 to 141 and 162 to 182 (Huismans and Van Dijk, 1990). NS3/3a can be found naturally in both n-linked glycosylated and non-glycosylated forms (Wu et
Glycosylation occurs at an asparagine in position 150 on the protein (Bansal et al., 1998). Hyatt et al. (1993) suggested NS3 facilitates the release of virus by causing local lesions in the cell membrane. NS3 and NS3a also contain the characteristic P(T/S)AP and PPXY membrane binding motifs found at residus 7-10 on the p6 region of the human HIV-1 GAG protein (Garrus et al., 2001) and in membrane-associated proteins of filo-, rhabdo-, and oncoretroviruses (Craven et al., 1999; Harty et al., 2000). The presence of these motifs also suggest a role for NS3/3a in progeny release through recruiting cellular proteins. Wirblich et al. (2006) have recently shown NS3 to recruit Tsg101, a component of the ubiquitinization ESCRT-1 complex, though this motif, and that depletion of Tsg101 through RNA interference inhibits the release of BTV from infected cells.

NS3, through yeast two-hybrid studies, has also been shown to associate with cellular protein p11 (calpactin light chain) though an N-terminal domain (Beaton et al., 2002). This protein is part of the annexin II complex involved in exocytosis.

NS3 also binds VP2 though a c-terminal domain (Beaton et al., 2002), suggesting NS3 may bridge association between assembled viral particles and exocytosis machinery. NS3 has also recently been shown to have viroporin like properties (Han and Harty, 2004). Viroporins are a group of hydrophobic transmembrane proteins that can form hydrophilic pores in a lipid bilayer. The formation of these pores would allow release of progeny virus by direct penetration of the cell membrane. Even though the functions of NS3 have not been definitively stated, it is certain that NS3 plays an important role in the release of viral progeny.
Measuring BTV RNA and Protein Expression

The 10 genes described previously all play an essential role in replication and changes in their expression can affect replication rates and fitness. Various methods have been developed to measure BTV RNA and protein expression. One way BTV RNA can be detected and quantitated is through quantitative real time PCR (qRT-PCR) (Buccambusco, 2005). This process involves purifying total RNA from a sample, followed by reverse transcription of RNA to complimentary DNA (cDNA) and subsequent PCR. The PCR reaction takes place in the presence of Cyber Green, a flourescent intercalating dye. Primers specific to each viral gene lead to specific amplification of the target gene. Amplification of the target gene results in more Cyber Green intercalation leading to a higher fluorescent signal. This signal can be used to quantify the amount of each viral gene present in the sample.

Available methods for quantifying BTV protein are not as automated as RT-PCR. BTV viral protein is measured by Western blot. Western blotting is the process of probing for a specific protein using enzyme labeled antibodies-specific to the protein of interest. Sample is resolved by polyacrylamide gel electrophoresis then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane is then blocked to prevent non-specific binding and incubated with BTV specific antibody. Antibody binding is detected by the addition of the linked enzyme’s substrate.

When antibodies to specific BTV proteins are not commercially available, antisera is used instead. BTV antiserum reacts to the major antigenic epitopes
within the viral capsids, predominantly VP2 followed by VP7, VP5, and VP3, respectively.

**Viral Replication Cycle**

BTV is a fully infectious virus, meaning it carries all of the proteins necessary to begin replication. This is necessary as the genome is dsRNA which must be processed by specialized proteins. BTV primarily infects endothelial cells of the vascular wall, *in vivo*. Utilizing VP2 attachment protein, the virus is taken into clathrin coated endosomes by endocytosis (Sturzenbecker *et al.*, 1987). In tissue culture, adsorption usually occurs within 20 minutes P.I. (Howell *et al.*, 1967). Within the endosome, as the pH is lowered, VP2 is lost and VP5 binds to the endosomal membrane where it promotes viral penetration into the cytoplasm (Dales, 1973; Tyler *et al.*, 1985). Once the outer capsid has been removed, the RNA-dependent RNA polymerase (VP1) becomes activated (Van Dijk and Huismans, 1980). The core particle then binds cellular intermediate filaments (Eaton and Hyatt, 1989) and begins to produce and release mRNA into the cytoplasm (Bowne and Jochim, 1967). New mRNA is 5' guanidine capped (Furwichi and Shatkin, 1977) by VP4 (Huang *et al.*, 1993), but not polyadenylated. This mRNA is used for translation and later condensed by NS2 (Zweerink *et al.*, 1971) into viral inclusion bodies where it associates with VP3 dimers (Kar *et al.*, 2004). Once associated with VP3, mRNA is then converted into viral genomic dsRNA (Acs *et al.*, 1971).

When all the necessary components are gathered, morphogenesis of the inner core takes place within the VIB (Morgan and Zweerink, 1975; Zarbl *et al.*, 1987).
After exiting the VIB, VP2, and VP5 are added to form the outer capsid. From there, virulent particles may either stay in the cell (Howell and Verwoerd, 1971) or be released through the aid of NS3/3a. Release can occur through direct penetration of the cell membrane (Hyatt et al., 1993) or though budding (Bowne and Ritchie, 1970; Foster and Jones, 1979). Both events have been seen, though viral release in mammalian cells through budding is noted in less than 5% of cases. If virus is released by budding, the membrane is lost shortly thereafter.

**Non-Ruminant Host Relationship**

BTV is known to infect a broad diversity of host species and the severity of disease is just as diverse across these species, though bluetongue disease does not occur in humans and mice. There is a rare BTV strain which has been reported to replicate in mice (Brewer and Osburn, 1998) though this has yet to be independently confirmed. Species specificity is less clear in vitro where BTV can replicate in a wide variety of cell lines (McPhee et al., 1982). It is normally cultured in hamster kidney (BHK-21) cells or in African green monkey kidney (Vero) cells.

Challenge of some human cell lines with BTV has recently been shown to induce apoptotic cell death (Xiao et al., 2004). This observation may be related to the transformed nature of the cells and other experiments have shown that BTV infection of human primary cells does not cause cell death (Hu et al., 2008). These findings suggest BTV may be an oncolytic virus, meaning the virus replicates more efficiently in cancer cells than in normal cells (Guo et al., 2005).
Cancer cells often make a good host for viruses for reasons that include mutated tumor suppressor genes and inactivated antiviral interferon pathways (Myers et al., 2005; Galanis et al., 2005). The mechanisms which allow BTV's selective oncolysis are unknown.

**Innate Immunity**

One mechanism by which BTV can induce cell death is through activation of innate immunity. Innate immunity includes viral defenses which are intrinsic to each cell independent of the host immune system. Innate defenses to dsRNA include toll-like receptors (TLRs), type I interferons (IFNα and IFNβ), and RNA-interference.

TLRs are molecular pattern recognition receptors (Aderem and Ulevitch, 2000). There are currently 13 recognized TLRs, each having affinity for a specific pattern commonly expressed by pathogens (Takeda et al., 2003). For example, lipopolysaccharide (LPS) is recognized by TLR4 leading to activation of cells and expression of numerous inflammatory cytokines (Ståhl et al., 2006). TLRs display differential tissue and cell expression highly dependent on the function of the receptor (Hopkins and Sriskandan, 2005). Though each TLR has its own specific function, virtually all utilize the same MyD88 cell signaling pathway leading to inflammation. The exception is TLR3, which utilizes a MyD88-independent signaling pathway (Meylan et al., 2004). TLR signaling is graphically represented in Fig. 1-3.
TLR3 specifically recognizes dsRNA (Hopkins and Sriskandan, 2005). This innate receptor is of particular interest with respect to BTV because the genome of BTV is dsRNA. Double-stranded RNA does not naturally exist in mammalian cells. If present, it is treated as a product of infection with an RNA virus. In the case of ssRNA viruses, dsRNA is generated as part of the genomic replication process as the RNA-dependent RNA polymerase makes replicate copies of the viral genome. The genome will be packaged as ssRNA, but dsRNA is an intermediate as well as a byproduct of incomplete packaging.

Unlike ssRNA viruses, BTV’s dsRNA genome can induce innate immunity through TLR3 or through cytoplasmic receptors such as retinoic acid-induced protein I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5) (Andrejeva et al., 2004; Yoneyama et al., 2004; Kato et al., 2005; Gitlin et al., ...
to cause cell death and cytokine expression (Hoebe et al., 2004), even without viral replication.

In addition to avoiding TLR's and cytoplasmic receptors, dsRNA must also avoid degradation of viral mRNA caused by activation of the cellular RNA-induced silencing complex (RISC) and simultaneous activation of the type I interferon response (Saha et al., 2006). RISC formation is part of the RNA interference (RNAi) process first characterized in the nematode worm Caenorhabditis elegans (Fire et al., 1998). Double-stranded RNA was later shown to induce the formation of the RISC complex which would target and destroy mRNA complementary to the dsRNA sequence (Sledz and Williams, 2005).

Double stranded RNA can also induce type I interferons which are autocrine and paracrine cytokines that induce transcription of numerous antiviral genes (Theofilopoulos et al., 2005). Both RNAi and type I interferon production are defense mechanisms intended to prevent the virus from completing replication. Even though BTV attempts to protect its dsRNA genome by sequestering it in the viral core particle during transcription, it is particularly vulnerable to this mechanism because dsRNA is prevalent within the cell at every stage of infection (Grimes et al., 1998).

Types of Viral-Induced Cell Death

BTV induces both apoptotic and necrotic cell death. Apoptosis is tightly controlled, programmed cell death. Necrosis encompasses any other type of cell death (Kerr et al., 1972). Apoptosis is specifically, an energy-dependent,
asynchronous, genetically controlled process by which unnecessary or damaged single cells self-destruct upon apoptotic activation (Martin, 1993; Earnshaw, 1995). One major characteristic of apoptosis is that an apoptotic cell has little impact on surrounding cells (Bar, 1996), while necrosis is characterized by widespread damage and inflammation (Kam and Ferch, 2000). This is because reactive oxygen species and toxic proteins are released from necrotic cells, causing localized tissue damage. In the animal host, BTV infection often causes secondary necrosis as a result of severe inflammation and vasoconstriction (DeMaula et al., 2002a; DeMaula et al., 2002b), but in mammalian cell culture, infection usually results in apoptosis (Nagaleekar et al., 2007).

Apoptosis is used by the host to regulate, renew, and protect itself. It is particularly important in viral host defense. This is because a virus relies on the cellular machinery to complete replication and a cell which undergoes apoptosis can no longer be used by the virus to replicate. If the apoptotic system is compromised, the host is more susceptible to viral infection as well as many other diseases, including cancer (Osaki et al., 2004). Some viruses directly inhibit apoptosis in their target cells to prolong host cell life thereby prolonging replication. Examples include Papilloma virus and Adenovirus which both encode p53 inhibitors (Zekri et al., 2006; Ying et al., 2007). P53 is an essential component in regulating the cell cycle as well as recombination after genomic damage. When p53 is inhibited, the cell continually replicates and is unable to undergo apoptosis (Zekri et al., 2006).
On a systemic level, there are three phases to apoptosis (Arends and Wyllie, 1991). First, the cell detaches from adjacent cells and the genomic DNA is digested by specific endonucleases and packaged into organelles (Wyllie, 1997). Second, the organelles are released as membrane-bound vesicles to be recycled by neighboring cells. Last, the remaining apoptotic body is phagocytosed by myeloid cells (Wyllie, 1997).

Apoptotic stimuli are broadly categorized into four major groups; including DNA damage; activation of death receptors (Fas receptor, TNF receptor); stimulation of apoptotic pathways (kinase inhibitors); and damage by UV, heat, or peroxides (Kam and Ferch, 2000). Inflammation is generally not a product of apoptosis though some transcription factors associated with viral-induced apoptosis are also associated with cytokine expression (Heylbroeck et al., 2000).

**BTV-Induced Cytokine Expression**

Within the body, an infected cell will recruit the help of the systemic immune system and send a warning to neighboring cells to minimize viral spread. This is done through the secretion of cytokines. The mechanisms of cytokine induction are diverse and often dependent on the infecting agent. The effects of cytokine induction are just as diverse as the mechanisms of induction.

Cytokines are hormone-like proteins produced by nearly all cells. They are the predominant mode of communication within the immune system. Through cytokines, the immune system regulates stem cell differentiation (Bishop et al., 2002), inflammation (Arai et al., 1990), angiogenesis (Carmeliet, 2000), as
well as many other events. Correspondingly, aberrant cytokine expression is also often involved in progression and pathogenesis of disease (Whicher and Evans, 1990).

Studying cytokines provides insight into how a cell is responding to infection and how the immune system as a whole is managing the infection. Cytokines often perform multiple functions and regularly share receptors (Akira et al., 1990). A single cytokine can elicit one effect on one cell type, a different effect on another cell type and a completely different response when administered concomitantly with other cytokines (Akira et al., 1990). This diversity of activity allows the immune system to control a great variety of events with a relatively small repertoire of signaling agents. This diversity also makes interpreting the effects of cytokine expression very difficult.

In the past, there have been few cytokine studies on BTV infection. This is due predominantly to the limited availability of testing materials for ruminant species. The use of RT-PCR has allowed researchers to do limited cytokine studies on an mRNA expression level (DeMaula et al., 2002a; DeMaula et al., 2002b). Though not as accurate as protein based tests in determining cytokine concentrations, mRNA studies have provided some information about the native host cytokine response to BTV infection.

Within native hosts, BTV infection results in severe pathological effects, including prominent swelling systemically, hemorrhaging, and inflammation-induced necrosis (Spruell, 1905; Chiang et al., 2006; DeMaula et al., 2002a). DeMaula et al. (2002a) found that BTV infection of ovine lung microvascular
endothelial cells (ECs) showed increased transcription of genes encoding IL-1β and IL-8, but little IL-6. Transcription of these same genes was markedly higher in bovine lung microvascular ECs. These differences between sheep and cattle are significant when considering that ovine ECs had lower incidence of infection and produced significantly less virus than did bovine ECs (DeMaula et al., 2001), but have much higher rate of mortality.

These experiments show that cytokine expression from infected cells in natural ovine and bovine hosts is greatly elevated and consistent with inflammation, though the diversity of cytokines tested was small. Recently Chiang et al. (2006) performed cytokine mRNA testing in human endothelial cells infected with BTV-10 and found mRNA expression increased for IL-1β, IL-6, IL-8, and TNFα. They also found that the stimulation of cells with polyinosinic:polycytidilic acid (poly I:C) resulted in expression of the same cytokines, suggesting that the cause of expression in these cells is due to innate immunity to dsRNA.

There are four classes of cytokines which can be observed in BTV-infected human carcinoma transformed cells. These include inflammatory cytokines, chemokines, TH1 type differentiators, and angiogenesis factors. Inflammatory cytokines are those which promote inflammation at the site of infection. Chemokines are a class of cytokines which are chemotactic for specific lymphocytes (Baggiolini et al., 1997). TH1 differentiators are cytokines which promote TH1 type T-cell activation as opposed to TH2 type activation. (Clerici and Shearer, 1993).
The last set of cytokines are promoters of angiogenesis. Angiogenesis is the process of creating these new blood vessels; and thus, it is an important component of human growth. As the body grows, the demand for oxygen increases. To accommodate for greater demand, new blood vessels must be constructed. This is a complex process incorporating breakdown of old tissue, cleanup of digested tissue, and formation of new vascular endothelial cells in its place (Carmeliet, 2000). Angiogenesis plays a critical role in tumor progression as well because the tumor’s oxygen demands increase dramatically the larger the tumor and the more aggressively the tumor is growing (Carmeliet and Jain, 2000; Patan, 2004). BTV infection may inhibit angiogenic factor expression which would affect the tumor’s ability to grow.

*Measuring Cytokine Expression*

Cytokine expression is determined by either quantifying cytokine mRNA using RT-PCR or cytokine protein by reporter-linked immunoassay. The most accepted immunoassay is a sandwich (capture) ELISA (Knight *et al.*, 2004). This process uses two antibodies. One antibody is bound to a solid phase (i.e. micro-well plate) and then incubated with sample. As the sample is incubated, cytokines in solution are captured by the anchored antibody. Following this, each well is incubated with a second enzyme-linked antibody. The second antibody binds to a different region on the cytokine. This binding is detected by monitoring the activity of the attached enzyme. Enzyme activity is proportional to the amount of cytokine present in the sample. One of the best ways to improve interpretation is to assay for multiple cytokines (Knight *et al.*, 2004; Leng *et al.*, 2004).
The profile of cytokine type and concentration helps to generate a clearer picture of the cellular response as well as the underlying cell signaling taking place. Conventional ELISAs, while allowing the researcher to test many samples at one time, can test only one type of cytokine in a single test. This makes cytokine profiling costly with respect to time, reagents, and sample volume. The expense of these factors makes multiplexed assays the preferred choice for profiling. Multiplex formats have included fluorescent bead-based assays as well as printed arrays (Morgan et al., 2004). Both methodologies allow the researcher to simultaneously measure multiple cytokines in a relatively small volume.

*Intracellular Signaling During BTV Infection*

Fundamental to any biological system is accurate communication. A biological system as a whole can communicate through various signals including: neuronal signals, chemical hormones, cytokines, and contact. Accurate intracellular communication is just as important. Within a cell, signals from the environment must be relayed through the membrane and then throughout the cell. This is necessary for the cell to properly respond to its environment. In addition, intracellular signals must be passed to the nucleus for proper regulation of gene expression. Finally, changes in gene activity or demand must be relayed to the rest of the system and properly accounted for in order to keep the system in homeostasis. When communication pathways are disrupted, pathology inevitably follows.

The cell is analogous to the modern automobile; equipped with sensors throughout the entire system, ready to notify the driver of any potentially
deleterious condition. Likewise, the cell employs a brigade of regulatory molecules responsible for monitoring and reporting the extracellular and intracellular environment. Also like the automobile, a simple change in one process, if not properly identified and accounted for can lead to greater problems.

The regulatory molecules discussed above are collectively classified as signaling molecules, with some responsible for detecting changes and others responsible for relaying messages to the proper system. Signaling molecules are a large and diverse group of proteins, but even with a large assortment of signaling proteins, the cell often utilizes the same protein in multiple signaling pathways and regularly cascades signals via multiple messengers (Kornblau et al., 2006; Chang and Karin, 2001; Pearson et al., 2001). The use of the cascading process allows the signal to be rapidly amplified and disseminated through multiple systems, such that small triggers can induce dramatic changes. Cascading also means slight alterations in the makeup of the signaling framework can have dramatic end results. It also makes it more difficult for researchers to distinguish and discern signaling pathways.

The most common way signals are relayed along the cascade is through reversible phosphorylation (Johnson and Lewis, 2001). The regulation of action by reversible phosphorylation was first discovered in 1952 by Edwin G. Krebs (Krebs, 1981) with the recognition that glycogen phosphorylase was reversibly regulated by phosphorylation. Though phosphorylation was first discovered in eukaryotes, bacteria were later shown to utilize similar phosphorylation signaling (Deutscher and Saier, 2005). Since its first discovery, scientists have discovered
500+ kinases, but have mainly succeeded in revealing the complexity of signal transduction and regulation (Manning et al., 2002).

Phosphorylation signals are relayed by transferring a phosphoryl group from ATP to the hydroxyl group of the amino acid. Because of the need for a hydroxyl group, there are only three candidate amino acids for phosphorylation: serine, threonine, and tyrosine (Johnson et al., 2001). Once covalently attached, the protein is referred to as a phospho-protein. Phosphate groups are transferred to proteins by kinases and removed by phosphatases (Mumby and Walter, 1993). Protein kinases make up the largest family of genes in eukaryotes (Rubin et al., 2000; Lander et al., 2001). The family is classified into three types: those which can phosphorylate serine and threonine, those which can phosphorylate tyrosine, and those which can phosphorylate any of the three (Kitamura et al., 1998; Hubbard and Till, 2000; Cobb and Goldsmith, 1995). Phosphorylation occurs after the protein has been folded, restricting phosphorylation sites to the surface of a protein.

The impact of phosphorylation is different for every protein, with effects dependent on the site(s) of phosphorylation. It is not uncommon for a protein to have multiple phosphorylation sites, with the pattern of phosphorylation defining the activity of the protein (Mayo et al., 2005; Xie et al., 2001).

Because of the import of intracellular signaling to homeostasis, aberrant intracellular signaling often leads to disease. Cancer is commonly attributed to mutations in apoptotic and cell cycle related signaling. For example, the protein kinase Ras is a known oncogene (Bos, 1989). Normally, it is a GTPase involved
in regulating various signaling pathways. One of the pathways it regulates is the MAPK pathway. When mutated to an overactive state, Ras can induce constitutive activation of the cell cycle causing cancer (Lodish et al., 2000).

In cases where cancer is the result of changes in intracellular signaling, treatment can be very complex because inhibition of the mutated kinase can have consequences on other known and unknown pathways. Even so, there are cancer drugs on the market which are specific kinase inhibitors (Reuter et al., 2000).

Protein kinase A, B, and C (PKA, PKB, and PKC, respectively) are three of the earliest kinases discovered with involvement in many signaling pathways (Newton, 2003). Each has a common conserved kinase core and a regulatory domain. The regulatory domain is where the kinase is regulated by phosphorylation and dephosphorylation but also helps target the kinase to the appropriate cellular location (Newton, 2003). PKB is of interest in this study as it is directly involved in cell survival.

PKB, plays a critical role in controlling the balance between survival and apoptosis (Yoeli-Lerner and Toker, 2006). There are 3 known isoforms which are generally considered to have the same function (Toker and Yoeli-Lerner, 2006). PKB promotes cell survival by inhibiting apoptosis through its ability to inactivate by phosphorylation several targets involved in apoptosis. Prolonged survivability is the direct result of constitutive over phosphorylation of PKB in numerous cancers (Yoeli-Lerner and Toker, 2006). Elevation in PKB-phosphorylation makes the cell more resistant to apoptosis while declines in
PKB-phosphorylation make the cell more susceptible to apoptosis (Jeong et al., 2007). One hypothesis is that PKB is constitutively phosphorylated in carcinoma cells promoting survivability and that infection with BTV triggers an independent apoptotic mechanism which causes a reduction in PKB phosphorylation, thereby allowing the immortalized cell to enter apoptosis.

Besides suppressing PKB phosphorylation, BTV is also hypothesized to be activating pro-apoptotic processes. One of the major pro-apoptotic signaling pathways associated with infection is the p38 mitogen-activated protein kinase (p38 MAPK) pathway (Porras et al., 2004). P38 MAPK is also a known oncogene as aberrant MAPK phosphorylation can lead to prolonged survivability (Huynh et al., 2003).

MAPKs include more than p38 MAPK. Other MAPKs influence cell growth, differentiation, apoptosis, as well as cellular responses to stress (Ashwell, 2006). Mutations in each member of the MAPK family can cause cancer. In addition, because of their involvement in the inflammation and cell cycle regulation, each member could also be involved in BTV-induced cell death and cytokine expression (Hersey et al., 2006).

In general, MAPKs are a set of serine/threonine kinases which are evolutionarily conserved (Widmann et al., 1999). The MAPK subfamily is activated by dual phosphorylation at a domain composed of a tyrosine two amino acids downstream of threonine in the TXXY motif (Ashwell, 2006). MAPKs are inactive in their unphosphorylated state and become active when phosphorylated by MAPK kinases (MKKs). MKKs are in turn phosphorylated by MAPK kinase
kinases (MKKKs) (Dodeller and Schulze-Koops, 2006). As a minimum, MAPKs require three sequential phosphorylations in order to be activated (Ray and Sturgill, 1988). Once active, each member has a specific effector that it in turn activates by phosphorylation (Raman et al., 2007).

The MAPKs include three subfamilies: Extracellular signal-regulated kinases ERK1 and ERK2, Src associated protein kinases (SAPK) also known as c-Jun NH2-terminal kinases (SAPK-JNK1, -2, and -3), and the four p38 MAPK enzymes p38α, β, γ, δ (Johnson and Lapadat, 2002). A fourth MAPK, ERK5, has also been identified though little is presently known about it (Zhou et al., 1995).

The MAPK subfamilies each have specific functions. ERK1 and ERK2 are involved in the regulation of mitosis and meiosis (Lloyd, 2006; Meloche and Pouysségur, 2007). They can be activated by stimuli including growth factors, cytokines, virus infection, transforming agents and carcinogens (Wang, 2007; Valerie et al., 2007). The ERK-1/2 path can also be activated by the proto-oncogene RAS (Dance et al., 2008). Mutated RAS persistently activates ERK-1/2 which has been known to contribute to the increased proliferation rate of tumor cells (Ruscica et al., 2007).

The SAPK-JNK subfamily members are stress-activated proteins often activated when protein synthesis has been inhibited (Kyriakis et al., 1995; Singh and Czaja, 2007). SAPK-JNKs phosphorylate the c-Jun transcription factor, which is part of the AP-1 transcription complex involved in transcription regulation, including cytokine gene expression (Salh, 2007). SAPK-JNK's are
also important in controlling apoptosis (Tournier et al., 2000; Takeda et al., 2007).

The third subfamily includes the p38 MAPKs. There are four known p38 MAPK isoforms with similar function. P38 MAPK was first discovered from its involvement in TNFα-induced inflammation (Grivennikov et al., 2006; Aggarwal et al., 2006). P38 MAPK is also involved in the expression of various other cytokines (Schindler et al., 2007; Patil and Kirkwood, 2007). In addition to the usual pathway of activation, p38 MAPK can also be activated by the non-enzymatic protein TAB1 (Ge et al., 2002), suggesting that there are multiple mechanisms of p38 MAPK regulation.

P38 MAPK is also known to be associated with innate defense against dsRNA (Williams, 1999; Williams, 2001; Servant et al., 2002) and is a strong promoter of inflammation upon microbial challenge (O'Neill, 2006). Viral-induced p38 MAPK activation generally leads to cell death as well as elevated cytokine production. Chiang et al. (2006) observed p38 MAPK phosphorylation in BTV challenged primary lung microvascular endothelial cells. They also found this activation could be reproduced using synthetic dsRNA. In their studies, dsRNA-induced the expression of the cytokines IL-1β, IL-6, and TNFα, consistent with p38 MAPK phosphorylation via innate immunity.

OBJECTIVES AND HYPOTHESIS

BTV preferentially replicates in transformed human cells relative to primary cells. Understanding this selective trophism may lead to future treatments for cancer. Our objectives were to determine how BTV replication in
carcinoma transformed human cells differs from replication in traditional host cells and how the human cells respond to BTV infection, including induction of apoptosis, changes in cytokine expression, and changes in intracellular signaling. Our hypothesis was that cell death and cytokine expression were directly related to viral replication and were the result of innate immune responses to BTV genomic dsRNA.

REFERENCES


CHAPTER 2

BLUETONGUE VIRUS (BTV)-INDUCED CELL DEATH CORRELATES DIRECTLY WITH BTV-INDUCED CYTOKINE EXPRESSION

ABSTRACT

Bluetongue virus (BTV) naturally infects ruminants, even though it is most commonly cultured in vitro in baby hamster kidney (BHK-21) and monkey kidney (Vero) cells. BTV does not normally replicate in human cells though BTV administration has been shown to cause cell death and apoptosis in human HEP-3G and A549 carcinoma transformed cell. In addition, studies suggest that BTV infection of human cell lines is selective for transformed cells. As a novel oncolytic virus, understanding the mechanism of cell death may lead to future therapies for cancer. To better understand the mechanism of BTV-induced cell death, we determined the rate of BTV-induced as well as polyinosinic-polycytidylic acid (poly I:C)-induced cell death in four cell lines (A498, HEP-G2, A549, and Vero). We also determined the profile and magnitude of BTV-induced and poly-I:C-induced cytokine expression in each cell line. Each cell line was found to express interleukin-6 (IL-6), IL-8, monocyte chemotactic protein 1 (MCP-1), regulated upon activation T-cell expressed and secreted protein (RANTES), tissue inhibitor of metalloproteinases 1 (TIMP-1), and vasoendothelial growth factor (VEGF). IL-6, IL-8, MCP-1, and RANTES were found to be inducible both by BTV and poly I:C. The magnitude of cytokine expression was cell line dependent. BTV-induced cytokine expression correlated directly with BTV-induced cell death.
INTRODUCTION

The ability of bluetongue virus (BTV) to cause disease is heavily species dependent. In cell culture, BTV’s ability to replicate and cause cell death is also quite variable. BTV causes cell death in some human carcinoma transformed lines while primary cultures are not susceptible to BTV-induced cell death (Xiao et al., 2004; Chiang et al., 2006). The ability of BTV to cause death in carcinoma transformed lines can be due to multiple mechanisms including innate immunity. Our objective was to determine the rates of cell death in 4 cell lines (A498, HEP-G2, A549, and Vero). We also determined which cytokines are expressed upon infection and the contribution of dsRNA to this cytokine expression.

Bluetongue virus (BTV), classified in the genus Orbivirus within the family Reoviridae, can cause disease in a variety of animals though only in ruminant species. Though BTV naturally infects ruminants, it is most commonly cultured in vitro in baby hamster kidney (BHK-21) or monkey kidney (Vero) cells. BTV does not naturally replicate in humans, but can infect human cells in vitro. Chiang et al. (2006) demonstrated viral S3 gene replication in BTV-serotype-10-treated human microvascular lung endothelial cells (ECs), and other studies have demonstrated BTV infectivity in transformed human cells (Xiao et al., 2004; Hu et al., 2008). Fig. 2-1 and 2-2 show evidence of BTV-induced cell death in carcinoma transformed HEP-3G and A549 cells.
Chiang et al. (2006) also observed elevated expression of TNFα, IL-1β, and IL-6 mRNA, in human microvascular lung ECs consistent with cytokine mRNA expression observed in bovine primary lung microvascular ECs (DeMaula et al., 2002). However, comparable cytokine expression could be induced with poly(inosinic):poly(cytidylic) acid (poly I:C), indicating BTV genomic dsRNA may be the cause of inducible cytokine expression (Chiang et al., 2006).

Our objective was to characterize cell death as well as cytokine and chemokine expression, in BTV-serotype-17-infected human cells transformed by carcinoma (A498, HEP-G2, and A549 cells) and determine if poly I:C could induce similar changes. We hypothesized that BTV would induce cell death in all tested cells in a time-dependent manner and that infection would lead to the production of pro-inflammatory cytokines.
MATERIALS AND METHODS

Cell Culture

Five cell lines were used in this study, including BHK-21 baby hamster kidney cells, A498 human kidney cells, HEP-G2 human liver cells, A549 human lung epithelial cells, and Vero African green monkey kidney cells (American Type Culture Collection, Manassas, VA). Designated CCL-10, HTB-4, HB-8065, CCL-185, and CRL-1587, respectively by the American Type Culture Collection. Each cell line was propagated in 75 cm² flasks seeded at 1X10⁶ cells and cultured in MEM/EBSS plus 10% FBS (Hyclone, Logan, UT) under a 5% CO₂ atmosphere.

BTV Stock Propagation

Original stocks were triple plaque purified and stored frozen in aliquots (Kowalik et al., 1990; Li and Yang, 1990). Seed virus was prepared by infecting BHK-21 cells with frozen viral stock. Cells were infected at 80% confluency and harvested by scraping three days post infection (P.I.). After scraping, cells were pelleted, washed, resuspended in fresh media, then gently sonicated three times using a VirSonic 50 sonicator (VirTis Inc., Gardiner, NY) prior to 0.22 μm filter sterilization. Viral titer was determined by plaque assay, using Vero cell monolayers (Fillmore et al., 2002; Hayama and Li, 1994). Mock infections were similarly prepared using uninfected BHK-21 cells.

Plate Preparation and BTV Infection

96-well plates were seeded one day prior to experimentation in MEM/EBSS plus 10% FBS (Hyclone Laboratories, Logan, UT), such that the
culture was approximately 50% confluent when starting the experiment. Prior to experimentation, plates were rinsed with MEM/EBSS. BTV infection occurred at an MOI of 1 using BTV serotype 17. Mock infections were performed using a comparable volume of uninfected BHK-21 cells, prepared as described above. Plates were incubated at 37 °C under 5% CO₂ in MEM/EBSS plus 5% FBS.

*Poly I:C Treatment of Cell Lines*

Stock poly I:C (Sigma, St. Louis, MO) was prepared at 2 mg/mL in MEM/EBSS and further prepared as serial half-log dilutions at 0, 0.1, 0.32, 1.0, 3.2, 10, 32, and 100 μg/mL. Three 96-well plates per cell line were prepared as described above and treated with poly I:C (n=4). Samples of the cell culture supernatant were taken daily for 3 days (stored at -80 °C) for cytokine analysis, and one plate from each cell line was stained on days 1, 2, and 3 with neutral red.

*Neutral Red Staining*

A 0.034% solution of neutral red (Fisher Scientific, Fairlawn, NJ) in saline was prepared and filter sterilized. Immediately before use, the solution was diluted to 0.011% in MEM/EBSS. All media were removed from the plate, 200 μL of the 0.011% neutral red solution was added, and plates were incubated at 37 °C under 5% CO₂ for two hours. Plates were then rinsed once with PBS, and neutral red was extracted by adding 100 μg/mL extraction buffer, made by combining 500 mL Sorensen citrate (0.1 M sodium citrate [Sigma, St. Louis, MO] and 0.1 M hydrochloric acid [Fisher Scientific, Fairlawn, NJ]) and 500 mL ethanol
Absorbance at 540 nm was read using a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA).

**Quantitation of Secreted Analyte Expression**

Supernatant samples, collected previously and stored at -80 °C, were thawed at room temperature. Cytokines were detected by multiplex immunoassay (Quansys Biosciences, Logan, UT). Samples were tested for: Interleukin 1 alpha (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), TNFβ, transforming growth factor beta 1 (TGF-β1), monocyte chemotactic protein 1 (MCP-1), regulated upon activation T-cell expressed and secreted protein (RANTES), angiotensin 2 (ANG2), hepatocyte growth factor (HGF), tissue inhibitor of metalloproteinases (TIMP), thrombopoetin (TPO), vasoendothelial growth factor (VEGF), platelet derived growth factor (PDGF), eotaxin (EO), macrophage inflammatory protein 1 alpha (MIP-1α), and C-reactive protein (CRP). Follow-up experimentation was performed only on IL-1α, IL-1β, IL-6, IL-8, IFNγ, TNFα, MCP-1, and RANTES. The chemiluminescent signal was detected using an Alpha Innotech 8900 Gel Documentation System (San Leandro, CA). Plates were exposed for one minute, and signal intensity was quantified using software provided by Alpha Innotech.

**Statistical Analysis**

P-values were calculated in Excel (Microsoft Corporation, Redmond, WA) using a two-tailed Student’s t-test.
RESULTS

*Human Tumor Cells Detach and Die Progressively Throughout BTV Infection*

Numerous viruses are oncolytic (Vidal et al., 2006), including BTV (Hu et al., 2008). In this study, we first explored cell death in three human cell lines transformed by carcinoma: A498, HEP-G2, and A549 to confirm the findings of Hu et al. (2008) and compare the effects of BTV in different types of tissue. Cell death continuously increased over the course of infection for all tested cell lines. The A498 cells were most susceptible to BTV-induced cell death, with only 12% viability on day 3, compared with mock-infected cells, followed by Vero cells with 43% viability on day 3. A549 cells were comparable to Vero cells with 45% viability on day 3 followed last by HEP-G2 cells with 70% viability on day 3. Surprisingly, BTV caused greater cell death in A498 cells than in the common laboratory host, Vero. Percent viability on day 3 in each cell line is shown in Fig. 2-3. Viability in each cell line was also determined when treated with poly I:C instead of BTV. Cells were treated at either 1 μg/mL or 50 μg/mL. One μg/mL poly I:C did not cause a statistically significant change in viability in any tested cell line. Fifty μg/mL poly I:C caused statistically significant cell death only in A498 cells, with death comparable to BTV-induced cell death (see Fig. 2-3).

*Inflammatory Mediators Are Elevated During BTV Infection*

Samples from the viability study were tested for 25 different cytokines and chemokines. Six were found to be expressed by these three cell lines.
FIG. 2-3. Percent viability of A498, HEP-G2, A549, and Vero cells as determined by neutral red staining 3 days P.I. Cells were either mock-infected, BTV-infected at an MOI of 1, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). * indicates conditions with statistically significant decreases in cell viability compared with the placebo.

Of the six, four (IL-6, IL-8, MCP-1 and RANTES) were found to be significantly elevated upon BTV infection, while the other two (VEGF and TIMP) were constitutively expressed by the cell but did not change significantly with infection (data not shown).

A new array with IL-6, IL-8, MCP-1, RANTES, IL-1α, TNFα, IL-10, IL-12p70, MIP-1α, and EO was prepared to further explore cytokine expression. Using this array, IL-6, IL-8, MCP-1, and RANTES were found expressed from all 4 cell lines with cytokine expression correlating directly with cell death. Concentrations of the four BTV-induced cytokines are shown in table 2-1. Also shown in table 2-1 are cytokine levels from cells treated with poly I:C at 1 μg/mL and 50 μg/mL. A498, A549, and Vero cells expressed cytokines in response to
TABLE 2-1

Cytokine Expression (pg/mL) from A498, A549 and Vero Cells
Seeded in 96-well Plates 3 Days Post Treatment (n=4)

<table>
<thead>
<tr>
<th></th>
<th>A498</th>
<th>A549</th>
<th>Vero</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/mL</td>
<td>STDEV</td>
<td>pg/mL</td>
</tr>
<tr>
<td>Mock-infected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>280</td>
<td>90</td>
<td>200</td>
</tr>
<tr>
<td>IL-8</td>
<td>1100</td>
<td>250</td>
<td>630</td>
</tr>
<tr>
<td>MCP-1</td>
<td>4650</td>
<td>620</td>
<td>1110</td>
</tr>
<tr>
<td>RANTES</td>
<td>150</td>
<td>50</td>
<td>190</td>
</tr>
<tr>
<td>BTV-infected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>3310</td>
<td>310</td>
<td>540</td>
</tr>
<tr>
<td>IL-8</td>
<td>1810</td>
<td>250</td>
<td>3100</td>
</tr>
<tr>
<td>MCP-1</td>
<td>8450</td>
<td>1230</td>
<td>2950</td>
</tr>
<tr>
<td>RANTES</td>
<td>390</td>
<td>120</td>
<td>5670</td>
</tr>
<tr>
<td>Poly I:C 1.0 μg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>3850</td>
<td>1180</td>
<td>390</td>
</tr>
<tr>
<td>IL-8</td>
<td>8900</td>
<td>890</td>
<td>1060</td>
</tr>
<tr>
<td>MCP-1</td>
<td>14900</td>
<td>530</td>
<td>1720</td>
</tr>
<tr>
<td>RANTES</td>
<td>3820</td>
<td>1060</td>
<td>1130</td>
</tr>
<tr>
<td>Poly I:C 50 μg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>13600</td>
<td>740</td>
<td>420</td>
</tr>
<tr>
<td>IL-8</td>
<td>13300</td>
<td>470</td>
<td>4590</td>
</tr>
<tr>
<td>MCP-1</td>
<td>18900</td>
<td>770</td>
<td>7080</td>
</tr>
<tr>
<td>RANTES</td>
<td>7580</td>
<td>1480</td>
<td>6050</td>
</tr>
</tbody>
</table>

poly I:C treatment while HEP-G2 cells did not express.

Prior studies by Chiang et al. (2006) showed that IL-1β and TNFα were secreted from BTV infected human lung primary ECs, but neither one was expressed during this study. It is possible that IL-1β and TNFα are responses specific to human primary lung microvascular ECs, MALT-associated lymphocyte carryover in the primary cell isolation process, or that the pathway is inactivated as a result of transformation by carcinoma.
Poly I:C Causes Cell Death in A498 Cells, but Induces Cytokine Expression in A498, A549, and Vero Cells

One of the possible mechanisms by which BTV could cause cytokine expression is through innate immunity. Chiang et al. (2006) used BTV-10 as a model for virus-induced vascular permeability in human ECs and found that the effects BTV had on the ECs could be replicated using synthetic dsRNA. To test for innate immunity’s role in the host cell response to BTV, each cell line was then treated with poly I:C in a dose dependent manner, ranging from 0.1 μg/mL to 100 μg/mL. Percent viability as a function of poly I:C concentration is shown in Fig. 2-4. This study demonstrated no statistically significant change in cell viability in HEP-G2, A549, or Vero cells treated with poly I:C, but a strong change was dose dependent between 1 and 100 μg/mL but displayed a second peak in cell death at 0.32 μg/mL.

Poly I:C treated samples were sampled at 24, 48, and 72 hours post treatment and assayed for cytokine expression. A498, A549, and Vero cells all expressed cytokines during poly I:C treatment while HEP-G2 cells did not. Both A498 and Vero cells had peak cytokine responses at 0.32 μg/mL and 100 μg/mL poly I:C, while A549 cells only showed poly-I:C-induced cytokine expression at concentrations above 3.2 μg/mL. Cytokine expression did increase slightly in A549 cells treated with 0.32 μg/mL, suggesting there may have been a second peak, though the change was not statistically significant. Interestingly, cytokine expression stopped or slowed dramatically 48 hours post treatment, also in a cell
line dependent manner. Fig. 2-5 through 2-16 show cytokine expression from A498, A549, and Vero cells as a function of poly I:C concentration and time.

**DISCUSSION**

Bluetongue disease was first characterized over 100 years ago in BTV’s natural host ruminants. It has only recently been shown to have selectively oncolytic effects in certain human cell lines (Xiao et al., 2004; Hu et al., 2008). Selective oncolysis by viruses has been observed previously in both DNA and RNA viruses, each employing different mechanisms (Strong et al., 1998; Vorburger et al., 2004; Vidal et al., 2006). Little is known about the mechanism by which BTV causes oncolysis. BTV is closely related to reovirus, another
FIG. 2-5. IL-6 expression in A498 cells as a function of poly I:C concentration, at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.

FIG. 2-6. IL-8 expression from A498 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.
FIG. 2-7. MCP-1 expression from A498 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.

FIG. 2-8. RANTES expression from A498 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.
FIG. 2-9. IL-6 expression from A549 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.

FIG. 2-10. IL-8 expression from A549 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.
FIG. 2-11. MCP-1 expression from A549 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.

FIG. 2-12. RANTES expression from A549 cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.
FIG. 2-13. IL-6 expression from Vero cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.

FIG. 2-14. IL-8 expression from Vero cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.
FIG. 2-15. MCP-1 expression from Vero cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.

FIG. 2-16. RANTES expression from Vero cells as a function of poly I:C concentration at 24, 48, and 72 hours P.I. Cells were treated with concentrations of poly I:C ranging from 0.1 to 100 μg/mL (n=4) and assayed for cytokine expression using supplies from Quansys Biosciences.
oncolytic virus, and may share a similar mechanism, though BTV is predicted to have particular advantages over reovirus because humans are seronegative to BTV (Wildner, 2003).

To further explore the therapeutic potential of BTV in cancer, we compared cell death and cytokine expression in A498, HEP-G2, and A549 cells during BTV infection and poly I:C treatment. Our hypothesis was that BTV infection causes cell line dependent death while simultaneously inducing cytokine expression. We showed elevated expression of pro-inflammatory cytokines from BTV-17 infected human carcinoma transformed cells (see Table 2-1) and that infected cells die as a direct result of infection (see Fig. 2-1). Cytokine expression and cell death directly correlated between cell lines and with time. The rate of cytokine expression and cell death were primarily cell line dependent, but continuously increased as long as there were viable cells, indicating that activation was continuous until death. All tested cell lines expressed the same cytokines, suggesting the mechanism of induction is similar in each cell line. We hypothesized that cell death and cytokine expression are related through conserved intracellular signaling events. Various intracellular pathways were tested for involvement and are discussed in Chapter 4.

We also hypothesized that innate immunity was the initiator of this intracellular signaling. Prior studies from our lab infecting A498, HEP-G2, and A549 cells with DNA and RNA viruses showed that all three cell lines responded to viral infection with the expression of IL-6, however, only cells infected with RNA viruses induced RANTES expression (data not shown). In this experiment,
RANTES was observed in all tested lines suggesting each cell line recognized the virus as an RNA virus and responded accordingly. Because every cell line responded with RANTES expression, we conclude that the event is conserved across each cell line and is functional in each cell line. The cytokine expression pattern is consistent with type-I interferon signaling through the transcription factors IRF3 and IRF7 leading to JAK-STAT signaling which generally results in the production of CXC chemokine ligand 10 (CXCL10), MCP-1, and RANTES. TLR3 is also known to activate the type-I interferon pathway as well as the nuclear translocation factor kappa B (NFκB) pathway, though the cytokine pattern is not fully consistent with this. We observe IL-6 and IL-8 expression, but not IL-1 or TNFα as is generally observed in NFκB associated signaling. The lack of IL-1 or TNFα may be carcinoma or cell type related.

Though there are multiple innate immunity mechanisms, focus was on immunity to dsRNA, since BTV's genome is dsRNA. To test the contribution of dsRNA to the observed cell death and cytokine expression, cells were treated with poly I:C. First, cells were treated with either 1 μg/mL or 50 μg/mL poly I:C. Treatment resulted in cell death only in A498 cells, and only at 50 μg/mL (Fig. 2-1), suggesting that BTV-induced cell death was not entirely the result of an innate immune response to BTV dsRNA; also evidenced by no concentration of poly I:C generating statistically significant changes in viability in HEP-G2, A549, or Vero cells.

A498 cells have a greater sensitivity to dsRNA than the other cell lines, which may explain why these cells are more susceptible to BTV-induced cell
death but does not entirely explain BTV’s mechanism of inducing cell death. The experiment was repeated using a broader range of poly I:C concentrations (Fig. 2-2). Like the previous study, only the A498 cells died in response to poly I:C treatment, but the study revealed two peaks in poly-I:C-induced cell death. One peak at 0.32 μg/mL and a second peak at 100 μg/mL. This type of curve suggested two different dsRNA response mechanisms. One functional only at low concentrations of poly I:C, and the other responding only at very high concentrations of poly I:C. When tested for cytokines, the same trend was observed in the A498 cells (Fig. 2-3 through 2-6). The similar patterns suggest that the same two mechanisms which induced cell death in A498 cells also induced cytokine expression. HEP-G2, A549, and Vero cells were also tested for cytokines. HEP-G2 cells did not express any cytokines in response to poly I:C, A549 cells expressed cytokines in a dose dependent manner mainly at concentrations greater 3.2 μg/mL, and Vero cells displayed a cytokine pattern similar to A498 cells. Vero cells having a pattern similar to A498 cells is not entirely surprising given that both cell lines were originally isolated from kidneys, suggesting the second mechanism may be more active in kidney derived cells. There remains, however, distinctions between A498 and Vero cells, including a lack of poly-I:C-induced cell death in Vero cells. Even though both anti-dsRNA mechanisms in Vero cells are recognizing the poly I:C, the response does not lead to apoptosis in Vero cells.

We conclude from these experiments, that BTV induces cytokine expression in immortalized human A498, A549, and HEP-G2 cells and
subsequently kills the infected cell with cell line dependent severity. The BTV-induced cytokine response is pro-inflammatory, including IL-6, IL-8, MCP-1, and RANTES. Double-stranded RNA can induce expression of the same cytokines but only causes cell death in A498 cells, and only at unnaturally high concentrations. We conclude that BTV-induced cell death is not the result of innate immune responses to BTV dsRNA, though observed cytokines can be partially attributed to innate immune responses to dsRNA.

REFERENCES


ABSTRACT

Bluetongue virus (BTV) is a dsRNA virus which must utilize its own replication machinery for transcription but must rely on host machinery for translation. Packaging of viral transcription proteins makes BTV less reliant on host mechanisms than other viruses, which may contribute to its diverse species tropism in vitro. Though BTV does not naturally cause disease in humans, it can cause cell death and cytokine expression in human carcinoma transformed cells. To better understand the mechanism of induced cell death, viral RNA and protein expression were quantified by qRT-PCR and Western blotting, respectively. Our hypothesis was that cell death and cytokine expression are a direct result of viral replication and thus RNA and protein expression would directly correlate with cell death and cytokine expression. BTV RNA and protein were found in all tested human carcinoma transformed cell lines, but RNA expression showed no correlation with cell death or cytokine expression while protein expression inversely correlated with cell death and cytokine expression. The data indicates that cell death and cytokine expression are not a direct result of the virus, but the result of the cell’s response to infection.
INTRODUCTION

Bluetongue virus (BTV) is a dsRNA virus with a genome composed of 10 segments (Verwoerd, 1970). Each segment codes for a different protein except segment 10 which codes for two proteins (NS3 and NS3a) via a non-overlapping open reading frame (Mertens and Sangar, 1985). During replication, proteins necessary in mRNA processing are usually produced first, followed by structural proteins and progeny genomic RNA (Roy, 1989). Prior to this study, BTV RNA expression of all ten genes in human cells had not been tested.

Protein expression was also compared between human cell lines. Viral proteins are classified into two general types: structural and non-structural. The latter type includes all proteins not normally found within the viral protein itself, but are synthesized during replication for the purpose of facilitating replication.

The objective of this study was to characterize viral RNA and protein expression in infected human cell lines and compare these results with the cell death and cytokine expression observed in Chapter 2. The hypothesis was that RNA expression and protein expression directly correlate between the tested human cell lines, and that cell death increases proportionately with accumulation of viral RNA and protein.

MATERIALS AND METHODS

Cell Culture

Five cell lines were used in this study, including BHK-21 baby hamster kidney cells, A498 human kidney cells, HEP-G2 human liver cells, A549 human
lung epithelial cells, and Vero African green monkey kidney cells (American Type Culture Collection, Manassas, VA). Designated CCL-10, HTB-4, HB-8065, CCL-185, and CRL-1587, respectively by the American Type Culture Collection. Each cell line was propagated in 75 cm² flasks seeded at 1X10⁶ cells and cultured in MEM/EBSS plus 10% FBS (Hyclone, Logan, UT) under a 5% CO₂ atmosphere.

**BTV Stock Propagation**

Original stocks were triple plaque purified and stored frozen in aliquots (Kowalik *et al.*, 1990; Li and Yang, 1990). Seed virus was prepared by infecting BHK-21 cells with frozen viral stock. Cells were infected at 80% confluency and harvested by scraping three days post infection (P.I.). After scraping, cells were pelleted, washed, resuspended in fresh media, then gently sonicated three times using a VirSonic 50 sonicator (VirTis Inc., Gardiner, NY) prior to 0.22 μm filter sterilization. Viral titer was determined by plaque assay, using Vero cell monolayers (Hayama and Li, 1994; Fillmore *et al.*, 2002). Mock infections were similarly prepared using uninfected BHK-21 cells.

**BTV mRNA Expression**

BHK-21, A498, HEP-G2, and A549 cells were seeded into separate 6-well plates at 1 X 10⁵ cells per well. Three wells from each cell line were infected with BTV serotype 17 at an MOI of 1, while the other three wells were mock infected. At 24, 48, and 72 hours, cells from one BTV infected well and one mock infected well were collected by scraping. Collected samples were centrifuged, washed once with PBS, and stored at -80C until all samples had been collected. Total
RNA was isolated from each sample using the Qiagen total RNA extraction kit (Valencia, CA) and reverse transcribed using the Bio-Rad reverse transcription kit and random hexamer primers (Hercules, CA). After reverse transcription, the presence and amount of each BTV gene was determined by quantitative real time PCR (qRT-PCR) as described by Buccambusco et al. (2005) using the primers listed in Table 3-1.

### TABLE 3-1

Primer Sequences and Gene Location Used in the Quantitation of Each BTV cDNA by qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 F</td>
<td>2107-2126</td>
<td>5’-ACAAAGGATTGCACACGCTT-3’</td>
</tr>
<tr>
<td>L1 R</td>
<td>2402-2383</td>
<td>5’-TTCATGTCCACACTTCGCA-3’</td>
</tr>
<tr>
<td>L2 F</td>
<td>1224-1347</td>
<td>5’-GACGACTTATGACAGCGGATACAT-3’</td>
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<td>5’-TGTTAGAGGCCCACCAGATAATG-3’</td>
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<td>1624-1643</td>
<td>5’-AAACGGCTGACGAACTGAAA-3’</td>
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<tr>
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<td>5’-CATTTTCACATGCCATGGA-3’</td>
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<td>565-589</td>
<td>5’-AGGCTTCAGAGAGATTCAGACGACG-3’</td>
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<td>5’-CGGTTGCTACAGCAGTGCCATA-3’</td>
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<td>5’-TCGAGATGCTTTTGTGAA-3’</td>
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<tr>
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<td>277-257</td>
<td>5’-GACTGTTTCCGCCATCATA-3’</td>
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<tr>
<td>S1 F</td>
<td>13-31</td>
<td>5’-AGCCATATGTTGAGTATA-3’</td>
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</table>
BTV Protein Western Blot

Cells were prepared as described by Li and Yang (1990). Briefly, two flasks of cells from each cell line were seeded separately at 1 X 10^6 cells/flask and allowed to adhere overnight. One flask from each cell line was infected with BTV serotype 17 at an MOI of 1, while the second flask was mock infected. Cells were harvested 72 hours P.I. by scraping. Samples were washed once in PBS and the cell pellet stored at -80 °C until use. Prior to electrophoresis, cell pellets were resuspended in PBS and lysed by sonication. The protein concentration of each sample was determined by BCA assay and each sample adjusted to 1 mg/mL protein using PBS. Samples were then diluted to a final concentration of 500 μg/mL in Lameli buffer with 5% 2-ME and boiled for 10 minutes. Boiled samples were electrophoresed on 10% polyacrylamide, and electrophoretically transferred to PVDF membrane (12V 1A 90 min). Membranes were blocked with 5% BSA for 30 minutes then incubated with rabbit anti-BTV17 antibody diluted 1:1000 in 5% BSA overnight at 4 °C with gentle shaking. Antibody binding was
detected by incubating the membrane with HRP-labeled goat anti-rabbit secondary antibody (Sigma, St. Louis, MO) diluted 1:5000 for 2 hours at room temperature with gentle shaking. HRP activity was visualized with TMB membrane substrate (Sigma, St. Louis, MO).

**Statistical Analysis**

P-values were calculated in Excel (Microsoft Corporation, Redmond, WA) using two-tailed Student’s t-test. Coefficient of variation (%CV) was calculated by dividing the standard deviation of the group by the group average.

**RESULTS**

*All BTV Genes are Transcribed During Infection*

Data from Chapter 2 showed that cell death in human cells is cell line dependent. To understand how this cell death related to viral replication, RNA for each gene was quantified by qRT-PCR. RNA for every BTV gene was found expressed in the tested human cell lines, consistent with active viral replication. The RT-PCR data shows that the detected viral RNA was not from the genome of the original infecting virus nor a cross reaction with cellular RNA. Fig. Tables 3-2 through 3-5 show the cycle threshold, c(T), values for each gene in BHK-21, A498, HEP-G2, and A549 cells 72 hours P.I. Figures 3-1 to 3-11 show the qRT-PCR amplification curves for each primer set.

Cell lines showed strong consistency in amount of each gene produced, with cycle threshold [c(T)] values between genes in the same cell line.
FIG. 3-1. qRT-PCR graphs for the L1 transcript 72 hours P.I. BTV17 L1 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-2. qRT-PCR graphs for the L2 transcript 72 hours P.I. BTV17 L2 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-3. qRT-PCR graphs for the L3 transcript 72 hours P.I. BTV17 L3 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-4. qRT-PCR graphs for the M1 transcript 72 hours P.I. BTV17 M1 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-5. qRT-PCR graphs for the M2 transcript 72 hours P.I. BTV17 M2 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-6. qRT-PCR graphs for the M3 transcript 72 hours P.I. BTV17 M3 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-7. qRT-PCR graphs for the S1 transcript 72 hours P.I. BTV17 S1 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-8. qRT-PCR graphs for the S2 transcript 72 hours P.I. BTV17 S2 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-9. qRT-PCR graphs for the S3 transcript 72 hours P.I. BTV17 S3 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-10. qRT-PCR graphs for the S4 transcript 72 hours P.I. BTV17 S4 transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
FIG. 3-11. qRT-PCR graphs for the GAPDH transcript 72 hours P.I. Human GAPDH transcript amplification by qRT-PCR in (A) BHK-21, (B) A498, (C) HEP-G2, and (D) A549 cells 72 hours post BTV (green line) or placebo (red line) infection. The cycle threshold is measured at the point at which the exponential rise in product crosses the threshold value set at 0.05.
maintaining an average %CV of 7.7%. In BHK-21, the average c(T) was 35.8 +/- 4.3 in mock-infected and 14.9 +/- 1.4 in BTV-infected cells. For A498, the average was 34.9 +/- 3.2 for mock-infected and 15.9 +/- 1.4 for BTV-infected cells. Average HEP-G2 mock-infected cell c(T) was 32.6 +/- 2.3 but 23.1 +/- 1.3 in BTV-infected cells, while A549 mock-infected cells averaged 34.9 +/- 2.3 but 29.9 +/- 2.2 in BTV-infected cells.

**TABLE 3-2**

**BHK-21 Cycle Threshold Values at 72 Hours P.I.**

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### TABLE 3-3

A498 Cycle Threshold Values at 72 Hours P.I.

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### TABLE 3-4

HEP-G2 Cycle Threshold Values at 72 Hours P.I.

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TABLE 3-5

A549 Cycle Threshold Values at 72 Hours P.I.

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<tr>
<td>GAPDH</td>
<td>19.7</td>
<td>86</td>
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</tbody>
</table>

To ensure consistency in the data, RNA concentrations in the purified RNA samples were determined spectrophotometrically at A\textsubscript{260} and A\textsubscript{280} absorbance and then adjusted to 50 \( \mu \)g/mL total RNA prior to reverse transcription. Furthermore, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured simultaneously to confirm normalization of the samples. The average %CV of GAPDH c(T) values between mock-infected and BTV-infected cells was 0.7%. Average GAPDH values between cell lines was 21.0 +/- 1.3 corresponding to a %CV of 6.1%, suggesting good consistency between samples.
**BTV Proteins Are Expressed During Infection**

In order to identify viral protein expression, Western blots were performed on mock-infected and BTV-infected cultures. Western blot results using purified rabbit anti-BTV17 antisera are shown in Fig. 3-12. Bands were identified in all 3 cell lines with molecular weights consistent with BTV proteins NS2 and VP7. Band intensity for the two putative BTV proteins was strongest in HEP-G2 cells, followed by A549 cells. BTV protein expression was weakest in A498 cells, with only one viral protein band clearly detectable on the Western blot (see Fig. 3-12).

**FIG. 3-12.** Western blot of mock-infected and BTV-infected cells. A498, HEP-G2, and A549 cells were either infected with BTV serotype 17. Cells were harvested 72 hours P.I. The samples were processed by sonication and protein content adjusted to 500 mcg/mL prior to PAGE analysis. Gel was blotted to a PVDF membrane and stained with rabbit anti-BTV17 antisera, and visualized with TMB substrate.
DISCUSSION

BTV has therapeutic potential in cancer because of its ability to kill human cancer cells and reduce human tumor size (Xiao et al., 2004). In this experiment BTV is shown to replicate in each cell, though the efficiency of replication is heavily cell line dependent.

RNA production was highest in the kidney derived A498 cells, followed by HEP-G2 and last by A549 cells. RNA concentrations in A498 cells were comparable to RNA concentrations observed in the laboratory host BHK-21 (Table 3-2). It may be that the high viral RNA expression is related to the tissue type as BHK-21 and A498 are both kidney derived cells. However, protein expression in A498 cells was severely limited in comparison to the other cell lines as determined by Western blotting with anti-BTV17 antisera. Even though A498 cells produced the most RNA of any cell, they produced the least protein. HEP-G2, on the other hand, showed strong RNA expression, though not nearly as high as A498 cells, but showed the most BTV protein (Fig. 3-12). A549 cells expressed the least amount of BTV RNA, with concentrations barely above controls, though every BTV gene was detected in A549 cells.

Within each cell line, all of the genes quantified in a narrow range of cycle threshold values (1.29-17.6 for BHK-21 cells, 14.6-18.8 for A498 cells, 21.0-25.4 for HEP-G2 cells, and 26.8-33.0 for A549 cells). This narrow range suggests there was no preferential over/under expression or over/under degradation of a particular gene.
Based on c(T) numbers, of the human cell lines, A498 cells produced the most viral RNA, with levels within the standard deviation of the BHK-21 cells. HEP-G2 produced the next highest level of viral RNA, though considerably less than A498. Human A549 cells showed the lowest amount of RNA, with levels clearly above background, but substantially lower than HEP-G2 and A498 (see Table 3-1 through 3-4). Testing was performed 72 hours P.I., so these values represent cumulative RNA expression over 3 days.

One possibility for the differences in RNA levels is differences in the rate of RNA degradation in each cell line, though BTV viral RNA would be expected to degrade at a faster rate than cellular mRNA due to its lack of polyadenylation. However, the testing was a measure of total viral RNA, and genomic dsRNA is not degraded by the host and is therefore expected to accumulate as a function of time and replication rate. Given the lack of a clear relationship between RNA and protein among the human cells, the differences in expression are probably not related to rates of RNA degradation but related to the replication rate being influenced by the intracellular environment. Specifically, the activity of antiviral defense mechanisms, such as type I interferon.

The role of the intracellular environment suggests that viral tropism may be as much related to innate immune system activity and usability of the host transcription/translation machinery as it is related to receptor expression. BTV may equally infect various tissues, but only concentrate in the tissues in which it replicates most efficiently.
Hence, BTV may equally infect primary and cancerous cells, but only cancerous cells are conducive to efficient viral replication. A strong possibility, since cancer cell expression is highly modified from its primary counterpart with various genes turned on and others, such as innate defense mechanisms, turned off.

The influence of the intracellular environment is supported by the inverse correlation between protein expression and cell death/cytokine expression. A498 cells expressed the most RNA but least protein. These cells also displayed the most cell death and cytokine expression. HEP-G2 which expressed the most protein was the least susceptible to BTV-induced cell death, and expressed the least amount of cytokines. A549 cells, which showed the least amount of RNA, showed modest protein expression, cell death, and cytokine expression.

This inverse relationship between cell death/cytokine expression and viral protein expression indicates that the cell death and cytokine expression are both functions of the cell’s response to infection rather than a direct result of the virus. If the virus was completely in control, all four variables would be expected to rise proportionately. As the virus replicates, RNA and protein expression would increase, and as replication completes, cell death would increase, and cytokine expression would increase steadily. In this scenario, differences in fitness between cell lines would lead to proportionate changes in all four variables. However, expression between the four variables is not proportionate indicating influences by the host cell.
Possible influences include innate immunity, as well as altered signaling. For example, the type-I interferon response leads to cytokine expression, activation of the caspase pathways, and phosphorylation of EIF-2 thereby shutting down protein synthesis (Watanabe, 2004). In the presence of a functioning type-I interferon pathway, viral protein production would remain low in the presence of high viral RNA and would be inversely related to cell death and cytokine expression, as was seen in A498 cells. The same pattern, however was not seen in every cell line, suggesting there may be other mechanisms present as discussed in Chapter 4.

In conclusion, the objective of this experiment was to correlate BTV RNA and protein expression to the cytokine and cell death data of the previous experiment. Our hypothesis was that cell death and cytokine expression correlates directly with viral RNA and protein expression consistent with cytokine expression and cell death the direct result of viral replication. We observed high levels of both RNA and protein in each cell line, showing that the virus is fully capable of infecting and replicating in each line; however, comparisons between the three human cell lines showed no obvious correlation between RNA and cell death or cytokine expression, and an inverse correlation between viral protein expression and cell death. The data does not support our initial hypothesis, but rather suggests that the magnitude of cell death and cytokine expression is cell line dependent and a function of the cell’s ability to respond to infection.

No matter the reason for BTV’s selective infectivity, it is clear that the virus is fully capable of utilizing human host machinery to begin replication; and though
the rate is not as efficient as in a native host, it is sufficient enough to induce cell
death and cytokine expression in carcinoma transformed cells.

REFERENCES

quantitative real-time polymerase chain reaction. In “A Kinetic Analysis of
Bluetongue Virus Messenger RNA and Protein” (M. Buccambusco, ed.),
pp. 16-54. Utah State University Press, Logan, UT.


CHAPTER 4

CELLULAR RESPONSES TO BLUETONGUE VIRUS INFECTION DO NOT INCLUDE ACTIVATION OF NFκB, P38 MAPK, OR JAK-STAT SIGNALING

ABSTRACT

BTV causes cell death and cytokine expression in human carcinoma transformed cells. The magnitude of cell death and cytokine expression are a function of the cell’s response to infection, not a direct result of viral replication. Our objective was to determine the contributions of major apoptotic and inflammatory signaling molecules to the observed cell death and cytokine expression. Our hypothesis was that the cell promotes apoptosis by dephosphorylating PBK and induces cytokine expression through activation of NFκB and p38 MAPK signaling. PKB, NFκB, MAPK family, and tyrosine kinase signaling contributions were all determined by selective inhibition of signaling activity. Phosphorylation levels of PKB and MAPK family members were subsequently measured by ELISA.

Inhibition of PKB, NFκB, and MAPK activity all had no effect on cell death or cytokine expression though PKB and p38 MAPK were both constitutively phosphorylated in A498 and A549 cells. Phosphorylation of PKB and p38 MAPK was subsequently lost upon infection with BTV. Tyrosine kinase signaling was found to be involved in both cell death and cytokine expression, involving EGFR, but not JAK-STAT signaling. A 35 kDa tyrosine phosphorylated protein with multiple phosphorylation states was isolated by two-dimensional gel
electrophoresis, though the identity of the protein remains unknown. We conclude that conventional pro-inflammatory signaling through NFκB, p38 MAPK, and JAK-STAT are not involved in BTV-induced cell death and cytokine expression, but that PKB, EGFR, and an unknown tyrosine kinase pathway are involved.

**INTRODUCTION**

In previous chapters, bluetongue virus (BTV) was shown to infect human carcinoma transformed cells, resulting in cytokine expression and cell death. Cell death and cytokine expression were not the direct result of viral infection, but the result of the cell’s response to infection. Even though the virus is fully replicating its genome in each cell line, cell death and cytokine expression were markedly disproportionate. This data suggested that host defense mechanisms are involved, though the data does not match up exactly with literature for any specific process (Mahalingam et al., 1999; Nociari et al., 2007). The fact that each cell line produced the same cytokine pattern and that cell death compared between cell lines directly correlated with the magnitude of cytokine expression suggested the same mechanisms are acting in each cell line and that cell death and cytokine expression are related through intracellular signaling.

PKB was chosen for this study because prior studies by our lab suggested changes in PKB signaling occur during viral infection. The MAPK family was chosen because of its role in apoptosis, inflammation, and innate immunity. In addition, Chiang et al. (2006) demonstrated activation of p38 MAPK signaling
during BTV infection of human primary endothelial cells. We also tested NFκB and tyrosine kinase signaling because of their involvement in inflammation and innate immunity. These various signaling molecules represent a broad spectrum of potential signaling molecules involved in the cell’s response to infection. The objective of this investigation was to determine the involvement of these signaling molecules in the human carcinoma transformed cell’s response to BTV infection. Our hypothesis was that the cell responds to infection by dephosphorylating PKB and by activating both NFκB and p38 MAPK signaling.

MATERIALS AND METHODS

Cell Culture

Five cell lines were used in this study, including BHK-21 baby hamster kidney cells, A498 human kidney cells, HEP-G2 human liver cells, A549 human lung epithelial cells, and Vero African green monkey kidney cells (American Type Culture Collection, Manassas, VA). Designated CCL-10, HTB-4, HB-8065, CCL-185, and CRL-1587, respectively by the American Type Culture Collection. Each cell line was propagated in 75 cm² flasks seeded at 1X10⁶ cells and cultured in MEM/EBSS plus 10% FBS (Hyclone, Logan, UT) under a 5% CO₂ atmosphere.

BTV Stock Propagation

Original stocks were triple plaque purified and stored frozen in aliquots (Kowalik et al., 1990; Li and Yang, 1990). Seed virus was prepared by infecting BHK-21 cells with frozen viral stock. Cells were infected at 80% confluency and harvested by scraping three days post infection (P.I.). After scraping, cells were
pelleted, washed, resuspended in fresh media, then gently sonicated three times using a VirSonic 50 sonicator (VirTis Inc., Gardiner, NY) prior to 0.22 μm filter sterilization. Viral titer was determined by plaque assay, using Vero cell monolayers (Hayama and Li, 1994; Fillmore et al., 2002). Mock infections were similarly prepared using uninfected BHK-21 cells.

Plate Preparation and BTV Infection

96-well plates were seeded one day prior to experimentation in MEM/EBSS plus 10% FBS (Hyclone Laboratories, Logan, UT), such that the culture was approximately 50% confluent when starting the experiment. Prior to experimentation, plates were rinsed with MEM/EBSS. BTV infection occurred at an MOI of 1 using BTV serotype 17. Mock infections were performed using a comparable volume of uninfected BHK-21 cells, prepared as described above. Plates were incubated at 37 °C under 5% CO₂ in MEM/EBSS plus 5% FBS.

Neutral Red Staining

A 0.034% solution of neutral red (Fisher Scientific, Fairlawn, NJ) in saline was prepared and filter sterilized. Immediately before use, the solution was diluted to 0.011% in MEM/EBSS. All media was removed from the plate, 200 mcL of the 0.011% neutral red solution was added, and plates were incubated at 37 °C under 5% CO₂ for two hours. Plates were then rinsed once with PBS, and neutral red was extracted by adding 100 μg/mL neutral red extraction buffer (1:1 ratio of Sorensen citrate buffer [0.1 M sodium citrate (Sigma, St. Louis, MO) and 0.1 M hydrochloric acid (Fisher Scientific, Fairlawn, NJ)] and absolute ethanol
(Pharmco-AAPER, Brookfield, CT). Absorbance at 540 nm was read using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA).

**Determination of Phosphorylated PKB Expression**

Rabbit anti-phospho-AKT (Ser473) antibody (Cell Signaling Technologies, Danvers, MA) was deposited onto the bottom of a 96-well plate in a defined location using a Genomic Solutions (Ann Arbor, MI) non-contact printing machine. Samples collected previously were diluted into cell lysis buffer (Cell Signaling Technologies, Danvers, MA) with 1 mM PMSF (Sigma, St. Louis, MO). Cells were then sonicated on ice 3 times for 10 seconds each using VirSonic 50 sonicator (Virtis Inc., Gardiner, NY), and clarified by centrifugation. Supernatant was incubated on the 96-well plates at room temperature on a lab rotator set at 120 RPM for 1 hour. Following TBST wash, plates were incubated with biotinylated mouse anti-phospho-AKT1 antibody (Cell signaling technologies, Danvers, MA) diluted in 5% BSA. Binding was detected by horse radish peroxidase (HRP) conjugated streptavidin (Biolegend Inc., San Diego, CA) diluted in 5% BSA and subsequently visualized with Pierce chemiluminescent substrate (Rockford, IL) on an Alpha Innotec 8900 gel documentation system (San Leandro, CA). Luminescent signal intensity was quantified using software provided with the Alpha Innotec camera.

**Determination of Phosphorylated p44/42 MAPK and p38α MAPK Expression**

Cell pellets, collected previously, were processed and subsequently assayed for expression with the PathScan Inflammation 4-plex array (Cell...
Signaling Technologies, Danvers, MA) according to the manufacturer’s recommended protocol.

**Determination of Phosphorylated SAPK/JNK Expression**

Cell pellets, collected previously, were processed and subsequently assayed for expression with the PathScan Phospho-SAPK/JNK (Thr183/Tyr185) Sandwich ELISA Kit (Cell Signaling Technologies, Danvers, MA) according to the manufacturer’s recommended protocol.

**Activity Inhibition of p38 MAPK, ERK-1/2, SAPK/JNK, and NFκB**

P38 MAPK kinase activity inhibitor SB-203580, the MEK-1/2 kinase activity inhibitor U-0126, SAPK/JNK kinase activity inhibitor SP600125, and NFκB activity inhibitor QNZ were acquired from Biomol International, Inc. (Plymouth Meeting, PA). Each was reconstituted to 2 mg/mL in DMSO (Sigma, St. Louis, MO) and tested at a final concentration of 1 μg/mL in MEM/EBSS plus 5% FBS. Each inhibitor stock was stored at -20 °C in DMSO when not in use, and working solutions were prepared immediately before use. Cells were seeded into 96-well plates as described previously. Inhibitor was loaded into each well and then either mock-infected, BTV-infected, treated with 1 μg/mL, or treated with 50 μg/mL of polyinosinic:polycytidylic acid (poly I:C) (n=4). Supernatant was harvested three days P.I., and the plates stained with neutral red.
Activity Inhibition of Tyrosine Kinase Families

General tyrosine kinase activity inhibitor Genistein, the Src kinase activity inhibitor PP2, JAK2, JAK3, and STAT-3 inhibitor AG490, platelet derived growth factor receptor (PDGFR) kinase activity inhibitor AG1278, and epidermal growth factor receptor (EGFR) kinase activity inhibitor AG1478 were acquired (Calbiochem, San Diego, CA). Each was reconstituted in DMSO and tested at a final concentration of 1 \( \mu \text{g/mL} \) in MEM/EBSS plus 5% FBS. Each inhibitor stock was stored at -20 °C in DMSO when not in use, and working solutions were prepared immediately before use. Cells were seeded into 96-well plates as described previously. Inhibitor was loaded into each well and either mock-infected, BTV-infected, treated with 1 \( \mu \text{g/mL} \), or treated with 50 \( \mu \text{g/mL} \) of poly I:C \((n=4)\). Supernatant was harvested three days P.I., and the plates stained with neutral red.

Quantitation of Secreted Analyte Expression

Supernatant samples, collected previously and stored at -80 °C, were thawed at room temperature. Cytokines were detected by multiplex immunoassay (Quansys Biosciences, Logan, UT). Samples were tested for: IL-1\( \alpha \), IL-1\( \beta \), IL-6, IL-8, IFN\( \gamma \), TNF\( \alpha \), MCP-1, and RANTES. The chemiluminescent signal was detected using an Alpha Innotech 8900 Gel Documentation System (San Leandro, CA). Plates were exposed for one minute, and signal intensity was quantified using software provided by Alpha Innotech.
Tyrosine Phosphorylation by Western Blot

Cell samples were diluted in Lameli buffer and protein concentration determined using the BCA method. After titration, samples were adjusted to a final concentration of 500 μg/mL in Lameli buffer, 2-ME added to each sample, and boiled for 10 minutes. Samples were then electrophoresed on 10% polyacrylamide, and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (12V 1A 90 min). Membranes were blocked with 5% BSA for 30 minutes. The PVDF membrane was incubated overnight at 4 °C with gentle shaking in 5% BSA containing Cell Signaling Technologies (Danvers, MA) biotinylated anti-phospho-tyrosine mouse monoclonal antibody. Antibody binding was detected by incubating the membrane with HRP-labeled streptavidin for 2 hours at room temperature with gentle shaking. Binding was visualized with TMB membrane substrate (Sigma, St. Louis, MO).

Two-Dimensional Gel Electrophoresis

Samples were prepared by diluting cells in water and boiling for 10 minutes followed by dilution into isoelectric focusing (IEF) rehydration buffer (8 M Urea, 2% CHAPS, and 50 mM DTT), and incubated at room temperature for 5 minutes. Samples were then clarified by centrifugation to remove the bulk of the genomic DNA and insoluble particulates. The supernatant was applied to Bio-Rad Laboratories (Hercules, CA) ReadyStrip™ IPG strips, and allowed to actively rehydrate for 12 hours. IEF was performed on the Bio-Rad Protean IEF cell apparatus. After focusing, IPG strips were reduced with DTT (6 M Urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2% w/v DTT) and free cysteines acetylated
with iodoacetamide (6 M Urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2.5% w/v iodoacetamide). Samples were then electrophoresed on 10% polyacrylamide. Mini gels were run at 100 volts 20 mA for 1 to 2 hours, while large gels were run at 150 to 300 volts 100 mA for 5-7 hours. After completion of the run, gels to be stained were incubated overnight with Coomassie blue stain (Sigma, St. Louis, MO). Gels to be transferred to PVDF were treated according to the protocols described for phospho-tyrosine Western blotting.

**Mass Spectroscopy**

Proteins resolved by two dimensional gel electrophoresis were excised from Coomassie blue stained gels. In gel trypsin digestion and mass analysis of digested peptides was performed by the Utah State University Center for Integrated Biosystems mass spectroscopy lab (Logan, UT).

**Statistical Analysis**

P-values were calculated in Excel (Microsoft Corporation, Redmond, WA) using two-tailed Student’s t-test.

**RESULTS**

*Inhibition of PKB Activity Does Not Affect Cell Death or Cytokine Expression*

PKB plays a key role in cell survivability and BTV infection leads to reduced survival rates in infected cells. To examine the contribution of PKB phosphorylation to cell death and cytokine expression, PKB activity in A498, HEP-G2, A549, and Vero cells was inhibited using Triciribine (n=4).
treatment resulted in no significant change in cell death compared to untreated cells (Fig. 4-1 through 4-4). Triciribine treatment also had no significant effect on cytokine expression. Fig. 4-5 through 4-8 compare cytokine expression from mock-infected, BTV-infected, and poly I:C treated cells. Our hypothesis was that even though Triciribine alone did not affect cell death or cytokine expression, Triciribine would contribute to cell death and cytokine expression in BTV-infected cells. While BTV-infected and poly I:C treated cells showed statistically significant increases in cell death and cytokine expression compared to mock infections, Triciribine treatment had no influence on the dependent variables (Fig. 4-1 through 4-8).

*Inhibition of NFκB Does Not Affect Cell Death or Cytokine Expression*

Using the same experimental design as used in PKB inhibition, cells were treated with QNZ inhibitor to block NFκB activity. NFκB plays an important role in both apoptosis and inflammation, by promoting cell survival while simultaneously inducing transcription of multiple pro-inflammatory cytokines and chemokines (Ghosh and Hayden, 2008). When cells were treated with QNZ, however, no significant change in cell death or cytokine expression occurred as observed in Fig. 4-1 through 4-8.

*Inhibition of MAPK Signaling Does Not Affect Cell Death or Cytokine Expression*

Using the same experimental design as used in PKB inhibition, MAPK signaling was inhibited. There are three groups in the MAPK family of signaling
molecules: p38 MAPK, ERK-1/2, and SAPK/JNK. Each group has separate functions but all have an important role either in cell viability or inflammation. Interestingly, none of the inhibitors resulted in a significant change in cell death as shown in Fig. 4-9 through 4-12. Though BTV and poly-I:C-induced cell death compared with mock-infected cells, the inhibitors given singly had no influence on cell death. Likewise, MAPK family inhibition had no effect on cytokine expression. Fig. 4-13 through 4-16 show cytokine expression from A498 cells. Comparisons between inhibitors is similar in HEP-G2, A549, and Vero cells (data not shown).

FIG. 4-1. Percent viability of A498 cells treated with Triciribine or QNZ 3 days P.I. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.
FIG. 4-2. Percent viability of HEP-G2 cells treated with Triciribine or QNZ 3 days P.I. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.

FIG. 4-3. Percent viability of A549 cells treated with Triciribine or QNZ 3 days P.I. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.
FIG. 4-4. Percent viability of Vero cells treated with Triciribine or QNZ 3 days P.I. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.

FIG. 4-5. IL-6 expression from A498 cells treated with either placebo, PKB inhibitor Triciribine, or NFκB inhibitor QNZ. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.
FIG. 4-6. IL-8 expression from A498 cells treated with either placebo, PKB inhibitor Triciribine, or NFκB inhibitor QNZ. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

FIG. 4-7. MCP-1 expression from A498 cells treated with either placebo, PKB inhibitor Triciribine, or NFκB inhibitor QNZ. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.
FIG. 4-8. RANTES expression from A498 cells treated with either placebo, PKB inhibitor Triciribine, or NFκB inhibitor QNZ. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

FIG. 4-9. MAPK inhibitor treated A498 cell viability 3 days P.I. Cells were treated with either placebo, SB-203580, U-0126, or SP600125. Treated cells were either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.
FIG. 4-10. MAPK inhibitor treated HEP-G2 cell viability 3 days P.I. Cells were treated with either placebo, SB-203580, U-0126, or SP600125. Treated cells were either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.

FIG. 4-11. MAPK inhibitor treated A549 cell viability 3 days P.I. Cells were treated with either placebo, SB-203580, U-0126, or SP600125. Treated cells were either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.
FIG. 4-12. MAPK inhibitor treated Vero cell viability 3 days P.I. Cells were treated with either placebo, SB-203580, U-0126, or SP600125. Treated cells were either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.

FIG. 4-13. IL-6 expression from A498 cells treated with either placebo, p38 MAPK inhibitor SB-203580, ERK-1/2 inhibitor U-0126, or SAPK/JNK inhibitor SP600125. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.
FIG. 4-14. IL-8 expression from A498 cells treated with either placebo, p38 MAPK inhibitor SB-203580, ERK-1/2 inhibitor U-0126, or SAPK/JNK inhibitor SP600125. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

FIG. 4-15. MCP-1 expression from A498 cells treated with either placebo, p38 MAPK inhibitor SB-203580, ERK-1/2 inhibitor U-0126, or SAPK/JNK inhibitor SP600125. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.
FIG. 4-16. RANTES expression from A498 cells treated with either placebo, p38 MAPK inhibitor SB-203580, ERK-1/2 inhibitor U-0126, or SAPK/JNK inhibitor SP600125. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

*BTV Infection Results in Loss of PKB and p38 MAPK Phosphorylation in vitro*

Previous experiments showed that neither PKB, NFκB, p38 MAPK, ERK-1/2, nor SAPK/JNK inhibition had any affect on cell death or cytokine expression. However, preliminary Western blots (data not shown) and current literature on MAPK (Chiang *et al.*, 2006) suggested both PKB and the MAPK family had involvement in BTV-induced cytokine expression. Therefore, PKB and MAPK phosphorylation levels were directly measured in A498, HEP-G2, and A549 cells to ensure inhibitor functionality three days P.I. and determine if lack of activity was a result of changes in downstream signaling.
None of the tested cell lines constitutively expressed phosphorylated ERK-1/2, while HEP-G2 cells did not express phosphorylated PKB or phosphorylated p38 MAPK. All of the cell lines expressed phosphorylated SAPK/JNK, while A498 and A549 cells expressed both phosphorylated PBK and phosphorylated p38 MAPK as shown in Fig. 4-17 and 4-18, respectively.

Interestingly, phosphorylation of both PKB and p38 MAPK declined significantly to near background levels when cells were infected with BTV as shown in Fig. 4-17 and 4-18. Poly I:C treatment did not have an effect on A549 cells, though there was a significant decline in PKB phosphorylation in poly I:C treated A498 cells, though not to levels observed during BTV-infection. Resting primary cells do not constitutively express phosphorylated PKB or any of the MAPKs, but are phosphorylated upon infection with BTV or upon treatment with poly I:C in vitro (Chiang et al., 2006). The herein observed levels of phosphorylated PKB and p38 MAPK are likely a consequence of the transformation by carcinoma, and the subsequent decline in p38 MAPK phosphorylation along with the lack of corresponding IL-1 and TNF$\alpha$ expression represents a clear distinction between infection of primary cells and carcinoma cells.

_inhibition of tyrosine kinase activity does not affect BTV-induced cell death_

Using the same experimental design as used in PKB inhibition, cells were treated with five different inhibitors of tyrosine kinase activity. AG1296 and AG1478 directly inhibit receptor tyrosine kinases, PP2 and AG490 inhibit
receptor associated tyrosine kinases, and Genistein is a pan tyrosine kinase activity inhibitor. Each has a major role in tyrosine based signal transduction.

Fig. 4-19 through 4-22 show the effects of each tyrosine kinase inhibitor on A498, HEP-G2, A549, and Vero cell viability, respectively.

Viability in the four cell lines was unaffected by treatment with inhibitors alone. Likewise, BTV-induced cell death was unaffected in A498, HEP-G2, and Vero cells. A549 cell viability, however, declined as a result of Genistein treatment (Fig. 4-21).

When cells were treated with poly I:C instead of BTV, the tyrosine kinase activity inhibitors protected the A498 cells from poly-I:C-induced cell death. This was only observed in A498 cells because they are the only cells which lose viability in the presence of poly I:C. In A498 cells, four of five tyrosine kinase inhibitors provided protection from poly-I:C-induced cell death (Fig. 4-19).

FIG. 4-17. Phosphorylation levels of PKB Ser473 in A498 and A549 cells. Cells were either mock-infected, BTV-infected, or poly I:C treated (n=6). S/N ratios were determined 3 days P.I. using supplies provided by Quansys Biosciences.
FIG. 4-18. Phosphorylation levels of p38 MAPK at Thr180/Tyr182 in A498 and A549 cells. Cells were either mock-infected, BTV-infected, or poly I:C treated (n=6). S/N ratios were determined 3 days P.I. using supplies provided by Quansys Biosciences.

Findings were followed up on by testing poly I:C in the presence of each tyrosine kinase inhibitor treated in a dose dependent manner. As observed before, A498 cells were affected by tyrosine kinase activity inhibition, as shown in Fig. 4-23 through 4-27. BTV-induced cell death was not protected by treatment with any of the inhibitors while all the inhibitors provided some protection from poly-I:C-induced cell death. Though all of the tyrosine kinase inhibitors were cytotoxic, every poly-I:C-treated curve experienced a rise in cell viability with treatment concentration before falling as a result of cytotoxicity. The most effective was Genistein, the general tyrosine kinase inhibitor where treatment at >3.2 μg/mL resulted in full protection from poly-I:C-induced cell death.
FIG. 4-19. Tyrosine kinase inhibitor treated A498 cell viability 3 days P.I. Vero cells treated were with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.

FIG. 4-20. Tyrosine kinase inhibitor treated HEP-G2 cell viability 3 days P.I. Vero cells treated were with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.


FIG. 4-21. Tyrosine kinase inhibitor treated A549 cell viability 3 days P.I. Vero cells treated were with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were either mock-infected, BTV-infected, 1 µg/mL poly I:C treated, or 50 µg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.

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FIG. 4-22. Tyrosine kinase inhibitor treated Vero cell viability 3 days P.I. Vero cells treated were with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were either mock-infected, BTV-infected, 1 µg/mL poly I:C treated, or 50 µg/mL poly I:C treated (n=4). Viability was determined by neutral red staining 3 days P.I.
FIG. 4-23. Genistein treated A498 cells 3 days P.I. A498 cells were treated with pan tyrosine kinase inhibitor Genistein in a dose dependent manner. Treated cells were either mock-infected, BTV-infected, or 50 μg/mL poly I:C (n=4) treated. Viability was determined by neutral red staining 3 days P.I.

FIG. 4-24. PP2 treated A498 cells 3 days P.I. A498 cells were treated with Src inhibitor PP2 in a dose dependent manner. Treated cells were either mock-infected, BTV-infected, or 50 μg/mL poly I:C (n=4) treated. Viability was determined by neutral red staining 3 days P.I.
FIG. 4-25. AG490 treated A498 cells 3 days P.I.  A498 cells were treated with JAK-STAT inhibitor AG490 in a dose dependent manner. Treated cells were either mock-infected, BTV-infected, or 50 μg/mL poly I:C (n=4) treated. Viability was determined by neutral red staining 3 days P.I.

FIG. 4-26. AG1296 treated A498 cells 3 days P.I.  A498 cells were treated with PDGFR inhibitor AG1296 in a dose dependent manner. Treated cells were either mock-infected, BTV-infected, or 50 μg/mL poly I:C (n=4) treated. Viability was determined by neutral red staining 3 days P.I.
FIG. 4-27. AG1478 treated A498 cells 3 days P.I. A498 cells were treated with EGFR inhibitor AG1478 in a dose dependent manner. Treated cells were either mock-infected, BTV-infected, or 50 μg/mL poly I:C (n=4) treated. Viability was determined by neutral red staining 3 days P.I.

*Genistein and AG1478 Treatment Reduces Cytokine Expression from Poly I:C-Treated, but Not BTV-Infected Cells*

In addition to measuring cell viability during treatments with tyrosine kinase inhibitors, cytokines were measured 3 days P.I. None of the tyrosine kinase inhibitors were found to significantly reduce resting cell cytokine expression, but Genistein treatment did significantly reduce cytokine expression from BTV-infected A549 cells, though Genistein also exacerbated BTV-induced cell death suggesting that the decline in cytokine expression was a result of fewer viable cells. BTV-infected Vero cells were also affected by tyrosine kinase inhibition, most notably from Genistein, but also from Src and EGFR inhibitors.
Cytokine expression from BTV-infected A498 and HEP-G2 cells was unaffected by treatment with Genistein or any other tyrosine kinase inhibitor.

Unlike BTV-infected cells, Genistein and AG1478 consistently reduced cytokine expression from poly-I:C-treated A498, A549, and Vero cells as shown in Fig. 4-28 through 4-39. This reduction suggests the involvement of EGFR signaling in the cell’s response to dsRNA and may also lead to cell death in some cell lines.

*Tyrosine Phosphorylation is Activated During Infection with BTV*

Previous testing demonstrated that tyrosine kinase signaling can have an influence on cell death and cytokine expression in human carcinoma transformed

![Graph showing IL-6 expression from A498 cells treated with different inhibitors and treatments](image)

**FIG. 4-28.** IL-6 expression from A498 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.
FIG. 4-29. IL-8 expression from A498 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

cells. Inhibition of signaling resulted in both protection from poly-l:C-induced cell death in A498 cells, and reduced cytokine expression in each cell line. To identify changes in tyrosine signaling during infection, Western blots were performed on both mock-infected and BTV-infected human carcinoma transformed cells. Fig. 4-40 and 4-41 are Western blots using anti-BTV17 antiserum and mouse anti phosphorylated-tyrosine monoclonal antibodies, respectively. There are distinct bands in Fig. 4-41 present only in BTV-infected cells indicating the presence of a tyrosine phosphorylated protein present only in BTV-infected cells (indicated by an arrow in Fig. 4-41). Band intensity was the strongest in HEP-G2 cells followed by A549 cells and not resolvable in A498
FIG. 4-30. MCP-1 expression from A498 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

cells. The bands in Fig. 4-41 were comparable in molecular weight to bands identified in Fig. 4-40 as probable BTV protein (compare to Fig. 4-40), with the 35 kDa band the most prominent.

Following these findings, each tyrosine kinase inhibitor tested previously was tested for its ability to prevent phosphorylation of the band identified in the above mentioned Western blot. HEP-G2 cells were selected for the study because band intensity was strongest in these cells. Cells were cultured in the presence of each MAPK inhibitor or each tyrosine kinase activity inhibitor and BTV. Three days P.I., cells were harvested and assayed by Western blot for phosphorylated tyrosine (Fig. 4-42). Phosphorylation of the 35 kDa protein
FIG. 4-31. RANTES expression from A498 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

remained in samples treated with MAPK inhibitors but was reduced by treatment with all tyrosine kinase inhibitors. Genistein, the general tyrosine kinase activity inhibitor was the most effective at inhibiting phosphorylation of 35 kDa protein but all tyrosine kinase activity inhibitors resulted in noticeable reductions in band intensity.

*Unknown Tyrosine Phospho-Protein is a 35 kDa Protein Displaying Multiple Isoelectric Points Between 6.5 and 7.5*

In addition to identifying the tyrosine kinase signaling pathways that can influence phosphorylation of the 35 kDa protein, two dimensional gel electrophoresis was performed to isolate the unknown protein. BTV-infected and mock-infected A549
FIG. 4-32. IL-6 expression from A549 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

cells were harvested three days P.I. from T75 flasks. Proteins from each sample were separated by two-dimensional gel electrophoresis and stained with Coomassie blue. Fig. 4-43 is the mock-infected sample without the 35 kDa spots (area where spots should be is indicated by an arrow), and Fig. 4-44 is the BTV-infected sample with 35 kDa spots indicated by the arrow. Replicate gels were blotted onto PVDF membrane and phosphorylated tyrosine containing proteins identified. Spots on the gel of the BTV-infected A549 cells were found near a molecular weight of 35 kDa, which correlated to the subject bands on Fig. 4-41. The 35 kDa protein displayed multiple isoelectric points between 6.5 and 7.5.
FIG. 4-33. IL-8 expression from A549 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

The spot with an isoelectric point of approximately 7.3 was the most intense and was excised for MALDI-TOF mass spectroscopic analysis. Peptide fragment molecular weights identified by MALDI-TOF mass spectroscopy can be seen in Fig. 4-45. Comparisons against known peptide fragment databases did not yield any credible predictions as to the identity of the unknown protein.

Though the identity of the 35 kDa protein remains unknown, the protein clearly displays multiple charge states, possibly the result of multiple phosphorylation sites. Testing also indicates that this 35 kDa protein is phosphorylated by a kinase capable of inhibition by multiple inhibitors or possibly can be phosphorylated by multiple tyrosine kinases.
FIG. 4-34. MCP-1 expression from A549 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

DISCUSSION

BTV was recently shown in human cells to replicate selectively in cancerous cells (Xiao et al., 2004). Understanding the mechanisms through which BTV causes cell death may provide new avenues of research and potential targets in the treatment of cancer. Chapter 2 demonstrated the involvement of cellular apoptotic and inflammatory pathways. The objective of this study was to determine the involvement of key inflammatory and apoptotic signaling molecules in BTV-induced cell death in carcinoma transformed cells.
FIG. 4-35. RANTES expression from A549 cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

The signaling molecule PKB was first explored because of its involvement in regulating cell survival. It is a known proto-oncogene, over-phosphorylated in various cancers, along with its upstream and downstream signaling molecules (Yuan and Cantley, 2008). We hypothesized the carcinoma transformed cell responds to BTV infection by shutting down PKB related signaling leading to cell death. Testing revealed phosphorylated PKB in mock-infected A498 and A549 cells, but not in HEP-G2 cells. Subsequent infection with BTV resulted in complete loss of phosphorylation while poly I:C treatment did not affect the level of phosphorylation. The observations for A498 and A549 cells is consistent with
FIG. 4-36. IL-6 expression from Vero cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

the hypothesis, though testing with PKB inhibitor, Triciribine, demonstrated that a loss of PKB phosphorylation alone is not sufficient to cause cell death. Triciribine treatment in mock-infected cells had no effect on cell death in any of the tested cell lines, indicating that additional signals are required to cause cell death.

BTV clearly supplies these additional signals during infection, possibly as a result of viral replication. BTV’s genome is dsRNA which can activate an innate immune response to the dsRNA, which can lead to cell death in primary and carcinoma transformed cells (Zhang and Samuel, 2007). Our hypothesis was that the reductions in phosphorylated PKB during BTV infection made it possible for dsRNA-induced pro-apoptotic signals to proceed. To test this,
FIG. 4-37. IL-8 expression from Vero cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

Triciribine treated cells were also treated with poly I:C. However, inhibition of PKB phosphorylation by Triciribine had no effect on poly-I:C-induced cell death (Fig. 4-1 to 4-4) disproving our hypothesis and indicating that BTV was also activating non-PKB, non-dsRNA related apoptotic pathways.

In addition to activating apoptosis, BTV also activates pro-inflammatory signaling pathways as evidenced by the expression of cytokine and chemokines during infection. Double stranded RNA activates the same or similar pro-inflammatory signaling pathways leading to expression of the same cytokines and chemokines (Chapter 2), suggesting it was possible that apoptosis and cytokine expression were activated through the same signaling procedure. One
signaling molecule involved in both inflammation and apoptosis is nuclear translocation factor kappa B (NFκB). NFκB plays a central role in inflammatory signaling in nearly all cells. Activation, however, leads to expression of interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα), in addition to the cytokines observed in Chapter 2. NFκB also promotes cell survival when activated. The lack of IL-1 and TNFα as well as the presence of apoptosis suggested NFκB was probably not involved during BTV infection.

NFκB involvement was tested using the inhibitor QNZ. BTV-infected and poly-I:C-treated cells were both treated with QNZ, and subsequently measured for cell death and cytokine expression (Fig. 4-1 through 4-8). NFκB was found to
FIG. 4-39. RANTES expression from Vero cells treated with either placebo, pan tyrosine kinase inhibitor Genistein, Src inhibitor PP2, JAK-STAT inhibitor AG490, PDGFR inhibitor AG1296, or EGFR inhibitor AG1478. Treated cells were subsequently either mock-infected, BTV-infected, 1 μg/mL poly I:C treated, or 50 μg/mL poly I:C treated (n=4). Cytokine expression was determined 3 days P.I. using supplies provided by Quansys Biosciences.

have no influence on the observed cell death or cytokine expression, indicating that BTV-induced cell death and cytokine expression involves either signaling through non-NFκB mediated pathways or other pathways are compensating for the inhibited NFκB pathway. The observed cytokines are known to be activated by multiple pathways leading us to conclude that NFκB signaling is not involved in the cell’s response to BTV infection.

One potential alternative pathway was MAPK signaling. MAPK signaling can lead to cytokine expression very similar to NFκB signaling. In addition, studies in human primary cells showed that BTV activates p38 MAPK during infection leading to the expression of cytokines (Chiang et al., 2006). Normal
FIG. 4-40. Western blot using anti-BTV17 antisera. Mock-infected and BTV-infected A498, HEP-G2, or A549 cells were harvested 3 days P.I., protein concentration adjusted to 500 μg/mL, ran on a 10% polyacrylamide gel, blotted to PVDF membrane and stained with rabbit anti-BTV17 antisera. Column (1) Bio-Rad prestained protein marker with weights indicated in kilodaltons, (2) mock-infected A498 cells, (3) BTV-infected A498 cells, (4) mock-infected HEP-G2 cells, (5) BTV-infected HEP-G2 cells, (6) mock-infected A549 cells, (7) BTV-infected A549 cells. Arrows indicate bands present only in BTV-infected samples.

p38 MAPK-induced cytokine expression, however, includes Il-1 and TNFα expression which are not observed in any of the tested human carcinoma transformed cells, suggesting that if the pathway is also activated in transformed cells, it is not functioning properly. ERK-1/2 and SAPK-JNK pathways were both
FIG. 4-41. Western blot using anti-phospho tyrosine antibodies. Mock-infected and BTV-infected A498, HEP-G2, or A549 cells were harvested 3 days P.I., protein concentration adjusted to 500 μg/mL, ran on a 10% polyacrylamide gel, blotted to PVDF membrane and stained with rabbit anti-BTV17 antisera. Column (1) Bio-Rad prestained protein marker with weights indicated in kilodaltons, (2) mock-infected A498 cells, (3) BTV-infected A498 cells, (4) mock-infected HEP-G2 cells, (5) BTV-infected HEP-G2 cells, (6) mock-infected A549 cells, (7) BTV-infected A549 cells. Arrows indicate bands present only in BTV-infected samples.

tested along with p38 MAPK for involvement during BTV infection and during poly I:C treatment, cell death or cytokine expression. Further testing revealed that p38 MAPK was constitutively phosphorylated in A498 and A549 cells, but is subsequently de-phosphorylated upon infection with BTV. A surprising observation that is markedly different from primary cells. P38 MAPK is not
Inhibition of p38 MAPK, ERK-1/2, and SAPK/JNK all had no significant effect on phosphorylated in mock-infected primary cells but is phosphorylated upon infection with BTV. This observation reveals p38 MAPK is a key differentiator between some primary and carcinoma transformed cells. The observation also suggests either that p38 MAPK signaling requires other signals to lead to cytokine expression or that the p38 MAPK signaling pathway is broken downstream of p38 MAPK in carcinoma transformed cells.

ERK-1/2 and SAPK/JNK were also tested for level of phosphorylation. ERK-1/2 was not phosphorylated in mock-infected or BTV-infected cells, while SAPK/JNK was constitutively phosphorylated in mock-infected cells but remained phosphorylated in mock-infected primary cells but is phosphorylated upon infection with BTV.
FIG. 4-43. Coomassie blue stained gel of mock-infected A549 cells 3 days P.I. Sample protein concentration was adjusted to 500 μg/mL, ran on a 10% polyacrylamide gel and subsequently stained with Coomassie blue. Arrow indicates the location where the 35 kilodalton protein displaying isoforms between 6.5 and 7.5 pl should be found.

unaffected by infection with BTV. Given the lack of a significant change in phosphorylation combined with no significant change in cell death or cytokine expression during inhibitor treatment, we concluded that ERK-1/2 and SAPK/JNK signaling are not involved in the cell’s response to BTV-infection. P38 MAPK signaling on the other hand is significantly affected by infection with BTV,
FIG. 4-44. Coomassie blue stained gel of BTV-infected A549 cells 3 days P.I. Sample protein concentration was adjusted to 500 μg/mL, ran on a 10% polyacrylamide gel and subsequently stained with Coomassie blue. Arrow indicates the location where the 35 kilodalton protein displaying multiple isoforms between 6.5 and 7.5 pI.

suggesting that altered p38 MAPK signaling plays a central role in cell transformation and BTV-oncotrophism. DeMaula et al. (2001) demonstrated that in bovine endothelial cells expressed greater levels of IL-1, IL-6, and TNFα relative to ovine endothelial cells which correlated with higher viral titers but less severe disease and pathology. The elevated expression indicates greater
FIG. 4-45. MALDI-TOF mass spectroscopy analysis of a 35 kDa protein isolated from BTV-infected A549 cells 3 days P.I. The ~7.3 pI isoform of the 35 kDa protein identified in Fig. 4-44 was excised from the polyacrylamide gel and was subject to in gel trypsin digestion. The unknown protein is a tyrosine phosphorylated protein displaying multiple isoelectric points.

activation of the NFκB and p38 MAPK pathways, as is also found in human primary endothelial cells. Greater activation of these pathways leads to reduced apoptosis, which in vivo reduces hemorrhage, and the corresponding coagulation and necrosis. However, in the tested carcinoma transformed cell lines, these pathways are not activated, as evidenced by the lack of IL-1 and TNFα expression, and are therefore not protected from apoptosis.

Though p38 MAPK signaling was affected by BTV-infection and may play an important role in preventing apoptosis and promoting IL-1 and TNFα
expression from primary cells, it is not the pathway through with cytokine expression occurs in carcinoma transformed cells. To further explore the signaling pathways utilized by carcinoma cells to induce apoptosis and cytokine expression, tyrosine kinase signaling was tested. Tyrosine kinase signaling is best known for its role in conveying extracellular signals through receptor tyrosine kinases (RTKs), though tyrosine kinase signaling is utilized by many systems including innate immunity. For example, type I interferons signal through the JAK-STAT pathway, which involves tyrosine kinase signaling (Schindler and Plumlee, 2008). To determine tyrosine kinase signaling involvement during infection, the profile of tyrosine phosphorylated proteins in BTV-infected cells were compared with mock-infected cells (Fig. 4-41). This Western blot suggested there was at least one protein selectively tyrosine phosphorylated during BTV-infection. These findings led us to acquire a panel of tyrosine kinase inhibitors. Each inhibitor was reduced to levels which were not cytotoxic and studies performed with mock-infected, BTV-infected, and poly-I:C-treated cells. None of the inhibitors were shown to have a significant impact on cell death or cytokine expression in mock-infected cells. BTV-infected cells also were not significantly affected by tyrosine kinase inhibition, except for A549 and Vero cells wherein Genistein, the general tyrosine kinase inhibitor, caused an increase in cell death. If a tyrosine kinase signaling pathway were conveying pro-inflammatory or pro-apoptotic signals, then inhibition would have protected the cells from death rather than increased cell death. Instead, it may be that the tyrosine kinases are conveying cell survival signals, and inhibition of the signaling
promoted apoptosis. For example, EGFR and Src, two tyrosine kinases, are upstream activators of phosphoinositol-3-kinase (PI-3K) mediated PKB phosphorylation. However, cell death was present only in the A549 and Vero cells indicating the increase in cell death is related only to some cell lines. Also, EGFR and Src kinase inhibitors did not have the same effect indicating the example pathway is not the pathway being affected by Genistein.

Unlike BTV-infected or mock-infected cells, poly-l:C-treated cells were affected by tyrosine kinase inhibition. A498 cells were protected from poly-l:C-induced cell death, suggesting that A498 cells respond to poly l:C through tyrosine kinase signaling. Interestingly, all of the tyrosine kinase inhibitors partially protected A498 cells from poly-l:C-induced cell death, suggesting that the inhibitors all cross inhibit the same pathway.

Poly-l:C-treated HEP-G2, A549, and Vero cell viability were unaffected by tyrosine kinase inhibition, but cytokine expression was affected. A498, A549, and Vero cells all showed reductions in cytokine expression during tyrosine kinase inhibition. The reductions were most prevalent in Genistein and AG1478 treated cells. AG1478 inhibits EGFR signaling which may be related to the reduction in PKB signaling shown previously during BTV infection. PKB involvement and the changes in cytokine expression observed during AG1478 treatment suggest some EGFR involvement during BTV-infection, possibly part of the innate immune response. In addition, cell death and cytokine expression from poly-l:C-treated cells may utilize the same signaling pathway(s), but protection from cell death is only observed in A498 cells because these cells are
the only ones in which poly I:C induced cell death. However, the more significant tyrosine kinase inhibitor is Genistein, which suggests that there remains additional tyrosine kinases, apart from the four major families tested, which are involved in both cell death and cytokine expression.

After determining the effect each inhibitor had on cell death and cytokine expression, each inhibitor was used to determine the identity of the tyrosine phosphorylated protein observed in Fig. 4-41. HEP-G2 cells were treated with each tyrosine kinase inhibitor and each MAPK inhibitor and then tested for changes in phosphorylation of the 35 kDa protein by Western blot. MAPK inhibitors were tested along with tyrosine kinase activity inhibitors because MAPK activation involves tyrosine phosphorylation in the activation site and needed to be excluded. Fig. 4-42 demonstrated, that in HEP-G2 cells, phosphorylation of the unknown protein was inhibited by every tested tyrosine kinase inhibitor, but not the MAPK inhibitors. Indicating that the unknown protein is not one of the MAPK proteins. The pathway of phosphorylation could not be determined as hoped, but instead suggests the observed protein is phosphorylated by a tyrosine kinase that can be inhibited by many compounds. The same conclusion was made from the A498 poly I:C data, raising the possibility that the same pathway that is protecting A498 cells from poly-I:C-induced death is the same protein identified here in HEP-G2 cells. This protein, however, was weakly phosphorylated in A498 cells and BTV-induced cell death is not protected by inhibition of this protein.
In an effort to identify the unknown protein and its function, BTV-infected and mock-infected A549 cells were resolved by two-dimensional gel electrophoresis. One set was stained with Coomassie blue (Fig. 4-43 and 4-44), and the other set was transferred to PVDF and tyrosine phosphorylated proteins determined by Western blot. The unknown tyrosine phosphorylated protein was found to be about 35 kDa in size and had multiple isoelectric points suggesting multiple phosphorylation states. The protein, however, could not be identified by mass spectroscopy leaving no answer to its function.

Findings in Chapter 2 suggested that cell death and cytokine expression were directly related. We hypothesized that this relationship was through conserved intracellular signaling. Our objective was to determine the role of key apoptotic and pro-inflammatory signaling molecules in the cell’s response to BTV infection. We conclude that both PKB and p38 MAPK play a role in transformation of the A498 and A549, but not HEP-G2 cell lines and that BTV induced cell death by inactivating these pathways. The inactivation of p38 MAPK during infection is one distinguishing characteristic of BTV infection of carcinoma transformed cells compared with BTV-infected human primary cells, and explains the lack of IL-1 and TNFα expression and the increased rate of apoptosis of carcinoma transformed cells relative to primary cells.

Other MAPK family members were not affected by BTV infections, but tyrosine kinase signaling is involved. BTV infection leads to increased tyrosine phosphorylation. This tyrosine signaling promotes cell death in A498 cells, promotes cell survival in BTV-infected A549 and Vero cells, and promotes
cytokine expression in A498, A549, and Vero cells. Tyrosine signaling involvement in cell death is highly variable between cell lines but utilizes a signaling pathway that is capable of cross inhibition by multiple classes of kinase inhibitors. Cytokine signaling occurs through both an unknown pathway and partially through EGFR signaling. The unknown pathway involved in cytokine expression may be the same pathway involved in cell death. Western blotting suggests this pathway involves a 35 kDa protein with multiple phosphorylation states, though the pathway remains unknown. However, this pathway does not involve PDGFR signaling or JAK-STAT signaling, and therefore does not involve type I interferon signaling. The other two conventional inflammatory pathways, p38 MAPK and NFκB are also not involved, leaving the mechanism by which carcinoma transformed cells respond to BTV infection distinct from primary cells and independent of conventional inflammatory pathways. BTV selectively kills carcinoma transformed cells because their cellular response to BTV infection does not include activation of NFκB, p38 MAPK, or JAK-STAT signaling.

REFERENCES


CHAPTER 5
SUMMARY AND CONCLUSION

SUMMARY

Bluetongue virus (BTV) is a dangerous pathogen to ruminants recently re-emerging in epidemic form in Europe, but it is BTV’s effects in human carcinoma transformed cells that is the subject of this study. In ruminants, like sheep, BTV causes bluetongue disease, a hemorrhagic disease that is often fatal. However, BTV does not cause disease in humans and the population remains largely seronegative, suggesting that not only are humans not compatible hosts, but that we don’t usually get infected. However, BTV will infect human cells in vitro. When primary cultures are infected, viral RNA can be detected but there is no cell death, though BTV will cause cell death in carcinoma transformed cells.

The study objectives were to determine infectivity of BTV serotype 17 in three human cell lines, quantify cell death and cytokine expression, and determine the involvement of key intracellular signaling molecules in the observed cell death and cytokine expression. First, BTV was found to induce cell death in all three human cell lines. Cell death was most significant in A498 cells followed by A549 cells and last by HEP-G2 cells (Fig. 2-3). Cytokine expression from the same cells was subsequently found elevated in all cell lines upon infection with BTV (Table 2-1). Cytokine expression is the cell’s primary means of communication with the immune system and consequently the expression profile provides great insight into the cell’s response to infection. Multiplex
cytokine testing methods were employed to examine multiple cytokines (25 in total). Four cytokines were found elevated upon infection, including IL-6, IL-8, MCP-1, and RANTES. All are pro-inflammatory cytokines which are expressed constitutively by cancer cells and naturally expressed by damaged or stressed cells. Cytokine production directly correlated with cell death between the cell lines which suggested that cell death and cytokine expression were both activated through the same signaling cascade, and that all three human cell lines utilized this same pathway. Our hypothesis was that this signaling pathway was activated by innate immunity’s response to BTV generated dsRNA.

To understand the involvement of innate immunity in viral induced inflammation and cell death, studies were repeated with polyinosinic:polycytosinic acid (poly I:C) treated cells. Poly I:C is a synthetic dsRNA molecule known to induce anti-dsRNA responses in vitro and in vivo. Studies following a similar procedure in primary lung endothelial cells found that synthetic dsRNA could induce cytokine expression and p38 MAPK phosphorylation commensurate with BTV infection. However, poly I:C treatment in human carcinoma transformed cells had no detectable effect on cell death in HEP-G2, A549, and Vero cells, and only resulted in cell death in A498 cells at abnormally high concentrations of poly I:C (Fig. 2-4). Poly I:C could induce cytokine expression in A498, A549, and Vero cells, though cytokine expression did not include IL-1 and TNFα as was seen in primary cells.

Viral RNA and protein expression were subsequently measured in each cell line. Our objective was to determine the relative extent of replication
between cell lines. Our hypothesis was that cell death and cytokine expression were directly related to viral replication. However, RNA expression from BTV was highest in A498 cells generating concentrations statistically indistinguishable from BHK-21 cells, while BTV generated the lowest concentrations of RNA in A549 cells. Surprisingly, viral protein expression did not directly correlate with RNA expression and was inversely proportionate, between cell lines, with cell death and cytokine expression. The data did not support the initial hypothesis, but suggested that cell death and cytokine expression are a function of the cell’s ability to detect and respond to infection rather than the virus rate of replication.

Protein and RNA studies in conjunction with cell death and cytokine testing suggested that the host cell is quickly recognizing the infection in a cell line dependent manner and is activating pro-apoptotic pathways. To better understand the intracellular events taking place, we acquired inhibitors to a number of signaling molecules central to inflammation and apoptosis. These included protein kinase B (PKB), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal regulated kinase (ERK-1/2), stress activated protein kinase (SAPK/JNK), nuclear translocating factor kappa B (NFκB), and the major tyrosine kinase families. The respective proteins were inhibited in mock-infected, BTV-infected, and poly I:C treated cells. However, only the tyrosine kinase inhibitors had any appreciable effect on cell death, which was observed only in poly I:C treated A498, and BTV-infected A549 cells. The tyrosine kinase inhibitors were also the only inhibitors to reduce cytokine expression. The effects
were most overt with the pan-tyrosine kinase inhibitor and the endothelial growth factor receptor (EGFR) inhibitor.

Inhibition studies suggested that none of the tested signaling proteins were singly involved in cell death or cytokine expression. It was expected that if there was involvement, there would have been differences in either cell death or cytokine expression upon treatment.

In addition to the inhibition studies, phosphorylation of PKB, p38 MAPK, ERK-1/2, and SAPK/JNK were directly measured. These direct measurements confirmed that the inhibitors were functioning, as the level of phosphorylation diminished in the presence of the respective inhibitor, thereby validating the results of the inhibition studies. Direct measurements also revealed that A498 and A549 cells constitutively expressed active (phosphorylated) PKB; but when infected with BTV, phosphorylation was inhibited. It was hypothesized that BTV reduced phosphorylation of PKB by activating alternate pro-apoptotic mechanisms; and that this dephosphorylation permitted normal apoptotic mechanisms to take place. However, there was no detectable change in cell death when uninfected cells were treated with PKB inhibitor suggesting that dephosphorylation of AKT alone is not sufficient to induce apoptosis.

P38 MAPK was also found to be constitutively phosphorylated in mock-infected cells. This level of phosphorylation is consistent with transformation by carcinoma, as p38 MAPK phosphorylation leads to protection from apoptosis. In these cells, infection with BTV caused p38 MAPK dephosphorylation. Prior studies found that p38 MAPK was not phosphorylated in primary cells, but then
phosphorylated during BTV infection. This phosphorylation lead to IL-1 and TNFα expression, which is not seen in carcinoma transformed cells. These discrepancies in p38 MAPK phosphorylation suggest that lack of functional p38 MAPK signaling in carcinoma transformed cells may be a primary reason for BTV’s oncotrophism. Other MAPK family members: ERK-1/2 and JNK were also tested for changes in phosphorylation upon infection, but were not significantly affected.

Lack of NFκB or p38 MAPK involvement in cytokine expression suggested possible involvement of tyrosine kinase signaling. Innate immunity to dsRNA is known to signal through tyrosine kinase signaling pathways, such as the Janus kinase (JAK-STAT) signaling pathway. Western blots using anti-phosphorylated tyrosine antibodies suggested that there was differential tyrosine phosphorylation between mock-infected and BTV-infected cells. One specific 35 kDa protein in BTV-infected cells was particularly phosphorylated. The tyrosine kinase inhibitors were all tested for their ability to phosphorylate this protein in HEP-G2 cells. Cells were treated with each tyrosine kinase and MAPK inhibitor and the proteins stained by Western blot with anti-phospho-tyrosine antibodies three days P.I. Surprisingly, each tyrosine kinase inhibitor caused a reduction in phosphorylation on the unknown protein, suggesting that the unknown protein is phosphorylated by a kinase to which many different inhibitors function. The identity of the protein could not be determined by mass spectroscopy but the inhibition of phosphorylation combined with the lack of changes in cell death or
cytokine expression in HEP-G2 cells during inhibitor treatment indicates that the 35 kDa protein is not integrally involved in the cell’s response to infection.

CONCLUSION

BTV is a dangerous ruminant pathogen which does not naturally cause disease in humans, but is capable of infecting and killing human carcinoma cells \textit{in vitro} while simultaneously inducing an immune response from the infected cell. The cellular immune response includes the production of pro-inflammatory cytokines and chemokines followed by apoptosis. The key differences in infection between carcinoma cells and primary endothelial cells includes extensive cell death in carcinoma cells; lack of IL-1 and TNF$\alpha$ expression; constitutive PKB and p38 MAPK phosphorylation, which is subsequently lost upon infection; and a lack of poly I:C inducible cytokine expression or p38 MAPK phosphorylation.

Cytokine production and cell death are the product of the cell recognizing and responding to infection. Hence, greater cell death correlates with reduced viral protein production. Both apoptosis and cytokine expression are related through intracellular signaling. The cell responds to infection by activating tyrosine kinase signaling leading to cytokine expression through a non-NF$\kappa$B pathway and subsequently dephosphorylating PKB and p38 MAPK, leading to apoptosis. Tyrosine kinases, PKB, and p38 MAPK are all related through EGFR signaling shown here to affect cytokine production and may be the primary cause of cell death, cytokine expression, and p38 MAPK dysfunction.
We conclude that BTV can infect many types of cells, including primary and carcinoma transformed cells, but that BTV-induced cell death is a function of the cell's response to infection and is therefore cell dependent. Carcinoma transformed cells are susceptible to cell death because they do not respond to infection through NFκB or p38 MAPK signaling which traditionally leads to protection from apoptosis. As a result, when a carcinoma cell is infected with BTV, the virus is recognized by innate immunity and responds by expressing cytokines and subsequently inducing apoptosis.

FUTURE RESEARCH

This study focused entirely on cells transformed by carcinoma. Future studies would involve direct comparisons between primary epithelial cells and carcinoma transformed cells. Additionally, p38-MAPK was shown to be dephosphorylated during BTV infection, contrary to observations in primary cells. We would want to inhibit p38 MAPK phosphorylation in primary cells, and inhibit p38 MAPK dephosphorylation in carcinoma cells to determine the contribution p38 MAPK signaling has on cell survival. Similar testing should also be done with NFκB.
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