ABSTRACT

POLLARA, JUSTIN JOSEPH. Characterization of the Prostaglandin E_2 Response to Poxvirus Infection and of a Novel Poxvirus Antiviral. (Under the direction of Dr. Ian T. D. Petty).

Prostaglandin E_2 (PGE_2) is an arachidonic acid-derived signaling molecule that has important roles in inflammation, and the adaptive immune response. Similar to cytokines, PGE_2 can constitute an important determinant of the host immune response to virus infection, or vaccination. In this study, we show that infection with the poxvirus vaccine strain, modified vaccinia Ankara virus (MVA), induces robust accumulation of PGE_2 in culture supernatants of both a mouse fibroblast cell line and murine bone marrow-derived dendritic cells. Furthermore, we describe several distinctions between MVA-induced PGE_2 production and the canonical model of prostaglandin synthesis in response to proinflammatory stimuli. Viral gene expression was required for accumulation of PGE_2, and for release of the PGE_2 precursor, arachidonic acid, and both of these processes were remarkably delayed relative to the onset of infection. MVA-induced PGE_2 production was dependent on the activity of cyclooxygenase-2, but liberation of arachidonic acid occurred independently of cytosolic phospholipase A_2. Additionally, we showed that other poxviruses varied in their ability to induce PGE_2 production depending on the particular combinations of virus and cell type assayed. Experiments to identify the underlying basis of these differences suggested that some poxviruses can suppress PGE_2 production in certain cell types. The results of this study provide insight into a novel aspect of MVA biology that likely affects the efficacy of MVA-based vaccines.

Although MVA has significant potential as a vaccine and vaccine vector, many related poxviruses are human pathogens. The use of antiviral agents can provide a crucial complement to vaccination for the prevention of human disease caused by pathogenic poxviruses. Currently, the Food and Drug Administration has licensed only one drug for use against poxvirus. Thus, there is a need to develop, or identify, additional poxvirus antivirals. In this study, we examine the ability of Terameprocol (TMP), a methylated derivative of nordihydroguaiaretic acid, to inhibit poxvirus growth in vitro. Our data show that TMP can potently inhibit the growth of cowpox virus and vaccinia virus in a variety of cell lines. Furthermore, our data suggests that TMP inhibits poxvirus growth by preventing the efficient spread of virus particles from cell to cell. Consistent with this interpretation, TMP treatment dramatically reduced the number of actin tails present on infected cells. These structures require complete poxvirus morphogenesis for their formation, and they are involved in viral cell-to-cell spread. Data presented in this study, together with the clinical safety record of TMP, support further evaluation of TMP as a poxvirus therapeutic.

Characterization of the Prostaglandin E_2 Response to Poxvirus Infection and of a Novel Poxvirus Antiviral

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Microbiology

Raleigh, North Carolina

2010

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DEDICATION

This work is dedicated to my wife Katie, and to friends and family, both here, and gone. All that I have done has only been possible because of your love and support.

BIOGRAPHY

Justin Joseph Pollara was born to a wonderful and supportive family in Binghamton, New York. Justin received a Bachelor of Science degree in Biology, with a minor degree in Biological Anthropology from Binghamton University. After graduation, Justin moved to Raleigh, North Carolina in the hopes of finding work and warmer weather. As an Environmental Technician, Justin traveled the small towns and back roads of rural North Carolina to evaluate various environmental impact sites. He then sought a career with a focus on biomedical research, and began work as a Research Specialist at Duke University under the guidance of Dr. Guido Ferrari. It was at Duke that Justin realized his penchant for research, and he began taking courses at North Carolina State University. Justin left Duke to pursue a doctoral degree at North Carolina State University, under the advisement of Dr. Tim Petty. Upon completion of his dissertation defense, Justin will continue his training as a postdoctoral scientist.

ACKNOWLEDGEMENTS

I would like to thank the following people, without whom this work could not have been possible, nor would I have been prepared to complete it:

First, I thank Dr. Tim Petty for his patient guidance and mentoring, I learned much from you, and I look forward to applying this knowledge to future endeavors. Thank you also to the rest of my advisory committee, Dr. Scott Laster, Dr. Frank Scholle, and Dr. Fred Fuller, for all of your advice and support. Finally, thank you to all of my fellow students; we depend on each other to get through this process, and I hope that I can repay the encouragement and camaraderie you have shown me.

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CHAPTER 1

Literature Review

Introduction

Prostaglandin E_2 (PGE_2) is a fatty acid-derived signaling molecule that mediates a diverse assortment of physiologic and pathologic processes. In common with other soluble cellular factors, such as interferons, chemokines, and cytokines, the accumulation of PGE_2 is a common host-response to a wide variety of pathogens. Although traditionally regarded as a crucial mediator of innate responses and inflammation (Kuehl and Egan, 1980; Portanova et al., 1996), more recent literature has defined important roles for PGE_2 in influencing both cell-mediated and humoral responses of the adaptive immune system (Harris et al., 2002). In most cases, PGE_2 functions as a normal component of many immune processes with both activating and regulatory roles. However, PGE_2 is a potent T helper type 2 (Th2) promoting signal, and improper regulation or hyperinduction of PGE_2 production and signaling has the potential to detrimentally imbalance the effector responses of the immune system.

Viruses are adept at evading or manipulating the immune responses of their hosts, and a considerable body of research has been conducted to elucidate the roles of these strategies in viral pathogenesis. Viruses of many types induce a PGE_2 response upon infection (Steer and Corbett, 2003). For many viruses, this response likely contributes to viral pathogenesis either via direct effects on virus replication, or via misdirection of host immune responses. Unsurprisingly,

examples of viral inhibition of the PGE_2 response have also been described (Culver and Laster, 2007; Savard et al., 2000), suggesting that some viruses have evolved the capacity to manipulate the PGE_2 response for their own advantage. Accordingly, a comprehensive understanding of the roles of PGE_2 signaling in the regulation of immune responses, and in viral pathogenesis, may facilitate the development of novel anti-viral therapies, or more effective prophylactic vaccines or vaccination regimens.

Prostaglandin E_2

Several biologically active prostaglandin (PG) subtypes have been described, however, the most thoroughly studied and best characterized is PGE_2. PGE_2 is produced by most types of cells and tissues in response to a wide variety of stimuli including direct mechanical stresses, soluble cellular factors such as cytokines and growth factors, mitogens, or as a response to various types of pathogens (Fidler et al., 1990; Jiang and Cheng, 2001; Kasai et al., 1987; Lin et al., 1992; Steer and Corbett, 2003; Wang and Chadee, 1995; Yokota et al., 1986). Notably, PGE_2 has potent regulatory activities and is the most abundant PG subtype produced during inflammatory and immune responses (Dubois et al., 1998; Harris et al., 2002; Kuehl and Egan, 1980; Phipps et al., 1991).

Biosynthesis of PGE_2

PG biosynthesis begins with the release of arachidonic acid (AA) from membrane glycerophospholipids (Smith, 1989). Multiple cellular phospholipases may be involved in AA release, however, several lines of evidence suggest that cytosolic phospholipase $A_2\alpha$ ($cPLA_2$) is crucial for PG biosynthesis in most circumstances. All members of the phospholipase A_2 (PLA_2) superfamily characteristically hydrolyze fatty acids from the *sn*-2 position of glycerophospholipids, and thus have the potential to liberate AA (Dennis, 1994). However, only $cPLA_2$ shows preferential selectivity towards AA in the *sn*-2 position over other fatty acids (Clark et al., 1991; Leslie et al., 1988). Additionally, $cPLA_2$ acts on phospholipid constituents of perinuclear membranes and is often co-localized with AA-metabolizing downstream enzymes (Murakami et al., 2003; Murakami et al., 2002; Ueno et al., 2001). Perhaps most significantly, studies conducted using cells obtained from $cPLA_2$ knock-out mice (Gijon et al., 2000; Sapirstein and Bonventre, 2000), or with a specific $cPLA_2$ inhibitor (Ono et al., 2002), have indicated severe deficiencies in PG production in response to a variety of stimuli, thus providing further evidence of the central role of $cPLA_2$ in PG synthesis. Secretory PLA_2 enzymes can also be involved in AA release and PG synthesis in some cell types, acting either to amplify $cPLA_2$ activity, or as the predominate effector of AA release (Balboa et al., 1996;

Kikawada et al., 2007; Ruiperez et al., 2009). Additionally, calcium independent PLA_2s, which are traditionally regarded as housekeeping enzymes responsible for maintaining phospholipid homeostasis, have been shown to contribute to AA release and subsequent PGE_2 production in a limited number of cell types (Akiba and Sato, 2004). Moreover, examples of cross-regulation between $cPLA_2$ and $sPLA_2$ have been described, which likely confounds many observations regarding sole dependence of AA release on either enzyme in a particular experimental situation (Balsinde et al., 1998). Nonetheless, the liberation of AA from phospholipids by $cPLA_2$, or by other members of the PLA_2 superfamily, represents the primary, and often rate-limiting, step in the synthesis of PGs.

Next, the released AA is converted into the intermediate prostaglandin H_2 by cellular cyclooxygenase (COX) enzymes (Smith et al., 2000). There are two predominant isoforms of COX, COX-1 and COX-2. Differentiation of the specific biological roles of both COX enzymes has largely been facilitated by the recognition and development of COX-inhibiting drugs. The activity of both COX enzymes can be broadly inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, while subtle differences in the active sites of COX-1 and COX-2 have allowed for the development of selective COX-2 inhibitors such as celecoxib and NS-398 (DeWitt, 1999). In general, the COX-1 isoenzyme is primarily utilized for homeostatic functions, and is constitutively expressed (Smith et al., 2000; Smith et al., 1996). COX-2, in

contrast, has low basal expression in most cells and tissues but is readily inducible (Smith et al., 2000; Smith et al., 1996; Tsatsanis et al., 2006). The induction of COX-2 is usually transient, and is regulated both at the transcriptional and posttranscriptional levels (Lasa et al., 2000; Ristimaki et al., 1994; Smith et al., 2000). Posttranscriptional regulation of COX-2 involves mRNA stabilization and is likely an important determinant of the duration of COX-2 induction (Lasa et al., 2000; Ristimaki et al., 1994). However, the contribution of this process to PGE_2 production in response to most stimuli has not been well established. Transcriptional regulation of COX-2 can involve a multitude of cellular transcription factors including NF-κB, AP-1, NFAT, CRE, and others (Iniguez et al., 2000; Kang et al., 2006). The contribution of each of these transcription factors to COX-2 regulation varies with particular types of cells and stimuli (Smith et al., 2000; Tsatsanis et al., 2006), but the involvement of NF-κB signaling has been implicated in COX-2 induction, and the accumulation of PGE_2, upon infection with several types of viruses (Alvarez et al., 2005; Murono et al., 2001; Waris and Siddiqui, 2005; Wu et al., 2009). Regulation by such a diverse set of transcription factors provides evidence that many signal transduction pathways control COX-2 expression, indicating the importance of this enzyme in a broad range of cellular responses.

Prostaglandin H_2 generated by COX activity is then converted enzymatically to PGE_2, or other PG subtypes, by specific PG synthases (Park et

al., 2006; Ueno et al., 2005). In particular, microsomal PG E-synthase-1 is often co-expressed with COX-2, and is typically involved in the production of PGE_2 in response to proinflammatory stimuli (Jakobsson et al., 1999; Murakami et al., 2000). Once produced, PGE_2 is released from cells either by passive diffusion or active transport (Chan et al., 1998; Schuster, 2002). PGE_2 signals in an autocrine or paracrine fashion via any of four specific receptors, EP1–4, which are each preferentially linked to particular G protein-coupled signal transduction pathways (reviewed by Sugimoto and Narumiya (2007)). Thus, the differential expression of these receptors by various cells and tissues, either constitutively, or as a response to a given stimulus, contributes to the diversity of biological responses mediated by PGE_2 (Ikegami et al., 2001; Sugimoto and Narumiya, 2007).

Roles of PGE_2 signaling in inflammation and innate immune responses

PGE_2 signaling has long been known to be a principal mediator of several hallmarks of inflammation including fever, swelling, and pain sensitization (Dubois et al., 1998; Ivanov and Romanovsky, 2004; Portanova et al., 1996; Ushikubi et al., 1998). Inflammation is an early component of innate immune responses, which are critical for initial control of pathogens, and for producing signals needed to activate adaptive immune processes. In the context of a viral

infection, cells of the innate immune system, including macrophages, natural killer cells, and plasmacytoid dendritic cells are activated to secrete interferons and other cytokines that are essential for the activation of cell-intrinsic antiviral responses, and for establishing an environment that will promote an appropriate adaptive immune response that is biased towards clearance of the virus. Studies conducted in vitro have demonstrated that PGE_2 can suppress the secretion of TNFα, IL-1β, and IL-12 by monocytes and macrophages (Hurme, 1990; Ikegami et al., 2001; van der Pouw Kraan et al., 1995), IFNγ by natural killer cells (Walker and Rotondo, 2004), and IFNα by plasmacytoid dendritic cells (Son et al., 2006). Collectively, these PGE_2-dependent effects would be expected to attenuate innate antiviral processes, and contribute to the priming of adaptive immune responses towards a Th2 phenotype. Thus, in this context, PGE_2 likely acts in a regulatory manner, and it can be envisaged that excessive or prolonged PGE_2 production may be detrimental to the host response to viral infection.

The effects of PGE_2 signaling on dendritic cells

PGE_2 has also been found to have important roles in shaping both cell-mediated and humoral adaptive immune responses, with functions complementary to those of more widely studied protein-based signaling

molecules. In general, PGE_2 is regarded as a Th2-biasing signal (Harris et al., 2002; Phipps et al., 1991). Dendritic cells (DCs) are an essential point of control for the activation and differentiation of adaptive immune responses, and these cells can produce PGE_2 as well as respond to PGE_2 signaling (Fogel-Petrovic et al., 2004; Harizi et al., 2001; Morelli and Thomson, 2003). Soluble mediators released during innate immune processes induce the maturation of DCs, providing them the unique ability to activate, and direct the differentiation of, naïve T-cells (Kapsenberg, 2003). Therefore, DCs serve as a functional bridge between innate and adaptive immunity, and are influenced both indirectly by PGE_2, through its effects on the innate immune response described above, or via direct effects of PGE_2 signal transduction in the DCs themselves (Harizi and Gualde, 2005). Direct effects of PGE_2 signaling in DCs include promotion of DC maturation (Kabashima et al., 2003), survival (Vassiliou et al., 2004) and migration (Legler et al., 2006; Scandella et al., 2004). However, the defining characteristic effect of PGE_2 signaling in DCs is the suppression of IL-12p70 production (Kalinski et al., 1997a). Two mechanisms for PGE_2-dependent suppression of IL-12 biological activity have been described. First, PGE_2 can induce production of the p40 subunit of IL-12 without coordinately inducing the p35 subunit (Kalinski et al., 2001). Thus, limited availability of IL-12p35 restricts formation of the bioactive IL-12p70 heterodimer. In such cases, the ability of cells to respond to IL-12 may also be reduced as the accumulation of IL-12p40

dimers and free monomers can antagonize IL-12 receptors (Gillessen et al., 1995; Ling et al., 1995). Second, PGE_2 can induce DCs to produce substantial amounts of IL-10, which can then provide a negative feedback signal resulting in transcriptional regulation of IL-12, further contributing to PGE_2-induced suppression of IL-12 (Harizi et al., 2002; Kalinski et al., 1997b). The involvement of multiple, or redundant, mechanisms for PGE_2-mediated suppression of IL-12 suggests that this constitutes a fundamental immunoregulatory role of PGE_2 signaling. Interestingly, Kalinski et al. (1998) demonstrated that human monocyte-derived DCs matured in the presence of PGE_2, IL-1β, and TNFα were deficient in IL-12 production, and this phenotype was stably maintained in the absence of additional PGE_2 for at least 48 hours. These data suggest that PGE_2-dependent inhibition of IL-12 is likely to be physiologically relevant in vivo, as the IL-12-inhibitory effect can persist long enough for DCs activated in the periphery to reach the secondary lymphoid organs. Consistent with expected results, it has been demonstrate in vitro that IL-12 deficient DCs preferentially induce Th2 cell differentiation (Kalinski et al., 1997a; Kalinski et al., 1997b; Kalinski et al., 1998). An effective Th2 response is important for the production of antibodies, which are often essential for the prevention of repeat infection. However, cell-mediated immune responses are important for clearance of an active virus infection, and these responses are not promoted by PGE_2 signaling at either the innate level, or in DCs.

The effects of PGE_2 signaling on B and T cells

The effector cells of the adaptive immune system, B cells and T cells, can also respond to PGE_2. Activated B cells express COX-2 and can produce PGE_2 (Ryan et al., 2005). Inhibition of COX-2 activity in B cells has been found to reduce IgM and IgG antibody (Ab) production both in vitro and in vivo (Bancos et al., 2009; Ryan et al., 2005). PGE_2 has also been shown to promote Ab class switching to Th2 isotypes, IgG1 and IgE (Roper et al., 1995; Roper et al., 2002; Roper and Phipps, 1992). When considered in combination with the propensity of PGE_2 to promote Th2 T cell differentiation, it becomes apparent that PGE_2 signaling generally contributes to the generation of humoral immune responses. In contrast, cytotoxic T cell (CTL) responses are suppressed by PGE_2. The suppression of CTL activity by PGE_2 can occur during T cell priming, as a result of impaired Th1 differentiation due to reduced production of IL-12 and IFNγ by DCs and other cells responding to PGE_2-biased innate immune signals. Alternatively, PGE_2 signaling can inhibit CTL activity by acting directly on $CD8^+$ T cells (Harris et al., 2002; Kapsenberg, 2003). PGE_2 inhibits IFNγ secretion by CTL (Ganapathy et al., 2000), and has also been implicated as a regulatory factor of T cell proliferation (Hendricks et al., 2000). In addition, PGE_2 signaling can limit T cell proliferation by inhibiting the secretion of IL-2 and IFNγ from

activated T cells (Betz and Fox, 1991; Katamura et al., 1995), through enhancement of indoleamine 2,3-dioxygenase (IDO) production by antigen presenting cells (von Bergwelt-Baildon et al., 2006), or by promoting the development and activity of regulatory T cells (Mahic et al., 2006).

The effects of PGE_2 signaling on T and B cells are consistent with those described for DCs and for innate immune processes. Namely, PGE_2 primarily promotes a Th2 bias, and attenuates many aspects of the immune system required for effective cell-mediated immune responses.

Virus infection and the host PGE_2 response

In most cases, the Th2-promoting effects of PGE_2 contribute to the normal regulation of immune processes, and are one of many factors used to properly direct and limit the host immune response. However, viruses have evolved countless strategies for immune evasion, and it is likely that viral exploitation of the host PGE_2 response constitutes one such mechanism used by viruses of many types to facilitate persistence and pathogenesis. In addition, PGE_2 has been shown to directly impact the replication, or gene expression, of many types of viruses in cell culture, suggesting that cell-autonomous effects of PGE_2 may also influence the outcome of a virus infection (Steer and Corbett, 2003). The multifaceted interplay of virus infection, PGE_2, and host anti-viral responses is

best illustrated by studies conducted with cytomegalovirus, herpes simplex virus type-1 (HSV-1), dengue virus, and respiratory syncytial virus (RSV).

The expression of COX-2 mRNA, accumulation of COX-2 protein, and production of PGE_2 are transiently induced in human foreskin fibroblasts (HFFs), human retinal pigment epithelial cells, primary human monocytes, and human coronary artery smooth muscle cells infected with human cytomegalovirus (HCMV) in vitro (Hooks et al., 2006; Nokta et al., 1996; Speir et al., 1998; Zhu et al., 2002). These data indicate that the production of PGE_2 is a common response to primary HCMV infection, presumably benefiting either the host, by contributing to anti-viral immune responses, or benefiting the virus, by promoting viral pathogenesis. In HFF cells, HCMV-induced production of PGE_2 was determined to be COX-2 dependent, and inhibition of PGE_2 synthesis with COX-2-specific inhibitors was associated with a significant decrease in the production of infectious progeny virus (high doses of COX-2 inhibitors) (Zhu et al., 2002), and inhibition of cell-to-cell spread of the virus (low doses of COX-2 inhibitors) (Schroer and Shenk, 2008). Exogenous addition of PGE_2 was sufficient to restore normal virus growth and relieve the inhibitory effects of COX-2 inhibition, thus confirming that PGE_2 had a direct affect on virus growth. Similarly, PGE_2 was found to augment viral replication in both retinal pigment epithelial cells and smooth muscle cells (Hooks et al., 2006; Speir et al., 1998). Collectively, these results suggest that HCMV-induced production of PGE_2 can contribute to the

pathogenesis of HCMV by promoting viral replication. In addition to these direct, cell-autonomous effects of PGE_2 on HCMV growth, HCMV-induced PGE_2 may further contribute to viral pathogenesis through its regulation of the immune system. In particular, acute HCMV infection is often associated with generalized immunosuppression (Rinaldo et al., 1980), which may, at least in part, involve a PGE_2-dependent mechanism. In vitro infection of human monocytes with HCMV caused the accumulation of free AA and PGE_2 in the cell culture supernatants (Nokta et al., 1996). These supernatants were shown to effectively inhibit the PHA-induced proliferation of peripheral blood mononuclear cells, suggesting that the supernatants contain a soluble factor capable of inhibiting T cell proliferation. Interestingly, supernatants collected from monocytes infected with HCMV in the presence of a COX inhibitor (indomethacin) were not inhibitory, and in fact, significantly enhanced cell proliferation (Nokta et al., 1996). Taken together, these data suggest that HCMV-induced production of PGE_2 can contribute to HCMV pathogenesis directly, via promotion of virus replication or cell-to-cell spread, and indirectly, via suppression of immune responses. Unfortunately, the lack of a satisfactory small animal study system prevents a more complete analysis of the contribution of these PGE_2-dependent effects to HCMV pathogenesis in vivo.

Rhesus cytomegalovirus (RhCMV) is closely related to HCMV, and both viruses cause similar disease pathology in their respective natural hosts.

However, unlike HCMV, RhCMV fails to induce the expression of cellular COX-2 upon infection of fibroblast cells in culture. Instead, RhCMV expresses a viral homolog of COX-2 (vCOX-2) (Rue et al., 2004). Experiments conducted with a RhCMV deletion mutant lacking the vCOX-2 gene indicated that vCOX-2 was dispensable for virus growth in fibroblast cells, but was indispensable for virus growth in endothelial cells (Rue et al., 2004). To date, no information regarding the enzymatic activity vCOX-2 has been described, nor has it been determined how vCOX-2 facilitates cell type-dependent virus growth. Although much work regarding the particular mechanisms involved remains, these data support a novel role for COX-2 (and possibly PGs) in determining cell tropism during virus infection.

Numerous studies have demonstrated that HSV-1 replication, cell-to-cell spread, and reactivation from latency are facilitated by prostaglandins (reviewed by Reynolds and Enquist (2006)). Consistent with these effects, COX inhibitors have been shown to have therapeutic benefits for the treatment of HSV infection in humans (Inglot and Woyton, 1971; Wachsman et al., 1990). In common with HCMV, the interplay between the PGE_2-response and HSV-1 pathogenesis is not limited to direct effects on virus replication. A recent publication by Theodoridis et al. (2007) demonstrated that mRNAs for the PGE_2 receptors EP2 and EP4 were dramatically reduced in human monocyte-derived DCs infected with HSV-1 when compared to mock-infected control cells. The reduction of EP2 and EP4

mRNAs was also observed in DCs infected with an HSV-1 mutant lacking the virion host shutoff gene, providing evidence that the effects on EP2 and EP4 mRNAs are independent of the general RNAse activity associated with HSV-1 infection. EP2 and EP4 are expressed by human DCs under normal conditions, and PGE_2 signaling transduced through these receptors is essential for CCR7-dependent migration of DCs along CCL19 and CCL21 gradients into secondary lymphoid organs (Legler et al., 2006; Scandella et al., 2004). In accordance with these known roles for PGE_2-dependent signaling, mature DCs infected with HSV-1 were unable to migrate towards CCL19 in transwell migration assays. Although it has not yet been determined if HSV-1 infection influences the expression of EP2 and EP4 in DCs in vivo, it is intriguing to speculate that this effect represents a novel, cell type-specific, immune evasion strategy. Presumably, most cell types infected by HSV-1 produce PGE_2, thus contributing to viral pathogenesis by facilitating efficient virus growth. By targeting the expression of PGE_2 receptors in DCs, HSV-1 infection can render these cells unresponsive to HSV-1 induced PGE_2 signaling, and prevent their migration to lymph nodes where adaptive immune processes will be initiated. By this method, HSV-1 benefits from PGE_2 augmentation of virus growth, while strategically inhibiting the ability of DCs to initiate an adaptive immune response.

Like HSV-1, dengue virus infection also induces COX-2-dependent production of PGE_2 in DCs (Liou et al., 2008; Wu et al., 2009). However, unlike

HSV-1, dengue virus replication appears insensitive to PGE_2 signaling (Liou et al., 2008), and infection of DCs with dengue virus does not lead to reduced expression of EP2 or EP4 receptors (Wu et al., 2009). Conversely, the expression of CCR7 was induced in dengue-infected DCs, partially by a PGE_2-dependent mechanism (Wu et al., 2009). Consistent with these findings, dengue-infected DCs efficiently migrated towards CCL21 in transwell migration assays (Wu et al., 2009). DC migration was suppressed when infections were conducted in the presence of a COX-2 inhibitor, or antagonists of EP2 and EP4, thereby confirming the role of PGE_2 signal transduction in virus-induced migration (Wu et al., 2009). Thus, as opposed to HSV-1, dengue virus is unable to interfere with PGE_2 signaling in DCs. Dengue pathogenesis in general remains poorly understood due to the lack of a representative animal model, therefore it is not yet known how dengue-induced PGE_2 production may play into the natural progression of dengue infection.

RSV infection has been shown to induce COX-2-dependent production of PGE_2 in an alveolar epithelial cell line, as well as in primary cord blood-derived macrophages and DCs (Bartz et al., 2002; Liu et al., 2005; Richardson et al., 2005). Additionally, elevated levels of PGE_2 have been measured in endotracheal aspirates from infants positive for RSV infection (Sznajer et al., 2004), and elevated levels of COX-2 have been measured in the lungs of RSV-infected cotton rats (Richardson et al., 2005), the small animal model system

used to study RSV pathogenesis. In common with HCMV and HSV-1, RSV-induced PGE_2 was found to be essential for efficient virus replication in vitro (Liu et al., 2005). However, and in marked contrast, virus titers in the lungs of indomethacin-treated and control cotton rats infected with RSV were comparable several days after infection, suggesting that PGE_2 was not essential for efficient virus replication in vivo (Richardson et al., 2005). Interestingly, although virus titers were near identical, histological analysis of indomethacin-treated cotton rats indicated significant reductions in RSV-induced lung pathology, suggesting that PGE_2 contributed to destructive inflammatory processes in the lungs of RSV-infected rats (Richardson et al., 2005). Similarly, a recently published report by Lee et al. (2008) linked the potent induction of COX-2 by pathogenic influenza viruses to the promotion of a proinflammatory cascade that is associated with influenza-induced lung pathology. It seems therefore, that in some cases, PGE_2-dependent promotion of immune responses can contribute to virus-induced pathology.

Collectively, these examples provide insight into several of the many potential mechanisms by which PGE_2 may contribute to the natural progression of virus infection, or the host anti-viral immune response. For many viruses, these interactions have not been thoroughly investigated, and there remains a need for additional research in this area of study.

PGE_2 and vaccines

As previously described, PGE_2 mediates a broad range of effects on both innate and adaptive immune processes. Several recent studies have investigated whether modulation of these PGE_2-dependent effects can enhance or reduce the efficacy of prophylactic and therapeutic vaccines. Because PGE_2 promotes Th2 immune responses, it is expected that PGE_2 signaling will generally contribute to the ability of a vaccine to generate neutralizing antibody responses, while attenuating vaccine-induced CTL responses. Thus, the contribution of PGE_2 signaling to vaccine efficacy is expected to vary based on the desired outcome of vaccination. This rationale is supported by data presented in the available literature.

Multiple studies have utilized pharmacologic inhibition of COX-2 to determine the contribution of PGs to the efficacy of anti-cancer therapeutic vaccines. In order to eliminate malignant cells, anti-cancer vaccines must provoke potent cell-mediated immune responses capable of eliciting tumor cell-specific CTL activity. Hence, the Th2-promoting effects of PGE_2 signaling are not expected to augment vaccine-induced tumorcidal activity, and are instead likely to reduce vaccine efficacy by attenuating Th1 responses. Accordingly, pharmacological inhibition of COX-2 in conjunction with antitumor vaccination has been shown to reduce circulating levels of PGE_2, increase tumor-specific CTL

activity, reduce tumor growth, and increase survival rates (Basu et al., 2006; Haas et al., 2006; Mukherjee et al., 2009; Sharma et al., 2005; Zeytin et al., 2004). A combination of factors was proposed to be mediating these beneficial effects. Importantly, in all studies, increased numbers of tumor cell-specific CTLs, and increased CTL activity, were observed in response to combination treatment with the vaccines and COX-2 inhibitors. Additionally, reduced levels of PGE_2 resulting from COX-2 inhibition were proposed to facilitate CTL-mediated tumor killing by reducing the immune suppression capabilities of the tumor microenvironment. In support of this, analysis of COX-2 inhibitor-treated mice used in these studies indicated elevated intratumoral levels of Th1 cytokines and chemokines (IFNγ, IL-12, CXCL10) (Haas et al., 2006; Sharma et al., 2005), reduced levels of IDO within tumors and DCs isolated from tumor-bearing mice (Basu et al., 2006; Mukherjee et al., 2009), and decreased T regulatory cell activity in the tumor microenvironment (Mukherjee et al., 2009). Taken together, the results of these studies demonstrate that COX-2 activity and PGE_2 signaling can attenuate the effectiveness of vaccines aimed at inducing CTL responses. However, it is important to note that PGE_2 has been shown to promote the growth of numerous types of tumors by inducing malignant cell proliferation, invasion, and tumor angiogenesis (Wang and Dubois, 2006). As a result, COX-2 inhibition can reduce tumor growth by a combination of factors, thus making it difficult to

determine with certainty that immune modulation was the sole factor contributing to vaccine augmentation in these studies.

In direct contrast, studies have shown that COX-2 activity and PGE_2 signaling are essential when the desired outcome of vaccination is robust production of Ag-specific Abs. Ryan et al. (2006) compared the antibody response generated by vaccination with human papilomavirus type 16 virus-like particles in wild type and COX-2-deficient mice. They observed marked decreases in total IgG, Ab secreting cells, and Ag-specific neutralizing Ab responses in vaccinated COX-2-deficient mice compared to wild-type controls. Unfortunately, COX-2-knockout mice have known deficiencies in both the B cell and T cell compartments of the immune system (Rocca et al., 1999; Ryan et al., 2005), making it likely that Ab production in mice lacking COX-2 was perturbed not only during the generation of a vaccine-induced immune response, but during immune system development as well. However, this study also demonstrated dose-dependent reductions of human memory B cell expansion and Ab secretion upon pharmacologic inhibition of COX-2 activity in assays conducted with PBMC isolated from human subjects vaccinated with human papilomavirus type 16 virus-like particles. The data presented provide evidence that COX-2 is essential for optimal production of neutralizing Abs, and for generation of memory B cell expansion. Similarly, the immune adjuvant, synthetic CpG oligodeoxynucleotides (ODN), induces COX-2 expression and production of PGE_2 in human B cells

(Bernard and Phipps, 2007). Studies conducted in vitro using COX-2 inhibitors demonstrated that the COX-2 activity induced by CpG ODN was required for optimal production of IgM and IgG, thus implicating the PG response as being a contributing factor to the adjuvant effects of CpG ODN (Bernard and Phipps, 2007). Collectively, these data verify the importance of COX-2 and PGE_2 in the provocation of humoral immune responses. Additionally, they suggest that the use of NSAIDs to relieve common side effects of vaccination (pain, swelling) may be contraindicated, as this may have untoward consequences to the outcome of vaccination. The outcomes of these studies provide evidence that intentional manipulation of the PGE_2 response may be a useful consideration for the design of new vaccines or vaccination regimens. Keeping in mind that the majority of viruses evaluated to date have been shown to induce the production of PGE_2 upon infection, the effects of PGE_2 signaling on vaccine-induced immune responses may require special attention when vaccines based on a live viral vector are used.

Poxviruses

The first, and most successful, live viral vaccines ever used for prophylaxis against a human pathogen were poxviruses. Poxviruses are large double-stranded DNA-containing viruses that replicate in the cytoplasm of host

cells. Several poxviruses are significant for human health including the causative agent of smallpox (variola virus), vaccinia virus (VAC), cowpox virus (CPXV), and monkeypox virus (Fields et al., 2007). CPXV was the original virus used by Jenner as a human smallpox vaccine (Mercer et al., 2007). However, over time, VAC replaced CPXV as the live-viral vaccine against smallpox, which was eradicated in 1979 (Fenner, 1988). The ancestral origin of VAC is unknown and complicated by repeated passage in various host animals throughout its history as a smallpox vaccine (Baxby, 1981). Although effective, vaccination with VAC has been associated with numerous adverse events, especially in immune-compromised individuals (Fulginiti et al., 2003). To address this complication, several attenuated derivatives of VAC have been produced. One of the most promising is modified vaccinia Ankara virus (MVA). MVA is an attenuated form of VAC strain Ankara produced by repeated serial passage in chick embryo fibroblasts (Sutter and Staib, 2003). As a consequence of attenuation by serial passage, the host-range of MVA is severely restricted, and infection of most non-avian cell types is abortive (Carroll and Moss, 1997; Mayr et al., 1978). MVA has been shown to be completely avirulent and highly immunogenic in humans, and has been shown to elicit cross-protective immunity against virulent poxviruses (Earl et al., 2004; Mayr et al., 1978; Wyatt et al., 2004). Due to its safety and immunogenicity, MVA is also a candidate live-viral vaccine vector for cancer immunotherapy and prophylaxis against various infectious diseases (Gomez et

al., 2008; Rimmelzwaan and Sutter, 2009; Sauter et al., 2005; Sutter and Staib, 2003).

Poxvirus life cycle and morphogenesis

The poxvirus replication cycle and most aspects of general poxvirus biology have been determined through studies conducted with the prototypical poxvirus, VAC. Although much research has been conducted, many aspects of poxvirus replication remain unknown or contentious.

The poxvirus life cycle begins with the binding of the virion to the surface of the cell. The exact determinants of virus–cell binding remain unknown, but based on the promiscuity of most poxviruses, it is expected that common constituents of cell membranes are recognized by a complement of virus proteins (Moss, 2006). Once attached, the outer virus membrane and cell membrane fuse, thus initiating entry of the virus core into the cytoplasm of the host cell (Moss, 2006; Vanderplasschen et al., 1998). For poxviruses, the process of entry is complicated by the ability of distinct forms of virions to initiate infection. There are four infectious types of poxvirus virions, intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV) (Smith et al., 2003; Smith et al., 2002). The crucial differences between these distinct infectious forms of virions are the

number of membranes surrounding the virus core, and the complement of virus proteins present in the outermost membrane. IMV particles are surrounded by one or two membranes (an unresolved issue amongst virologists), IEV are surrounded by two more layers of membrane than IMV, and CEV and EEV have one fewer layer than IEV (Smith et al., 2002). The outer membranes of IEV, CEV, and EEV contain viral proteins that are absent in IMV (Smith et al., 2003), and it has been demonstrated that one result of these differences is that IMV enters cells by a pH-independent process while entry of other virion forms is dependent on low pH (Vanderplasschen et al., 1998). Upon release of the virus core into the host-cell cytoplasm, transcription of virus genes is initiated by the combined activities of the viral RNA polymerase and viral transcription factors that are packaged within the virus core (Broyles, 2003). Early viral mRNAs are transcribed within the core, and then extruded through pores into the cytoplasm (Kates and Beeson, 1970). These early viral mRNAs are translated by host cell machinery into early viral proteins including the viral DNA polymerase, viral transcription factors, and proteins involved in evasion of host immune responses (Fields et al., 2007). Some early proteins together with unknown host factors then act to uncoat the viral core and release the double-stranded DNA virus genome into the cytoplasm, thereby initiating concatemeric DNA replication (Fields et al., 2007; Schramm and Locker, 2005). The replicating genomes serve as templates for temporally-regulated transcription, resulting in stepwise

expression of intermediate and late-stage viral proteins (Broyles, 2003). The late-stage viral proteins include viral structural proteins and components of the viral transcriptosome that will be packaged into the viral core. Viral concatemers are then resolved and packaged into immature virions (Condit et al., 2006). These immature virions gain one or two lipid membranes by an unknown mechanism, forming the first infectious morphogenic progeny virus, IMV (Roberts and Smith, 2008; Smith et al., 2003). Some IMV are wrapped with additional cellular membranes obtained from either the *trans*-Golgi network, or early endosomes, to form IEV (Schmelz et al., 1994; Tooze et al., 1993). IEV are transported via microtubules to the surface of the cell (Sanderson et al., 2000). At the surface, the outer IEV membrane fuses with the plasma membrane, resulting in the loss of one outer layer of membrane and exposing a virus particle at the cell surface (Smith et al., 2002). Particles that remain associated with the cell are termed CEV, while those that are released are termed EEV (Smith and Law, 2004; Smith et al., 2003; Smith et al., 2002). CEV and EEV particles are distinct only by association with the cell. For some CEV, specific viral proteins associated with the CEV particle concentrate at the edge of the plasma membrane and trigger a signal cascade that mimics a tyrosine kinase signaling pathway involved in normal cellular control of actin polymerization (Frischknecht et al., 1999; Reeves et al., 2005). The activation of this process by CEV-associated proteins results in the polymerization of actin filaments, which thereby

propel the CEV away from the cell, thus facilitating efficient cell-to-cell spread (Blasco and Moss, 1992; Cudmore et al., 1995). In contrast, EEV particles are freely released from the surface of infected cells, and are primarily involved in long distance virus spread and systemic dissemination (Payne, 1980).

Poxvirus strategies for modulation of host immune responses

Highly conserved poxvirus genes including those needed for replication and assembly of progeny viruses lie near the middle of the linear double-stranded DNA genomes (Upton et al., 2003). Conversely, the ends of the genome are poorly conserved and encode many gene products important for allowing virus growth in various host cells, and for viral pathogenicity in vivo (Fields et al., 2007; Upton et al., 2003). Many of these ancillary genes encode a diverse repertoire of gene products that function to disrupt inflammatory and immune signaling. Several poxvirus proteins, including chemokine binding proteins, IL-18 and IFN-α/β binding proteins, and secreted viral receptor homologues for TNF-α, IL-1β, and IFN-γ, can act as competitive antagonists of cellular soluble signaling molecules (Haga and Bowie, 2005; Seet et al., 2003). Alternatively, other poxvirus proteins target intracellular components of activated signaling pathways including NFκB, MyD88, PKR, and TRIF (Oie and Pickup, 2001; Stack et al., 2005; Willis et al., 2009). Poxviruses can also subvert other

immune processes by reducing the potential for host detection of an active infection. This strategy includes sequestering double strand RNA to prevent protein kinase R (PKR) activation, production of complement binding proteins, and expression of NKG2D antagonists that can prevent NK cell activation (Campbell et al., 2007; Davies et al., 1993; Kotwal et al., 1990). As a result of cell passage-mediated attenuation, MVA has lost many accessory gene products found in related poxviruses, and therefore often differs greatly it its capacity to disrupt host responses (Antoine et al., 1998; Blanchard et al., 1998). Because of the potential for widespread use of MVA as a vaccine, or vaccine vector, in human populations, there is a need to better understand the unique biology of MVA, and how it differs from the more wild-type poxviruses during host-virus interactions. Further research into this area may also identify accessory genes found in related poxviruses, but missing in MVA, that can be engineered into MVA vaccine vectors to elicit targeted effects on host immune responses with the aim of increasing vaccine efficacy.

Poxviruses and prostaglandins

Although substantial research has been conducted on the diverse immune modulation strategies of poxviruses, the PG response following poxvirus infection has received comparatively little attention. In vitro experiments conducted in

mouse L cells indicated that exogenous addition of E-series PGs had no effect on the replication of VAC, however no attempt was made to determine the possible effects of endogenous PGs in the culture system (Santoro et al., 1982). The ability of poxvirus-infected cells to metabolize AA and produce PGE_2 was first described in early experiments conduced in monkey kidney cells. When these cells were treated with a calcium ionophore (A23187), and fed with radiolabeled eicosanoid precursors, PG synthesis was found to be enhanced specifically during infection with several poxviruses, including MVA (Palumbo et al., 1994; Palumbo et al., 1993). These investigators did not, however, test whether poxvirus infection was sufficient to induce PG biosynthesis, or the release of PGE_2 from infected cells, in the absence of exogenous stimulus or precursor molecules. Additionally, the PG response to poxvirus infection was tested only in a monkey kidney cell line; no biologically relevant primary cells were tested, and no detailed analysis of poxvirus-dependent induction of the PG biosynthesis pathway was conducted. As a result, considerable gaps remain in our understanding of this important poxvirus–host interaction. Data presented in this study describe key aspects of poxvirus-induced production of PGE_2 with particular emphasis on the vaccine strain, MVA. Our data show that infection with MVA results in copious production of PGE_2 by murine bone marrow-derived DCs and a murine fibroblast cell line in vitro. Related poxviruses, VAC and CPXV, vary in their ability to induce PGE_2 production by infected cells, and we

determined that viral suppressive mechanisms are active in a virus and cell type-dependent manner. Additionally, data presented demonstrate that unlike other viruses, including HSV-1, RSV, and HCMV, poxvirus growth in vitro is unaffected when endogenous PG production is inhibited, or when PGE_2 is added exogenously. We propose that the production of PGE_2 in response to infection with MVA is likely a contributory determinant of the immune response generated by MVA-based vaccines, and targeted manipulation of this response should be considered in the development of strategies aimed at increasing vaccine efficacy.

Poxvirus antiviral agents

Unlike MVA, several related poxviruses are pathogenic in humans, and thus constitute threats to human health. Because widespread public vaccination against smallpox was discontinued, the majority of the current population has no protection against smallpox and related diseases. If the general public were to be exposed to pathogenic poxviruses, via natural or criminal means, the use of antiviral drugs would provide a crucial complement to vaccination in limiting the extent of human disease (Neyts and De Clercq, 2003).

To date, the Food and Drug Administration has approved only one investigational new drug for use against poxviruses, a nucleoside analog called cidofovir (CDV). More specifically, CDV is an acyclic nucleoside phosphonate

(De Clercq, 2003). This compound contains a stably-linked phosphate-mimetic group that allows CDV to be readily phosphorylated to the active diphosphate form by cellular kinases present in uninfected, or virus-infected cells. CDV can then serve as an alternate, chain-terminating, substrate for DNA polymerases, with a high affinity for viral DNA polymerases (De Clercq, 2003). In this manner CDV inhibits the replication of many DNA viruses, and has been shown to be effective in a diverse assortment of animal models that represent major DNA virus infections common in humans (De Clercq, 2003). Unfortunately, CDV is dose-limited by its nephrotoxic effects, and poxviruses with resistance to CDV have been isolated (Smee et al., 2002). Accordingly, there is a need for novel antiviral agents that target other aspects of poxvirus growth using mechanisms of action that are distinct from those used by CDV.

One recently described compound, ST-246, is readily bioavailable following oral administration and has recently been evaluated in a phase I clinical trial (Jordan et al., 2008; Yang et al., 2005). Unlike CDV, ST-246 does not inhibit poxvirus replication in a cell-autonomous manner. Alternatively, ST-246 has been shown to interact with a specific viral protein (encoded by the F13L gene in vaccinia virus and the V061 gene in cowpox virus) that is required for the additional membrane wrapping steps needed to form IEV particles (Blasco and Moss, 1991). Thus, ST-246 interferes with poxvirus morphogenesis, and prevents the formation of IEV, CEV, and EEV particles. These particles are

involved in virus cell-to-cell spread and systemic dissemination (Smith et al., 2002). A recently conducted study determined that orally administered ST-246 protected non-human primates from smallpox and monkeypox challenge (Huggins et al., 2009). However, in vitro studies have indicated resistance to ST-246 in poxviruses containing a single point mutation (Yang et al., 2005), providing evidence that there is a need to further develop, or identify, new poxvirus antiviral agents.

Data presented in this study identifies Terameprocol, a proprietary derivative of nordihydroguaiaretic acid, as a novel poxvirus antiviral agent. We show that Terameprocol can inhibit the growth of poxviruses in a diverse panel of cell lines by inhibiting the efficient spread of infectious particles from cell to cell. The data presented in this study are sufficient to support experiments to determine the ability of Terameprocol to prevent, or reduce, poxvirus-induced disease in established animal models.

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CHAPTER 2

Cell type and virus-specific factors affect prostaglandin E_2 production in response to infection with modified vaccinia Ankara virus and other poxviruses

ABSTRACT

Prostaglandin E_2 (PGE_2) is a lipid signaling molecule that has important roles in inflammation and the adaptive immune response. Although poxviruses utilize a diverse repertoire of strategies to interfere with host innate and adaptive immune processes, the effect of poxvirus infection on PGE_2 production has not been well defined. In this study, we show that infection with the poxvirus vaccine strain, modified vaccinia Ankara virus (MVA), induces robust accumulation of PGE_2 in culture supernatants of both a mouse fibroblast cell line and murine bone marrow-derived dendritic cells. Furthermore, we observed several distinctions between MVA-induced PGE_2 production and the canonical model of prostaglandin synthesis in response to proinflammatory stimuli. Viral gene expression was required for accumulation of PGE_2, and for release of the PGE_2 precursor, arachidonic acid, and both of these processes were remarkably delayed relative to the onset of infection. MVA-induced PGE_2 production was dependent on the activity of cyclooxygenase-2, but liberation of arachidonic acid occurred independently of cytosolic phospholipase A_2. Additionally, we showed that other poxviruses varied in their ability to induce PGE_2 production depending on the particular combinations of virus and cell type assayed. Experiments to identify the underlying basis of these differences suggested that some poxviruses can suppress PGE_2 production in certain cell types. The results of this study

provide insight into the novel characteristics of MVA, and point to strategies that may lead to engineered MVA vaccine vectors capable of optimizing prostaglandin-mediated immune responses for increased vaccine efficacy.

INTRODUCTION

Prostaglandins (PGs) are a group of fatty acid-derived molecules that regulate a myriad of physiological and pathological processes. One of the most ubiquitous and best-characterized PG subtypes is prostaglandin E_2 (PGE_2). The biological responses associated with PGE_2 signaling have long been known to include key roles in the mediation of inflammation, including the promotion of fever, vessel dilation, and pain sensitivity (Dubois et al., 1998; Ivanov and Romanovsky, 2004). More recently, PGs have also been found to have important roles in shaping both cell-mediated and humoral immune responses. In most cases, PGE_2 has been shown to polarize the immune system towards generation of a Th2 response (Harris et al., 2002; Phipps et al., 1991). Examples of PGE_2-mediated effects include the reduction of Th1 cytokine production by T cells, macrophages, and NK cells, and the promotion of IgG1 and IgE class switching by B cells (Ganapathy et al., 2000; Ikegami et al., 2001; Katamura et al., 1995; Roper et al., 1995; Roper et al., 2002; Walker and Rotondo, 2004). Additionally, both human and murine dendritic cells (DCs) have been shown to produce PGE_2, as well as respond to PGE_2 signaling (Fogel-Petrovic et al., 2004; Harizi et al., 2001; Harizi et al., 2002; Morelli and Thomson, 2003). PGE_2 promotes migration and survival of DCs, suppresses their ability to produce IL-12p70, and can function as a negative regulator of plasmacytoid DCs (Harizi et

al., 2002; Kalinski et al., 2001; Legler et al., 2006; Sheibanie et al., 2004; Son et al., 2006; Vassiliou et al., 2004). Thus, PGE_2 plays a critical role during adaptive immune responses and has functions complementary to those of more widely studied, protein-based signaling molecules.

The canonical pathway for inducible PGE_2 synthesis begins with the release of arachidonic acid (AA) from membrane glycerophospholipids (Smith, 1989). Several cellular phospholipases may be involved, however, cytosolic phospholipase A_2 ($cPLA_2$) is often regarded as crucial because cells obtained from $cPLA_2$ knock-out mice are severely deficient in PG production in response to a variety of stimuli (Gijon et al., 2000; Sapirstein and Bonventre, 2000). Calcium-independent PLA_2 is traditionally regarded as a housekeeping enzyme involved in the maintenance of phospholipid homeostasis (Murakami and Kudo, 2002), whereas group V secretory phospholipase A_2 is inducible, and has been shown to amplify $cPLA_2$-dependent AA release (Ruiperez et al., 2009). The AA released by phospholipases is then converted into the intermediate prostaglandin H_2 by cellular cyclooxygenase (COX) enzymes. There are two predominant forms of COX, COX-1 is most often constitutively expressed, and has roles in tissue homeostasis, while COX-2 typically has low basal expression, but is readily inducible (Smith et al., 1996; Tsatsanis et al., 2006). Prostaglandin H_2 generated by COX activity then is converted enzymatically to PGE_2, or other PG subtypes, by specific PG synthases (Smith, 1989). In particular, microsomal PG

E-synthase-1 (mPGES-1) is often co-expressed with COX-2, and is typically involved in the production of PGE_2 in response to proinflammatory stimuli (Park et al., 2006). Once produced, PGE_2 signals in an autocrine or paracrine fashion via specific G protein-coupled receptors (Sugimoto and Narumiya, 2007).

Among the poxviruses significant for human health are smallpox (variola) virus, vaccinia virus (VAC), and cowpox virus (CPXV) (Fields et al., 2007). CPXV was the original virus used by Jenner as a human smallpox vaccine, however, over time, VAC replaced CPXV as the vaccine against smallpox (Mercer et al., 2007). Although effective, vaccination with VAC is associated with adverse events, especially in immune compromised individuals (Fulginiti et al., 2003). To address this issue, attenuated derivatives of VAC have been generated. Modified vaccinia Ankara virus (MVA) is a highly attenuated form of VAC strain Ankara that has been shown to be completely avirulent in humans (Moss et al., 1996), yet remains effective as a vaccine against virulent poxviruses (Earl et al., 2004; Mayr et al., 1978; Wyatt et al., 2004). Due to its safety and immunogenicity, MVA is also a candidate vaccine vector for cancer immunotherapy, and for prophylaxis against various infectious diseases (Gomez et al., 2008; Rimmelzwaan and Sutter, 2009; Sutter and Staib, 2003).

Poxviruses have relatively large genomes and encode a diverse repertoire of gene products that function to disrupt inflammatory and immune system signaling. Several poxvirus proteins, including chemokine binding proteins and

secreted receptor homologues can act as competitive antagonists of cellular cytokines (Haga and Bowie, 2005; Seet et al., 2003). Other poxvirus proteins target intracellular components of activated signaling pathways, such as NFκB, MyD88, and TRIF (Oie and Pickup, 2001; Stack et al., 2005). Poxviruses also avoid detection by the immune system by such strategies as sequestering dsRNA to prevent protein kinase R activation, producing complement binding proteins, or expressing antagonists that can interfere with antigen presentation and prevent NK cell activation (Campbell et al., 2007; Davies et al., 1993; Kotwal et al., 1990; Seet et al., 2003). During its attenuation by repeated passage in cell culture, MVA has lost many accessory gene products found in related poxviruses, and therefore it often differs greatly from them it its capacity to disrupt host responses (Antoine et al., 1998; Blanchard et al., 1998). Because of the potential for widespread use of MVA in human populations as a vaccine, or vaccine vector, there is a need to increase our understanding of the unique characteristics of MVA, and how MVA differs from other poxviruses during its host–virus interactions.

Although substantial research has been conducted on the diverse immune modulation strategies of poxviruses, the PG response following poxvirus infection has received comparatively little attention. In early experiments, when monkey kidney cells were treated with a calcium ionophore and fed with radiolabeled eicosanoid precursors, PG synthesis was found to be enhanced specifically

during infection with several poxviruses, including MVA (Palumbo et al., 1994; Palumbo et al., 1993). These investigators did not, however, test whether poxvirus infection was sufficient to induce PG biosynthesis in the absence of exogenous stimulus or precursors. In the current study, we addressed this question directly and describe molecular aspects of poxvirus-induced production of PGE_2 that occur during infection with MVA, and other poxviruses. Our data show that MVA infection results in copious production of PGE_2 by a murine fibroblast cell line, as well as by murine bone marrow derived DCs in vitro. Related poxviruses, VAC and CPXV, vary in their ability to induce PGE_2 production by infected cells. Experiments to uncover how these three viruses differentially activate PGE_2 synthesis revealed strategies that may facilitate the design of engineered MVA vaccine vectors capable of optimizing PG-mediated immune responses for increased vaccine efficacy.

MATERIALS AND METHODS

Reagents and antibodies

All FBS, cell culture media, and cell culture reagents were purchased from Gibco unless otherwise stated. Recombinant GM-CSF and IL-4 were purchased from R&D Systems. PGE_2 and the COX-2-specific inhibitor NS-398 were purchased from Cayman Chemical Company. The specific $cPLA_2$ inhibitor (N-{(2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl) was purchased from Calbiochem. Phorbol 12-myristate 13-acetate (PMA) was purchased from Biomol International. Radiolabeled [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid ([^{3}H]AA) was purchased from Perkin Elmer. COX-2-specific rabbit polyclonal Ab was purchased from Cayman Chemical. Rabbit polyclonal Ab specific for mPGES-1 (Jakobsson et al., 1999) was a generous gift from Dr. Per-Johan Jakobsson (Karolinska Institutet). Murine mAb specific to β-actin was purchased from Sigma-Aldrich. Ascites fluid containing murine mAb (TW2.3) specific to the VAC early gene product E3L (Yuwen et al., 1993) was a generous gift from Dr. Jack Bennink (National Institutes of Health). Tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse, and FITC conjugated goat anti-rabbit Abs were obtained from

Sigma-Aldrich. Secondary Abs for use with the Odyssey Infrared Imaging System, goat anti-mouse IRDye 680 and goat anti-rabbit IRDye 800CW, were purchased from Li-Cor Biosciences.

Cell culture and generation of murine bone marrow derived DCs

C3HA murine fibroblasts (Gooding, 1979) were cultured in DMEM supplemented with 1 mM sodium pyruvate and 5% FBS. 143B cells, CHO-K1, BHK-21 and RAW 264.7 cells were from the American Type Culture Collection (CRL-8303, CCL-61, CCL-10, and TIB-71 respectively). 143B cells were cultured in MEM supplemented with 5% FBS, CHO-K1 cells were cultured in F-12K medium supplemented with 10% FBS, BHK-21 cells were cultured in MEM-alpha with 5% FBS, and RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere. Bone marrow cells were harvested from 12-24 week old female C57BL/6 mice (Charles Rivers Labs). Procedures for the use and care of mice were conducted in accordance with the National Institutes of Health guidelines, and protocols approved by Duke University's Institutional Animal Care and Use Committee. Bone marrow cells were cultured to generate DCs, as described by Lee et al. (Lee et al., 2005). Briefly, bone marrow progenitor cells were cultured in DC medium (RPMI 1640 supplemented with 5% heat-inactivated FBS, 100 u/ml

penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, 1X MEM non-essential amino acids, 0.5X MEM essential amino acids, 10 mM HEPES, 2 mM L-glutamine, 55 mM β-mercaptoethanol and 10 ng/ml each of recombinant mouse GM-CSF and IL-4). After three days, the adherent cells were washed and replenished with fresh DC medium, and cultured for three additional days. Non-adherent, immature DCs, were harvested at day six, and cultured in DC medium without cytokines for all experiments.

Viruses and infections

Viruses employed in this study were MVA (ATCC VR-1566), VAC strain Western Reserve (ATCC VR-1354), and CPXV strain Brighton red (ATCC VR-302). Recombinant MVA/ATI-EGFP contains a gene encoding enhanced GFP (EGFP), under control of the CPXV ATI gene promoter, inserted into intergenic region 4 (Timm et al., 2006). Unless otherwise indicated, all virus infections were conducted at 5 PFU/cell for 1 h at 37°C in medium containing 2.5% FBS. Where indicated, virus preparations were heat inactivated by incubation for 1 h at 55°C.

Quantitative PGE_2 ELISA

Accumulation of PGE_2 in cell culture supernatants was determined using

specific immunoassays (Assay Designs). Briefly 2.5 X 10^4 C3HA cells were plated into 24-well flat bottom tissue culture plates (Corning Incorporated) and incubated overnight at 37°C to allow the cells to adhere. When DCs were used, 5 X 10^5 cells per well were plated, followed by incubation for 1 h at 37°C to allow the cells to adhere. Virus infections and experimental treatments were conducted as indicated, and cell supernatants were collected at various times after infection, or treatment. Supernatants were briefly centrifuged to remove large debris, and immunoassay analysis was performed according to the manufacturer's instructions. Absorbance was determined using a PolarStar microplate reader (BMG Labtechnologies) and concentration of PGE_2 was determined by comparison to a standard curve. In experiments conducted using the COX-2 specific inhibitor, NS-398, cells were plated, infected, and incubated in media supplemented with the drug (1 μM), or the solvent control (DMSO).

Immunofluorescence microscopy

8-well glass chamber slides (Nalge Nunc International) were seeded with 2 X 10^4 C3HA cells in 2.5% FBS DMEM and incubated at 37°C overnight. Cells were then infected with MVA or MVA/ATI-EGFP and incubated at 37°C for the times indicated. Cells were fixed in 4% formaldehyde PBS for 20 min at room temperature and washed twice in 3% BSA PBS. Cells were then permeabilized

with 0.1% Triton X-100 PBS for 5 min, washed twice in 3% BSA PBS, and incubated with primary Abs in 0.3% BSA for 1 h. Cells were then washed again twice in 3% BSA PBS and incubated with secondary Abs in 0.3% BSA PBS for 30 min, washed twice in 3% BSA PBS, and mounted in anti-fade slide mount containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc.). Microscopy was conducted on a Zeiss Axioskop 2 Plus and images were captured using Spot Advanced software (Diagnostic Instruments, Inc.). Matched exposure times were used within each set of experiments.

[^{3}H]AA release assays

For each experimental condition a total of 5 X 10^5 C3HA cells were seeded into T25 tissue culture flasks (Becton Dickinson) and incubated at 37°C overnight in medium containing 0.1 μCi/ml [^{3}H]AA. After labeling, the cells were washed two times with HBSS. Fresh medium supplemented with 2.5% FBS was added to the flasks and the cells were incubated at 37°C for 2 h to allow for spontaneous release of the radiolabel. The cells were then washed again with HBSS and infected as indicated for 1 h at 37°C. Following infection, 2.5% FBS DMEM was added to each flask to a final volume of 5.5 ml and the cultures were incubated at 37°C. At the indicated times after infection, 250 μL of supernatant were collected from each flask and briefly centrifuged to remove debris. Liquid

scintillation counting was performed on 200 μL samples of this supernatant using a Beckman Coulter model LS 5801.

Quantitative Immunoblotting

Monolayers of mock-infected, or virus-infected, C3HA cells were solubilized in lysis buffer (50 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 2 mM PMSF, and 0.5% SDS) and collected by scraping. Total protein concentration of the samples was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc). Equal amounts of total protein (10 μg) were loaded onto 12% or 16% polyacrylamide Tris-glycine gels and separated by electrophoresis on a Novex MiniCell System (Invitrogen Life Technologies). Proteins were transferred to Immobilon-FL PVDF membranes (Millipore) and blocked for 1 h in Odyssey Blocking Buffer (Li-Cor Biosciences). Primary Abs were diluted in 0.1% Tween-20 Odyssey Blocking Buffer and incubated with membrane overnight at 4°C (COX-2 and mPGES-1), or for 1 h at room temperature (β-actin). Membranes were washed extensively in PBS containing 0.1% Tween-20. Secondary Abs specifically designed for use with the Li-Cor Odyssey system were diluted in 0.1% Tween-20 Odyssey Blocking buffer and incubated for 45 min at room temperature, followed by extensive washing with PBS containing 0.1% Tween-20. Band visualization and quantification was

completed on a Li-Cor Odyssey scanning system running Odyssey 2.1 software (Li-Cor Biosciences). The integrated intensity of each specific band of interest was measured and normalized to the respective β-actin control band to account for any minor variations in total protein loads.

Virus growth assays

To determine the effect of endogenously produced PGE_2 on poxvirus replication, C3HA cells were treated with 1 μM NS-398 or DMSO solvent control and incubated overnight. Cells were infected (0.1 PFU/cell) with CPXV or VAC and incubations were continued in the presence of 1 μM NS398 or DMSO solvent control. Total virus was collected by freeze-thaw lysis of infected cells 24 h after infection. Virus titer was measured by standard plaque assay on 143B cells (VAC and CPXV) or BHK-21 cells (MVA). To determine the effect of exogenously added PGE_2 on poxvirus replication, RAW 264.7 cells were infected (0.1 PFU/cell) with CPXV or VAC and incubated for 24 h in media supplemented with 0.1 μM, 1.0 μM, or 10 μM PGE_2 (Cayman Chemical) or DMSO solvent control. Total virus was collected after 24 h, and virus titer was determined as described above.

Reactivation of Heat-Inactivated (HI) virus

Reactivation experiments were conducted in order to confirm that heat-treated virus preparations were inactivated without being thermally destroyed. For reactivation, MVA was used as a helper virus in mixed infections with HI VAC or HI CPXV. Monolayers of C3HA cells were infected with VAC or CPXV alone, HI VAC or HI CPXV alone, or HI virus in combination with MVA. All virus inoculations were conducted at 5 PFU/cell. Total virus preparations were collected by freeze-thaw lysis for each experimental condition 24 h after infection. Virus titer was determined by standard plaque assay on C3HA cells.

Statistical analysis

Data are presented as mean and standard error of the mean (SEM). Group analysis of variance was compared by one-way ANOVA followed by multiple comparisons of means using Tukey-Kramer post-tests. All statistical analyses were performed using GraphPad Prism (version 5.01) software (GraphPad Software).

RESULTS

Characterization of MVA infection in C3HA mouse fibroblasts

We have previously used the C3HA mouse fibroblast cell line to study PGE_2 production and AA release in response to adenovirus infection (Culver and Laster, 2007). In order to establish the usefulness of this model for investigations of poxvirus-induced PG synthesis, we first determined the ability of MVA to infect C3HA cells. As a result of attenuation by serial passage in chick embryo fibroblasts, MVA has lost the ability to reproduce in most other cell types. Initial experiments revealed that MVA infection indeed was non-productive in C3HA cells (data not shown), as described for other mouse fibroblast cell lines (Carroll and Moss, 1997). However, MVA enters the preponderance of cell types, and can express both early and late viral genes, which are features that contribute to its usefulness as a vaccine vector (Sutter and Moss, 1992). We used fluorescence microscopy to evaluate the ability of MVA to express early and late viral gene products in C3HA cells that were infected with MVA/ATI-EGFP, a recombinant MVA that expresses EGFP under the control of the CPXV ATI gene promoter. Expression of the ATI gene, or heterologous proteins under control of the ATI promoter, is restricted to the late stage of poxvirus replication (Patel and Pickup, 1987). At various times post infection (p.i.) the cells were fixed,

permeabilized, and immunostained with an antibody specific to the viral E3L protein. E3L typically is expressed rapidly after infection and persists to late stages of viral replication, making it a useful marker for both confirmation of viral infection and for monitoring viral early gene expression (Watson et al., 1991). E3L could be detected in all MVA-infected cells from 2 h to 24 h p.i., and its abundance increased with time (Fig. 1). In contrast, late gene expression, exemplified by expression of EGFP regulated by the ATI promoter, was mostly absent at 2 h and 6 h p.i. But, by 12 h (and 24 h) p.i. an increase in the number of EGFP-expressing cells, and an increase in the abundance of EGFP was apparent. We therefore conclude that although MVA does not productively infect C3HA cells, both early and late viral genes are expressed. This mirrors the profile described in many other cell types, and supports the use of C3HA cells as a representative study system.

As controls, we also investigated the replicative capacity of the related, non-attenuated poxviruses, VAC and CPXV, in C3HA cells. Both VAC and CPXV have a broad host range, and can productively infect many cell types in vitro (Fields et al., 2007). As expected, we found that C3HA cells supported productive infection of both VAC and CPXV, which further validated this cell line as a model study system (data not shown).

MVA infection of C3HA cells induces PGE_2 production

We next evaluated whether PGE_2 is produced in response to MVA infection of C3HA cells. PGE_2 accumulation in the culture supernatant of infected cells was measured by ELISA and was found to be minimal until approximately 12 h p.i. (Fig. 2A). Whereafter, between 12 h and 24 h p.i. there was a dramatic increase in PGE_2 production. The long delay between MVA infection and the onset of PGE_2 accumulation was in marked contrast to the response seen in control cells treated with PGE_2-inducing ligands, such as PMA or LPS (Fig. 2A, and data not shown). To determine whether other poxviruses also could induce PGE_2 production, C3HA cells were infected with either VAC, or CPXV. Accumulation of PGE_2 in supernatants of VAC-infected C3HA cells was much lower than that for MVA-infected C3HA cells (Fig. 2B), and by 24 h p.i. the difference between MVA- and VAC-infected cells was highly significant ($P<0.001$ by ANOVA and Tukey-Kramer post-test). In contrast, CPXV-infected C3HA cells produced large amounts of PGE_2, comparable to those produced by MVA-infected cells. Interestingly, PGE_2 accumulated more rapidly during CPXV infection than during MVA infection, with the preponderance occurring between 2 h and 12 h p.i. (Fig. 2C). The differences in the kinetics and extent of PGE_2 production in response to infection by MVA and other poxviruses suggest that

there are interesting, and previously undescribed, virus-specific components to the poxvirus-induced PG response.

Unlike the response to well studied, fast-acting stimuli, such as PMA or LPS, the accumulation of PGE_2 following poxvirus infection was relatively slow, with levels remaining at background for up to 2 h p.i. (CPXV), or longer (MVA, VAC). This suggested that production of PGE_2 in response to poxvirus infection might not be mediated through interaction of the virus particles with a cellular receptor, but instead might require entry of the virus into the cell and expression of viral gene products. To address this question, inoculations were performed with inactivated virus preparations. Mild heat-treatment (55°C, 1 h) of poxviruses prevents gene expression by inactivating the viral DNA-dependent RNA polymerase present within the virus particles, but does not disrupt their structure. Control, reactivation experiments verified that heat-treated virus preparations were fully inactivated without being compromised for viral attachment or entry (Supplemental Fig. S1). Infection of C3HA cells with heat-inactivated (HI) MVA, HI VAC, or HI CPXV resulted in levels of PGE_2 accumulation comparable to those of mock-infected control cells (Fig. 2D), indicating that viral gene expression is required for the production of PGE_2 in response to infection by MVA and other poxviruses.

Delayed PGE_2 production follows the kinetics of AA release in MVA-infected C3HA cells

In a previous report, BS-C-1 cell cultures that were provided with exogenous eicosanoid precursors accumulated similar amounts of PGE_2 regardless of whether they were infected by MVA, VAC, or CPXV (Palumbo et al., 1994). This suggested to us that differences in the release of the endogenous PGE_2 precursor, AA, from cellular membranes might contribute to the virus-specific differences in PGE_2 production that we observed in our experiments. To evaluate this possibility, the liberation of AA from virus-infected cells was measured by liquid scintillation counting of [^{3}H]AA accumulation in the culture supernatant of radiolabeled C3HA cells at various times after infection. Release of [^{3}H]AA from MVA-infected cells was markedly delayed relative to the onset of infection, with the preponderance occurring between 12 h and 24 h p.i. (Fig. 3A). Notably, the kinetics of [^{3}H]AA release in response to MVA infection closely paralleled those of PGE_2 accumulation. Infection with either of the other poxviruses caused more rapid release of [^{3}H]AA, with the preponderance occurring between 0 h and 12 h p.i. (Figs. 3B and 3C). The robust release of [^{3}H]AA from VAC-infected cells was in striking contrast to their low level of PGE_2 accumulation. Taken together, these results indicate that a step in PGE_2 biosynthesis downstream from AA release must be blocked in VAC-infected C3HA cells. In contrast, the kinetics of AA release during CPXV infection were

similar to those of PGE_2 accumulation, as was seen for MVA. This suggests that for MVA, as well as for CPXV, the release of AA is the rate-limiting step in PGE_2 biosynthesis by infected C3HA cells.

Experiments conducted with HI virus preparations showed that, as for the production of PGE_2, the release of [^{3}H]AA from infected cells was almost entirely dependent on viral gene expression (Fig. 3A-C). The dependence on viral gene expression for the initiation of [^{3}H]AA release likely accounts for the relatively slow response to poxvirus infection when compared to the responses induced by treatment of C3HA cells with proinflammatory ligands, such as PMA or LPS (Fig. 3A, and data not shown). These ligands (Barbour et al., 1998; Gijon et al., 2000), as well as the few viruses that have been investigated (Culver and Laster, 2007; Liu et al., 2005), cause AA release via activation of $cPLA_2$. This prompted us to evaluate the role of $cPLA_2$ in poxvirus-induced [^{3}H]AA release. A cell-permeable, $cPLA_2$-specific inhibitor is available (Seno et al., 2000), and in initial control experiments we determined that this inhibitor remained fully effective for at least 24 h after its addition to cell cultures (Supplemental Fig. S2). Surprisingly, when added to MVA-infected C3HA cells, the $cPLA_2$ inhibitor had no effect on the accumulation of [^{3}H]AA in culture supernatants (Fig. 3D). A similar result also was found for VAC-infected C3HA cells (Fig. 3E). In contrast, [^{3}H]AA accumulation in supernatants of CPXV-infected cells was significantly reduced by the $cPLA_2$ inhibitor (Fig. 3F), although it was not blocked completely. Overall, the

results of these inhibitor studies imply that poxvirus-induced AA release, either wholly (MVA, VAC), or in part (CPXV), involves a phospholipase, or phospholipases, other than $cPLA_2$.

MVA-induced PGE_2 production requires COX-2 activity and COX-2 accumulates in infected C3HA cells

Of the two predominant COX isoforms, COX-2 is typically associated with inducible PGE_2 synthesis (Smith et al., 1996). We used a cell-permeable, COX-2-selective inhibitor, NS-398, to evaluate the contribution of COX-2 activity to the production of PGE_2 during poxvirus infection. We found that NS-398 effectively suppressed the accumulation of PGE_2 in poxvirus-infected cell cultures, as well as in PMA-stimulated control cultures (Fig. 4A). These results show that essentially all of the PGE_2 production by poxvirus-infected C3HA cells is dependent on the activity of COX-2.

Next, we utilized immunofluorescence microscopy to determine if COX-2 protein accumulation was induced specifically in MVA-infected C3HA cells. By 24 h after infection with MVA (5 PFU/cell), C3HA cells had noticeably elevated COX-2 protein accumulation compared to mock-infected controls (Fig. 4B). That the cells were infected with MVA was confirmed by detection of the viral E3L protein. To determine whether COX-2 accumulation was induced in a cell-

autonomous manner by the virus infection, or whether uninfected bystander cells in the culture also accumulated COX-2, cells were infected with MVA at 0.01 PFU/cell. Accumulation of COX-2 above background levels occurred in virus-infected cells, but not in uninfected cells in the culture (Fig. 4B). This result shows that accumulation of COX-2 is induced specifically during MVA infection of C3HA cells. Further, in combination with our results showing that COX-2 activity was required for poxvirus-induced PGE_2 synthesis, it suggests that the PGE_2 detected in culture supernatants is produced predominantly by virus-infected cells.

To evaluate the time-course of COX-2 accumulation during MVA infection, and to determine whether COX-2 accumulation was a general characteristic of poxvirus infection, we applied quantitative immunoblotting to C3HA cell lysates. This revealed that the level of COX-2 protein present in mock-infected cells declined steadily during a 24 h incubation in medium containing low serum (Fig. 4C). In contrast, in MVA-infected cells the level of COX-2 began to rise by 6 h p.i., and by 24 h p.i. it was elevated ~80-fold relative to the level in mock-infected cells. Thus, quantitative immunoblotting confirmed the results obtained by immunofluorescence microscopy. As seen for MVA, C3HA cells infected by CPXV contained elevated levels of COX-2, consistent with the efficient PGE_2 production we observed. In CPXV-infected cells the abundance of COX-2 rose between 2h and 24 h p.i., by which time levels were ~60-fold higher than those in

mock-infected control cells (Fig. 4C). In contrast, COX-2 protein levels in VAC-infected C3HA cells did not rise steadily over 24 h (Fig. 4C). At 6 h p.i., the level of COX-2 in VAC-infected cells was similar to those in MVA- or CPXV-infected cells, so all three viruses were able to induce COX-2 accumulation. At 12 h p.i., however, the level of COX-2 in VAC-infected cells was less than that found in cells infected with the other poxviruses. By 24 h p.i., VAC-infected cells contained significantly less COX-2 than cells infected with MVA or CPXV. Although VAC-infected cells appeared to contain sufficient COX-2 at early times after infection, they were unable to produce PGE_2, suggesting that VAC can block PGE_2 production independently of effects on COX-2. Additionally, the inability of VAC-infected cells to maintain their COX-2 levels likely contributes to the lack of PGE_2 accumulation at late times after infection. It appears, therefore, that VAC may interfere with PGE_2 production through at least two mechanisms. One mechanism, that already is active early in infection, is independent of COX-2 accumulation, while a second mechanism, that acts late after the onset of infection, affects the level of COX-2 protein in infected cells.

mPGES-1 does not accumulate in C3HA cells infected with MVA or other poxviruses

Interestingly, the foregoing results showed that both COX-2 enzyme and its substrate, AA, were present in VAC-infected C3HA cells, yet only low levels of PGE_2 were produced. Consequently, we asked whether the inducible PGE_2 synthase, mPGES-1, accumulated to different levels upon poxvirus-infection of C3HA cells. Quantitative immunoblotting indicated that basal mPGES-1 was readily detectable in mock-infected C3HA cells under our experimental conditions, and the enzyme did not accumulate much more during infection with either MVA, VAC, or CPXV (Fig. 5). These results indicated that mPGES-1 accumulation is not required for MVA-induced PGE_2 production, and that a deficit of mPGES-1 is not responsible for the lack of PGE_2 production by VAC-infected C3HA cells.

Infection with VAC actively prevents PGE_2 production by C3HA cells in response to both viral and non-viral stimuli

Since the basis of the low PGE_2 production by VAC-infected C3HA cells remained elusive, we turned instead to a genetic approach. We reasoned that the phenotype of VAC could result either from active inhibition of PGE_2

production, or simply from failure to stimulate the host cell to make PGE_2. To determine whether the phenotype of VAC was dominant or recessive to the high level PGE_2 producing phenotypes of MVA or CPXV, we measured PGE_2 production during mixed virus infections of C3HA cells. Co-infections of MVA plus VAC, or CPXV plus VAC, in each case resulted in low levels of PGE_2 accumulation that were comparable to those of VAC infection alone (Fig. 6A). In the mixed infection between MVA and VAC, a VAC-like PGE_2 production phenotype is consistent with *trans*-complementation of genetic defects present in MVA by co-infecting VAC. In contrast to MVA, however, CPXV is not defective, so the VAC-like phenotype seen in mixed infections between CPXV and VAC indicates that VAC actively suppresses PGE_2 production in CPXV-infected C3HA cells. VAC did not affect PGE_2 production indirectly through interference with the growth of CPXV, because control experiments confirmed that the growth of CPXV was similar whether inoculated alone, or as a mixture with VAC (Supplemental Fig. S3). We also examined COX-2 protein levels during mixed virus infections of C3HA cells since we had previously observed an effect of VAC infection on COX-2 accumulation. We found that the accumulation of COX-2 protein 24 h after co-infection of C3HA cells with MVA plus VAC, or CPXV plus VAC, was comparable to that in cells infected with VAC alone (Fig. 6B). Taken together, these results show that the phenotype of VAC is dominant in mixed

poxviral infections and they suggest that VAC infection inhibits PGE_2 production that would normally occur in response to infection with either MVA or CPXV.

Finally, we investigated whether VAC infection could suppress the production of PGE_2 in response to an exogenous, non-viral stimulus, such as phorbol ester. We found that simultaneous PMA stimulation and infection of C3HA cells with VAC resulted in the inhibition of PMA-induced PGE_2 production (Fig. 6C). This suggests that VAC infection can cause a generalized inhibition of PGE_2 production by C3HA cells, regardless of the initiating stimulus.

MVA infection induces PGE_2 production by murine DCs

Because MVA is a candidate vaccine, and vaccine vector, we were interested in investigating its effect on PG production by cells that are directly involved in generation of vaccine-induced immune responses. DCs mediate critical roles in initiating and influencing adaptive immune responses, and it has recently been shown that MVA preferentially targets DCs over other subsets of hematolymphoid cells, both in vitro and in vivo (Liu et al., 2008). We therefore investigated whether infection of murine DCs with MVA could induce a PGE_2 response similar to that seen in C3HA cells. Murine DCs were generated in vitro from bone marrow cells, and were either mock infected, or infected with MVA, or other poxviruses, each at 5 PFU/cell. Consistent with previous reports (Bronte et

al., 1997; Liu et al., 2008), we found that MVA and VAC were taken up by murine DCs and expressed viral genes, but were non-permissive for viral replication. Similar results also were obtained for CPXV (data not shown). The PGE_2 produced by murine DCs was measured in culture supernatants 24 h after infection. We found that MVA-infected DCs produced abundant PGE_2 (Fig. 7), which showed that MVA is a strong inducer of PGE_2 production in DCs, as well as in C3HA cells. Strikingly, although infection of either DCs or C3HA cells with the vaccine vector MVA caused high level production of PGE_2, VAC and CPXV had opposite effects in these two cell types (compare Fig. 2 and Fig. 7). PGE_2 was produced efficiently by VAC-infected DCs, but not by VAC-infected C3HA cells. Conversely, PGE_2 was produced by CPXV-infected C3HA cells, but not by CPXV-infected DCs. These contrasting results show that the production of PGE_2 in response to poxvirus infection is not only dependent on virus-specific factors, but is influenced by host cell-specific factors as well.

DISCUSSION

Our study expands upon previous reports (Palumbo et al., 1994; Palumbo et al., 1993), and demonstrates for the first time that poxvirus infection can result in PGE_2 production in the absence of exogenous PG precursors. This study is the first to describe release of the endogenous PGE_2 precursor, AA, from cellular membranes in response to poxvirus infection. We also show for the first time that COX-2 accumulation is induced during poxvirus infection, and that COX-2 is required for PGE_2 production by poxvirus-infected cells. Our elucidation of a role for COX-2 provides a mechanistic explanation for the enhanced conversion of exogenous precursors into PGE_2 that occurs in poxvirus-infected cells (48). Importantly, we found that the extent of PGE_2 production varied depending on the combinations of virus and cell type assayed, which implicates interplay of virus- and host-specific factors in determining the overall phenotype.

In particular, our study revealed that MVA infection caused robust PGE_2 production in a murine fibroblast cell line (C3HA) and in primary murine immune system cells (DCs). In common with other systems (Smith, 1989; Smith et al., 1996), we found that the kinetics of virus-stimulated [^{3}H]AA release paralleled those of PGE_2 accumulation, and inducible COX-2 was required for PGE_2 production in MVA-infected C3HA cells. However, [^{3}H]AA release and PGE_2 accumulation were delayed by approximately 12 h relative to the onset of

infection, and [^{3}H]AA release was found to be cPLA$_2$-independent. The delayed release of [^{3}H]AA observed upon MVA infection is in remarkable contrast to the more rapid, cPLA$_2$-dependent, release described following infection with adenovirus (Culver and Laster, 2007), or stimulation with PMA, or LPS. Although it remains to be determined whether MVA employs other characterized, or uncharacterized cellular phospholipases, the results of this study suggest that MVA activates a non-canonical mechanism for AA release and initiation of PGE$_2$ synthesis that may represent an important pathway for eicosanoid production during chronic viral infection.

We investigated poxvirus-induced activation of the PG synthesis pathway to determine why the PGE$_2$ response elicited by MVA infection differed from those elicited by closely related poxviruses. The C3HA cell line was used as a model study system for these experiments because we have used it previously to analyze PG production in response to adenovirus infection (Culver and Laster, 2007). Similarly to MVA, CPXV infection of C3HA cells caused potent, COX-2-dependent production of PGE$_2$. However, when compared to cells infected with MVA, CPXV induced a more rapid release of [^{3}H]AA, leading to more rapid production of PGE$_2$. Accumulation of COX-2 protein occurred either prior to [^{3}H]AA release (MVA), or concurrent with [^{3}H]AA release (CPXV), which suggests that for both viruses the liberation of AA is the rate-limiting step in PG synthesis. The liberation of [^{3}H]AA in response to CPXV infection was shown to be largely

dependent on the activity of $cPLA_2$, although additional cellular or viral phospholipases might also be involved. Thus, in C3HA cells infected with CPXV, PGE_2 production appears to follow the canonical model for induced PG responses. In contrast, MVA-infected cells are set apart by the $cPLA_2$-independence of [^{3}H]AA release, as well as by the unusual kinetics of both [^{3}H]AA release and PGE_2 production.

Unlike C3HA cells infected with MVA or CPXV, VAC-infected cells did not produce high levels of PGE_2. Although reduced accumulation of COX-2 may contribute to this phenotype late in infection, we showed that neither AA availability, nor accumulation of mPGES-1, were likely to be limiting for PGE_2 production. The VAC-specific, low level PGE_2 production phenotype was dominant in mixed infections with either MVA, or CPXV. The ability of VAC to block the response to a non-viral stimulus such as PMA, as well as to the other poxviruses, suggests that VAC-infected C3HA cells may have a general defect that prevents PGE_2 production. Some reports have described co-localization of PLA_2, COX, and terminal PG synthases at intracellular membranes during PG synthesis (Murakami et al., 2003; Murakami et al., 2000; Schievella et al., 1995). It is possible that the rapid and dramatic cytopathic effects and cytoskeletal rearrangements associated with VAC infection (Schepis et al., 2006) prevent proper localization of key enzymes, and/or reduce access to their respective

substrates. Alternatively, VAC might directly inhibit the activity of COX, or of PG synthases, within infected C3HA cells.

Although derived from a VAC ancestor, MVA apparently lacks the ability to suppress PG production in C3HA cells. The results of our mixed infection experiments provided evidence that genetic information present in VAC, but missing from MVA, is involved in the inhibition of PGE_2 production. This suggests that the ability to induce efficient PGE_2 production may have arisen fortuitously during the attenuation of MVA, and, consequently, this property may not be common to other VAC-based vaccine vectors.

MVA induced high level production of PGE_2 in either C3HA cells or DCs, but cell type specificity in the PGE_2 response to poxvirus infection was evident for both VAC and CPXV. The underlying causes of such cell type specificity are likely to be multifactorial, and our results provide evidence for at least two possible mechanisms. Although COX-2 accumulation is induced rapidly by MVA, with kinetics similar to those of VAC and CPXV, the release of [^{3}H]AA from MVA-infected C3HA cells is markedly delayed relative to [^{3}H]AA release from cells infected with the other poxviruses. This disparity indicates that [^{3}H]AA release and induction of COX-2 expression likely are activated via separate, or divergent, signal transduction pathways in response to poxvirus infection. Failure to activate either one of these signal transduction pathways might result in a cell type-specific lack of PGE_2 production. In addition to the potential for differential

activation, or upregulation, of various components of the biosynthetic pathway, active suppression of PGE_2 production by poxviruses appears to be cell type specific. Our results demonstrate this for VAC, which can suppress PGE_2 production in C3HA cells, but not in DCs. Thus, cell type-specific factors contribute either to the effectiveness, or to the expression, of the VAC suppressive mechanism. In addition, although PGE_2 is not produced by CPXV-infected DCs, this phenotype can be reversed by deletion mutations in the viral genome (unpublished results). These observations imply that specific CPXV gene products normally act to prevent PGE_2 production in infected DCs. Thus, mechanisms for active suppression of PG production in certain cell types may be present generally among the poxviruses.

PGE_2 has been shown to have direct effects, either negative or positive, on the replication in vitro of several viruses, which include respiratory syncytial virus, CMV, HSV-1, adenoviruses, and measles virus (Liu et al., 2005; Steer and Corbett, 2003). In contrast, replication of dengue virus is insensitive to PGE_2 in vitro, although PGE_2 is produced by the infected cells (Liou et al., 2008). We likewise found no effect on growth in vitro of VAC or CPXV, either by inhibition of endogenous PGE_2 production, or by addition of exogenous PGE_2 (Supplemental Fig. S4). This is consistent with a previous report that exogenously added E series PGs had no effect on in vitro replication of VAC (Santoro et al., 1982). Although PGE_2 apparently is without effect on virus growth in vitro, poxvirus-

mediated modulation of PGE_2 production nevertheless may be important in determining the outcome of an infection in vivo.

The robust production of PGE_2 in response to MVA infection is particularly interesting because of the potential for widespread use of MVA as a vaccine, and vaccine vector. Vaccine-induced production of PGE_2 by both fibroblasts and professional immune system cells, as would be expected for MVA-based vaccines, may have roles in influencing the overall immune response that is generated. This is consistent with the results of a study in which the humoral and cellular immune responses induced by MVA- and VAC-based vaccines were compared directly (Ramirez et al., 2000). Cytokine profiles measured in mouse spleen homogenates one and two days after inoculation were markedly different for MVA and VAC. Inoculation with MVA resulted in reduced levels of IL-12 and IFN-γ, and increased levels of IL-6, compared to inoculation with VAC. Additionally, at high inoculum doses, MVA preferentially induced production of IgG1, a Th2 Ab isotype, over IgG2a. These results correlate with known effects of PGE_2 signaling (Harris et al., 2002; Hinson et al., 1996), and suggest that manipulation of MVA-induced PGE_2 production might allow customization of the immune responses generated by MVA-based vaccines.

Recent studies have shown that PGs can mediate both beneficial and deleterious effects on vaccine-induced immune responses. In several reports, inhibition of COX-2 used in combination with various anti-cancer vaccines has

been shown to significantly augment vaccine efficacy (Basu et al., 2006; Haas et al., 2006; Mukherjee et al., 2009; Zeytin et al., 2004). It is thought that COX-2 inhibition, via the concomitant decrease in PGE_2 production, leads to reduced activation of indoleamine 2,3-dioxygenase and increased Th1 cytokine production, which facilitates a more effective CTL response. In contrast, following vaccination with human papillomavirus type 16 virus-like particles, COX-2 activity was shown to be essential for the generation of an optimal neutralizing Ab response, and for memory B-cell expansion (Ryan et al., 2006). These results provide evidence that the intentional manipulation of COX-2 and PGs may be a useful consideration in the design of new vaccines, or vaccination regimens.

Global COX-2 inhibition can have undesirable, non-specific consequences, such as impairment of IgG and IgM antibody production by B-lymphocytes (Ryan et al., 2005). Consequently, the most effective approach to modifying the PG phenotype of MVA-based vaccines likely will be to incorporate changes into the vaccine vector itself. This strategy has been tested for the manipulation of cytokine signaling (Abaitua et al., 2006), and is expected to restrict the effects to virus-infected cells and their immediate neighbors. The results described here point to some possible strategies that might be used to manipulate the PG phenotype of MVA-based vaccines. In this study, we have shown that COX-2 is essential for production of PGE_2 following MVA infection.

One approach for creation of a low PG producing, or PG-null, MVA-based vaccine vector might be to incorporate a COX-2-specific small-interfering RNA cassette into the MVA genome. It has previously been reported that RNA silencing systems remain functional in poxvirus-infected mammalian cells, and RNA silencing can be used to reduce expression of cellular genes during poxvirus infection (Lantermann et al., 2007). Conversely, it may also be possible to enhance PG production by MVA-based vaccines. In this study, we determined that the rate-limiting step of MVA-induced PG synthesis likely was the liberation of AA. Therefore, a plausible strategy for the augmentation of MVA-induced PG production might be to incorporate the coding sequence for a constitutively active PLA_2 into the vaccine vector. The availability of both high and low PG-inducing MVA-based vectors should allow optimization of PG-mediated effects to tailor the immune response to a given Ag, and increase the efficacy of recombinant vaccines.

ACKNOWLEDGEMENTS

The author thanks April Spesock for assistance with dendritic cell experiments, Dr. Jack Bennick and Dr. Per-Johan Jakobsson for providing antibodies, and Dr. David Pickup for providing cowpox deletion mutants, and for a critical review of the chapter. This work was funded in part by a grant from the National Institutes of Health (Grant #CA-59032) awarded to Dr. Scott Laster, and by the College of Agriculture and Life Sciences, North Carolina State University.

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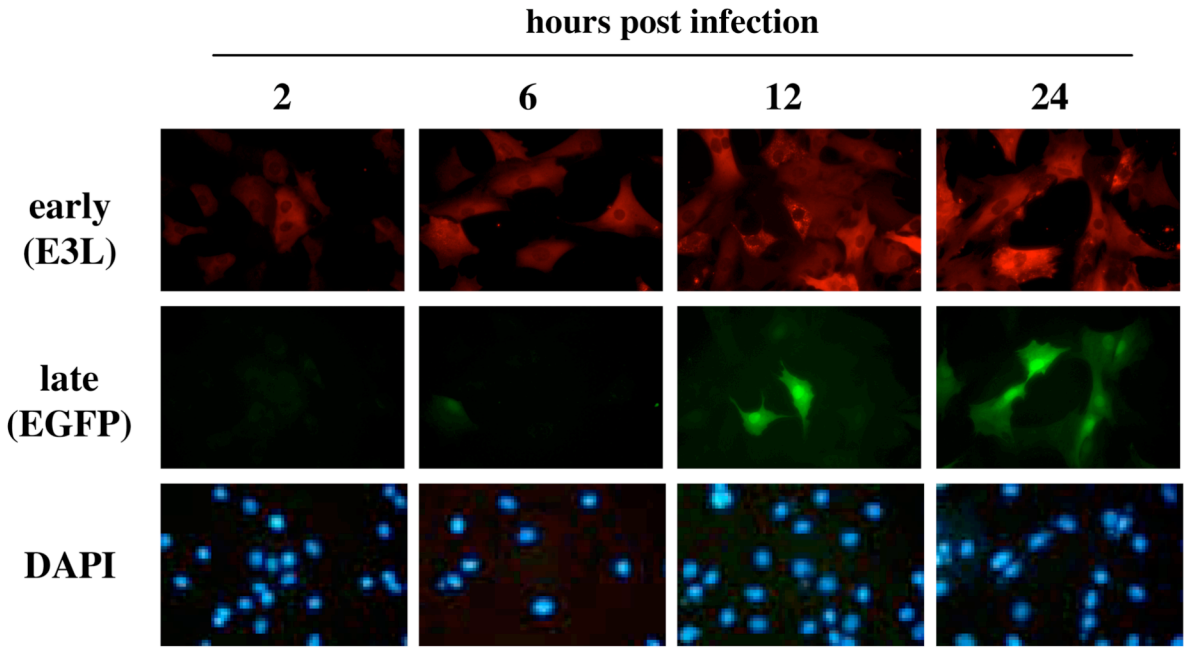


FIGURE 1. Time course of MVA infection in C3HA murine fibroblasts. Cells were infected with MVA/ATI-EGFP (5 PFU/cell). At 2, 6, 12, or 24 h after infection, cells were fixed, permeabilized, and immunostained with anti-E3L primary mAb and TRITC-labeled secondary Ab to detect poxviral early gene expression. Poxviral late gene expression was detected by EGFP autofluorescence, and DAPI staining was used to visualize uninfected as well as infected cells. Images were captured with constant exposure times to show intensity differences, and representative fields are shown.

FIGURE 2. PGE_2 production by murine fibroblasts in response to poxvirus infection. *A – C*, C3HA cells were mock infected, treated with PMA (10 ng/ml), or infected with MVA (*A*), VAC (*B*), or CPXV (*C*), each at 5 PFU/cell. Culture supernatants were collected 2, 6, 12, or 24 h after infection and concentrations of PGE_2 were measured by ELISA. Representative data from three independent experiments are shown. *D*, Cells were mock infected, or infected with MVA, VAC, CPXV, or HI virus preparations, each at 5 PFU/cell. Culture supernatants were collected 24 h after infection and concentrations of PGE_2 were determined by ELISA. Data are means and SEM from two independent experiments.

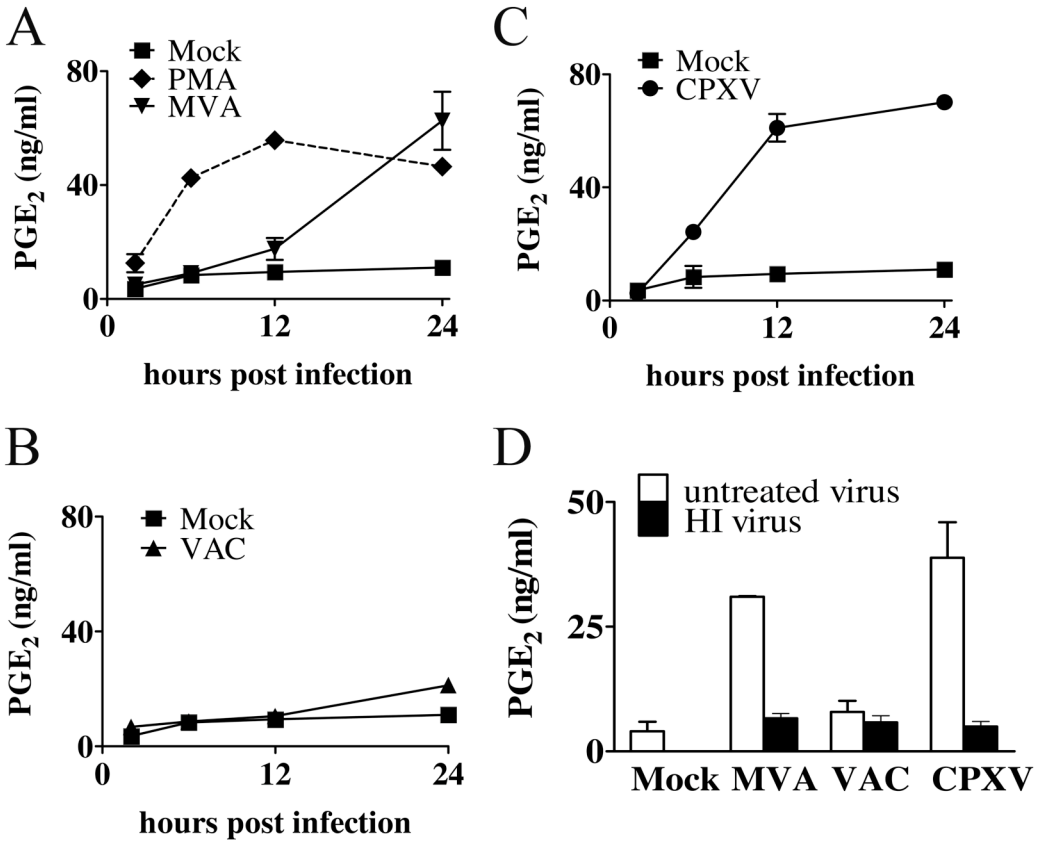

A
Mock
PMA
MVA
80
40
0
PGE2 (ng/ml)
0
12
24
hours post infection
C
Mock
CPXV
80
40
0
PGE2 (ng/ml)
0
12
24
hours post infection
B
Mock
VAC
80
40
0
PGE2 (ng/ml)
0
12
24
hours post infection
D
untreated virus
HI virus
50
25
0
PGE2 (ng/ml)
Mock
MVA
VAC
CPXV

FIGURE 3. Poxvirus infection stimulates AA release from murine fibroblasts. *A – C*, C3HA cells were radiolabeled with [^{3}H]AA overnight and then mock infected, treated with PMA (10 ng/ml), or infected with MVA (*A*), VAC (*B*), or CPXV (*C*), or HI virus preparations, each at 5 PFU/cell. Culture supernatants were collected 0, 2, 6, 12, and 24 h after infection and 200 μL aliquots were subjected to liquid scintillation counting to determine release of [^{3}H]AA (CPM). Data plotted are means and SEM, from six independent experiments for mock-infected and virus-infected cultures, and from two independent experiments for each HI virus. *D – F*, C3HA cells were radiolabeled with [^{3}H]AA and infected with MVA (*D*), VAC (*E*), or CPXV (*F*), in the presence of cPLA$_2$ inhibitor (2.5 µM), or solvent control (DMSO). Infected cells were incubated for 24 h in medium supplemented with cPLA$_2$ inhibitor, or DMSO, and 200 μL aliquots were subjected to liquid scintillation counting to determine release of [^{3}H]AA (CPM). Data are means and SEM from two (*D* and *E*), or three (*F*), independent experiments.

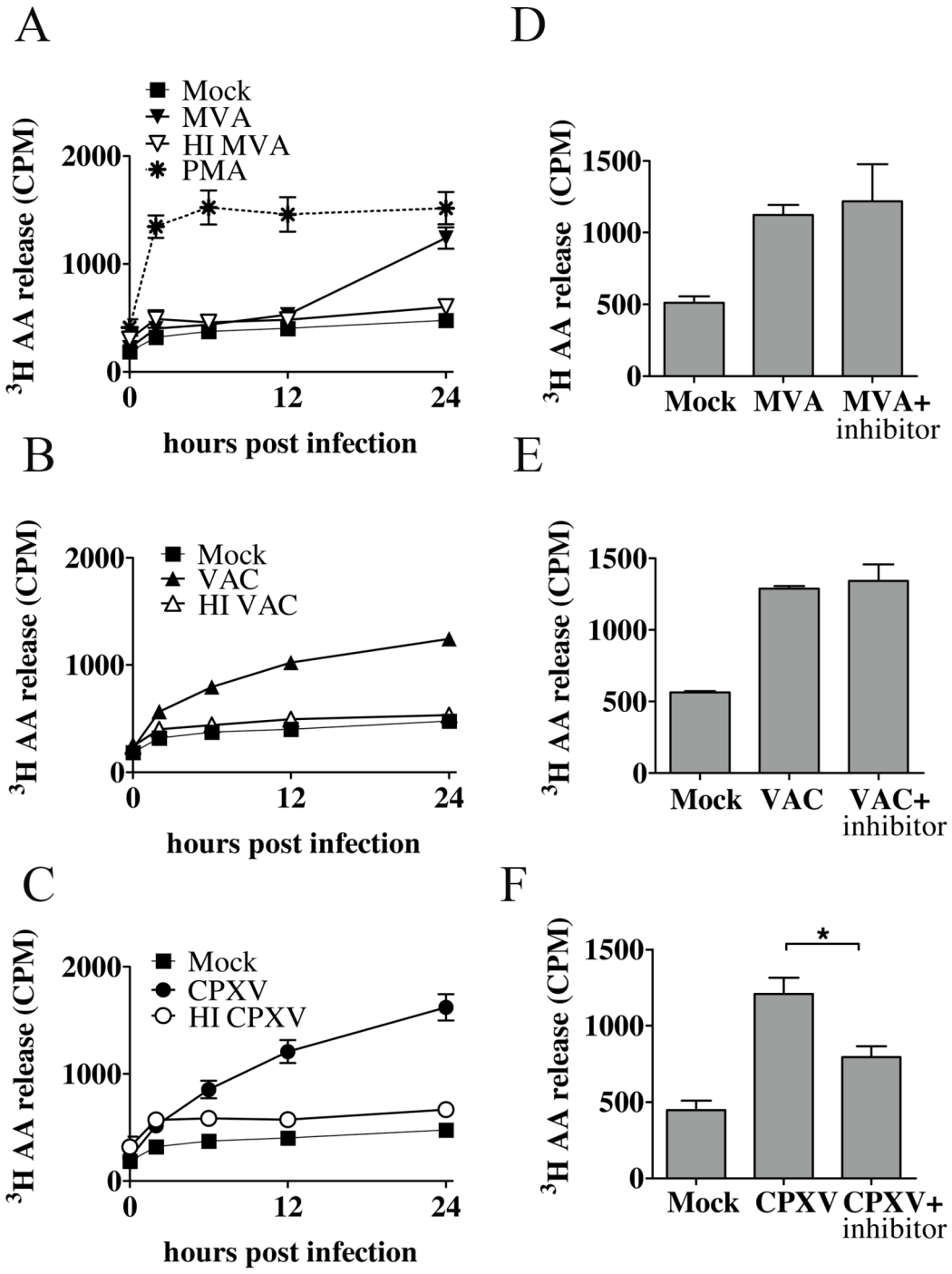
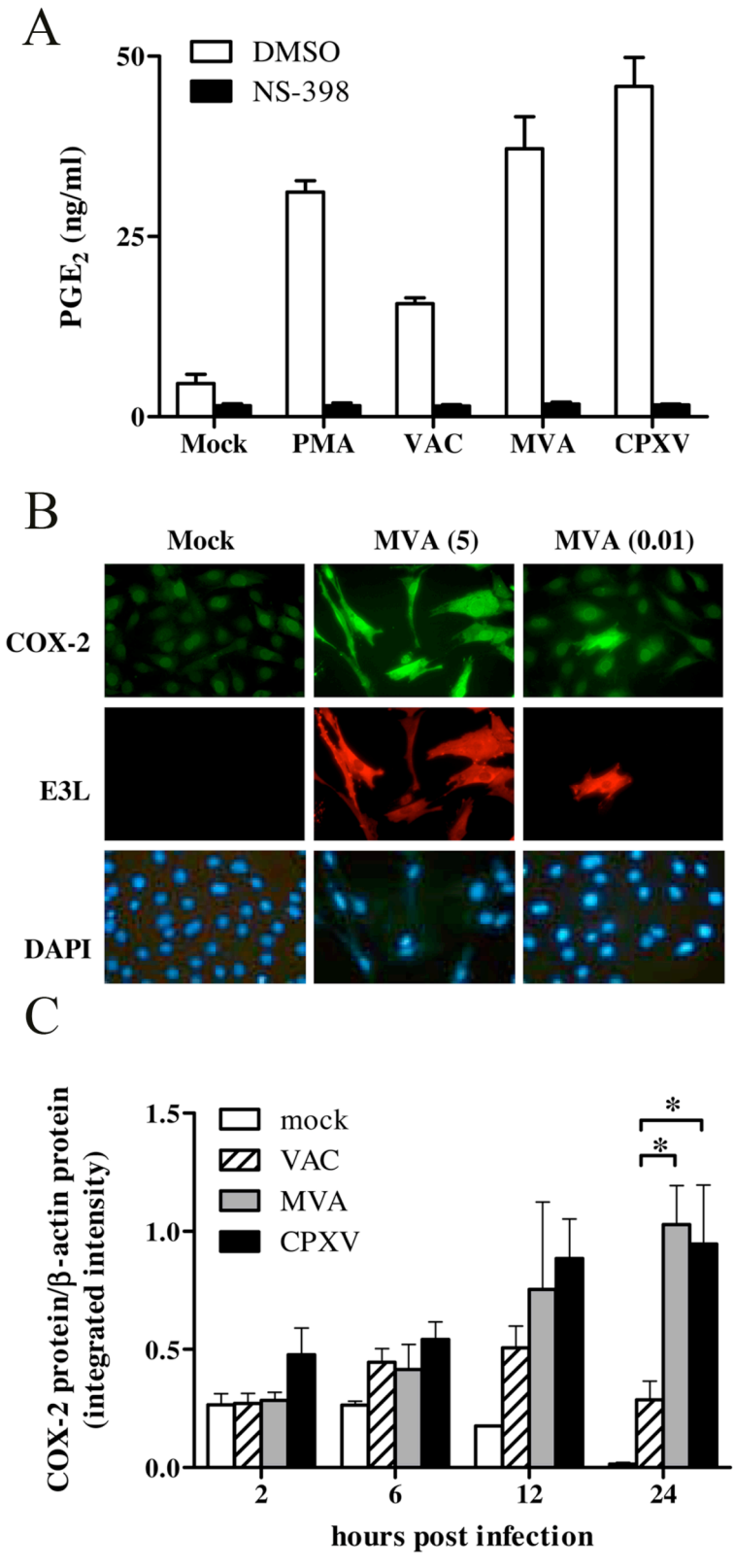

A
Mock
MVA
HI MVA
PMA
2000
1000
0
0
12
24
^{3}H AA release (CPM)
hours post infection
D
1500
1000
500
0
Mock
MVA
MVA+
inhibitor
^{3}H AA release (CPM)
B
Mock
VAC
HI VAC
2000
1000
0
0
12
24
^{3}H AA release (CPM)
hours post infection
E
1500
1000
500
0
Mock
VAC
VAC+
inhibitor
^{3}H AA release (CPM)
C
Mock
CPXV
HI CPXV
2000
1000
0
0
12
24
^{3}H AA release (CPM)
hours post infection
F
*
1500
1000
500
0
Mock
CPXV
CPXV+
inhibitor
^{3}H AA release (CPM)

FIGURE 4. Poxvirus-induced COX-2 is required for PGE_2 production by infected murine fibroblasts**.** *A*, C3HA cells were mock infected, treated with PMA (10 ng/ml), or infected with VAC, MVA or CPXV, each at 5 PFU/cell in the presence of solvent control (DMSO), or the COX-2-specific inhibitor, NS-398 (1 μM). Culture supernatants were collected 24 h after infection and PGE_2 concentrations were measured by ELISA. Data are means and SEM from three independent experiments. *B*, C3HA cells were infected with MVA at 5 PFU/cell (5), or 0.01 PFU/cell (0.01), and 24 h after infection, cells were fixed, permeabilized, and immunostained with anti-E3L primary mAb and TRITC-labeled secondary Ab to detect infected cells, and anti-COX-2 primary Ab and FITC-labeled secondary Ab to measure COX-2 accumulation. DAPI staining was used to visualize uninfected as well as infected cells. Images were captured with constant exposure times to show intensity differences, and representative fields are shown. *C*, C3HA cells were mock infected, or infected with VAC, CPXV, or MVA, each at 5 PFU/cell. Cell lysates were prepared 2, 6, 12, or 24 h after infection. Lysates (10 μg) were resolved by SDS-PAGE and immunoblotted to detect COX-2 and β-actin. The near infrared fluorescence intensity (integrated intensity) of each COX-2-specific band was measured with the LiCor Odyssey system, and was normalized to respective β-actin controls. Protein quantification data are means and SEM from three (2 h, 6 h, 12 h), or five (24 h), independent experiments. Differences indicated (*) are significant ($P<0.05$ by ANOVA and Tukey-Kramer post-test).


A
50
25
0
DMSO
NS-398
PGE2 (ng/ml)
Mock
PMA
VAC
MVA
CPXV
B
Mock
MVA (5)
MVA (0.01)
COX-2
E3L
DAPI
C
COX-2 protein/β-actin protein
(integrated intensity)
1.5
1.0
0.5
0.0
mock
VAC
MVA
CPXV
*
*
2
6
12
24
hours post infection

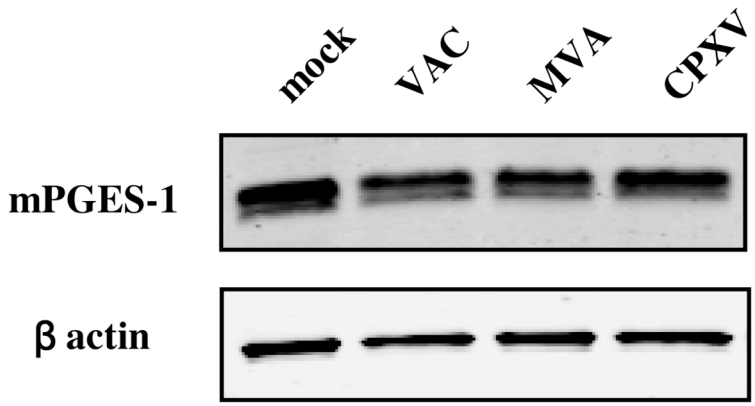
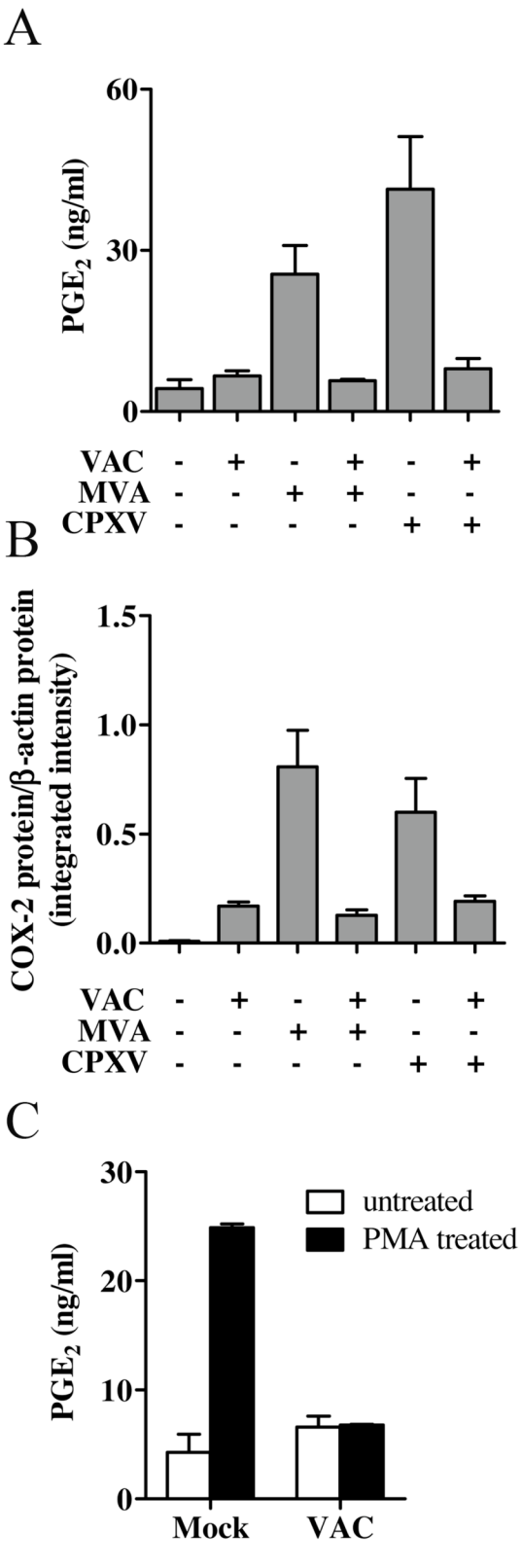


FIGURE 5. Detection of mPGES-1 in murine fibroblasts. C3HA cells were mock infected, or infected with VAC, CPXV, or MVA, each at 5 PFU/cell. Cell lysates were prepared 24 h after infection. Lysates (10 μg) were resolved by SDS-PAGE and immunoblotted to detect mPGES-1 and β-actin with the LiCor Odyssey system. A representative immunoblot image from two separate experiments is shown.

FIGURE 6. VAC infection prevents PGE_2 production in response to viral and non-viral stimuli. *A*, C3HA cells were mock infected (–), or infected with viruses (+), each at 5 PFU/cell, in the combinations indicated. Culture supernatants were collected 24 h after infection and PGE_2 concentrations were measured by ELISA. Data are means and SEM from two independent experiments. *B*, C3HA cells were infected as described above and cell lysates were prepared 24 h after infection. Lysates (10 μg) were resolved by SDS-PAGE and immunoblotted to detect COX-2 and β-actin. The near infrared fluorescence intensity (integrated intensity) of each COX-2-specific band was measured using the LiCor Odyssey system, and was normalized to respective β-actin controls. Data are means and SEM from three independent experiments. *C*, C3HA cells were mock infected, treated with PMA (10 ng/ml), infected with VAC (5 PFU/cell), or infected with VAC (5 PFU/cell) and treated with PMA (10 ng/ml). Culture supernatants were collected 24 h after infection and PGE_2 concentrations were measured by ELISA. Data are means and SEM from two independent experiments.


A
60
30
0
PGE2 (ng/ml)
VAC - + - + - +
MVA - - + + - -
CPXV - - - - + +
B
1.5
1.0
0.5
0.0
COX-2 protein/β-actin protein
(integrated intensity)
VAC - + - + - +
MVA - - + + - -
CPXV - - - - + +
C
30
20
10
0
PGE2 (ng/ml)
untreated
PMA treated
Mock
VAC

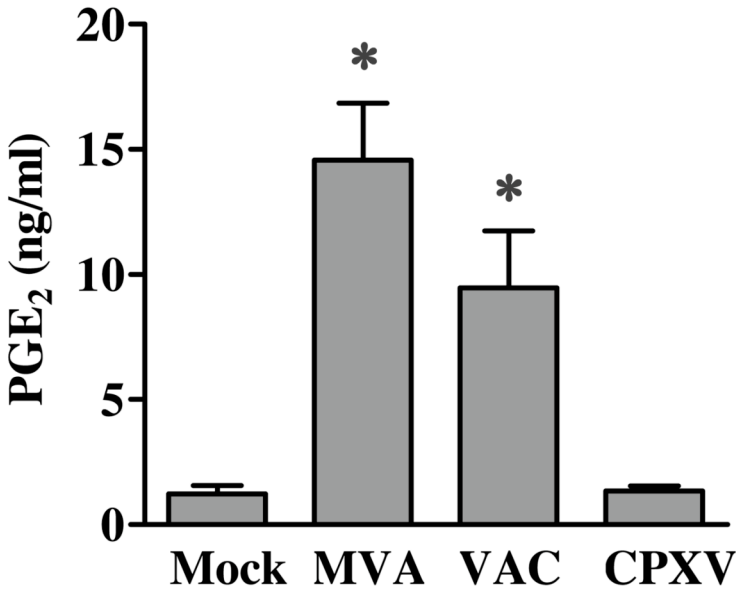
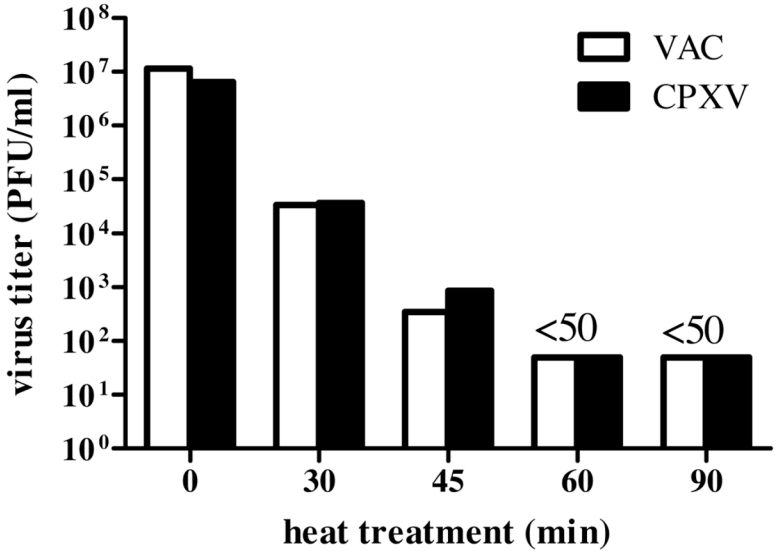


FIGURE 7. PGE_2 production by murine DCs in response to poxvirus infection. DCs were mock infected, or infected with MVA, VAC, or CPXV, each at 5 PFU/cell. Culture supernatants were collected 24 h after infection and PGE_2 concentrations were measured by ELISA. Data are means and SEM from separate experiments conducted with five independent preparations of DCs. Differences indicated (*) are significant compared to mock infected controls (P<0.05 by ANOVA and Tukey-Kramer post-test).

FIGURE S1. Heat-inactivation curve for VAC and CPXV and helper virus reactivation of heat-inactivated (HI) virus. *A*, Preparations of VAC and CPXV were incubated at 55°C for the times indicated. Virus titer after heat treatment was determined by standard plaque assay on C3HA cells (limit of detection 50 PFU/ml). *B*, The capacity of HI virus to regain replicative ability was assessed by co-infection of HI virus preparations with MVA helper virus. C3HA cells were inoculated with VAC or CPXV alone, HI VAC or HI CPXV alone, or each HI virus plus MVA. All infections were conducted at 5 PFU/cell, or equivalent volumes for HI virus preparations. Total virus from each tested condition was collected by freeze-thaw lysis 24 h after infection, and virus titers were determined by standard plaque assay on C3HA cells. Data are means and SEM from three independent experiments.

A


VAC
CPXV
virus titer (PFU/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
<50
<50
0
30
45
60
90
heat treatment (min)

B

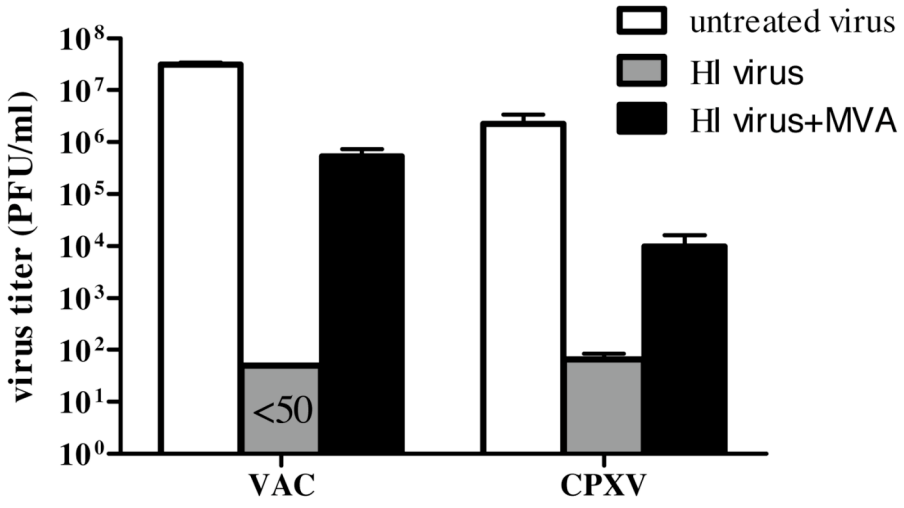
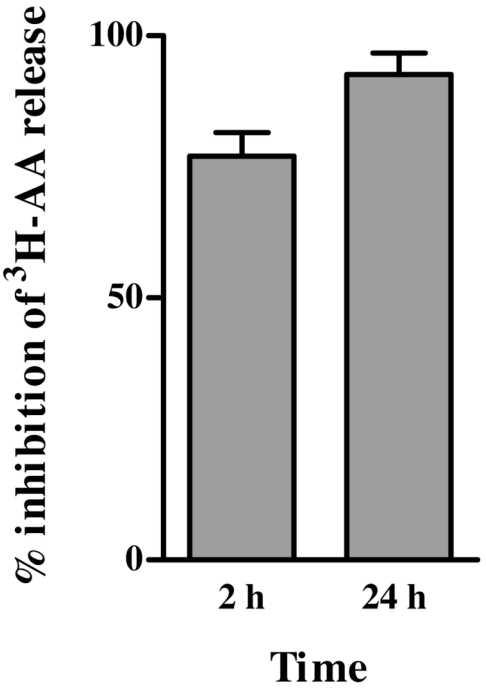

untreated virus
Hl virus
Hl virus+MVA
virus titer (PFU/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
<50
VAC
CPXV

FIGURE S2. Validation of $cPLA_2$ inhibitor effectiveness throughout 24 h incubation. Identical cultures of C3HA cells were labeled with [^{3}H]AA and then treated with either $cPLA_2$ inhibitor (2.5 μM), or solvent control (DMSO), and incubated at 37°C. The relative effectiveness of the $cPLA_2$ inhibitor between 0–2 h of incubation, or between 22-24 h of incubation, was determined by PMA (10 ng/ml) stimulation of matched inhibitor-treated and control cultures. For 0–2 h measurements, PMA was added 15 min after the $cPLA_2$ inhibitor, and following a further 1 h 45 min of incubation the release of [^{3}H]AA into culture supernatants was determined by liquid scintillation counting. For 22-24 h measurements, PMA was added 22 h after the $cPLA_2$ inhibitor, and following a further 2 h of incubation the release of [^{3}H]AA was determined as above. The ability of the $cPLA_2$ inhibitor to block PMA-stimulated [^{3}H]AA release is expressed as percent inhibition for each time point. Data are means and SEM from three independent experiments.


% inhibition of ^{3}H-AA release
100
50
0
2 h
24 h
Time

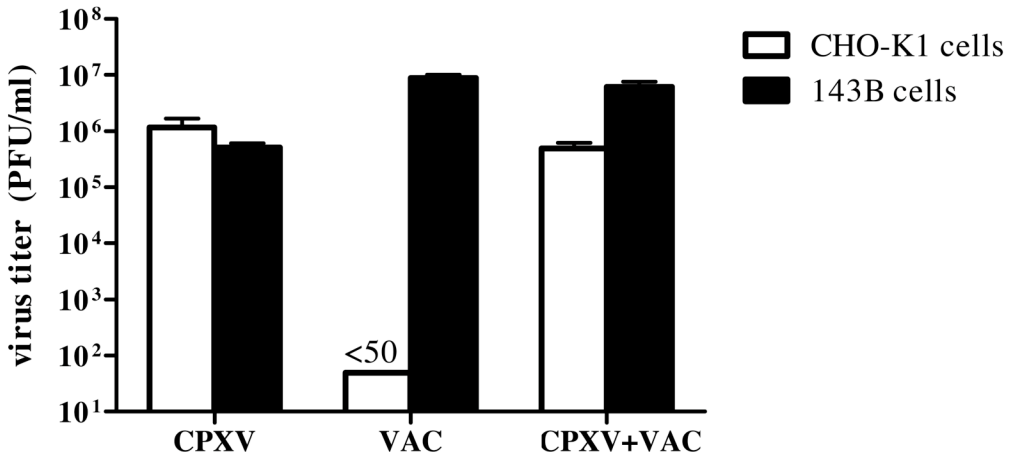
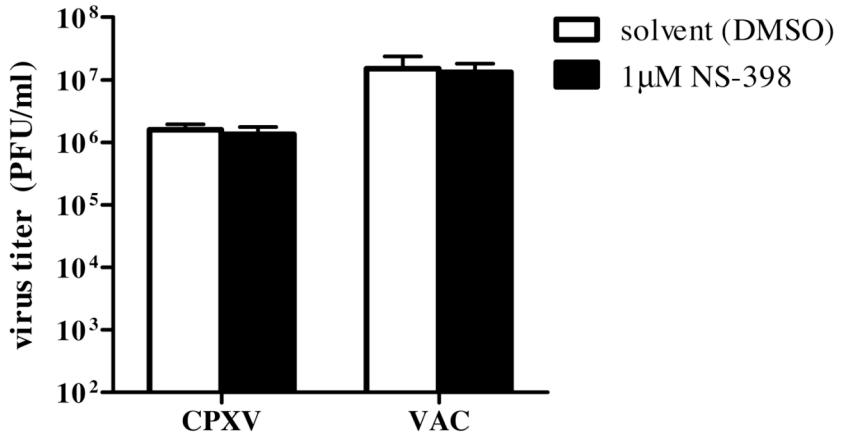
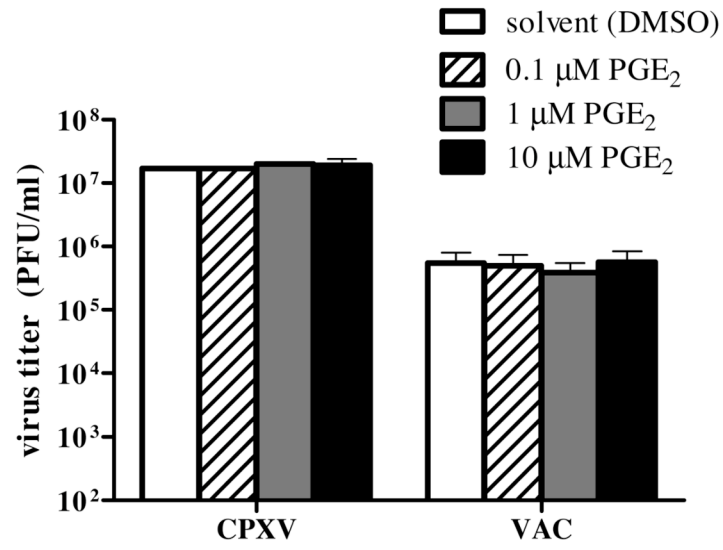


FIGURE S3. Both VAC and CPXV replicate efficiently during mixed infection of murine fibroblasts. Cultures of C3HA cells were infected with CPXV alone, VAC alone, or a combination of CPXV and VAC. All inoculations were conducted at 5 PFU/cell. At 24 h p.i., virus preparations were made from each culture by freeze-thaw lysis. For each preparation, virus titers were determined by standard plaque assay on both 143B cells (detects both VAC and CPXV) and CHO-K1 cells (detects only CPXV). Data are means and SEM from two independent experiments.

FIGURE S4. PGE_2 has no discernable effect on growth of VAC or CPXV in vitro. *A*, C3HA cells were treated with DMSO or 1 μM NS-398 (to inhibit endogenous production of PGE_2) and were infected with CPXV or VAC, each at 0.1 PFU/cell. Virus preparations were made from each culture by freeze-thaw lysis 24 h after infection and virus titers were determined by standard plaque assay on 143B cells. *B*, RAW 264.7 cells were treated with DMSO control or PGE_2 at the concentrations indicated, and infected with either CPXV or VAC, each at 0.1 PFU/cell. Total virus preparations were collected by freeze-thaw lysis 24 h post infection and virus titers were determined by standard plaque assay on 143B cells. RAW 264.7 cells were used for this experiment because they can respond to PG signaling, but do not produce endogenous PGE_2 in response to infection with VAC, or CPXV (data not shown). Data are mean and SEM from two independent experiments.


solvent (DMSO)
1μM NS-398
virus titer (PFU/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
CPXV
VAC


solvent (DMSO)
0.1 μM PGE_2
1 μM PGE_2
10 μM PGE_2
virus titer (PFU/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
CPXV
VAC

CHAPTER 3

Inhibition of poxvirus growth by Terameprocol, a methylated derivative of nordihydroguaiaretic acid

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Department of Microbiology, North Carolina State University, Raleigh, NC

ABSTRACT

Terameprocol (TMP) is a methylated derivative of nordihydroguaiaretic acid, a phenolic antioxidant originally derived from creosote bush extracts. TMP has previously been shown to have antiviral and anti-inflammatory activities, and has been proven safe in phase I clinical trials conducted to evaluate TMP as both a topical, and parenteral therapeutic. In the current study, we examined the ability of TMP to inhibit poxvirus growth in vitro. Our data show that TMP can potently inhibit the growth of cowpox virus and vaccinia virus in a variety of cell lines. Although TMP treatment did not substantially inhibit the synthesis of infectious progeny viruses in individual infected cells, it was highly effective at reducing infectious virus yield in multi-step virus growth assays. These results suggest that TMP inhibits poxvirus growth by preventing the efficient spread of virus particles from cell to cell. Consistent with this interpretation, TMP treatment dramatically reduced the number of actin tails present on infected cells. These structures require complete poxvirus morphogenesis for their formation, and they are involved in viral cell-to-cell spread. Data presented in this study, together with the clinical safety record of TMP, support further evaluation of TMP as a poxvirus therapeutic.

INTRODUCTION

Widespread public vaccination against smallpox was discontinued following the 1980 declaration by the World Health Organization that the disease had been eradicated. Indeed, vaccination in some countries, including the United States, was halted even earlier. As a result, it is likely that the majority of the current population has no protection against this disease, and that herd immunity has been lost. This has caused concern that smallpox may now constitute a dangerous biological weapon, and as such, the Centers for Disease Control and Prevention has identified the causative agent, variola virus, as among the highest potential bioterrorism threats. Another concern is the prospect of increased exposure to monkeypox virus due to human encroachment, globalization, or weaponized use (Parker et al., 2007). Although the currently licensed smallpox vaccine provides protection against both smallpox and monkeypox (Edghill-Smith et al., 2005), the development of a diverse arsenal of poxvirus antiviral agents is paramount for preparedness in the event of a potential outbreak. If widespread exposure of the general public were to occur, poxvirus antiviral agents would provide a crucial complement to vaccination (Neyts and De Clercq, 2003).

Poxvirus replication occurs in the cytoplasm of host cells and results in the formation of four distinct types of infectious virus particles. Intracellular mature virus (IMV) constitutes the primary infectious virion type produced in poxvirus-

infected cells (Roberts and Smith, 2008). IMV particles are already enveloped, but some subsequently become wrapped with additional cellular membranes, obtained from either the *trans*-Golgi network or early endosomes, to form so-called intracellular enveloped virus (IEV) (Schmelz et al., 1994; Tooze et al., 1993). IEV particles are transported via microtubules to the surface of the cell (Sanderson et al., 2000). At the surface, the outer IEV membrane fuses with the plasma membrane, thus exposing a virus particle at the cell surface. Particles that remain associated with the cell are termed cell-associated enveloped virus (CEV), while those that are released are termed extracellular enveloped virus (EEV) (Smith and Law, 2004; Smith et al., 2003; Smith et al., 2002). CEV and EEV particles are distinct only by association with the cell, and by pathogenic functions. CEV particles can induce actin polymerization at the surface of cells, forming actin tails that propel individual CEV particles outward toward adjacent cells, thus facilitating efficient cell-to-cell spread (Blasco and Moss, 1992; Cudmore et al., 1995). In contrast, EEV particles are involved in long distance virus spread and systemic dissemination (Payne, 1980). The complex morphogenesis of poxviruses provides additional targets for antiviral compounds beyond the replicative portion of the virus life cycle.

The Food and Drug Administration has approved only one investigational new drug for use against poxviruses. The drug, cidofovir (CDV), is a nucleoside analog that effectively inhibits the replication of many DNA viruses (De Clercq,

2003). Unfortunately, poxviruses with resistance to CDV have been isolated (Smee et al., 2002), demonstrating the need for novel antivirals that target other aspects of poxvirus growth. One recently described compound, ST-246, is readily bioavailable following oral administration, is effective against both variola virus and monkeypox virus in nonhuman primates, and has recently been evaluated in a phase I clinical trial (Huggins et al., 2009; Jordan et al., 2008; Yang et al., 2005). Unlike CDV, ST-246 does not inhibit poxvirus replication, but rather interacts with a specific viral protein needed for the formation of IEV. However, resistance to ST-246 has been described in poxviruses containing a single point mutation (Yang et al., 2005). Thus, there is a need to develop, or identify, additional antiviral agents. Many such investigations have been described, but given the difficulties associated with the clinical trials process, the continued identification of putative compounds is warranted.

Many of the medicinal effects associated with extracts prepared from the creosote bush, *Larrea tridenta*, have been attributed to the phenolic antioxidant nordihydroguaiaretic acid (NDGA), and related molecules (Arteaga et al., 2005). Previous studies have shown that methylated derivatives of NDGA can inhibit replication, and Tat-regulated transactivation, of human immunodeficiency virus (HIV) in vitro (Gnabre et al., 1995). Screening of eight different methylated derivatives of NDGA for increased ability to inhibit HIV transactivation identified tetra-O-methyl NDGA, also known as M4N, EM-1421, or terameprocol (TMP), as

having the highest activity (Hwu et al., 1998). Subsequently, TMP has also been shown to block the replication of herpes simplex viruses (HSV), and to prevent gene expression from the human papillomavirus type 16 P_{97} early promoter (Chen et al., 1998; Craigo et al., 2000). Based on this spectrum of antiviral activities, TMP has been evaluated in phase I/II clinical trials for use as a vaginal microbicide, and has been well tolerated (Khanna et al., 2008; Khanna et al., 2007). TMP is also under evaluation as a cancer therapeutic, and multiple phase I clinical trials have addressed safety and maximum tolerated dose following intravenous infusion in human volunteers (www.Clinicaltrials.gov).

In the current study, we examined the ability of TMP to inhibit poxvirus growth in vitro. Our data show that TMP potently inhibits the growth of both cowpox virus (CPXV) and vaccinia virus (VACV) in a variety of cell lines. Mechanistic studies revealed that TMP modestly reduced virus protein expression and DNA synthesis, but had little effect on the accumulation of infectious progeny virus in individual infected cells. However, TMP dramatically inhibited CPXV cell-to-cell spread, and reduced the formation of CPXV-induced actin tails. These results, when considered in combination with the established clinical safety of TMP, support the further evaluation of TMP as a poxvirus therapeutic.

MATERIALS AND METHODS

Reagents and antibodies

All fetal bovine serum (FBS), cell culture media, and cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Ascites fluid containing murine monoclonal antibody (Ab) TW2.3, specific to the VACV early gene product E3L (Yuwen et al., 1993), was a generous gift from Dr. Jack Bennink (National Institutes of Health, Bethesda, MD). Rabbit polyclonal Ab specific to the CPXV A-type inclusion (ATI) protein has been previously described (Patel et al., 1986) and was a gift from Dr. David Pickup (Duke University, Durham, NC). Murine monoclonal Ab specific to β-actin, and tetramethylrhodamine B isothiocyanate (TRITC) conjugated phalloidin were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor® 488 conjugated goat anti-mouse IgG Abs were obtained from Invitrogen Corporation (Carlsbad, CA). Secondary Abs for use with the Odyssey Infrared Imaging System, goat anti-mouse IRDye® 680 and goat anti-rabbit IRDye® 800CW, were purchased from Li-Cor Biosciences (Lincoln, NE). All chemicals, including dimethyl sulphoxide (DMSO) and cytosine arabinoside (AraC) were purchased from

Sigma-Aldrich (St. Louis, MO). TMP in DMSO solvent was obtained from Erimos Pharmaceuticals (Raleigh, NC).

Cell Lines

Cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). 143B cells (ATCC CRL-8303) were cultured in Minimum Essential Medium (MEM) supplemented with 5% FBS. BS-C-1, 293, HEP-G2, and A431 cells (ATCC CCL-26, CRL-1573, and HB-8065, respectively) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. CCD-1138SK cells (ATCC CRL-2707) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS. C3HA murine fibroblasts (Gooding, 1979) were cultured in DMEM supplemented with 1 mM sodium pyruvate and 5% FBS. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

Viruses

Viruses employed in this study were CPXV strain Brighton Red (ATCC VR-302) and VACV strain Western Reserve (ATCC VR-1354). Virus titers were determined by plaque assays in 143B cells.

CPXV plaque-reduction assay

143B cells or BS-C-1 cells were plated to near confluence in 6-well tissue culture plates. The cells were then mock infected with 200 μL of serum-free medium, or infected with approximately 200 plaque forming units (PFU) of CPXV in 200 μL of serum-free medium per well. The virus was allowed to adsorb for 45 minutes (min) with shaking every 10 min. The inoculum was removed and replaced with cell culture medium supplemented with 3.125 μM, 6.25 μM, or 12.5 μM TMP, or the solvent control (0.1% DMSO). Plaques were allowed to develop for 30 hours (h). The resultant plaques were visualized by negative staining with 0.1% crystal violet in 20% ethanol solution. Plaque size (mm^2) was measured using Bio-Rad GelDoc XR plaque analysis software (Bio-Rad Laboratories, Hercules, CA).

Virus growth assays and determination of virus yields

Low- and high-multiplicity of infection (MOI) growth assays were conducted to determine the effects of TMP treatment on poxvirus growth. For low-MOI growth assays, cells were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h. The treated cells were then infected with CPXV or VAC at

0.01 PFU/cell in serum-free medium containing 25 μM TMP or 0.1% DMSO. Virus was allowed to adsorb for 1 h with shaking every 15 min. Following adsorption, incubations were continued in cell-appropriate growth media containing 25 μM TMP or 0.1% DMSO. Total virus yield was collected 5 h, 12 h, 24 h, and 48 h after infection for CPXV growth assays, and 48 h after infection for VACV growth assays. Total virus yield was collected by freeze-thaw lysis of infected cells and culture media, followed by further disruption by sonication. Virus titers were measured with standard plaque assays on 143B cells.

For CPXV high-MOI growth assays, cells were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h. The treated cells were then infected with CPXV at 5 PFU/cell in serum-free medium containing 25 μM TMP, 10 μg/ml AraC, or 0.1% DMSO. Virus was allowed to adsorb for 1 h with shaking every 15 min. Following adsorption, incubations were continued in cell-appropriate growth media containing 25 μM TMP, 10 μg AraC or 0.1% DMSO. Total virus yield was determined 24 h after infection by freeze-thaw lysis of infected cells and culture media, followed by further disruption by sonication, and measurement of virus titer by standard plaque assays on 143B cells.

Quantitative Immunoblotting

BS-C-1 cells were infected with CPXV at 5 PFU/cell and cultured in growth

medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Cell monolayers were solubilized in lysis buffer (50 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 2 mM PMSF, 5% β-mercaptoehanol and 0.5% SDS) and collected by scraping at the indicated times post infection. Total protein concentration of the lysates was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories). Equal amounts of total protein (5 μg) were loaded onto 10% polyacrylamide Tris-glycine gels and separated by electrophoresis on a Novex MiniCell System (Invitrogen Corporation). Proteins were transferred to Immobilon-FL PVDF membranes (Millipore, Bedford, MA) and blocked overnight in Odyssey Blocking Buffer (Li-Cor Biosciences). The primary Abs (anti-ATI, and anti-β-actin) were diluted in 0.1% Tween-20 Odyssey Blocking Buffer and incubated with membrane for 1 h at room temperature. Membranes were washed extensively in PBS containing 0.1% Tween-20. Secondary Abs specifically designed for use with the Li-Cor Odyssey system were diluted in 0.1% Tween-20 Odyssey Blocking buffer and incubated for 45 min at room temperature, followed by extensive washing with PBS containing 0.1% Tween-20. Band visualization and quantification was completed on a Li-Cor Odyssey scanning system running Odyssey 2.1 software (Li-Cor Biosciences). The integrated intensity of each specific band of interest was measured and normalized to the respective β-actin control band to account for any minor variations in total protein loads.

Isolation and quantification of viral DNA

BS-C-1 cells were infected with CPXV at 5 PFU/cell and cultured in growth medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Cells were harvested by scraping at 6 h and 12 h post infection. Total cellular and viral DNA was isolated using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. CPXV DNA synthesis was measured by real-time PCR amplification of a portion of the CPXV CP77 gene using the primers 5´-GGA AAA TGA GGA GGT GGC TCT CGA TG-3´ and 5´-GAT AAT CGT CAT TTT GTG ATG GAT ATT CTT-3´ in a Bio-Rad iCycler running MyIQ System Software Version 1.1.410. A portion of the host cellular GAPDH gene was amplified with the primers 5´-GGA TTT GGT CGT ATT GGG CG-3´ and 5´-TGG AAG ATG GTG ATG GGA TTT C-3´ for use as a DNA load control. All primers were purchased from Integrated DNA Technologies (Coralville, IA). Reactions were carried out in 25 μL volume, using Quantace SensiMix *Plus* SYBR® & Fluorescein (Quantace, London, UK) with 5 ng of the isolated DNA per reaction. Copy number was established by comparison to a standard curve that was generated using DNA templates amplified with the use of cognate primer sets for the CP77 gene and GAPDH gene. Thermal cycling was performed as follows: 1.5 min at 95°C, followed by 50 cycles each of 95°C

for 0.5 min, 59°C for 0.5 min, and 72°C for 2 min, followed by melt-curve data collection and analysis. Final RT-PCR data is expressed as the ratio of CP77 copy number per 100 copies of GAPDH. The experiment was conducted twice, and all reactions were performed in triplicate for each experiment.

Immunofluorescence microscopy

For evaluation of actin tail formation, 8-well glass chamber slides (Nalge Nunc International, Naperville, IL) were seeded with 5 X 10^4 BS-C-1 cells and incubated at 37°C for approximately 8 h to allow cell attachment. Cells were then mock-infected or infected with CPXV at 5 PFU/cell in TMP- or DMSO-treated serum free medium and incubated for 1 h at 37°C to allow adsorption of the virus. The inoculum was then removed and incubations were continued in 2.5% FBS DMEM with 25 μM TMP or 0.1% DMSO. At 14 h post infection, the cells fixed in 4% formaldehyde PBS for 20 min at room temperature and washed twice in 3% BSA PBS. Cells were then permeabilized with 0.1% Triton X-100 PBS for 5 min, washed twice in 3% BSA PBS, and incubated with TRITC-phalloidin and anti-E3L Ab (TW2.3) in 0.3% BSA for 1 h. Cells were then washed again twice in 3% BSA PBS and incubated with Alexa Fluor® 488 conjugated goat anti-mouse Abs in 0.3% BSA PBS for 30 min, washed twice in 3% BSA PBS, and mounted in anti-fade slide mount containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector

Laboratories, Burlingame, CA). For evaluation of virus spread mechanisms at the leading edge of CPXV plaques, 8-well chamber slides were seeded to near confluence with BS-C-1 cells or C3HA cells in 2.5% FBS DMEM. Cells were then infected with CPXV at 0.001 PFU/cell in 2.5% FBS DMEM, and plaques were allowed to develop for 20 h. The slides were then prepared and immunostained as described above. All microscopy was conducted on a Zeiss Axioskop 2 Plus at 1000-fold magnification and images were captured using Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis

Data are presented as standard error of the mean (SEM). Group analysis of variance was compared by Kruskal-Wallis test followed by Dunn's multiple comparison test of log-transformed data. Proportion analysis was conduced using Fisher's exact test. All statistical analyses were performed using GraphPad Prism (version 5.01) software (GraphPad Software, La Jolla, CA).

RESULTS

TMP reduces the size of plaques formed by CPXV

We first evaluated the ability of TMP to reduce poxvirus growth by performing a CPXV plaque-reduction assay. The formation of a plaque requires the combination of both virus replication, and virus cell-to-cell spread. Thus, effects on plaque size can be used to screen for antiviral activity directed at either, or both, of these processes. Plaque-reduction assays were conducted on confluent monolayers of 143B cells in 6-well plates, infected with approximately 200 PFU of CPXV per well. Plaques were allowed to develop in culture media containing 2-fold increasing concentrations of TMP (3.125 μM, 6.25 μM, 12.5 μM) or the solvent control (0.1% DMSO). The resultant plaques in a representative experiment are shown in Figure 1A, and average plaque sizes for each condition are presented in Figure 1B. These data clearly show that TMP treatment caused a dramatic, dose-dependent decrease in CPXV plaque size, as well as reducing the number of plaques visible to the naked eye. In wells treated with 6.25 μM TMP, the reduction in the number of visible plaques was confirmed to be a result of substantial reductions in plaque size as many plaques could be detected only by microscopy (data not shown). At the highest tested concentration of TMP (12.5 μM) plaques were longer detected. Similar results were obtained from

plaque-reduction assays conducted with BS-C-1 cells (data not shown). These results provide evidence that TMP can inhibit the growth of CPXV in a dose-dependent manner.

TMP only has minimal effect on synthesis of CPXV proteins, DNA, and infectious virus particles.

We next sought to determine whether TMP treatment inhibited virus growth in a cell-autonomous manner. For this, we investigated the effect of TMP treatment on the yield of infectious progeny virus obtained from high-MOI (one-step) growth assays. BS-C-1 cells are a standard cell line for in vitro propagation of poxviruses, and therefore were used for this experiment, as well as for subsequent experiments to elucidate the mechanisms of TMP antiviral activity against CPXV. For these, and all subsequent experiments, TMP was used at a concentration of 25 μM, as pharmacokinetic and tissue distribution studies have shown that TMP can be detected at, or above, this concentration in the plasma, intestines, kidneys, lungs, hearts, and spleens of mice following repeated oral administration (Park et al., 2005). We evaluated the dose-dependent toxicity of TMP by trypan blue dye-exclusion of solvent control (0.1 % DMSO) and TMP treated BS-C-1 cells over a 48 h incubation. We observed no apparent reduction

of cell growth, or increase in cell death, when BS-C-1 cells were cultured in media supplemented with 25 μM TMP (data not shown).

For one-step growth assays, BS-C-1 cells were treated with DMSO, TMP, or the nucleoside analog AraC, and were infected with CPXV at 5 PFU/cell. Infected cells and culture medium were combined and collected 24 h post-infection, and virus titers were determined by standard plaque assays. As indicated in Figure 2A, TMP treatment did not cause a significant reduction of CPXV yield. In marked contrast, and as expected, treatment of BS-C-1 cells with the poxvirus DNA replication inhibitor, AraC, resulted in a significant ($P<0.05$), near three-log (99.9%), decrease in viral yield compared to solvent control-treated cells.

To confirm that TMP treatment had little effect on CPXV in individual infected cells, we also evaluated viral protein expression and DNA synthesis. Quantitative immunoblot analysis was used to measure the accumulation of the CPXV ATI protein in infected cells. Poxvirus gene expression is temporally regulated, and the expression of the CPXV ATI protein is restricted to the late stage of virus replication (Patel and Pickup, 1987). Therefore, the accumulation of the late stage-restricted ATI protein can serve as an indicator of unperturbed expression of all temporal classes of poxvirus genes. We applied quantitative immunoblotting to CPXV-infected BS-C-1 cell lysates, and compared the accumulation of the ATI protein in DMSO-treated (solvent control) cells to that of

TMP-treated cells, and AraC-treated cells. As expected, the data shown in Figure 2B indicate that AraC treatment caused a significant reduction in the accumulation of the ATI protein when compared to control-treated cells 12 h and 24 h post-infection. ATI protein levels were also reduced in TMP-treated cells, however the data clearly demonstrate that TMP does not inhibit late-stage viral protein accumulation to the extent seen for AraC, a bona fide inhibitor of poxvirus DNA replication.

We next investigated directly whether poxvirus DNA synthesis was reduced in TMP-treated BS-C-1 cells. Viral DNA synthesis was measured by real-time PCR quantification of the CPXV CP77 gene, normalized to host-cell GAPDH copy number. As shown in Figure 2C, TMP-treated cells contained fewer copies of the CPXV CP77 gene at 6 h and 12 h post-infection compared to cells treated with DMSO, indicating that TMP treatment reduced viral DNA synthesis. However, as described for viral protein accumulation, TMP has much less effect on viral DNA synthesis than the control, AraC.

Taken together, the results of these experiments show that treatment of CPXV-infected cells with TMP can modestly reduce both viral protein expression and DNA synthesis, but these effects are not sufficient to prevent virus replication, or the synthesis of new infectious virions at near normal levels. These results implicate TMP-dependent inhibition of virus spread from infected

cells to non-infected cells as the cause of the potent antiviral effects observed in plaque-reduction assays.

TMP inhibits cell-to-cell spread of CPXV in BS-C-1 cells

Low-MOI growth assays were used to test directly the ability of TMP to inhibit the cell-to-cell spread of CPXV. BS-C-1 cells were treated with DMSO or TMP, and were infected with CPXV at 0.01 PFU/cell. Infected cells and culture medium were combined and collected at 5, 12, 24, or 48 h after infection, and virus titers were determined by standard plaque assays. As shown in Figure 3, TMP treatment caused a dramatic reduction in CPXV growth when compared to solvent control-treated cells. At 48 h post-infection, TMP-treated cells had a reduction in virus yield of nearly three-logs (99.8%). These results show that TMP reduces virus growth by inhibiting the cell-to-cell spread of CPXV, and provide a mechanistic explanation for the ability of TMP to reduce plaque size, as shown in Figure 1.

TMP treatment prevents the CPXV-induced formation of actin tails in infected cells

Poxvirus infection can result in the formation of four different types of infectious virions: IMV, IEV, CEV, and EEV. Of these, the CEV form is thought to be the most critical for efficient cell-to-cell spread (Blasco and Moss, 1992). CEV induces the formation of actin tails that project the CEV particles outward from the surface of infected cells to potentially penetrate, and infect, neighboring cells (Cudmore et al., 1995). We evaluated the effect of TMP treatment on actin tail formation in CPXV infected BS-C-1 cells using fluorescence microscopy. To visualize cellular actin and actin tails, the cells were fixed, permeabilized, and stained with TRITC-labeled phalloidin. That the cells were infected with CPXV was confirmed by immunostaining for viral antigen (E3L). As shown in Figure 4A, normal actin central spanning fibers are apparent in both TMP- and DMSO-treated control cells, indicating that TMP treatment does not disturb the distribution and organization of actin filaments in uninfected cells. As expected, no actin tails were present in the mock-infected BS-C-1 cells. However, actin tails were readily discernable, and found in copious amounts, in CPXV-infected BS-C-1 cells that were treated with DMSO solvent control (Figure 4B, left panel). In striking contrast, cells infected with CPXV and treated with 25 μM TMP lacked actin tails (Figure 4B, right panel). Comparisons of ten random microscopy fields

per condition indicated that 75% (91 of 121) of CPXV-infected BS-C-1 cells in wells treated with DMSO were positive for the presence of actin tails, while actin tails were present in only 4.5% (4 of 88) of CPXV-infected BS-C-1 cells in wells treated with TMP. Thus, these data demonstrate that TMP treatment caused a significant reduction in the formation of actin tails by CPXV-infected BS-C-1 cells ($P<0.0001$ by Fisher's exact test). Comparatively, we observed no difference in the expression of the viral antigen, E3L, between CPXV-infected cells treated with DMSO or TMP. These results indicate that TMP may interfere with the cell-to-cell spread of infectious virions by blocking viral morphogenesis, or perhaps by direct inhibition of actin tail formation.

TMP inhibits poxvirus growth in a variety of cell lines

We next evaluated whether TMP could effectively inhibit CPXV growth in a panel of different cell lines chosen to represent biologically relevant tissues from mice and humans. Low-MOI growth experiments were conducted in 293 human kidney cells (Figure 5A), C3HA mouse kidney cells (Figure 5B), Hep-G2 human liver cells (Figure 5C), A431 human skin cells (Figure 5D), and CCD-1138SK human skin cells (Figure 5E). As described for BS-C-1 cells, none of the tested cell types indicated any loss of cell growth, or increase in cell death, when cultured in media supplemented with 25 μM TMP (data not shown). For each cell

type, the virus yield at 5, 12, 24 and 48 h post infection was determined, and the calculated percent inhibition of CPXV growth in TMP treated cells compared to DMSO treated cells after 48 h is indicated in the lower-right portion of each graph. As shown in Figures 5A–E, TMP inhibited CPXV growth in all cell lines tested, with efficacies of inhibition ranging from 30% to over 99.9% at 48 h post infection.

To determine whether TMP exhibits antiviral activity against other poxviruses, we conducted low-MOI growth assays with VACV in the same panel of cell lines. The VACV growth experiments were performed similarly to those described above for CPXV, except that total virus preparations were collected at 48 h post-infection only, and each experiment was conduced in duplicate. As shown in Figure 5F, TMP inhibited VACV growth in all cell types tested. Similar to the results obtained with CPXV, the efficacies of VACV growth inhibition by TMP ranged from 30%, to over 99.9%.

Predominant mechanisms of virus spread vary amongst cell types in vitro

Interestingly, the effectiveness of TMP varied in a cell type-dependent manner. TMP was much less effective at inhibiting poxvirus growth in C3HA cells, and CCD-1138SK cells, than in the other cell lines tested (Figure 5). Our results with BS-C-1 cells showed a specific effect of TMP on CPXV cell-to-cell

spread mediated via actin tail formation. Accordingly, we considered that the reduced effectiveness of TMP in C3HA cells and CCD-1138SK cells might be a reflection of poxvirus spread by an alternate mechanism in these cells. To test this hypothesis, we compared actin tail formation during CPXV plaque formation in C3HA cells and BS-C-1 cells. At the leading edge of an expanding plaque, virus spread from infected to uninfected cells is occurring, so we determined the number of actin tails formed on infected cells at this boundary. Monolayers of BS-C-1 and C3HA cells were grown in chamber slides, and were infected with 0.001 PFU CPXV/cell. Plaques were allowed to develop for 20 h, and the slides were then prepared, and immunostained, as previously described. As shown in Figure 6A, actin tails were readily discernable in CPXV-infected BS-C-1 cells at the leading edge of expanding plaques. In contrast, actin tails were not apparent in CPXV-infected C3HA cells at the leading edge of otherwise similar plaques (Figure 6B). These results indicate that at least one non-canonical mechanism of poxvirus cell-to-cell spread operates in certain cell lines in vitro. Importantly, they also establish that the effectiveness of TMP as an antiviral agent correlates with poxvirus cell-to-cell spread mediated via actin tails, and in animal models, poxvirus mutants that are defective in inducing actin tail formation have been shown to be highly attenuated (Parkinson and Smith, 1994).

DISCUSSION

In this report, we have shown that TMP, a methylated derivative of NDGA, has potent antiviral activity against both CPXV and VACV in a variety of cell lines, and was effective at a concentration found to be attainable in a pharmacokinetic study conducted in mice (Park et al., 2005). TMP was highly effective in reducing virus growth in multi-step (low-MOI) virus growth assays, and plaque-reduction assays. However, TMP did not prevent the synthesis of infectious progeny in one-step growth assays, and we showed that TMP had only modest inhibitory effects on viral protein expression and DNA synthesis. Interestingly, we found that the formation of virus-induced actin tails was markedly reduced when poxvirus-infected cells were treated with TMP. Taken together, the results of this study suggest that TMP inhibits poxvirus growth by preventing efficient spread of poxviruses from cell to cell.

Previous studies have shown that TMP has antiviral activity against HSV and HIV in vitro (Chen et al., 1998; Gnabre et al., 1995). For both these viruses, TMP was shown to inhibit replication by blocking the binding of the host cell transcription factor, Sp1, to viral promoters. Poxviruses genomes are largely devoid of putative Sp1 high-affinity binding sites, and the transcription of poxvirus genes depends on transcriptional activator proteins encoded by the viral genome, although some host-cell nuclear proteins have been identified in cytoplasmic

virus factories (Oh and Broyles, 2005). When considered in combination with our results indicating that TMP does not effectively prevent the replication of CPXV or VAC in high-MOI growth assays, it seems unlikely that TMP inhibits poxvirus growth by interfering with Sp1-dependent transcription of viral genes as described for other viruses. It remains possible, however, that TMP might interfere with Sp1-dependent transcription of a cellular factor that is required for virus cell-to-cell spread.

Our determination that TMP does not effectively inhibit the synthesis of infectious poxvirus progeny may seem at odds with the inhibition of virus growth we measured in low-MOI growth assays conducted in the presence of TMP. However, the multiple types of infectious virions produced by poxvirus-infected cells can explain these seemingly opposed observations. The formation of IEV, CEV and EEV first requires successful production of IMV (Smith and Law, 2004; Smith et al., 2003; Smith et al., 2002). Although infectious, IMV is not sufficient for efficient virus cell-to-cell spread or systemic dissemination, which comprise the two main functions of CEV and EEV, respectively (Blasco and Moss, 1992; Payne, 1980). Our results showing that TMP does not significantly reduce the total yield of infectious virus per cell suggest that IMV is produced in TMP-treated cells. Therefore, TMP must inhibit a subsequent step in viral morphogenesis, or virus release. Remarkably, although it is chemically dissimilar to TMP, the promising antiviral candidate ST-246 also targets the morphogenesis of

poxviruses (Yang et al., 2005), thus providing evidence that steps beyond viral replication can serve as practical, drugable, targets. Antiviral activity against CPXV for the chemically similar compound NDGA, has also been reported, however NDGA was found to directly inhibit viral replication, and potential effects on cell-to-cell spread were not investigated (Palumbo and Buller, 1991).

Our data demonstrate that TMP treatment prevents the formation of actin tails by poxvirus-infected cells. Production of CEV is a prerequisite for actin tail formation (Smith and Law, 2004; Smith et al., 2003; Smith et al., 2002). Specific viral proteins associated with the CEV particle concentrate at the edge of the plasma membrane and trigger a signal cascade that mimics a tyrosine kinase signaling pathway involved in normal cellular control of actin polymerization (Frischknecht et al., 1999; Reeves et al., 2005). The activation of this process by CEV-associated proteins results in the polymerization of actin filaments, which thereby propel the CEV away from the cell.

The lack of actin tail formation in poxvirus-infected cells treated with TMP suggests two possible mechanisms by which virus spread may be inhibited. First, TMP may inhibit the morphogenic pathway between IMV and CEV, thereby preventing actin tail formation indirectly. Intracellular membrane wrapping of IMV creates IEV particles, which are then transported to the plasma membrane where a membrane fusion event occurs, leaving exposed CEV on the surface of the cell (Smith and Law, 2004; Smith et al., 2003; Smith et al., 2002). The cellular

microtubule network facilitates transport of IMV and IEV particles during this morphogenic process (Sanderson et al., 2000). Although TMP has not been reported to affect the microtubule network, the related compound NDGA has been shown to inhibit microtubule-dependent processes along both the secretory and endocytic pathways, and has been reported to act as a microtubule-stabilizing agent (Nakamura et al., 2003; Tagaya et al., 1996). Recently, NDGA has also been implicated in over-stimulating dynein-dynactin microtubule motor activity (Arasaki et al., 2007). If TMP similarly perturbs microtubule functions, it could be envisaged that TMP might inhibit the formation of IEV, or the transport of IEV particles to the surface of the cell, either of which would serve to prevent the formation of CEV and subsequent actin-dependent spread of the virus. It also remains possible that TMP may interact directly with a viral protein that is essential for poxvirus morphogenesis, as is the case for ST-246.

A second potential mechanism by which TMP may act to inhibit actin tail formation is direct interference with poxvirus-induced actin polymerization. In some cell types, NDGA has been shown to disrupt the actin cytoskeleton, evident by a loss of central-spanning stress fibers (Seufferlein et al., 2002). Although in our experiments we found no such effect in cells treated with TMP, it remains possible that TMP has more subtle effects on cellular actin that can prevent poxvirus-induced actin polymerization and actin tail formation.

In this study, we found that TMP can inhibit the cell-to-cell spread of poxviruses by the canonical mechanism involving the formation of actin tails that propel virus particles away from the surface of infected cells. We also determined that poxvirus cell-to-cell spread can occur independently of actin tail formation in certain cell types in vitro, and that TMP was correspondingly less effective as a poxvirus antiviral agent in these specific cell types. The extent of poxvirus growth inhibition mediated by TMP in these cell lines (30.3% in C3HA cells and 60.4% in CCD-1138SK cells) was consistent with the cell-autonomous effect of TMP on virus yield that we observed in BS-C-1 cells (60.9%). Nevertheless, the effect of TMP on poxvirus cell-to-cell spread mediated via actin tails has the most impact on virus yield in vitro. Thus, the success of TMP as a therapeutic likely will depend on the importance of this mechanism of cell-to-cell spread for poxvirus pathogenesis in vivo. It is worth noting that the antiviral agent ST-246, which inhibits poxvirus morphogenesis, and so would also be expected to prevent cell-to-cell spread mediated via actin tails, is effective in animal models (Huggins et al., 2009; Yang et al., 2005). Also, poxvirus mutants that are defective specifically in actin tail formation are highly attenuated in vivo (Parkinson and Smith, 1994), providing direct evidence for the relative importance of this mechanism of cell-to-cell spread for poxvirus pathogenesis.

The results of this study have identified TMP as a novel antiviral agent that effectively prevents poxvirus growth in a variety of cell lines. When considered in

combination with the multiple phase I clinical trials conducted to address tolerated dose and safety, these data suggest that further development of TMP as a poxvirus therapeutic is warranted.

ACKNOWLEDGEMENTS

The authors would like to thank Rebecca Hansen Curry and Dawn Eads for their expert technical assistance, David Pickup and Jack Bennick for generously providing antibodies, and Mike Sikes for a critical review of the manuscript. This work was funded in part by the College of Agriculture and Life Sciences, North Carolina State University, and by a grant from Erimos Pharmaceuticals, LLC.

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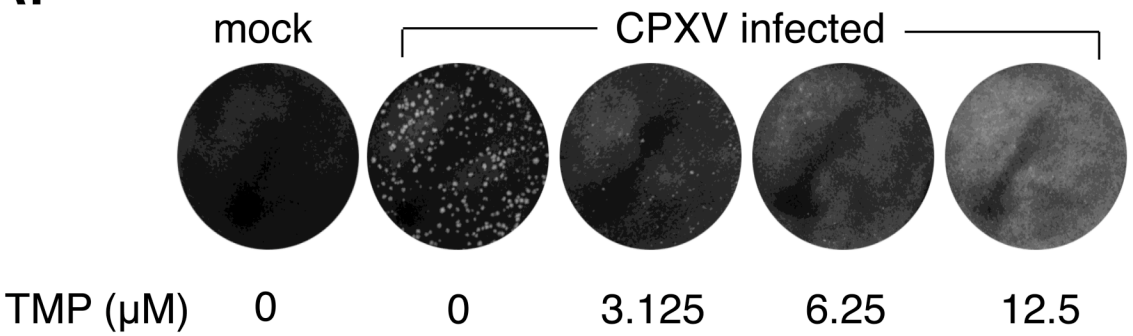
Figure 1. TMP reduces the size of plaques formed by CPXV. Near-confluent monolayers of 143B cells grown in 6-well tissue culture plates were mock infected (200 μL of serum free medium), or infected with approximately 200 PFU/well CPXV. Plaques were allowed to develop in cell growth medium supplemented with the indicated concentrations of TMP, or with the solvent control, 0.1% DMSO (0 μM TMP). (A) Cell monolayers were stained with 0.1% crystal violet in a 20% ethanol solution at 30 h post infection. Well images are shown. (B) Plaque size (mm^2) was determined using Bio-Rad Gel-Doc XR plaque analysis software. Data are means and SEM for each test condition.

A.


mock
CPXV infected
TMP (μM)
0
0
3.125
6.25
12.5

B.

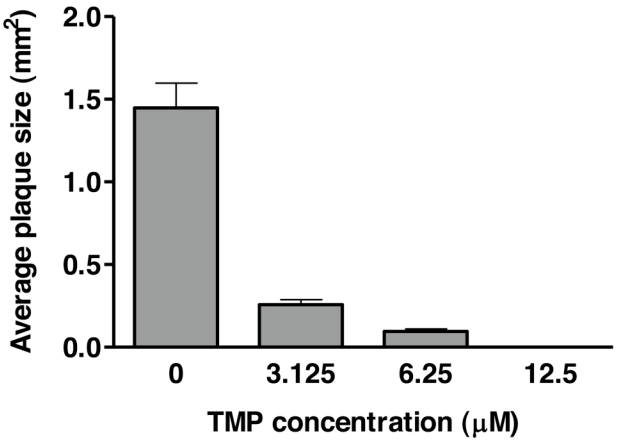
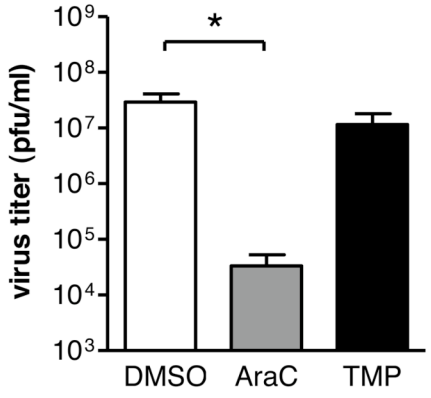

Average plaque size (mm2)
2.0
1.5
1.0
0.5
0.0
0
3.125
6.25
12.5
TMP concentration (μM)

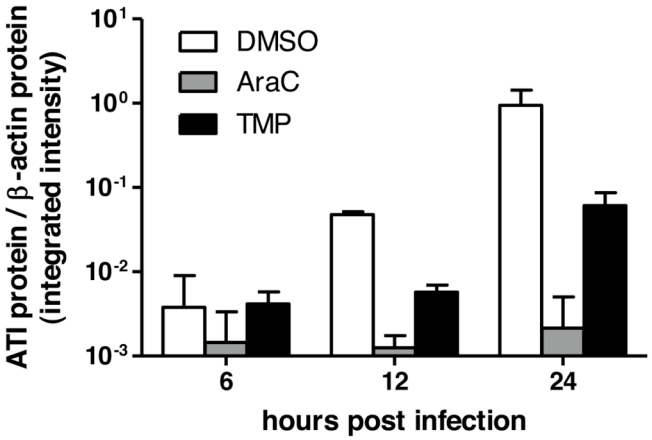
Figure 2. TMP only has minimal effect on synthesis of CPXV proteins, DNA, and infectious virus particles. (A) BS-C-1 cells were infected with CPXV at 5 PFU/cell and cultured in cell growth medium containing 25 μM TMP, 10 μg/ml AraC, or solvent control (0.1% DMSO). Total virus yield (PFU/ml) was determined at 24 h after infection by freeze-thaw lysis of cells and culture media, and standard plaque assay on 143B cells. Data are means and SEM from five, (DMSO and TMP treated) or three (AraC), independent experiments. Differences indicated (*) are significant ($P<0.05$ by Kruskal-Wallis test with Dunn's multiple comparison test of log-transformed data). (B) BS-C-1 cells were infected with CPXV at 5 PFU/cell, and cultured in growth medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Cell lysates were prepared 6, 12, or 24 h after infection. Lysates (5 μg) were resolved by SDS-PAGE and immunoblotted to detect the CPXV ATI protein and host-cell β-actin. The near infrared fluorescence intensity (integrated intensity) of each ATI-specific band was measured with the LiCor Odyssey system, and was normalized to respective β-actin controls. Protein quantification data are means and SEM from two independent experiments. (C) BS-C-1 cells were infected and treated as described above. Total cellular and viral DNA was collected at 6 and 12 h post infection. Real-time PCR amplification of a portion of the CPXV CP77 gene and the host-cell GAPDH gene was conducted. Copy number was established by comparisons of PCR amplification data to appropriate standard curves. Data are expressed as the ratio of CPXV CP77 copies per 100 copies of GAPDH. Data are

means and SEM from two independent experiments. ATI protein levels (B), and CP77 copy number (C), in AraC-treated cells were significantly reduced (P<0.1 by Kruskal-Wallis test with Dunn's Multiple comparison test of log-transformed data) when compared to DMSO treated controls.

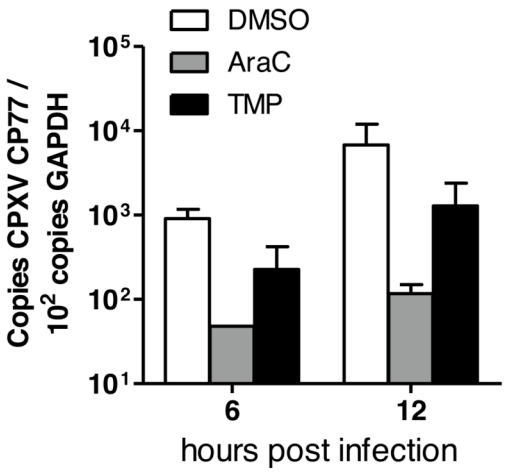
A.


virus titer (pfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
*
DMSO
AraC
TMP

B.


ATI protein / β-actin protein (integrated intensity)
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
DMSO
AraC
TMP
6
12
24
hours post infection

C.


Copies CPXV CP77 / 10^2 copies GAPDH
10^5
10^4
10^3
10^2
10^1
DMSO
AraC
TMP
6
12
hours post infection

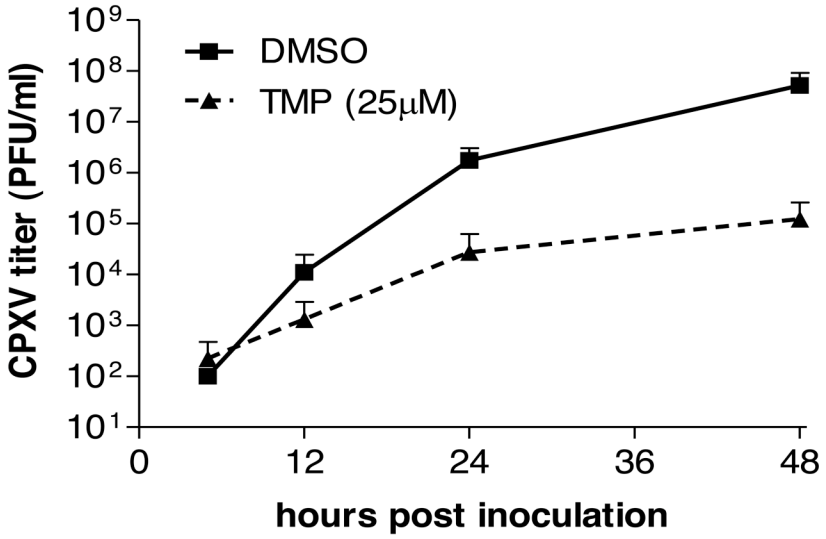


Figure 3. TMP inhibits cell-to-cell spread of CPXV. (A) BS-C-1 cells were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h. The cells were then infected with CPXV at 0.01 PFU/cell, and cultured in cell growth medium containing 25 μM TMP or 0.1% DMSO. Total virus yield was collected at 5, 12, 24, and 48 h after infection by freeze-thaw lysis of cells and culture media, and virus titer (PFU/ml) was determined by standard plaque assay on 143B cells. Data are means and SEM from two independent experiments.

Figure 4. TMP treatment prevents the formation of actin tails by CPXV-infected cells. BS-C-1 cells were mock infected (A), or infected with CPXV at 5 PFU/cell (B), and were then cultured in 2.5% FBS DMEM containing 25 μM TMP or solvent control (0.1% DMSO) for 14 h. Cells were then fixed, permeabilized, and immunostained with anti-E3L mAb and AlexaFluor® 488 secondary Ab to detect viral antigen, TRITC conjugated phalloidin to detect F-actin, and DAPI staining to visualize nucleic acids. (A) Representative fields of phalloidin-stained mock infected cells treated with DMSO or TMP as indicated. DAPI staining is shown in the figure insets (B) Representative fields of phalloidin-stained CPXV infected cells treated with DMSO or TMP as indicated. White arrows identify examples of actin tails. E3L immunostaining is shown in the figure insets.

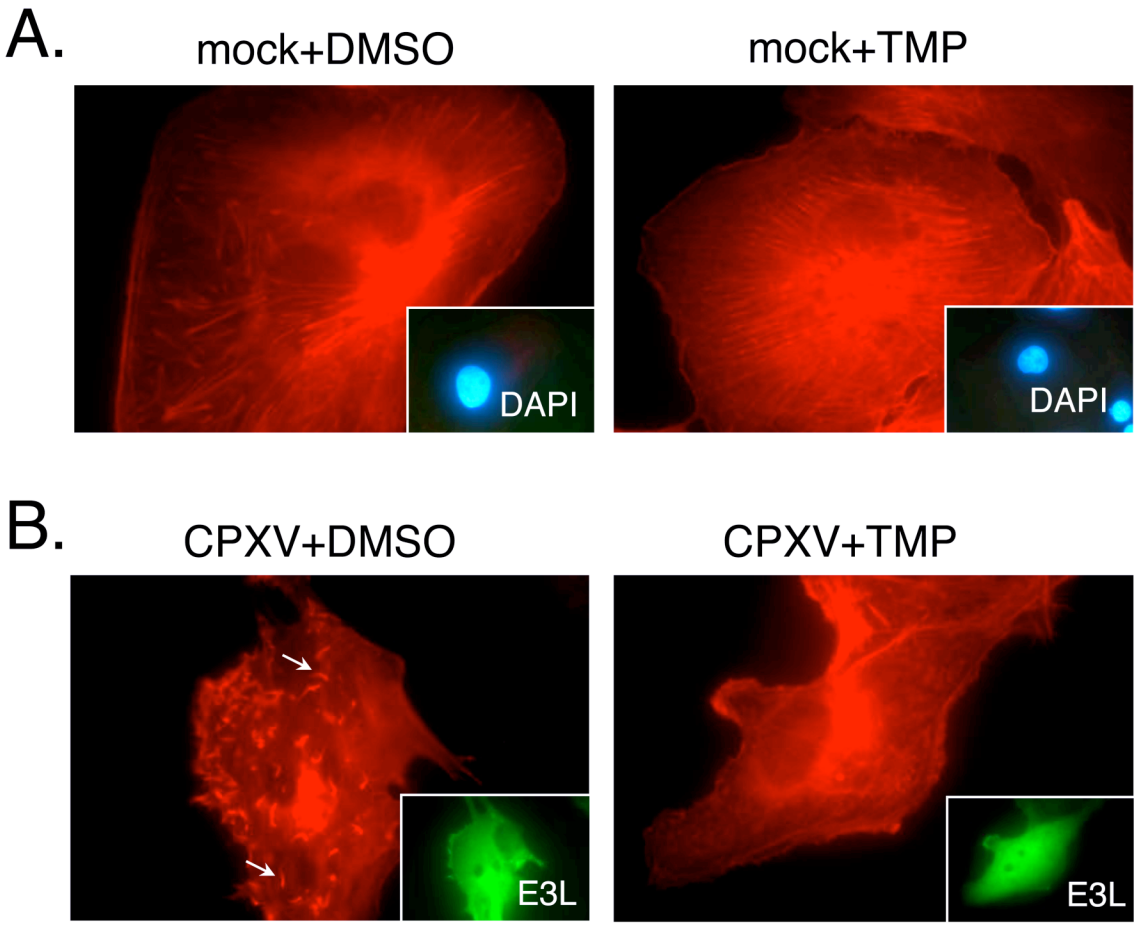

A.
mock+DMSO
mock+TMP
DAPI
DAPI
B.
CPXV+DMSO
CPXV+TMP
E3L
E3L

Figure 5. TMP inhibits poxvirus growth in a variety of cell lines. (A–E) Cells of each type indicated were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h. The cells were then infected with CPXV at 0.01 PFU/cell, and cultured in cell growth medium containing 25 μM TMP or 0.1% DMSO. Total virus yield (PFU/ml) was determined at 5, 12, 24, and 48 h after infection by freeze-thaw lysis of cells and culture media, followed by disruption by sonication, and standard plaque assay on 143B cells. The percent inhibition of CPXV growth in TMP-treated cells compared to DMSO treated cells at 48 h post infection is indicated in the lower-right portion of the graph. (F) BS-C-1 cells, 293 cells, C3HA cells, HEP-G2 cells, A431 cells, and CCD-1138SK cells were pre-treated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h, and then infected with VACV at 0.01 PFU/cell and cultured in cell growth medium containing 25 μM TMP or 0.1% DMSO. Total virus yield was determined as described above. Data are means and SEM from two independent experiments.

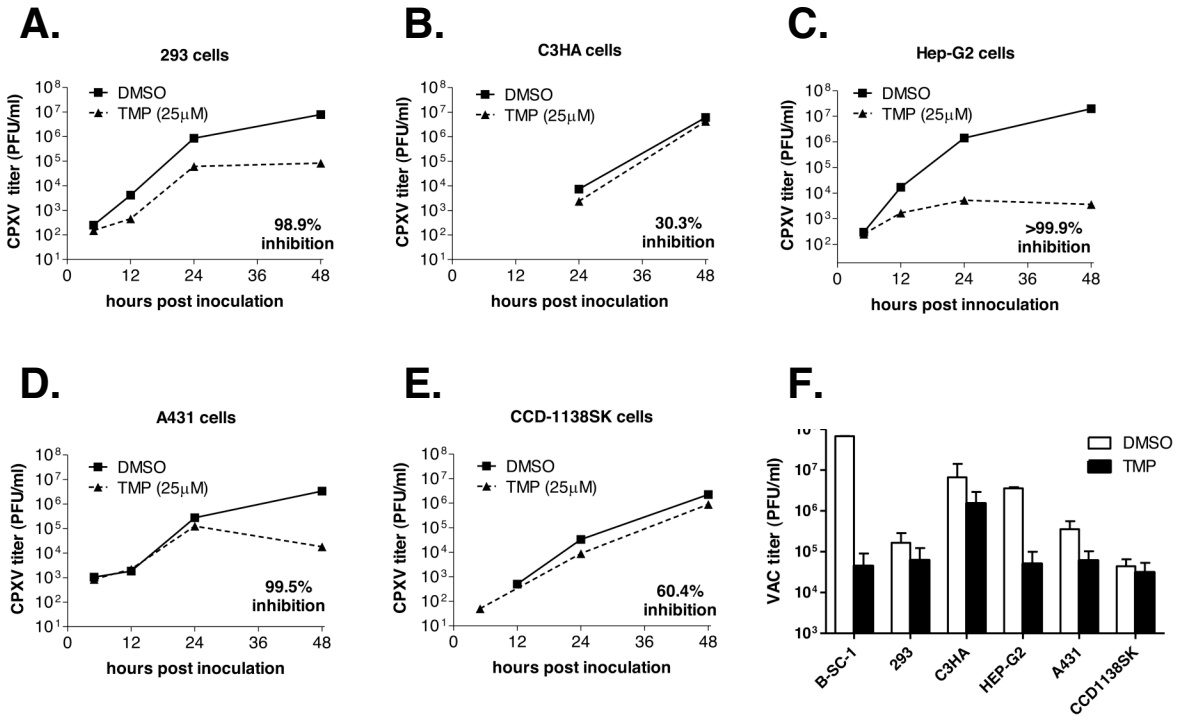
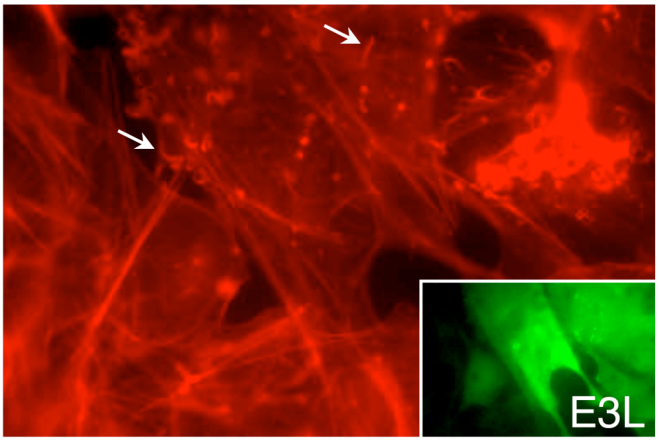

A.
293 cells
DMSO
TMP (25μM)
CPXV titer (PFU/ml)
98.9% inhibition
hours post inoculation
B.
C3HA cells
30.3% inhibition
C.
Hep-G2 cells
>99.9% inhibition
hours post innoculation
D.
A431 cells
99.5% inhibition
E.
CCD-1138SK cells
60.4% inhibition
F.
VAC titer (PFU/ml)
B-SC-1
293
C3HA
HEP-G2
A431
CCD1138SK

Figure 6. Mechanisms of virus spread may vary amongst cell types. BS-C-1 or C3HA cells were infected with CPXV at 0.001 PFU/cell and were then cultured in 2.5% FBS DMEM for 20 h. Cells were then fixed, permeabilized, and immunostained with anti-E3L mAb and AlexaFluor® 488 secondary Ab to detect viral antigen, and TRITC conjugated phalloidin to detect F-actin. (A) Representative fields of the leading edge of a CPXV plaque formed on a BS-C-1 cell monolayer. (B) Representative field of the leading edge of a CPXV plaque formed on a C3HA cell monolayer. E3L staining is shown in the figure insets.

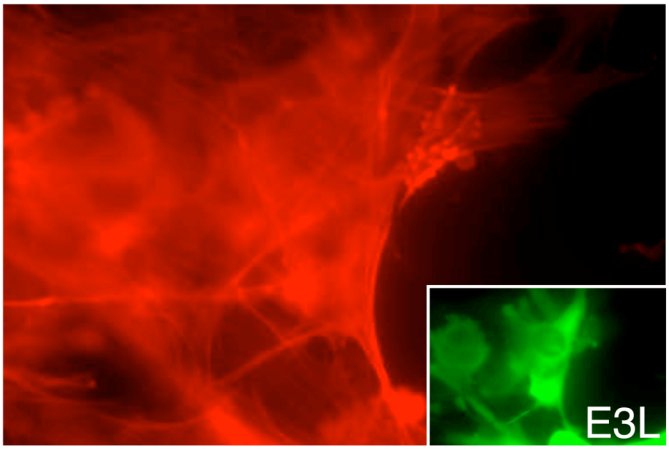
A.

BS-C-1 cells



B.

C3HA cells



CHAPTER 4

Summary

The highly attenuated poxvirus, modified vaccinia Ankara virus (MVA), is a promising live-viral vaccine, and vaccine vector. With further development, MVA-based vaccines have the potential to prevent, or help alleviate, human diseases caused by various infectious agents, or malignancies. Data presented in Chapter 2 of this dissertation characterizes the prostaglandin E_2 (PGE_2) response to infection with MVA, and related poxviruses. These data expand our understanding of the biology of MVA, and provide insight into an important virus–host interaction that is likely a contributing determinant of the immunogenicity of MVA-based vaccines.

In Chapter 2, we demonstrated that infection with MVA resulted in copious accumulation of PGE_2 in culture supernatants of murine dendritic cells, and a murine fibroblast cell line. We expect that the ability of MVA to induce the production of PGE_2 may contribute to the initiation of T helper type-2 responses by MVA-based vaccines. Therefore, manipulation of MVA-induced PGE_2 production might allow for customization of the immune responses generated by MVA-based vaccines. In this study, we determined that cyclooxygenase-2 is essential for production of PGE_2 following MVA infection, and production is rate-limited by the availability of arachidonic acid. Thus, strategies aimed at inhibiting or augmenting these steps in the PG biosynthesis pathway may foster the development of MVA-based vaccines with enhanced, or reduced ability to induce T helper type-2 responses, depending on whether humoral or cell-mediated

immunity is the desired outcome of vaccination. Experiments conducted with vaccinia virus and cowpox virus indicated that these viruses vary in their ability to induce the production of PGE_2 in a cell-type dependent manner. With regards to cowpox virus, we found that specific gene products were acting to prevent PGE_2 production in infected dendritic cells. Future studies aimed at identifying the gene (or genes) involved, and introducing this gene (or genes) into MVA, will likely provide one strategy for modulating the MVA-induced PGE_2 response. The availability of otherwise similar MVA-based vaccine platforms, differing only in their ability to induce PGE_2 production, should allow for a detailed investigation of the contribution of PGE_2 signaling to the immune responses generated by MVA vaccination. In addition, data collected in this study indicated that MVA, vaccinia virus, and cowpox virus, vary in their dependence on cytosolic phospholipase A_2 for arachidonic acid release and initiation of PGE_2 production. Additional research will be required to determine if other characterized, or uncharacterized cellular or viral phospholipases are involved in poxvirus-induced arachidonic acid release.

Unlike MVA, several related poxviruses are pathogenic in humans, and thus constitute threats to human health. Antiviral drugs can provide a crucial complement to vaccination for the prevention of human disease caused by pathogenic poxviruses. The data presented in Chapter 3 of this dissertation identifies Terameprocol (TMP), a proprietary derivative of nordihydroguaiaretic

acid, as an antiviral effective against poxviruses, and support further investigations of TMP as an anti-poxvirus agent.

In Chapter 3, we determined that TMP treatment prevents poxvirus growth in a variety of cell lines, predominantly by inhibiting the spread of virus particles from cell to cell. Consistent with these findings, we found that TMP prevents the formation of actin tails by poxvirus-infected cells. These structures are formed by a particular morphogenic form of poxviruses, termed cell-associated enveloped virus (CEV). Actin tails propel the CEV particles outward, facilitating efficient spread of the virus to nearby cells. The lack of these structures in poxvirus-infected cells treated with TMP provides a mechanistic explanation for the ability of TMP to inhibit poxvirus cell-to-cell spread. Additional research will be required to determine if TMP prevents actin tail formation by blocking a prerequisite step in viral morphogenesis required for the production of CEV, or by direct inhibition of actin tail formation. Nonetheless, the data presented in this study, when considered in combination with the clinical safety record of TMP in phase I clinical trials, support additional studies aimed at investigating TMP as a poxvirus antiviral agent. Specifically, the data presented are sufficient to support experiments to evaluate the ability of TMP to prevent, or reduce, poxvirus-induced disease in established animal models.