ACTIVATION OF MACROPHAGES BY ASTROVIRUS THROUGH A REPLICATION-INDEPENDENT MECHANISM

by

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ABSTRACT

Astroviruses are small, non-enveloped, single stranded RNA viruses known to be one of the leading causes of acute gastroenteritis world wide. Our understanding of astrovirus pathogenesis and disease resolution is limited. This was due to the lack of a small animal model for in depth study of basic astrovirus biology. To increase our understanding of astrovirus disease, we developed a small animal model in young turkeys, infected with turkey astrovirus type-2 (TAstV-2). Through these studies, we described TAstV-2 pathogenesis including viral distribution, kinetics of replication, and cellular histopathology. Additionally, we examined the immune response to primary TAstV-2 infection. These experiments demonstrated that TAstV-2 stimulated avian macrophages (MΦs) to produce nitric oxide (NO). We hypothesized that *TAstV-2 stimulated MΦ production of NO through a replication independent manner*. To test this hypothesis we used the well established avian MΦ cell line HD11. These experiments verified that TAstV-2 specifically bound to HD11 cells through an unidentified surface protein, was internalized, and stimulated NO in a replication-independent manner. Additionally, recombinant capsid protein alone is sufficient for NO stimulation suggesting that exposure of MΦs to astrovirus leads to activation and expression of NO. NO is a known antiviral factor. To determine if NO is involved in primary TAstV-2 clearance or disease, we first asked if NO levels increased during infection. *In vivo* experiments indicated increased expression of NO in the intestines of TAstV-2 infected embryos but not age-matched controls. Additionally, embryos infected with TAstV-2 in the presence of NO donors had limited viral replication as determined by real time RT-PCR, while the use of NO inhibitors increased the viral titers. These studies suggested that the presence of NO influences viral replication *in vivo*. These data are the first experimental evidence of an interaction between astroviruses and MΦs, and suggested that NO and the innate immune response was critical in the control of astrovirus during primary infection.

INDEX WORDS: Astrovirus, turkey astrovirus type 2/North Carolina/034/1999, TAstV-2, enteritis, poult enteritis mortality syndrome, turkey, macrophage, inducible nitric oxide synthase, nitric oxide, Real-Time RT-PCR, baculovirus

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INTRODUCTION

Gastroenteritis is one of the most common illnesses of all mankind, and has the greatest impact on the very young and the very old (6). It is estimated that there are 267 million episodes of gastroenteritis in the United States each year, leading to approximately 3000 deaths (7). In the countries of the developing world these numbers are much higher, an estimated 2.4-2.8 million children under the age of five die yearly from gastroenteritis. This number accounts for 25% of all deaths of children under the age of five (2). In the United States, children experience between 1.5-2.5 episodes of diarrhea per year, resulting in two million doctors' visits with 160,000 hospitalized (7). Currently several viruses are known to be medically relevant causes of gastroenteritis, including rotaviruses, caliciviruses, astroviruses, adenoviruses, enteroviruses, toroviruses, picobirnaviruses, hepatitis A virus, picornaviruses, and coronaviruses (4, 6, 7, 12). Because many of the enteric viruses are difficult to propagate in cell culture, the majority of what we know about enteric viruses is based on epidemiological studies and surveillance programs.

Our laboratory is interested in understanding the pathogenic mechanisms and basic biology of astroviruses. Astroviruses are small round, non-enveloped viruses, typically 28-30 nm in diameter (11). The name astrovirus comes from "astron" (Greek for star) describing the characteristic five- or six-pointed star-like surface projections detected by negative stained electron microscopy (EM). Astroviruses were first described by Madeley *et al*, as the cause of gastroenteritis in infants (10).

Astrovirus infection in humans typically leads to a mild transient diarrhea which is not as prolonged or severe as rotavirus infection, and therefore less likely to result in hospitalization (11). However, it is considered the most frequent cause of diarrhea in children less than 3 months of age (5). There are also reports of astrovirus infections in the elderly and the immune compromised (3, 8, 13). Because of this biphasic age range it is speculated that protection against astrovirus infection is primarily antibody (Ab) mediated (11). Unfortunately very little is known about astrovirus pathogenesis, or the host factors involved in viral clearance and disease resolution. Based on the observation that infection occurs most often in those whose acquire immunity machinery is non-functional or severely impaired, suggests a greater understanding of the role of innate immunity in the host response to astrovirus may lead to enhanced anti-viral therapies.

In depth studies of the astrovirus pathogenesis and astrovirus immunity have been extremely limited due to a lack of a small animal model for astrovirus disease. We isolated and characterized a novel turkey astrovirus (turkey astrovirus type 2/North Carolina/034/1999, TAstV-2), and developed an *in vivo* system for studying astrovirus pathogenesis (1, 9, 14). Using this system, we demonstrated that TAstV-2 induced acute diarrhea and severe weight loss in infected poults. TAstV-2 replication was only detected in the intestines of infected birds, but infectious virus could be isolated from multiple tissues and blood suggesting a viremic stage to disease. Preliminary studies of the basic host response to astrovirus infection demonstrated limited production of TAstV-2-

specific antibodies, and no detectable change in lymphocyte counts, or $CD4^+/CD8^+$ T cell ratios. Furthermore, these animals were not protected from subsequent astrovirus infection, suggesting the adaptive immune response in young turkeys is not critical for viral clearance and disease resolution. These initial experiments demonstrated an increased production of nitric oxide (NO) by splenocytes of infected animals over that of mock infected controls, suggesting that the innate immune response may be the first line of protection against astrovirus infection. To investigate the potential role of innate immunity and NO in astrovirus disease we initially examined the ability of astrovirus to active macrophages (MΦs), using the well established avian MΦ cell line HD11. These experiments demonstrated that TAstV-2 induced the expression of iNOS in HD11 cells. Based on these observations we defined the mechanism of MΦ activation by TAstV-2 *in vitro* and the role of NO in TAstV-2 disease *in vivo.*.

We hypothesized that *TAstV-2 binds to macrophages (chicken MΦ cell line HD11) and upregulates expression of the inducible NO (iNOS) gene in a replication independent manor.* To test this hypothesis we focused on four specific aims. 1) Determine the role of the virus in up-regulation of iNOS, by determining if viral replication is involved, and/or if TAstV-2 capsid protein was capable of stimulating iNOS production. 2) Determine the cellular factors involved in TAstV-2 mediated iNOS expression, by determining if TAstV-2 binds to HD11 cells and what general cell surface structure may be involved.

Our experiments demonstrated the expression of iNOS by HD11s was independent of productive viral replication. Cells were inoculated with TAstV-2 and examined for viral replication by RT-PCR, immunocytochemistry, *in situ* hybridization, and Real-Time RT-PCR. There was no detectable increase in viral titers, indicating that HD11 cells did not support productive viral replication. These results, did not rule out the possibility of abortive replication, or some low levels of viral gene expression. Thus, we expressed the TAstV-2 capsid protein in baculovirus, developed a purification method, and demonstrated that recombinant capsid also stimulated NO activity in HD11. These studies clearly demonstrated that the binding of the capsid protein alone was sufficient to elicit NO activity.

To investigate the nature of TAstV-2-HD11 cell interaction we examined the TAstV-2 binding to HD11 cells by flow cytometry. These experiments demonstrated that TAstV-2 specifically binds to HD11 cells. Through the use of specific chemical treatments, we determined that TAstV-2 did not bind to sialic acid, heparan sulfate, or chondroitin sulfate residues like many viruses. Following binding, the upregulation of iNOS was dependent on virus internalization. Together these results demonstrated that TAstV-2 bound specifically to a HD11 cell surface protein and that stimulation with capsid protein alone was sufficient for activation of NO.

NO is an important response to many viral infections and can influence both viral replication and disease. To understand the significance of increased NO activity in context of TAstV-2 infection, we examined infected turkey embryos for evidence of increased NO activity, as well as the effect of NO donors and iNOS inhibitors on viral replication. Evidence of increased NO levels were detected in the intestines of TAstV-2 infected embryos, as measured by 3-nitrotyrosine staining. The increases in tyrosine-nitrated proteins, correlated with decreases viral replication. At day 5 post inoculation there was intense staining of the lamina propria for 3-nitrotyrosine as compared with mock infected embryos. The cellular source of the increased NO is unknown but may be elicited by resident macrophages or the intestinal epithelial cells. The addition of the NO donor compound SNAP to the TAstV-2 inoculum demonstrated that NO dramatically limits viral replication *in vivo*, while *in vivo* inhibition of iNOS activity lead to higher viral titers than that of the positive control. These experiments demonstrated that TAstV-2 replication is inhibited by NO. This is the first report, to our knowledge, detailing the role of NO in astrovirus disease. These studies are the first experimental evidence of an interaction between astroviruses and MΦs, and suggest NO is an important aspect of the host response to primary infection in the young and immuno-compromised host.

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CHAPTER 2

LITERATURE REVIEW

VIRAL GASTROENTERITIS

Acute gastroenteritis is one of the world's most significant disease problems. An estimated 3 to 5 million people die each year from gastroenteritis, mostly in the developing world (31). In the United States, viral gastroenteritis is one of the most common acute illness, second only to viral respiratory diseases (31). Our understanding of the viruses associated with enteritis has been expanding at an ever-increasing rate (21, 22). Thirty years ago the first viral cause of gastroenteritis was discovered, the Norwalk agent (49). Since then, an increasing number of viruses have been isolated and implicated in causing enteric disease. Although several viruses cause gastroenteritis, the most clinically relevant include rotaviruses, caliciviruses, astroviruses, and enteric adenoviruses (25).

Viral gastroenteritis occurs in both an endemic and epidemic fashion, based on the routes of transmission and host response. The most common endemic viruses are group A rotaviruses, enteric adenoviruses, astroviruses and the Sapporo-like viruses (caliciviruses) (31). These infections are virtually universal in the first years of life. It is believed that during early childhood, immunity develops to these agents providing protection against recurring infection and explaining the decrease in cases in older children and adults (52, 53, 74). The route of transmission for endemic viruses is not clearly understood, but person-to-person contact and fomites are believed to be involved (31, 68). Epidemic viruses are best characterized by the Norwalk-like viruses (calicivirus) and the group B rotaviruses. These viruses affect people of all ages, and outbreaks are typically linked to contaminated water and/or food (32). Astroviruses are also implicated in epidemics, usually associated with an institutional setting like a hospital, retirement community, or military base and they have been isolated from shellfish linked to food borne disease (68).

ASTROVIRIDAE

Astroviruses are small round, non-enveloped viruses, typically 28-30 nm in diameter (68). The name astrovirus comes from "astron" (Greek for star) describing the characteristic five- or six-pointed star-like surface projections detected by negative stained electron microscopy (EM) (64). Astroviruses contain a single stranded positive sense RNA genome typically 7-8 kb in length (60). Their genome is organized into 3 open reading frames (ORFs) designated ORF1a, ORF1b, and ORF2 (102). The 5' reading frame, ORF1a is predicted to encode nonstructural proteins including a viral protease believed to be important in processing and maturation of each of the poly-proteins encoded in this first reading frame (29, 30, 50, 101). ORF1a and ORF1b are separated by a frame shift motif described as essential for expression of ORF1b (56-58, 67). This site establishes a -1 ribosomal frame shift, which brings the ORF1b into frame with ORF1a resulting in the transcription of a single polyprotein (67). ORF1b encodes an RNA-dependent RNA polymerase (55), which is liberated from the polyprotein by the serine protease from ORF1a (55). The final ORF, ORF2, encodes the viral structural protein (20). This region encodes a precursor protein with a mass between 75 kilodalton (kDa) and 90 kDa (depending on species) (46, 99). Currently, the intracellular processing of this sole structural precursor is not well understood (10, 72). It is known that ORF2 is transcribed into a subgenomic message, which is one of the key features, along with the ribosomal frameshift, which lead to the classification of astroviruses into their own family (78).

ASTROVIRUS DISEASE

Astroviruses were first described by Madeley *et al*, as the cause of gastroenteritis in infants (64). Ironically this was not the first case of astrovirus disease in humans. The first case was reported earlier that same year by Appleton & Higgins but this isolate did not exhibit the characteristic morphology and was only identified as an astrovirus in a retrospective study (6, 68). Presently, astroviruses have been reported to cause acute disease in the young of multiple species, including humans, cattle, sheep, cats, dogs, deer, chickens, turkeys, and ducks (17, 34, 40, 64, 69, 93, 98, 103, 104). Amongst the known astroviruses, multiple serotypes have been described for human, bovine, and turkey astroviruses, and complete genomic sequence for human (102), porcine, ovine (47), mink (AY179509), turkey (51), and chicken (43) are available through GenBank.

Astrovirus disease in humans typically involves; diarrhea and vomiting, and can be accompanied by abdominal distention and mild dehydration (74). These signs typically last approximately 4 days (68). Very little is known about the pathologic mechanisms involved in human astrovirus disease. Much of what we have learned about how astroviruses cause diarrhea and the histologic changes associated with infection have come from studies in animals. Astrovirus infections have been best characterized in gnotobiotic lambs (68). Gray *et al*, showed virus particles in the cytoplasm, lysosomes, and in the apical pits and tubules in the villus epithelial cells of the mid-gut (35). Additionally, virus particles were detected in lysosomal organelles of macrophages (MΦs) in the lamina propria (35).

TURKEY ASTROVIRUS

In poultry, astroviruses are more commonly recognized as a problem in turkeys. Turkey astrovirus (TAstV) was first described by McNulty *et al*, in poults in the United Kingdom (UK) suffering from diarrhea and increased mortality (69). In the United States, TAstV was first identified in the 1980s (TAstV-1), and shown to be widely distributed (86, 88, 89). Reynolds *et al,* demonstrated that astroviruses could be isolated from 78% of diseased turkey flocks, more than any other virus identified (88). TAstV is generally associated with self-limiting mild enteritis, transient growth depression, moderate increases in mortality (48, 51, 69, 85, 88, 106) and malabsorption (86, 87, 95, 96). Thouvenelle *et al*, has speculated that TAstV-induced malabsorption is linked to a reduction in activity of the intestinal enzyme maltase (96).

Recently, we isolated and characterized a TAstV associated with Poult Enteritis Mortality Syndrome (PEMS), which is genetically and immunologically distinct from previously described isolates (51). PEMS is a multifactorial, infectious disease that affects young turkeys, typically between 7 and 28 days of age. The disease was first described in 1991 in an area along the western North Carolina/South Carolina border (8). Currently a PEMS-like disease has been described in most turkey producing states across the United States (8, 16), and has been estimated to cost the turkey industry over $100 million (16). A great deal of research efforts have focused on identifying an etiologic agent(s) for PEMS. Several different viruses, bacteria, and parasites were isolated from PEMS-affected flocks; however, none of these agents alone reproduces PEMS (19, 26, 39). This suggests that the etiologic agent has not been isolated or more likely that PEMS is a multifactorial disease. We isolated and characterized a novel turkey astrovirus (TAstV-2) from turkey poults affected with PEMS and demonstrated that it causes a clinically similar disease (51). The PEMS- associated TAstV, TAstV-2, was isolated from the thymus of infected poults (91). Experimentally infected poults exhibited thymic and bursal atrophy, and viremia, although replication appeared to be limited to the intestines (11).

ASTROVIRUS IMMUNITY

The host response to astrovirus and immune components involved in clearance and protection is vastly understudied. This is primarily due to the lack of a small animal model of astrovirus disease. Results from astrovirus infection studies using human volunteers have led many to speculate that antibodies (Abs) are the key mediators of astrovirus protection. In these studies it was observed that healthy adults, who had pre-existing Ab titers against astrovirus, did not show signs of disease (53). This has led many to suggest Abs are the key mediators of immunity, and may explain why infants, young children, the elderly, and the immune compromised are susceptible. This idea appears to be supported by studies by Bjorkholm *et al,* who described the successful use of intravenous Ab therapy to eliminate acute astrovirus gastroenteritis in a 78-year-old male who was on immunosuppressive therapy to control Waldenstrom's macroglobulinemia (13). In addition to virus specific Ab responses, Molberg *et al*, demonstrated that normal adult small intestinal biopsies contain virus specific $CD4^+$ T cells with Th1-like properties (75, 76). The presence of virus specific T cells and serum Ab levels in healthy adults suggests routine exposure to the astrovirus (76). The effector cells of the adaptive immune response are likely involved in providing older children and adults protection from repetitive infection with astrovirus, however given the short duration of disease (1-4 days) it is likely that other host factors, such as innate immunity, participate in, and are required for, viral clearance and disease resolution.

INNATE IMMUNITY

Innate immunity represents an ancient mechanism for host defense with components conserved between both plants and animals (70). The innate immune system encompasses all components of an individual which work toward preventing infection without prior exposure to a given agent (27). This includes physical barriers which prevent exposure of vulnerable tissues as well as cellular and molecular mechanisms capable of responding to insult within minutes of detection. Recent advances in our understanding of the innate immune response has lead to the realization that it is far more specific than first thought, and potentially every bit as complex as acquired immunity (15). In recent years the study of innate immunity has focused on families of proteins both internal and external capable of recognizing molecular patterns unique to microorganisms (66). These germline encoded detection mechanisms are found, to varying degrees, in immune cells, such as MΦs, neutrophils, natural killer (NK) cells, as well as somatic cells. It is through these molecular pattern recognition receptors (PRRs) that the host is able to quickly detect the presence of bacterial cell wall components, parasites, tumor formation, or viral infection (45). Several classes of cellular receptors have been recognized as PRRs. These include the mannose receptor (MR), integrins such as CD11b/CD18, scavenger receptors (SR), and the Toll-like receptors (TLRs) (3, 45, 81-83). Each of these PRRs exhibit distinctive ligand-binding properties and recognize a vast array of microbial products.

The beginnings of our understanding of the innate immune response to viral infections began in 1957 with the first description of interferons (44). The observation that infected cells could detect viral infection, and produce compounds which inhibited replication and spread was one of the first discoveries of a non-adaptive response to infection. Since its initial discovery, interferons are now known to be important in the response to a variety of pathogens (54). Further investigation of the interferon pathways following viral infection lead to the discovery of the double-stranded (ds) RNA-dependent protein kianse (PKR), which is known to detect viral replication through binding to dsRNA intermediates of replicating RNA viruses (23). However, in addition to detection of viral infection inside host cell, there is increasing evidence that PRRs are capable of binding a wide range of viruses in the extra-cellular space. In the past few years, it has been recognized that the Toll-like receptor (TLR) family is capable of binding a variety of viruses, and/or viral products, and that TLR signaling is linked to interferon expression (9). TLR4 binds human respiratory syncytial virus (41) and mouse mammary tumor virus (84). Measles virus and human cytomegalovirus bind TLR2 (12, 24), while TLR3 has been shown to bind dsRNA (5)

With increasing evidence that the initial innate immune response determines the adaptive response (71), any concept of viral immunity is incomplete without a greater appreciation for this instinctive response. Determining the role of innate immune cells and how they interact with viral agents is critical to our understanding of the humoral and cellular anti-viral response. NK cells, dendtritic cells, neutrophils, and macrophages have all been demonstrated to play key roles in various viral diseases, either as mediators of viral clearance, or as hosts (37, 45, 65). Of these cells MΦs play a very central role in control of both the innate response and both humoral and cellular aspects of adaptive immunity (33).

MACROPHAGES

MΦs are systemically located leukocytes that arise from bone marrow stem cells, mature into monocytes, and then enter the blood stream (2). Under normal conditions, circulating monocytes randomly enter tissues and become resident MΦs. These resident MΦs remain in tissues for 2 to 3 months and function in immune surveillance and tissue homeostasis (27). Following antigenic-stimulation, MΦs produce cytokines and chemokines, such as interleukin-1 (IL-1), IL-6, IL-10, IL-12, and tumor necrosis factor-α (TNF-α), which modulate T cell and B cell activation, initiate the inflammatory response, and recruit more immune cells into the area (33, 94).

Activated macrophages are larger and have increased surface expression of major histocompatibility factor II (MHC II), co-stimulatory molecules, and cytokine receptors, such as interferon-γ (IFN-γ) which is the best described macrophage-activating factor (2). Once activated, MΦs are committed to antigen elimination and the increased surface expression of MHC II makes them more efficient antigen presenting cells (APC) (27). The role of MΦϕs as antigen presenting cells, their involvement in activating $CD4^+$ T cells, driving B cell antibody production and isotype switching is well documented (1). In this capacity MΦs are the initiators or triggers for adaptive immunity (2). However, MΦs are also important in innate immunity.

MΦ RESPONSE TO VIRUSES

Historically, immunity to viral infections was attributed to the humoral response and the presence of neutralizing Abs with little consideration give to the cellular and innate systems (79). This point of view is slowly changing, as we gain a respect of the various mechanisms viruses employ to evade detection and elimination (100). MΦs are a critical link between all three aspects of the anti-viral response. They aid humoral and cellular immunity through the production of various cytokines which drive B cells and T cells differentiation, and determine the subtype of Ab and effector cells used in that response (33, 100). They are also important in removing infected and lysed cells through phagocytosis and presenting digested antigens to B cells and T cells (3, 36). The pivotal role MΦs play in the immune response makes them a tempting target for pathogen exploitation. MΦs have been implicated in the systemic spread of influenza virus, rotavirus, adenovirus, and HIV, among others (18, 37, 61, 63, 92). The interaction between viral infection and responding MΦ can lead to an enhanced disease state, as well as viral clearance (2, 63).

NITRIC OXIDE

Stimulation and activation of MΦ by cytokines such as IFN-γ, or by signaling via a PRR typically leads to an upregulation and release of biologically active compounds (71, 94). Most notably these include the pro-inflammatory cytokines such as TNF-α and the multi-functional effector molecule nitric oxide (NO) (14). NO is the simplest biologically active compound known, and is an important molecule in multiple systems (97). NO is produced by an enzymatic reaction, which converts oxygen and $_L$-arginine to NO and $_L$-citrulline (63). There are three isoforms of the NO producing enzyme known as NO synthase (NOS), corresponding to each of the three main functions of NO. Endothelial NOS (eNOS) is involved in hemostatis, neuronal NOS (nNOS) is involved in neurotransmission, and inducible NOS (iNOS) is expressed following stimulation by cytokines and inflammatory signals (77). Both eNOS and nNOS are constitutively expressed at low levels, and are dependent on the availability of intracellular calcium for enzyme activity (14). iNOS differs from the other two isoforms in that, it is not present under normal conditions, but its expression is rapidly up regulated following signaling (63, 97). In addition, iNOS activity is independent of Ca^{2+}, and is mediated by a binding interaction with calmodulin. The interaction of iNOS with calmodulin results in greater enzymatic activity, which allows iNOS to produce large amounts of NO over a greater period of time, in response to specific stimuli making iNOS a useful immune system effector molecule (63, 97). iNOS has been identified in mice, rats, humans, cattle, and chickens, suggesting its role in the immune response has been conserved through evolution (97).

During viral infection, MΦs respond by increasing NO production in response to either direct binding of MΦ surface proteins by viral proteins, by detection of intracellular double stranded RNA, and/or by responding to interferons and other panic signals released by infected cells (42, 45, 59, 63). Studies using iNOS knockout mice have suggested it is important in limiting replication and conferring resistance to a variety of viral agents (28, 38, 62, 63, 107). However the increased NO activity in the lungs of influenza infected mice is believed to contribute to the severity of pneumonia (4). Understanding the role and significance of NO activity during viral infections is a critical link to our understanding of viral immunity as a whole.

CELLULAR RECEPTORS FOR VIRUSES

The first step in viral infection involves binding of the virion to its host cell via a cell surface protein (105). The nature of this interaction can be as varied as the viruses and possible disease outcomes themselves, and may involve one receptor or multiple co-receptors (73). The virus-receptor interaction may play a role in determining tissue tropism and host range. However, it should be noted that the distribution of a given receptor is generally wider than the observed tropism. This is best illustrated by influenza virus which has been shown to bind to sialic acid residues which is found on most cells however replication is primarily restricted to the respiratory tract (90).

Unfortunately, our understanding of virus-receptor binding is specific to individual viruses, and in most cases not well defined. Many broad classes of cellular receptors have been shown to be involved in binding to several distinct virus types, suggesting that many viruses may use similar strategies to achieve initial binding.

Newcastle disease virus, influenza virus, reovirus, canine parvovirus, Sendai virus, and some rotaviruses, have been shown to bind to sialic acid residues (7, 105). Vacinia virus, herpesviruses, adenoviruses, papillomavirus, andeno-associated virus, HIV, foot-and mouth disease virus, Denge virus, bovine viral diarrhea virus, Sindbis virus, bovine respiratory syncytial virus, and human respiratory syncytial virus have all been shown to bind to heparan sulfate (73). Similarly, adenoviruses, enteroviruses, rotaviruses, foot-and-mouth disease virus, hantavirus, coxsackie virus, and echoviruses have been shown to use integrins (73, 80). Given the ability of viruses from different families, with unique structural properties to recognize similar receptors, while resulting in distinct disease and tissue trophisms, indicates that intracellular events play an equally important part in viral disease. There have been no studies, to our knowledge, that suggest possible astrovirus receptors or binding events.

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CHAPTER 3

REVIEW: AVIAN ASTROVIRUSES[1]

[1]Koci, M. D. and S. Schultz-Cherry. 2002. *Avian Pathology*. 31: 213-227
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SUMMARY

As poultry becomes more important in the world economy, it is increasingly important to fully understand the mechanisms of disease and poor production that affect the industry. In order to more accurately and reasonably treat these diseases, a more sophisticated understanding of interrelatedness is required. This review addresses the history, diagnosis, treatment and control of avian astroviruses (AAstVs), with an emphasis on the recent advances in our understanding of AAstV molecular biology. Astroviruses have been shown to cause disease in ducks (duck astrovirus-1, DAstV-1), turkeys (turkey astrovirus-1 and –2, TAstV-1 and -2) and chickens (avian nephritis virus, ANV). Historically, diagnosis has relied heavily on electron microscopy and immune electron microscopy. To provide more rapid and sensitive detection of astroviruses in the field, molecular based diagnostic tests are needed to provide a more accurate picture of the overall impact of astroviruses in poultry disease. This review compares the three fully sequenced AAstVs, ANV, TAstV-1 and TAstV-2, in order to identify conserved regions and motifs that could be targets for pan-reactive AAstV tools. In addition a nomenclature for astroviruses is also proposed, based on: host species-astrovirus-type number/country(state)/reference number/year of isolation.

INTRODUCTION

Because of the growing importance of poultry in world economics it has become imperative to establish rapid and accurate diagnostics in treating poultry diseases (Lowenthal *et al*., 1999). As an increasing number of "small round viruses" (SRVs) are implicated in decreased production and increased mortality it is crucial that they be characterized to completely understand distribution and design effective control mechanisms (Asplin, 1965b; Gough *et al*., 1984; Reynolds *et al*., 1987a; Reynolds *et al*., 1987b; Johnson, 1990; Saif *et al*., 1990; Swayne *et al*., 1990; Guy & Barnes, 1991; Cavanagh, 1992; Qureshi *et al*., 1997; Imada *et al*., 2000; Koci *et al*., 2000b; Qureshi *et al*., 2000; Schultz-Cherry *et al*., 2000; Todd, 2000; Yu *et al*., 2000a; Yu *et al*., 2000b; Cavanagh, 2001; Todd et al., 2001). SRVs typically fall into one of five viral families, *Parvoviridae, Circoviridae, Picornaviridae*, *Calciviridae,* or *Astroviridae*, each with characteristic morphologies visible by electron microscopy (Caul & Appleton, 1982). Viruses are not static biological entities, but rather a collection of genetically diverse quasi-species capable of adaptation (Schneider & Roossinck, 2001). Therefore, diagnosis and classification must involve a collection of characteristics and not rely completely on morphology (van Regenmortel *et al*., 2000). As new genera and subgroups emerge, it is possible that characteristic physical properties may change or become less prominent. For this reason it is necessary to use replication strategy and genome organization along with biochemical properties and particle structure to properly assign an isolate to a viral family (van Regenmortel *et al*., 2000). In this review, we will discuss the recent advances in our understanding of avian astroviruses (AAstVs).

Astroviruses are small round, non-enveloped viruses, typically 28-30 nm in diameter (Matsui & Greenberg, 2001). The name astrovirus comes from “astron” (Greek for star) describing the characteristic five- or six-pointed star-like surface projections detected by negative stained electron microscopy (EM) (Madeley & Cosgrove, 1975). However, visualization of the “definitive” star-like structure is pH dependent and can vary greatly between isolation protocols (Caul & Appleton, 1982; Matsui & Greenberg, 2001). Because of this variability, there is a risk of miss-classification, where astrovirus isolates could be labeled picornavirus, picornavirus-like, enterovirus, enterovirus-like, small round virus, small round structured virus, etc (Reynolds, 1991; van Regenmortel *et al*., 2000). This was most recently demonstrated by the re-classification of avian nephritis virus (ANV) from that of picornavirus to astrovirus, after the genome was fully characterized (Imada *et al*., 2000). This demonstrated that proper viral order and family discrimination of viral isolates must be based primarily on molecular characteristics as described by the International Committee on Taxonomy of Viruses (ICTV) (van Regenmortel *et al*., 2000).

ASTROVIRUS DISEASE

Astroviruses were first described by Madeley & Cosgrove (1975) as the cause of gastroenteritis in infants. Ironically this was not the first case of astrovirus disease in humans. The first case was reported earlier that same year by Appleton & Higgins (1975) but this isolate did not exhibit the characteristic morphology and was identified as an astrovirus in a retrospective study (Appleton & Higgins, 1975; Matsui & Greenberg, 2001). The role of astroviruses in birds pre-dates that of Appleton & Higgins (1975). In

1965, a disease in ducklings was described (Asplin, 1965a; Asplin, 1965b) that was eventually identified in 1984 as an astrovirus (Gough *et al*., 1984; Gough *et al*., 1985). Presently, astroviruses have been reported to cause acute disease in the young of multiple species, including humans, cattle, sheep, cats, dogs, deer, chickens, turkeys, and ducks (Madeley & Cosgrove, 1975; Snodgrass & Gray, 1977; Woode & Bridger, 1978; Bridger, 1980; McNulty *et al*., 1980; Williams, 1980; Tzipori *et al*., 1981; Gough *et al*., 1984; Harbour *et al*., 1987). Astrovirus disease in most species causes gastroenteritis which is usually mild and self-limiting, however more severe diseases have been described in poultry (Matsui & Greenberg, 2001).

Turkey astrovirus

In poultry, astroviruses are more commonly recognized as a problem in turkeys, and can be accompanied by a moderate increase in mortality (McNulty *et al*., 1980; Reynolds *et al*., 1987b; Reynolds, 1991; Jordan & Pattison, 1996; Koci *et al*., 2000b; Yu *et al*., 2000a). Turkey astrovirus (TAstV) was first described by McNulty et al. (1980), and was associated with turkey poults in the United Kingdom (UK) suffering from diarrhea and increased mortality. In the United States (US) TAstV was first identified in the 1980s (TAstV-1) and shown to be widely distributed (Saif *et al*., 1985; Reynolds & Saif, 1986; Reynolds *et al*., 1987b). Reynolds *et al*., 1987b demonstrated that astroviruses could be isolated from 78% of diseased turkey flocks, more than any other virus identified. Recently, we have isolated and characterized a TAstV associated with poult enteritis mortality syndrome (PEMS) which is genetically and immunologically distinct from previously described US isolates (Koci *et al*., 2000b). The PEMS associated TAstV (TAstV-2) was originally isolated from the thymus of infected poults (Schultz-Cherry *et* *al*., 2000). Experimentally infected poults exhibit thymus and bursal atrophy, and virus can be isolated in other tissues, although replication is only routinely detected in the intestines (Behling-Kelly *et al*., 2001).

Duck astrovirus

Unlike other species, astroviruses in ducks have been associated with a fatal hepatitis, historically known as duck hepatitis virus type II (DHV type II) (Asplin, 1965b; Gough *et al*., 1984; Gough *et al*., 1985; Woolcock & Fabricant, 1991). This disease was first described in the UK by Asplin in 1965, associated with duck flocks vaccinated for DHV type I, which is believed to be a picornavirus (Asplin, 1965a; Woolcock & Fabricant, 1991). This new disease was not neutralized by anti-DHV type I sera (Asplin, 1965a), and vaccination resulted in little cross-protection between type I and type II (Asplin, 1965b). It was postulated that DHV type II represented the emergence of a new serotype (Asplin, 1965b). Several years later, (Gough *et al*., 1984) described another outbreak of fatal hepatitis in ducklings in the UK. Examination of livers from affected duckling revealed the presence of astrovirus-like particles (Gough *et al*., 1984). Vaccination of ducklings with DHV type II vaccine strains described by Asplin (1965b) protected against this new isolate (Gough *et al*., 1985). Therefore, DHV type II was declared an astrovirus and it was proposed that the name be changed to duck astrovirus (DAstV), while DHV type I, and a later described type III isolated in the US, are still classified as picornaviruses (Woolcock & Fabricant, 1991).

Avian nephritis virus

Avian nephritis virus (ANV) was first isolated from rectal contents of normal broiler chicks (Yamaguchi *et al*., 1979). Experimental infections demonstrated that ANV primarily results in a sub-clinical disease (Imada *et al*., 1979; Maeda *et al*., 1979; Yamaguchi *et al*., 1979; Imada *et al*., 1983; Jordan & Pattison, 1996), although mild growth depression and mortality has been reported with the G-4260 strain (Imada *et al*., 1979; Shirai *et al*., 1991a; Reece *et al*., 1992). ANV typically causes histological changes in the kidneys (Shirai *et al*., 1989; Shirai *et al*., 1991b; Shirai *et al*., 1992; Jordan & Pattison, 1996), although viral antigens can be detected in the liver, spleen, pancreas, kidney, jejunum, and rectum (Imada *et al*., 1979; Imada *et al*., 1983). Young chicks are the most susceptible, with resistance to disease developing after the first month of life (Imada *et al*., 1981). Antibodies against ANV have been found in chicken and turkey flocks throughout the UK and Japan, suggesting a broad distribution (Nicholas *et al*., 1988; Takase *et al*., 2000). ANV was initially classified as a picornavirus, based on EM (Maeda *et al*., 1979; Yamaguchi *et al*., 1979). However, this classification was changed following the complete sequencing of the viral genome (Imada *et al*., 2000). ANV was shown to have all the molecular properties and gene organization consistent with the *Astroviridae* family (Imada *et al*., 2000; Matsui & Greenberg, 2001).

GENOME ORGANIZATION AND MOLECULAR BIOLOGY

Astroviruses have a positive-sense, single stranded (ss), RNA genome, 6.8-7.9 kb in length (Matsui & Greenberg, 2001). The complete sequence of five human astroviruses (HAstVs) isolates (Jiang *et al*., 1993; Lewis *et al*., 1994; Willcocks *et al*., 1994) (GenBank accession AF141381, AF260508), two turkey isolates (Jonassen *et al*., 1998; Koci *et al*., 2000b), ANV (Imada *et al*., 2000), and a sheep astrovirus (OAstV) (Jonassen *et al*., 1998) are available in GenBank. The basic organization and replication strategy is conserved among all of the astroviruses sequenced. The astrovirus genome includes a 5' untranslated region (UTR), followed by three open reading frames (ORFs), a 3' UTR, and a poly-A tail (Figure 3.1). There is a retrovirus-like frameshift structure between ORF1a and ORF1b, and ORF2 is expressed from a subgenomic RNA (Figure 3.1). The lengths of each of these features varies between species and serotypes. The specific details of the mammalian astroviruses (MAstVs) are thoroughly reviewed in the current edition of *Fields Virology* (Matsui & Greenberg, 2001) therefore this review will focus on properties of the AAstVs.

Among the three AAstVs there is some variation in the overall lengths of the genomes and their respective internal components (Table 3.1, Figure 3.1). In addition to variation in ORF lengths, there are also differences in the expression strategies for ORF2. Most MAstVs (except HAstV-8) have an overlap of approximately 8 nucleotides (nt) between the stop codon of ORF1b and the start codon of ORF2, which is in the same reading frame as ORF1a. However, the AAstVs deviate from this somewhat in their genome structure. The start codon for ORF2 of ANV is 19 nt downstream of the stop codon of ORF1b, though ORF2 is still in the same frame as ORF1a (Figure 3.1). The space between the ORF1b stop codon and ORF2 start site for both TAstVs is 18 nt (Figure 3.1), placing the TAstV ORF2 in the same frame as ORF1b (Figure 3.1). There are also some differences among the AAstVs toward the end of the genome. Sequence analysis of the last 19 nt of ORF2 and adjacent 3'UTR by (Jonassen *et al*., 1998; Jonassen *et al*., 2001) described a conserved sequence and predicted secondary structure present in all astrovirus isolates sequenced, except for TAstV-2 (Figure 3.1). This conserved motif is also present in infectious bronchitis virus (a coronavirus) and equine rhinovirus type 2 (a picornavirus), which the authors suggested was evidence of a recombination events between these viruses (Jonassen *et al*., 1998).

SEQUENCE ANALYSIS AND TRANSLATION STRATEGIES

Nonstructural Proteins

Analysis of the polypeptides of ORF1a and ORF1b from HAstVs indicate that these ORFs likely encode nonstructural proteins (Gibson *et al*., 1998). Examination of HAstV ORF1a has identified 4 potential transmembrane helical motifs, a serine protease, a putative bipartite nuclear localization signal (NLS), and a region referred to as the immune response element (IRE) identified by antiserum produced against purified particles (Gibson *et al*., 1998; Willcocks *et al*., 1999). ORF1a is translated as one polyprotein, which is post-translationally cleaved into functional peptides by the serine protease (Matsui & Greenberg, 2001). The presence and function of these peptides in the AAstVs have only been characterized by sequence analysis (Figure 3.1), although many of these motifs have been identified (Imada *et al*., 2000; Koci *et al*., 2000b).

The overall ORF1a sequence similarities between the AAstVs and the MAstVs is quite low ranging from 20-25% nt identity (12-15% amino acids, aa). However, it is the presence of astrovirus-like nonstructural motifs that is most important. ORF1a is also the most conserved among the HAstVs, and has been used to define two distinct genogroups (Belliot *et al*., 1997). This is not the case for the AAstVs sequenced to date. There is a greater relatedness among the HAstVs, and to lesser extent sheep astrovirus (OAstVs), than among AAstVs (Figure 3.2). This suggests AAstV non-structural proteins are allowed greater flexibility in sequence variation than their mammalian counterparts. This may be related to differences in host range (Schneider & Roossinck, 2001). There is no evidence that the MAstVs cross species line (Matsui & Greenberg, 2001). However based on surveillance studies of chicken and turkey farms, antibodies against ANV were isolated from both chickens and turkeys suggesting either support ANV replication (Nicholas *et al*., 1988; Cavanagh, 1992). Having greater genetic flexibility may increase the likelihood of replicating in whatever poultry species is available, so long as the overall functional motif is conserved (Schneider & Roossinck, 2001). This hypothesis has not been tested experimentally, and it should be pointed out that more isolates need to be sequenced in order to fully understand its significance.

The best-described protein encoded in ORF1a is the serine protease (Willcocks *et al*., 1994; Gibson *et al*., 1998). This viral protease is similar to chymotrypsin-like proteases of other positive sense RNA viruses, although it differs in that a serine residue has been substituted for a cysteine in the third catalytic position (Gorbalenya *et al*., 1989; Matsui & Greenberg, 2001). Alignments of the 3 AAstV ORF1a predicted amino acid (aa) sequences allowed for identification of a putative serine protease. When compared to the MAstV serine protease sequence, the three predicted catalytic residues can be identified and are conserved (Figure 3.3). There is a one-residue shift of the second catalytic aa (aspartic acid) between the AAstVs and the MAstVs; the significance of this is unknown. However the serine residues do align, as well as many of the residues predicted to be important in substrate binding (Figure 3.3).

Downstream of the serine protease, ORF1a is believed to encode a nuclear localization signal (NLS). This putative NLS is 664 aa from the N-terminus of the ORF1a polyprotein of HAstV1 (Willcocks *et al*., 1999). The need or function of an NLS in an

RNA virus is still unclear, but several investigators described limited nuclear staining for astrovirus antigen (Aroonprasert *et al*., 1989; Willcocks *et al*., 1999). A similar motif was identified for ANV, corresponding to aa positions 719-735 (Imada *et al*., 2000). Similar aa sequences can be found in both TAstVs, but none of the putative AAstV NLSs have been tested experimentally.

ORF1a most likely encodes for several other non-structural proteins that have not been identified. Sequence analysis of all the astroviruses have not identified the presence of a VPg or a helicase, both being proteins that conventional wisdom would suggest were essential (Willcocks *et al*., 1994; Gibson *et al*., 1998; Matsui & Greenberg, 2001).

Another distinct feature of the astrovirus genome is its translation machinery for ORF1b (Marczinke *et al*., 1994). Sequence analysis of ORF1b does not yield a clear picture of the overall translation strategy. The first start codon of ORF1b for the HAstVs is found more than 400nt inside the reading frame, in a suboptimal position according to Kozak's rules (Matsui & Greenberg, 2001). The ORF1a/ORF1b overlap region contains a heptameric shift sequence (A AAA AAC) and the potential for the formation of a downstream stem-loop and possible pseudoknot that would provide a ribosomal frameshift mechanism (Willcocks *et al*., 1994; Lewis & Matsui, 1995; Lewis & Matsui, 1996; Imada *et al*., 2000; Koci *et al*., 2000b). This mechanism is similar to that used by retroviruses and coronaviruses, however unlike those viruses the pseudoknot is not required for the astrovirus frameshift to occur (Lewis & Matsui, 1997). This heptameric sequence, and predicted secondary structure has been identified in all three AAstVs (Figure 3.4).

It is believed that this frameshift structure allows for the translation of ORF1a and ORF1b to occur as one polyprotein that is then cleaved into functional subunits. Analysis of ORF1b, indicates that it encodes for an RNA dependent RNA polymerase (RdRp) (Poch *et al*., 1989; Ishihama & Barbier, 1994; Lewis *et al*., 1994; Marczinke *et al*., 1994). This region of the astrovirus genome is the most conserved between the MAstVs and the AAstVs, as well as among the AAstVs (Figure 3.5).

Structural proteins

ORF2, is translated from a subgenomic message, and encodes the viral capsid protein (Monroe *et al*., 1993). The capsid protein is translated as one long precursor protein approximately 73 kDa (TAstV-1), 80 kDa (TAstV-2), or 74 kDa (ANV), which is post-translationally cleaved to form mature virion subunits in a mechanism that is not understood (Bass & Qiu, 2000). Both nt and aa analysis of all the astrovirus capsid genes (Figure 3.6) demonstrate that the MAstVs are more closely related than the AAstVs (Jonassen *et al*., 2001). Analysis of ORF2, by different groups, showed that the N-terminal end of the capsid gene is generally more conserved than the C-terminal end (Jonassen *et al*., 2001; Wang *et al*., 2001). This observation may be useful in the design of oligonucleotide primers for a diagnostic RT-PCR test.

DIAGNOSIS

Until recently the most common method to identify astrovirus infection in birds was EM (Reynolds, 1991). However, only 10% of particles may exhibit the 5- or 6- pointed star-like morphology making it difficult to accurately identify astroviruses using direct EM, especially when there are very few viral particles present (Caul & Appleton, 1982;

Reynolds, 1991; Matsui & Greenberg, 2001). Because of this limitation, Reynolds (1991) suggested, using immune EM (IEM) to encourage viral aggregation. This is a reasonable alternative, though it should be pointed out that the addition of purified antibody (Ab) or convalescent sera to a virus sample can actually mask the characteristic physical features or fail to detect new serotypes (Matsui & Greenberg, 2001). IEM can be an effective diagnostic tool if the Ab and the antigen it recognizes are completely characterized. For example, Guy & Barnes (1991) described the isolation and partial characterization of a small enterovirus-like virus isolated from turkeys with enteritis. Using a monoclonal Ab developed against that virus (generous gift from James Guy, North Carolina State University) we determined that it recognized recombinant TAstV-2 capsid protein by western blot analysis, ELISA, and immunofluoresces in transfected cells (unpublished observation). This suggests that TAstV-2 has been associated with diseased turkey flocks as early as 1991.

Diagnosis of both ANV and DAstV include growth in embryonated eggs, as well as various serological tests (Asplin, 1965b; Gough *et al*., 1985; Nicholas *et al*., 1988; Decaesstecker & Meulemans, 1991; Woolcock & Fabricant, 1991; Jordan & Pattison, 1996; Takase *et al*., 2000). These tests can be very accurate and rapid, though they are strain specific and, similar to IEM, risk miss-diagnosis of new serotypes. TAstV can also be isolated in embryonated eggs, though no tools to detect the presence of antibodies against TAstV-1 or TAstV-2 have been described (Reynolds, 1991; Koci *et al*., 2000b). Furthermore, ANV is the only AAstVs shown to replicate in cell culture (Imada *et al*., 1981).

Accurate detection of new AAstV isolates genetically similar to those already in GenBank is best accomplished using RT-PCR primers specific for each virus. By designing primers with knowledge of genome organization and conservation, one can select sites that are conserved amongst similar serotypes and potential new serotypes. We have previously described an RT-PCR protocol for the detection of TAstV-2 in field samples (Koci *et al*., 2000a). These primers have been used by our lab and others to detect TAstV-2 positive flocks in several states across the US. The ultimate diagnostic goal is the design of primers, or a panel of primers, that could be used to detect any AAstV from a clinical sample.

Analysis of the AAstV sequences suggests that a few areas may be useful for primer design. One potential site for primer design is the conserved sequence and RNA structure described in the 3' end of the genome (Jonassen *et al*., 1998). This area has been described in almost all astroviruses to date, although TAstV-2 does not have this sequence (Koci *et al*., 2000b; Jonassen *et al*., 2001). Because this motif was not present in TAstV-2, many were not willing to accept that it was an astrovirus until it had been completely sequenced (Koci *et al*., 2000b). In addition, this conserved site is part of a stem loop structure, which makes the design and selection of primers without hairpins difficult. Primers specific to regions of the capsid gene are functionally more reliable, but would not be useful pan-specific diagnostic tools because of the large amount of sequence divergence among the AAstV capsid genes (Figure 3.6). However, capsid based primers may be important in detection of specific serotypes.

Analysis of the most conserved gene of the AAstVs (ORF1b) indicates that there are potential priming sites that should cross-react between any two of the three viruses.

However potential sites specific for all three are not apparent. These three sequences have only 50% nt identity, and the likelihood that a fourth would match all three in exactly the same two sites is unknown. Variation in this most conserved region suggests that degenerate primers may be the only solution for pan-reactive AAstV primers. Based on predicted amino acid alignments ORF1b, several regions of conserved motifs can be identified which could be potential degenerate priming sites (Figure 3.7). However, in order to determine the most reliable and economic diagnostic technique more AAstV isolates need to be fully characterized.

To ensure proper diagnosis and classification of any new SRV isolated, molecular characterization will be required. The failure of previously described astrovirus specific primers to detect an isolate does not infer the isolate is not an astrovirus. Classification needs to include determination of genome composition, gene organization and sequence similarities (van Regenmortel *et al*., 2000). Overlap in properties such as diameter, surface projections, buoyant density, and capsid proteins among the SRVs, presented in Table 3.2, demonstrates that molecular properties are the most reliable characteristics for classification. If an isolate is determined to contain a ssDNA genome (Table 3.2) it is either a parvovirus or circovirus (van Regenmortel *et al*., 2000). These two viruses are distinguished (Figure 3.8 A & B) by the presence of a circular DNA genome in circoviruses and the larger linear DNA genome of parvovirus (Berns *et al*., 2000; Todd, 2000; Todd *et al*., 2000, Todd *et al*., 2001). Conversely, isolates with ssRNA genomes are most likely to be picornavirus, calicivirus, astrovirus, or the part of the genus “Hepatitis E-like viruses” (van Regenmortel *et al*., 2000). All of these viruses have positive ssRNA genomes of similar size. The major differences among these viruses are in their replication strategies and order of their genes. In *Picornaviridae* (Figure 3.8C), which includes the enteroviruses, the genome is translated into one polyprotein that is then cleaved into the individual structural and non-structural proteins (King *et al*., 2000). The caliciviruses and astroviruses differ from picornaviruses in that their genomes have distinct ORFs, each translated separately. *Caliciviridae* has been divided into 4 genera, Norwalk-like, Sapporo-like, *Lagovirus*, and *Vesivirus* with differences in reading frame usage (Figure 3.8D). The first ORF encodes the nonstructural proteins including the polymerase. This is followed by the capsid gene and a small 3' ORF that encodes a small basic minor structural protein. Similar to astroviruses the capsid gene is transcribed into a subgenomic message (Green *et al*., 2000a; Green *et al*., 2000b). The unclassified Hepatitis E-like viruses, also have three reading frames. The first ORF codes for the nonstructural proteins, the second encodes the capsid protein that is at the 3' end of the genome, similar to astroviruses (Figure 3.8E & F). There is a third ORF (Figure 3.8F) that overlaps both the first and second ORFs that encodes a protein of unknown function (Berke & Matson, 2000; Green *et al*., 2000c). By defining these molecular characteristics of any new isolate, that virus can be definitively assigned to a viral family, or be demonstrated to be unique, suggestive of a new viral family. This ultimately leads to a more complete understanding of both AAstVs as well as virology at large.

TREATMENT AND CONTROL

Strict containment is the only known method of preventing and controlling infections with any of the known astroviruses. Infected flocks, especially those that exhibit severe loss in viability and production, need to be treated with the utmost concern for biosecurity strictly adhering to the principles discussed in *Diseases of Poultry* (Zander & Mallinson, 1991). Astroviruses are extremely stable in the environment and resistant to inactivation by most routinely used disinfectants (Kurtz *et al*., 1980; Abad *et al*., 1997; Schultz-Cherry *et al*., 2001) similar to chicken anemia virus or foot-and-mouth disease virus. Studies in our laboratory with TAstV-2 demonstrated that partially purified astrovirus remained infectious following treatment with a panel of commercial disinfectants, including 10% bleach. The only products completely effective at inactivation were 0.3% formaldehyde, 1.5% Virkon S, 0.1% β-propriolactone, and 90% methanol. TAstV-2 is also very heat stable, resisting inactivation following treatment at 60°C for 10 min, and resistant to low pH (Schultz-Cherry *et al*., 2001). These findings suggest that, once a poultry production facility has been infected with astrovirus, complete sanitation of all materials and restricted access to facilities by personnel is required to contain the outbreak to an affected farm. To eliminate astrovirus infections contaminated farms should be thoroughly disinfected. All the litter and manure should be removed and disposed in a manner that ensures runoff does not contaminate the driveways or entrances to poultry houses. The floors, walls, fans, feeders, watering systems and all equipment should then be adequately scrubbed and disinfected using compounds and procedures proven useful at eliminating highly stable SRVs. Additionally, service personnel and attending veterinarians should be mindful of which farms are affected and those that are not, and schedule their visits to these properties to minimize the risk of transporting the virus to healthy flocks either on their person or on their vehicles (Zander & Mallinson, 1991).

The combination of age susceptibility and highly stable virions, suggest that multiple age farms may help prolong the period of poor production as older birds may recover and no longer exhibit clinical signs but still harbor virus. There is no experimental evidence that affected poultry develop a protective immune response. This may explain why new poults routinely develop enteritis soon after being placed in "cleaned" houses on farms with multiple aged birds (Edens & Doerfler, 1999). These factors also suggest that there is little hope for development of an effective vaccine strategy. The most practical prevention method is to use strict biosecurity prophylactically. A nominal investment of time and energy spent on keeping each farm pathogen-free will greatly reduce the likelihood of contracting an astrovirus infection, and likewise periods of prolonged poor production. This strategy is also advantageous for the control of most other poultry diseases, as procedures successful in the inactivation of astroviruses also inactivates other pathogens (Brunet, 1997).

NOMENCLATURE

The family *Astroviridae* is tentatively divided into two genera representing mammalian and avian astroviruses. The species within these genera are defined based on the animal that they infect (Table 3.3). There have been two different serotypes described for bovine astroviruses (BAstV), eight serotypes for HAstV, and two serotypes for TAstV. Serotypes are defined by a twenty-fold, or greater, difference in cross-reaction of neutralization titres, and are assigned numbers (e.g. TAstV-1, TAstV-2). The ICTV number designation is based on order by which they were characterized. Currently there is no established nomenclature scheme for new isolates within *Astroviridae*. We propose that nomenclature should follow a model similar to that used for influenza virus (e.g. host astrovirus - serotype number/ country (state or municipality)/ isolate reference number/ year of isolation). For example, the PEMS-associated TAstV should be listed as, turkey astrovirus-2/United States (North Carolina)/034/1999 (TAstV-2/US(NC)/034/1999).

CONCLUSIONS

Astroviruses infect and cause disease in several animal species, but their overall impact on animal health and economics is not fully understood in any system (Matsui & Greenberg, 2001). Very few astroviruses have been adapted to propagate in cell culture, and there is no established animal model for astrovirus disease. It most systems astrovirus infection results primarily in mild-to-moderate gastroenteritis with no observed pathologic changes outside the intestines. Astrovirus infections in birds have been reported to affect several different organs. TAstV, historically, has been described to cause gastroenteritis, growth depression, and a slight increase in mortality (McNulty *et al*., 1980; Saif *et al*., 1985; Reynolds & Saif, 1986; Reynolds *et al*., 1987a; Reynolds *et al*., 1987b; Thouvenelle *et al*., 1995a; Thouvenelle *et al*., 1995b). More recently TAstV-2 has been associated with PEMS and isolated from non-intestinal tissues (Koci *et al*., 2000b; Schultz-Cherry *et al*., 2000). ANV was described to cause sub-clinical pathologic changes to the kidneys of infected chicks (Yamaguchi *et al*., 1979), while DAstV infection can cause a fatal hepatitis in ducklings (Asplin, 1965b; Gough *et al*., 1985). Each of these viruses can be cultured in embryonated eggs, which makes studying the AAstVs the most promising model for unlocking some of the unknowns about

Astroviridae (Asplin, 1965b; Yamaguchi *et al*., 1979; Imada *et al*., 1982; Woolcock & Fabricant, 1991; Jordan & Pattison, 1996; Imada *et al*., 2000; Koci *et al*., 2000b).

Genomic alignments of the three AAstVs completely sequenced suggest there is far less conservation in nucleotide sequence than that detected among the MAstVs. This may change as new AAstVs are isolated and characterized. Until recently, there has not been an active, ongoing, survey for poultry flocks for astroviruses. This is partly due to a lack of tools specific for the detection of astroviruses. It is possible that more diseases of poultry, currently attributed to picornaviruses (enteroviruses), will be determined to be due to astroviruses. This requires efforts by other groups to add to the AAstV sequence database. The poultry field is in a position to greatly impact our overall knowledge of astroviruses and our understanding of basic virology.

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Table 3.1. Comparison of the nucleotide lengths of the AAstV genome regions.

	Number of nucleotides in					
Avian astrovirus	5' UTR	ORF 1a	ORF 1b	ORF 2	3' UTR	Total[a]
ANV	14	3012	1527	2052	305	6927
TAstV-2	21	3378	1584	2175	196	7325
TAstV-1	11	3300	1539	2016	130	7003nt

[a]excluding the poly-A tail

Table 3.2. Comparison of morphologic and biochemical properties of the small round virus families

Family	Diameter (nm)	Surface Sturcture	Chloroform Resistance	Stable at 60°C for 10 min	Number and size of expected proteins	Bouyant Density (g/ml))	Genome
Circovirus	12-26	None	Yes	Yes	1 capsid protein in some 50-36kDa 3 proteins in others 15-26kDa	1.33-1.37	Circular –ssDNA 1.7-2.3kb
Parvovirus	18-26	None	Yes	Yes	2 to 4 major capsid proteins VP1: 96-80kDa VP2: 85-64kDa VP3: 75-60kDa VP4: 52-49kDa	1.39-1.42	Linear ssDNA 4-6kb
Picornavirus	28-30	None	Yes	Some strains	4 capsid VP1,2,3: 41-24kDa VP4: 13.5-5.5kDa VPg: 2.4kDa	1.33-1.45	Linear +ssRNA 7-8kb
Calicivirus	30-38	Cup-shaped depressions (Not seen in Norwalk viurs)	yes	Some strains	1 major capsid protein 71-59kDa 1 minor protein in some viruses 30-28kDa VPg: 15-10kDa	1.33-1.40	Linear +ssRNA 7.4kb-7.7kb
Astrovirus	28-30nm	5-6 pointed star (only seen in ~10% of virions)	Yes	Yes	At least 2 major proteins maybe 3 39-29kDa possible smaller proteins 36-13kDa	1.36-1.39	Linear +ssRNA 7.2-7.9kb

Table 3.3: Genus and species described in the family Astroviridae.

Astrovirus species	Abbreviation
Mammalian astrovirus	
Bovine astrovirus	BAstV
Bovine astrovirus 1	BAstV-1
Bovine astrovirus 2	BAstV-2
Feline astrovirus	FAstV
Feline astrovirus 1	FAstV-1
Human astrovirus	HAstV
Human astrovirus 1	HAstV-1
Human astrovirus 2	HAstV-2
Human astrovirus 3	HAstV-3
Human astrovirus 4	HAstV-4
Human astrovirus 5	HAstV-5
Human astrovirus 6	HAstV-6
Human astrovirus 7	HAstV-7
Human astrovirus 8	HAstV-8
Ovine astrovirus	OAstV
Ovine astrovirus 1	OAstV-1
Porcine astrovirus	PAstV
Porcine astrovirus 1	PAstV-1
Avian astrovirus	
Duck astrovirus	DAstV
Duck astrovirus 1	DAstV-1
Turkey astrovirus	TAstV
Turkey astrovirus 1	TAstV-1
Turkey astrovirus 2	TAstV-2
Chicken astrovirus	CAstV
Avian nephritis virus	ANV

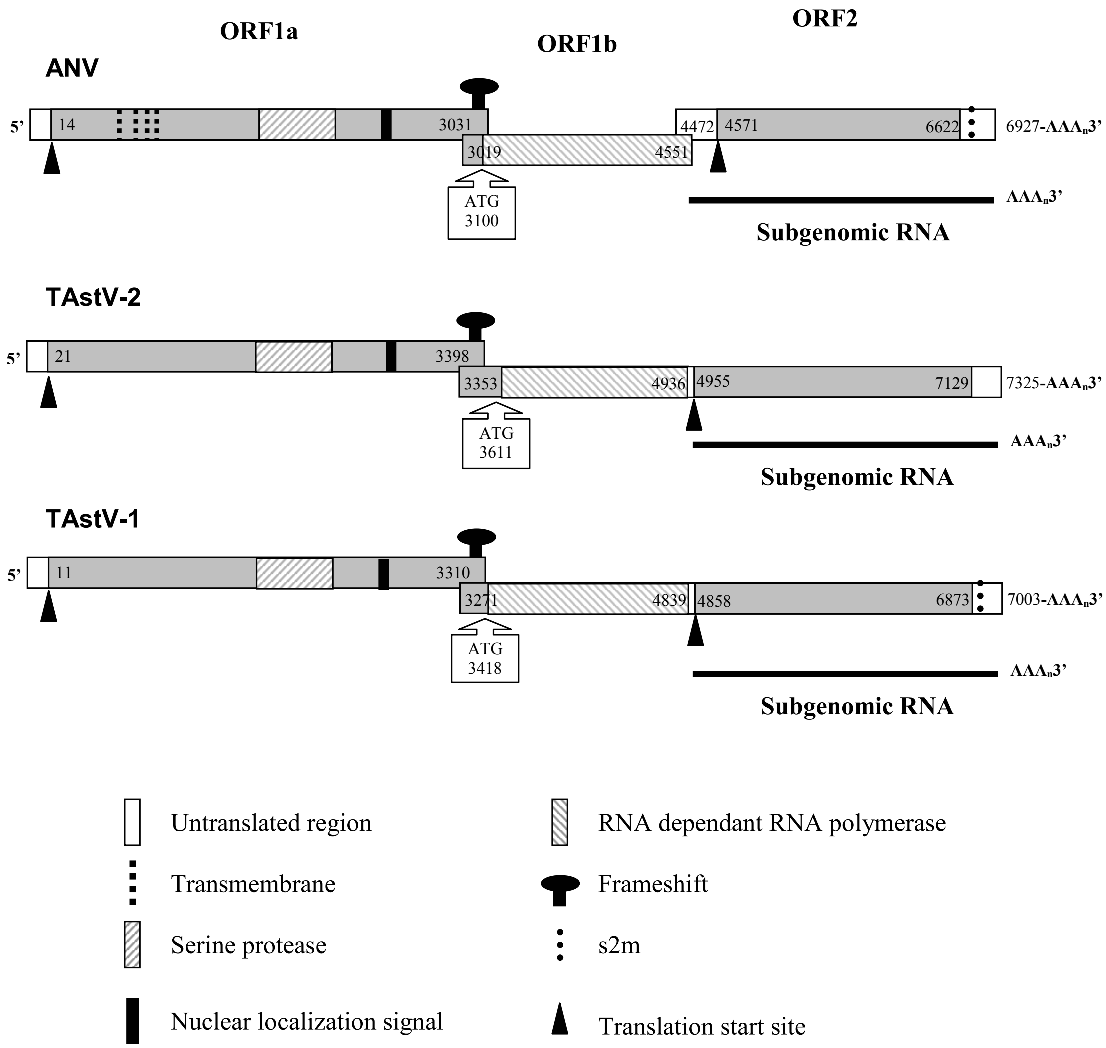

Figure 3.1. Diagram of the AAstV genome organization and predicted amino acid motifs. The nucleotide positions of the start and stop of each open reading frame are shown relative to the beginning of the genome. The first ATG site of ORF1b is indicated by the arrowed text box.

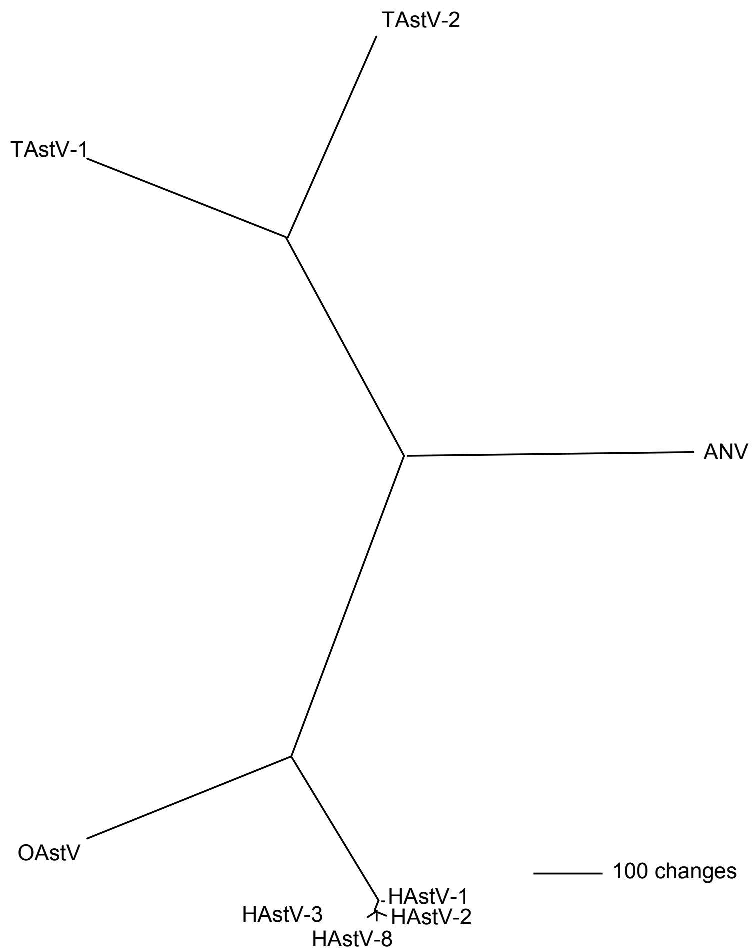

Figure 3.2. Phylogenetic analysis of astrovirus ORF1a. The predicted amino acid sequence of ORF1a from HAstV-1 (accession number NC_001943), HAstV-2 (accession number L13745), HAstV-3 (accession number AF141381), HAstV-8 (accession number AF260508), OAstV (accession number NC_002469), ANV, TAstV-1, and TAstV-2 were aligned using DNASTAR (Madison WI). An unrooted heuristic search was completed using PHYLIP.

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TAstV-2 T A E H V V Q G S D (20) I F E S V D N V A V (50) N N S F D T K F G N 697
TAstV-1 T A G H V V Q G S K (20) L F E C V D T L V E (50) F N S F N T Q F G N 647
ANV     T A G H V V G E A K (20) L P L F T D T L A R (50) N A P F E T Y A G T 621
HAstV-1 T A A H V V G N N T (16) Y M - P E K D I A F (48) S Y A V R T Q D G M 551
OAstV   T S K H V V G S D D (16) Y R H P T K D I A L (49) T Y S V A T R N G M 544

TAstV-2 S G A P Y C D H D G      R L V G I H L G T Q                       717
TAstV-1 S G A P Y V D S D G      R L V G M H L G S Q                       667
ANV     S G S P I I N R D G      R M L G V H F G S N                       641
HAstV-1 S G A P V C D K Y G      R V L A V H Q T N T                       571
OAstV   S G A P I T T V D G      R V I A V H Q T N T                       564
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Figure 3.3. Alignment of the putative astrovirus serine protease. The predicted aa sequence of the serine protease from ORF1a of TAstV-1, TAstV-2, ANV, HAstV-1, and OAstV were analyzed using DNASTAR. Residues in bold are conserved in all 5 sequences. The suspected catalytic triad for each virus is underlined. Numbers in parenthesis's are number of residues not shown. Numbers at the end of each row are aa positions from the N-terminus.

A

	G	U	U	C	A	A	A	A	A	A	C	U	A	A	A	U	G	A	G	C	Consensus
										10										20	
3005	A	G	U	C	A	A	A	A	A	A	C	U	A	A	A	U	G	**A**	**G**	**C**	ANV
3364	G	U	C	A	A	A	A	A	A	A	C	U	A	A	U	A	G	**A**	**G**	**G**	TAstV-2
3287	G	U	U	C	A	A	A	A	A	A	C	U	A	G	A	U	A	G	U	**C**	TAstV-1
	V	C	C	C	U	G	H	G	V	C	A	A	G	C	A	A	G	G	C	R	Consensus
										30										40	
3026	**C**	**C**	**C**	**C**	**U**	**U**	**C**	**G**	**G**	**G**	**G**	**G**	**G**	**C**	**U**	**A**	**C**	**A**	**C**	**A**	ANV
3385	**G**	**G**	**C**	**C**	**U**	**G**	**U**	**G**	**A**	**C**	**A**	**A**	**C**	**A**	**A**	**A**	**G**	**G**	**C**	**-**	TAstV-2
3308	**A**	**C**	**C**	**C**	**U**	**G**	**A**	**G**	**C**	**C**	**A**	**A**	**G**	**C**	**A**	**G**	**G**	**G**	**U**	**G**	TAstV-1
	C	Y	C	U	A	B	C	C	C	C	G	U	V	C	H	U	B	W	U	D	Consensus
										50										60	
3047	C	-	C	U	G	U	C	C	C	U	G	A	C	C	A	U	C	U	U	A	ANV
3406	**C**	**C**	**C**	**U**	A	C	C	C	C	C	G	U	A	C	C	A	G	A	U	U	TAstV-2
3329	U	U	C	C	A	G	C	C	U	C	U	U	G	A	U	UU	-	U	G		TAstV-1

B

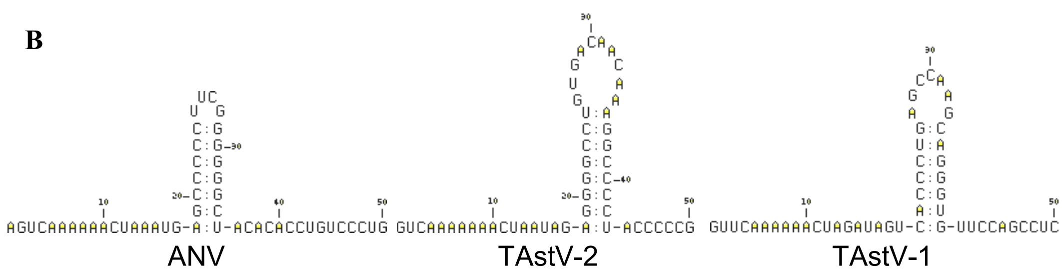

Figure 3.4 Analysis of the heptameric shift sequence and predicted frameshift structures of the AAstVs. A: The predicted frameshift sequences of ANV, TAstV-1, and TAstV-2 were analyzed using DNASTAR. The heptameric "slippery sequence" is outlined by a black box. Nucleotides shown in bold are predicted to be part of the retrovirus-like frameshift structure. B: The RNA secondary structure of ANV, TAstV-1, and TAstV-2 was predicted using RNAfold (Scientific & Educational Software).

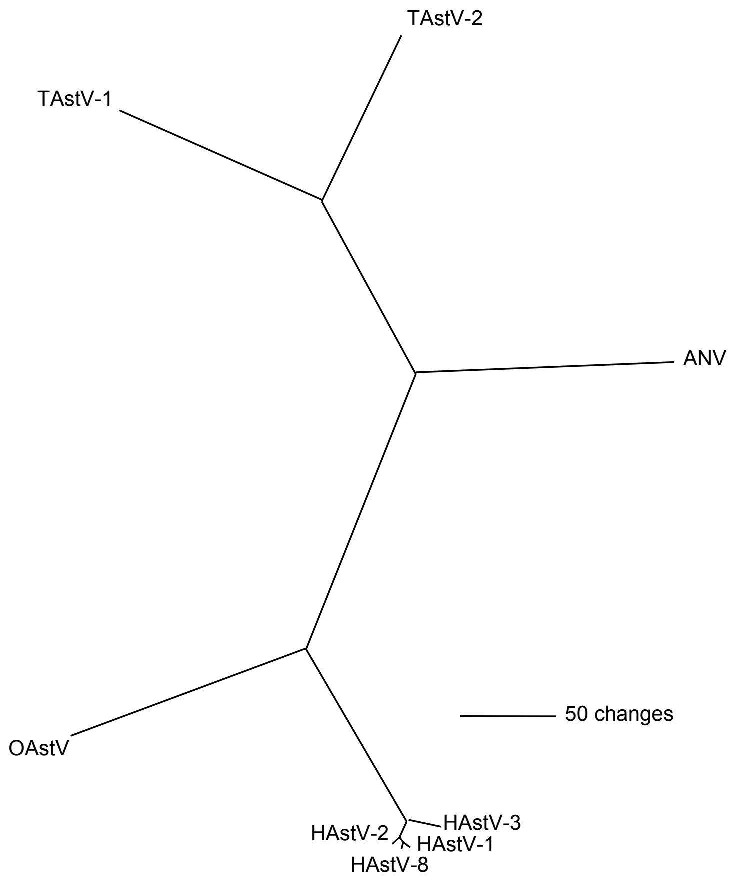

Figure 3.5. Phylogenetic analysis of astrovirus ORF1b. The predicted amino acid sequence of ORF1b from HAstV-1, HAstV-3, HAstV-8, OAstV, ANV, TAstV-1, and TAstV-2 were aligned using DNASTAR (Madison WI). An unrooted heuristic search was completed using PHYLIP.

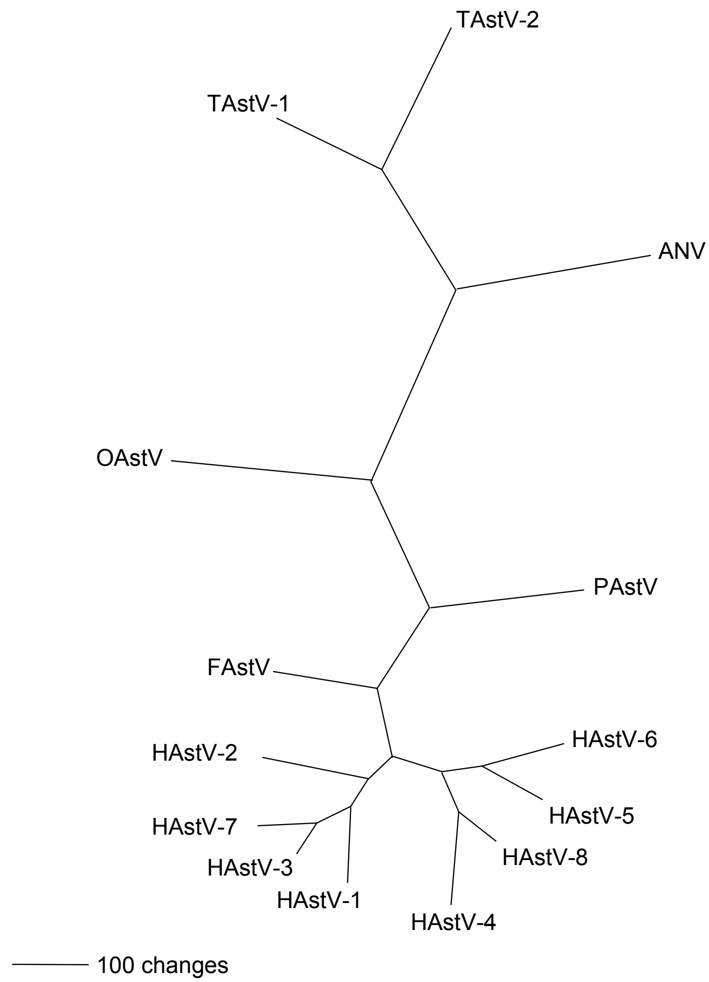

Figure 3.6. Phylogenetic analysis of astrovirus ORF2. The complete predicted aa sequence of HAstV-1, HAstV-2, HAstV-3, HAstV-4 (accession number AB025801), HAstV-5 (accession number AB037274), HAstV-6 (accession number Z46658), HAstV-7 (accession number AF248738), HAstV-8, OAstV, FAstV (accession number AF056197), PAstV (accession number Y15938), ANV, TAstV-1, and TAstV-2 were aligned using DNASTAR (Madison WI). An unrooted heuristic search was completed using PHYLIP.

```
ANV       K K L N E P P S G G   - - Y T P V P D H L   R W N N W Q I Y M E   P L D L R I T V P E   38
TAstV-2   K K L I E G P V T T   K A P T P V P D W L   K I F A W E D D I L   P P E G K T A L P E   40
TAstV-12  K K L D S H P E P S   R V F Q P L D F G L   G I F D W R F D L Q   P I R H H V A V P M   40

ANV       N Y P I L G H I A I   D K L V E R K K K V   N D P L L K M L E Q   P K C E G F T S T T   78
TAstV-2   N V T L I G H I P V   D K L V S R T K K V   Q D P L L G L V T P   W K Q D M Y D S T T   80
TAstV-1   N V E V L G Y I P V   D R L V E R R N V I   T D P L L K L V E P   W R Q E T Y G P A V   80

ANV       W T R K A Y T K S F   E K F D Y G D A V D   F V Q D Y P E L T A   F A D A A V L A E V   118
TAstV-2   W T V K A Y T K M F   E K F H Y H D P V D   F V E Q Y A E F V L   L C D N M V L R E H   120
TAstV-12  W T I K A Y N K M F   E K F F Y S E P L E   F A Q L D S S I L N   L A D S Y C L Q E H   120

ANV       G Y M E G T H V I P   I Q E T S K N M D S   T P A F P K M L D F   D S E R D Y L E A H   158
TAstV-2   D Y M A N S N I T P   I M S T E K N V N S   T P A Y P K F Q A Y   D S E A E Y L E D C   160
TAstV-1   D Y M S G S Q I V P   I T S T E K N L D S   T P G Y P K F K V F   S T E R E Y L S T C   160

ANV       G M K E Y I D T - Q   L G V Q S G - - - K   P L W W C F L K N E   I L K E K K V S E D   194
TAstV-2   G W Q E Y L D V - -   V S D P E T I N R R   P L W W C F L K N E   V L K R E K I E D S   198
TAstV-12  G W D E Y K T V W Q   V G P R E - - - - K   P L W W C F L K T E   V L K L A K I E Q D   196

ANV       D I R I I T C S D P   V I T R L G A S F D   S E Q N E R M K E R   T E T H H A Q V G W   234
TAstV-2   D I R M I L C T D P   I F T R I G A M F E   Q D Q N N R M K Q Q   T E I R S A Q V G W   238
TAstV-1   D I R M I L C T D P   V F T R I G A A F E   Q H Q N S L M K L E   T E N H H A Q V G W   236

ANV       T P F F G G L D K R   V R R I T S C G R T   Q V L E L D W T R F   D G T I P V Q L F Q   274
TAstV-2   T P F F G G L D R R   V R R L Y G D G D R   Y F V E M D W T R Y   D G T I P K S L F W   278
TAstV-1   S P F F G G I H R R   A T R L Y G E - H R   Y Y V E L D W T R F   D G T I P P E L F R   275

ANV       R M R E L R K F F L   - - - - - T R R S R   R R Y G K L L D W Y   N A Q L T D R I T L   309
TAstV-2   R I R Q I R F F F L   H D S H K T P K M R   R L Y - - - - N W Y   V K N L L E K I I L   314
TAstV-12  R I K L M R F F L L   D P K Y K T P E N R   D R Y - - - - N W Y   V E N L I D K V V L   311

ANV       L P T G E V T H V K   K G N P S G Q F S T   T V D N N L V N E W   L T A F E F G Y Q H   349
TAstV-2   L P T G E V C Q V K   K G N P S G Q F S T   T V D N N M I N V W   L T T F E V S Y L F   354
TAstV-12  L P T G E V C K I Y   G G N P S G Q F S T   T V D N N F V N V W   L T V F E L A Y L F   351

ANV       L E N H G I I P T V   R D Y R A N V D F L   C Y G D D R L L A F   N P S F V N - Y D P   388
TAstV-2   F K Q R G R L P T E   K E L Q E N C S M I   C Y G D D R L L S I   R K G F V E - Y E P   393
TAstV-1   Y K E H N R L P T I   C E I K K H T D W I   C Y G D D R L L A V   D K R F I N S Y D T   391

ANV       Q V T I D M Y K N I   F G M W V K P E N I   K L F D S P T G S S   F C G F T L V K P H   428
TAstV-2   D T V I D M Y K N I   F G M W V K R N N I   K I Q D T P E G L S   F C G L T I V K S S   433
TAstV-1   A A V I A M Y K D V   F G M W V K P D N I   K V F P S L E G V S   F C G M V W T K R K   431

ANV       - G Q W V G V V N V   N K L L Q S L K T P   T R R L P D L E S L   W G K L V S L K I M   467
TAstV-2   T G A Y V G V P N V   N K I L S T L E N P   V R R L P D V E S L   W G K L V S L R I L   473
TAstV-1   - G Q Y V G K P N V   D K I L S T L S D P   V S R L P D I Q S L   W G K L V S L R L L   470

ANV       C Y H S D P E A V S   Y L S N Q I R R V E   E Y A R A E G I E L   P E V G P D F Y R K   507
TAstV-2   C E N A P S N V K H   F L D E Q I S N V E   E F A A R E N I Q L   P E V G P D F Y S R   513
TAstV-1   C E N E S D E V V D   Y L D K Q I E S V S   R H A K E A G I A L   P K I G P D F Y A E   510

ANV       I W .                                                                                   510
TAstV-2   I W .                                                                                   516
TAstV-1   I W .                                                                                   513
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Figure 3.7. Amino acid alignment of AAstV ORF1b. The aa sequence of ORF1b of TAstV-1, TAstV-2, and ANV were analyzed using DNASTAR. Positions which are conserved in all three sequences are shown in bold. Regions of ORF1b with 6 or more consecutive conserved residues, which could be potential degenerate priming sites, are underlined.

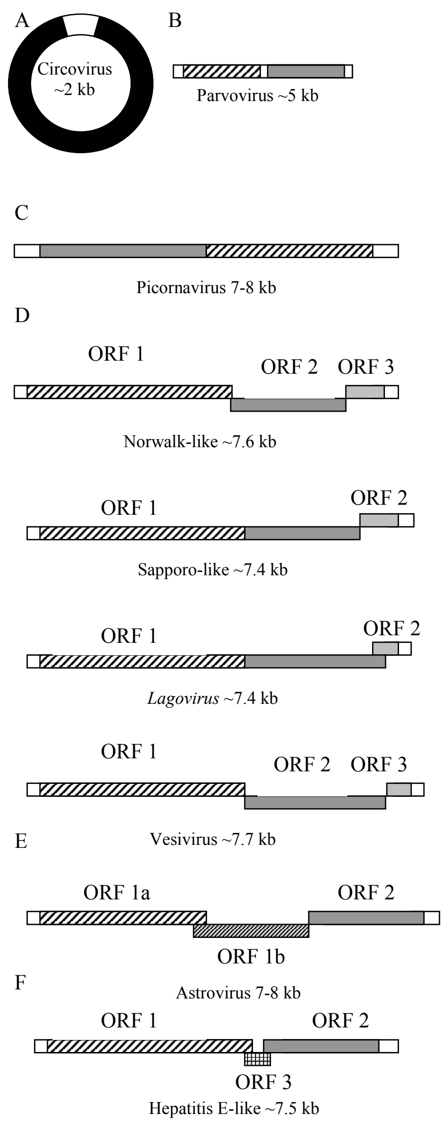

Figure 3.8. Diagram of basic small round virus genome organization. A, circovirus; B, parvovirus; C, picornavirus; D, calicivirus; E, astrovirus; F, hepatitis–like virus. Untranslated regions are shown as unshaded regions. Nonstructural proteins are indicated with diagonal lines. Structural proteins are shown with solid shading. Unknown reading frames are indicated by cross hatching.

CHAPTER 4

ASTROVIRUS INDUCES DIARRHEA IN THE ABSENCE OF INFLAMMATION AND CELL DEATH[1]

[1]Koci, M. D., L. A. Moser, L. A. Kelley, D. L. Larsen, C. C. Brown, and S. Schultz-Cherry. 2003. *Journal of Virology*, In Press.
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ABSTRACT

Astroviruses are a leading cause of infantile viral gastroenteritis worldwide. Very little is known about the mechanisms of astrovirus-induced diarrhea. One reason for this is the lack of a small animal model. Recently, we isolated a novel strain of astrovirus (TAstV-2) from turkeys with the emerging infectious disease, Poult Enteritis Mortality Syndrome (PEMS). In the present studies, we demonstrate that TAstV-2 causes growth depression, decreased thymic size, and enteric infection in infected turkeys. Infectious TAstV-2 can be recovered from multiple tissues, including the blood, suggesting there is a viremic stage during infection. In spite of the severe diarrhea, histopathologic changes in the intestine were mild and there was a surprising lack of inflammation. This may be due to the increased activation of the potent immunosuppressive cytokine, transforming growth factor-β (TGF-β) during astrovirus infection. These studies suggest that the turkey will be a useful small animal model to study astrovirus pathogenesis and immunity.

INTRODUCTION

Astroviruses are small round, non-enveloped viruses, typically 28-30 nm in diameter (33). The name astrovirus comes from "astron" (Greek for star) describing the characteristic five- or six-pointed star-like surface projections detected by negative stained electron microscopy (EM). Astroviruses were first observed in 1975 in association with outbreaks of gastroenteritis in infants (2, 31). Since then, astroviruses are known to be one of the leading causes of infantile viral gastroenteritis worldwide (15, 16). A longitudinal study in rural Mexico found that astrovirus was the most common cause of infantile gastroenteritis, suggesting the burden of astrovirus disease in developing countries may be especially high (32). Astrovirus is an endemic cause of diarrhea in infants, but is also capable of causing outbreaks in day care centers, hospitals, and other institutions (14, 17, 39-41, 55). Astrovirus-induced gastroenteritis has also been reported in association with food-related illnesses in the United States (35, 36) and is an important cause of gastroenteritis in immunocompromised individuals (5, 9, 10, 18, 27, 66, 70).

Astroviridae family members have been described to cause diarrhea and enteritis in several mammalian and avian hosts (13, 19, 31, 34, 58, 64, 67). Unfortunately very little is known about astrovirus pathogenesis, or the host factors involved in viral clearance and disease resolution. Of the non-human astroviruses, only the bovine, ovine, and turkey astroviruses have been studied experimentally (47, 48, 57, 58, 62, 63, 67-69). Although limited, these studies have generated some insight into the potential mechanisms of astrovirus infection and disease. In gnotobiotic lambs, astrovirus infected the mature enterocytes of the small intestine and infected cells were sloughed and replaced by cuboidal epithelial cells (57). The resulting slight villous atrophy was transient, lasting less than 5 days, and resembled a mild rotavirus infection. Unlike gnotobiotic lambs, which exhibited diarrhea, gnotobiotic calves infected with bovine astrovirus alone showed no illness (69). However, a unique type of infection was observed. Astrovirus appeared to target the M cells overlying jejunal and ileal Peyer's patches and mononuclear inflammatory cells and eosinophils were observed atop the infected dome epithelial cells (69). In turkeys, astrovirus infection can be accompanied by a moderate increase in mortality (24, 34, 46, 47). Limited studies demonstrated that astrovirus infection causes only mild histopathology while inducing severe osmotic diarrhea (62, 63).

Although important data was gleaned from these animal experiments, significant questions remain unanswered. The gap in our understanding in astrovirus biology is partly due to the fact that none of the previous systems were fully developed into an animal model. Prior studies did not address the kinetics of astrovirus replication, the cells supporting infection, the effect of infection on cell death, or the host response to astrovirus infection. Recently, we isolated and characterized a genetically and immunologically distinct turkey astrovirus strain, turkey astrovirus type 2/North Carolina/034/1999 (TAstV-2), associated with an emerging disease in turkeys (4, 22-24, 53, 54). In the present studies we describe an *in ovo* system to culture virus and report the pathogenesis of TAstV-2 in infected turkey poults and embryos, including clinical disease and viral localization. These are the first studies, to our knowledge, to evaluate viremia, apoptosis, and the immunomodulatory cytokine transforming growth factor-β (TGF-β) during astrovirus infection. Results from these studies suggest the turkey will be an important animal model for understanding the mechanism of astrovirus pathogenesis.

MATERIALS AND METHODS

TAstV-2 Propagation. TAstV-2 was isolated and propagated as described (24, 54). Briefly, the thymus or intestines from infected turkey poults were homogenized, 0.2 µm filtered, and inoculated into the yolk sac of 20-day-old specific pathogen-free (SPF) turkey embryos (from a closed flock of Small Beltsville White turkeys housed at Southeast Poultry Research Laboratory). Viral replication in embryo intestines was monitored by *in situ* hybridization at 1, 3, and 5 days post-inoculation (dpi). Virus was harvested at 5 dpi. Intestines were removed, homogenized, 0.2 µm filtered and centrifuged at 150 x g for 10 min. Additionally, embryo intestinal fluid was collected separately, 0.2 µm filtered and centrifuged at 500 x g for 10 min.

RNA Isolation and RT-PCR. Total RNA was isolated from purified virus, embryo intestines, or from tissues excised from experimentally-inoculated or control turkeys using Trizol™ following manufacturer instructions (Invitrogen, Carlsbad CA). RT-PCR was performed as previously described (23).

TAstV-2 Quantitation. Viral load was assessed by developing a TAstV-2-specific competitive quantitative RT-PCR (CQ RT-PCR) system. Briefly, total RNA, isolated from 100 µl of infectious material, was analyzed by one-step RT-PCR (Qiagen, Valencia CA) in the presence of a competitor RNA (cRNA). The cRNA was generated by modifying a plasmid (pTAstVpol18) which contains nucleotides 2863 to 5296 of the TAstV-2 genome. pTAstVpol 18 was digested with *Sca* I following the manufacturer's instructions (Invitrogen), then two 30bp randomly generated oligonucleotides were ligated to the cut plasmid to generate a construct with TAstV-2 pol gene with 60bp of additional sequence (pTAstVpolC). This new construct was then digested with *Sst* I and *Not* I following the manufacturer's instructions (Invitrogen) and ligated into the corresponding sites in pGEM T-Easy vector (Promega, Madison WI). This final construct pTAstVpolCQ, was then used to generate positive sense cRNA using the RNA polymerase SP6 (Roche Molecular, Indianapolis IN). cRNA was purified, and copy numbers quantitated using spectrophotometry as described (50). TAstV-2 polymerase gene specific primers, flanking the modified region in pTAstVpolCQ, were designed {CQ RT-PCR Fwd (CCATGATATGCTACGGGGAT) and CQ RT-PCR Rev (GACTCAACATCTGGTAGCCT)}. Sample RNA was added at a uniform concentration to each tube of a serial log dilution of cRNA, and amplified under the following conditions; 50º C for 30 min, 95º C for 15 min, 30 cycles of 94º C for 30 s, 55º C for 30 s, 72º C for 30 s, and final 72º C extension for 1 min, using the Qiagen OneStep RT-PCR Kit (Qiagen, Valencia CA) in a total reaction volume of 25 μl. Products were then separated by electrophoresis in an agarose gel and the amplification products visualized with ethidium bromide. The copy numbers of viral RNA in the sample/ml were calculated using Kodak Imaging Software densitometry and plotting against the standard curve of the competitor as previously described (12).

Animals. Two-day-old unvaccinated British United Turkey of America poults (male and female) were obtained from a commercial hatchery. Control and infected poults were housed in separate BL2 containment facilities in individual Horsfall units with HEPA filtered inlet and exhaust air valves. Birds were fed routine turkey starter from the

University of Georgia and given free access to clean water. After a brief acclimation period, five-day-old poults were weighed (day 0) and randomly assigned to either a control group or a group infected with astrovirus (n = 60 per group). Poults were orally inoculated with ~10^6 genomic units of astrovirus in 200 µl total volume, or phosphate buffered saline (PBS) alone. Birds were monitored daily for signs of clinical disease and weighed on 0, 3, 5, 9, and 12 dpi. On days 1, 2, 3, 4, 5, 7, 9, and 12 pi, five random poults per group were euthanized by cervical dislocation and the small intestine, bursa, spleen, pancreas, thymus, liver, kidney, bone marrow, skeletal muscle (breast), feces and blood were collected. All tissues were stored at -70^{o} C or placed in 10% phosphate-buffered formalin. Blood was collected in syringes containing heparin, incubated overnight at 4^{o} C and then separated into red cell, lymphocyte, and plasma fractions using Histopaque 1077 (Sigma Chemicals, St. Louis MO). The bursa, spleen, and thymus from each group were weighed to the nearest milligram prior to processing.

To perform RT-PCR analysis and virus isolation studies, the individual tissues at each time point were pooled, homogenized, and aliquoted for RNA isolation using Trizol™ or inoculation into 20-day-of-age turkey embryos. The animal experiments were repeated five times with different groups of poults with similar results. All animal experiments were approved by the USDA Animal Care and Use Committee and complied with all federal guidelines.

In situ Hybridization. The TAstV-2-specific riboprobe was generated as described (4). Briefly, TAstV-2 plasmid p25.5 containing a 1.5 kb segment of the extreme 3' end of the TAstV-2 genome (24) was digested with *BamH* I and transcribed with T7 RNA polymerase and digoxigenin labeled UTP (Roche Molecular), creating an antisense riboprobe of approximately 1.6 kb in length. Digoxigenin incorporation was verified by dot-blot. *In situ* hybridization was performed according to previously described techniques (8). Briefly, tissue sections were deparaffinized with Citrisolv (Fisher Scientific, Norcross GA), digested with 35 μg/ml Proteinase K for 15 min at 37^{o} C, and hybridized overnight at 42^{o} C, using approximately 35 ng of digoxigenin-labeled riboprobe per slide in 5X standard sodium citrate (SSC), 50% formamide, 5% modified milk protein (Roche Molecular), 1% N-lauroylsarcosine, and 0.02% SDS. The following day, slides were washed in increasingly stringent solutions – 2X SSC with 1% SDS for 30 min at 50º C, 1X SSC with 0.1% SDS for 30 min at 50º, 1X SSC for 15 min three times at room temperature, and 0.1XSSC for 15 min at room temperature. After the post-hybridization washes, sections were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular) for 2 hr at 37^{o} C and developed with nitroblue tetrazolium and bromcresylindolyl phosphate for 1 to 3 hr. Sections were counter-stained lightly with hematoxylin and coverslipped with Permount for a permanent record. Each group of slides was processed with a positive control tissue consisting of a section of positive embryo intestine, and negative control sections from uninfected poults.

Histopathology. Tissues from control and infected poults were fixed in 10% phosphate-buffered formalin overnight, then processed, embedded, sectioned (0.3 μm), and stained with hematoxylin and eosin and examined by light microscopy.

Detection of TAstV-2 Antigen by Immunofluorescence. The distribution of TAstV-2 was monitored using a rabbit polyclonal antibody generated to a peptide sequence in the TAstV-2 capsid protein (K_{676} – R_{691}) (ResGen, Carlsbad CA), accession# AAF18464. Briefly, tissue sections from turkeys sacrificed at 1, 2, 3, 4, 5, 7, 9, and 12 days post-inoculation (dpi) were processed as described above, deparaffinized with Citrisolv, antigenic sites exposed by microwaving the tissues for 5 min in a citrate buffer, then incubated with primary antibody diluted 1:500 in phosphate buffered saline containing 0.1% Tween-20 (PBST) overnight at 4^{o} C. After incubation in primary antibody, the slides were washed in PBST, incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame CA) for 30 min at room temperature (RT), washed in PBST, then incubated with a Alexa488-streptavidin-labeled antibody (Molecular Probes, Eugene OR) diluted 1:200 in PBST for 1 hr at RT. Slides were mounted in PBS+glycerol and fluorescence was examined on a motorized Zeiss Axioplan IIi equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/FITC/Texas Red) emission cube, and a Zeiss AxioCam B&W CCD camera. Fluorescence images were pseudocolored, and merged using OpenLabs 3.0 software (Improvision Inc., Lexington MA).

Co-Localization of TAstV-2 Antigen and Apoptosis. To determine if TAstV-2 induced cell death, intestinal sections from control or TAstV-2-infected turkey poults were deparaffinized and antigenic sites exposed as described above, then incubated with terminal deoxynucleotide transferase labeled with tetramethylrhodamine red fluorescence (*In situ* End Labeling TUNEL analysis, Roche Molecular) for 1 hr at 37^{0} C following manufacturer's instructions. Immediately following TUNEL staining, the sections were washed three times with PBST and stained for TAstV-2 as described above.

TGF-B Analysis.

NRK Soft Agar Assay.

TGF-B activity was assessed by determining the colony forming activity of normal rat kidney cells (NRK-49, CRL-1570, American Type Culture Collections , Manassas VA) in the presence of epidermal growth factor (EGF) in soft agar, as described previously (51). Briefly, 5% Noble agar (Difco) was diluted 10-fold in 5% calf serum (CS, Fisher Scientific) in Dulbecco's modified eagle media (DMEM), and 0.5 ml of this 0.5% agar dilution was added per well to a 24-well tissue culture plate and allowed to solidify. 100 μl of serum from PBS inoculated controls or TAstV-2 inoculated birds taken at days 1, 3, 5, and 12 pi containing EGF (1 ng, EMD Biosciences, San Diego CA) was combined with 0.6 ml of 0.5% agar and 0.2 ml (2 x 10^3 cells) of an NRK suspension in 5% CS in DMEM, and 0.5 ml of this 0.3% agar sample solution was added to the cooled base layer. The samples were incubated for 3-5 days at 37°C in 5% CO_2, and the total number of colonies greater than 62 μm was quantified with an inverted microscope. Colony formation is indicative of the presence of activated TGF-B (51). All conditions were performed in triplicate.

Mink Lung PAI Luciferase Assay.

Mink lung fibroblasts (Mv1Lu) stably transfected with the TGF-β responsive plasminogen activator inhibitor (PAI) luciferase reporter (Mv1Lu-PAI) were a kind gift from Dr. Daniel Rifkin (New York University) and used to assay for TGF-β activity as described (1). Briefly, Mv1Lu-PAI cells were plated at 1.6 x10^4 cells per well in a 96-well tissue culture plate and allowed to attach for 7 hr. After the attachment period, cells were cultured for 16 to 18hr at 37°C in 5% CO_2 in 100 μl/well of DMEM + 0.1% bovine serum albumin (BSA, Invitrogen) containing; recombinant active TGF-β_1 (6.25 pM, R&D Systems, Minneapolis MN), 321.25 μg of protein from homogenized embryo intestinal filtrate from PBS or TAstV-2-infected embryos 5 dpi, or embryo intestinal filtrate pre-incubated (40 min at RT) with a polyclonal anti-TGF-β neutralizing antibody

(Clone 1D11 R&D Systems). Cells were then lysed and luciferase activity was assessed using the Promega Luciferase System (Promega) and the Turner Luminometer (Turner Biosystems, Sunnyvale CA).

Statistics. Data comparing body weights and lymphoid organ weights were analyzed by one-way analysis of variance (ANOVA) and pairwise multiple comparison using the Student Newman-Keuls method (SigmaStat, Jandel Scientific, San Rafael, CA). Significance level was defined at $P \leq 0.05$.

RESULTS

Propagation of TAstV-2 in Embryos.

Attempts to propagate TAstV-2 in cell culture using primary turkey embryo fibroblast, turkey embryo kidney cells, chicken embryo fibroblast, chicken embryo kidney cells, African Green Monkey kidney cells (Vero), mink lung epithelial cells (Mv1Lu), Madin-Darby canine and bovine kidney cells (MDCK and MDBK), a human colorectal adenocarcinoma cell line (Caco-2), and an ileocecal colorectal adenocarcinoma cell line (HCT-8) were unsuccessful. Therefore, specific pathogen-free (SPF) turkey embryos at 20 embryonic days of age were inoculated with a tissue filtrate prepared from healthy or TAstV-2-infected turkey poults and incubated for 1, 3 or 5 days at 39^{o} C. Intestines were removed and tested for TAstV-2 RNA and replication by RT-PCR and *in situ* hybridization respectively. RT-PCR analysis on embryo intestines was positive for TAstV-2 at days 1 through 5 post inoculation. *In situ* hybridization showed extensive viral replication within 1 dpi. TAstV-2 replication increased until 3 dpi (Fig. 4.1 A) and then began to decrease by 5 dpi. No TAstV-2 *in situ* staining was detected in the control embryos (Fig 4.1 B). Interestingly at 5 dpi, TAstV-2-infected embryo intestines were enlarged, thin-walled, and distended. An immense accumulation of intestinal fluid was also observed in the intestines of TAstV-2-infected embryos (Fig. 4.1 C) but not the controls (Fig. 4.1 D). These results demonstrate that turkey embryos support TAstV-2 replication and are a valuable source for *in vitro* propagation.

TAstV-2-Induced Disease.

Clinical Signs and Gross Lesions

Inoculation of naïve poults with 10^6 genomic units of TAstV-2 resulted in 100% of the infected birds developing diarrhea within 24 hrs of challenge that continued throughout the course of the 12 day experiment (Fig. 4.2 A). Diarrhea was watery, yellow, frothy, mucus-filled, but did not contain undigested food or blood. Control animals had no diarrhea. In addition to the diarrhea, infected birds exhibited statistically significant growth depression as compared to uninfected controls (Fig. 4.2 A, $p<0.05$). At 5 dpi, there was a ~27% difference in the growth, and a 38% difference by 12 dpi (Fig. 4.2 A). The TAstV-2-infected birds remained smaller throughout experiments extended to 28 dpi (data not shown).

Upon necropsy, the intestines of infected poults were distended, dilated, and gas-filled. The intestines appeared to be three to five times the size of those of the non-infected controls. In addition to the macroscopic changes seen in the intestines, we noted that the bursa and thymus, and to a lesser extent the spleens, of the infected animals appeared reduced in size. To examine this further, these organs were removed, weighed, and compared to those of the mock-infected poults. Birds infected with TAstV-2 had a statistically significant decrease in the size of the thymus beginning 3 dpi and continuing through 9 dpi (Fig. 4.2 B, $p<0.05$). Calculating the differences as a ratio of organ weight to body weight we found, at 3 dpi, the thymus of the TAstV-2-infected group was 36% smaller than the control group and 52% smaller at 9 dpi. However, by 12 dpi, there was no difference in the relative thymic size suggesting these changes were transient. There were no statistically significant differences in the sizes of the bursa or spleen as compared to controls.

Histopathological Lesions

To investigate the histologic changes resulting from TAstV-2 infection, tissues were examined by routine hematoxylin and eosin staining and light microscopy. In spite of the severe diarrhea, the intestinal lesions were mild. By 2 dpi, there were scattered single degenerating villous epithelial cells, predominantly in the basal portions of the villi (Fig. 4.3 A). These degenerating cells were present through 9 dpi. Crypt hyperplasia was very mild at 3 dpi and continued through 12 dpi. By 5 dpi there was a minimal amount of mononuclear inflammatory infiltrate in the lamina propria that resolved by 12 dpi. Because of the gross changes seen in the thymus we also examined extra-intestinal tissues; bursa, spleen, pancreas, thymus, liver, kidney, bone marrow, skeletal muscle, and blood. No remarkable histologic changes were noted in any of these tissues. No lesions were seen in any of the control tissues (Fig. 4.3 B). These findings demonstrate that TAstV-2 infection resulted in severe diarrhea, growth suppression, and reduction in thymic mass in the absence of widespread inflammation or cellular damage.

Localization of TAstV-2.

We originally isolated TAstV-2 from the thymus suggesting that TAstV-2 is present outside the intestines (53). However, no studies to date have examined the distribution of astrovirus during infection. Therefore, we examined the distribution of

TAstV-2 at different times post-infection by RT-PCR, isolation of infectious virus, immunofluorescence, and *in situ* hybridization (Table 4.1). Not surprisingly, infectious virus could be isolated from the feces and intestines at all time points in the experiment from day 2 onward; however, the levels of virus in the feces at 1 dpi were below the level of detection by RT-PCR. TAstV-2 RNA was also detected by RT-PCR in the thymus, bursa, spleen, liver, kidney, pancreas, skeletal muscle, bone marrow, and in the plasma fraction of infected birds, generally at 3 and 5 dpi; and the thymus and spleen were still positive at 7 dpi (Table 4.1). Infectious virus could be isolated from all of the samples generally between 3 to 7 dpi. The presence of TAstV-2 outside the intestines was also detected by immunofluorescence. Mild, limited TAstV-2 capsid staining was detected in all tissues examined, most consistently between 3 and 5 dpi (Fig. 4.4 A, C, E). No staining was observed in control tissues (Fig 4.4 B, D, F). Although there was infectious virus and viral antigen staining in extra-intestinal tissues, *in situ* hybridization data suggested that astrovirus replication was limited to the intestines (Fig. 4.5 A). No replicating virus was detected in representative extra-intestinal tissues (thymus, bursa, and spleen) (Fig. 4.5 B, C, D). *In situ* staining of the TAstV-2 genome in the intestines was generally found in the deep edges of the villi and not in the crypts (Fig. 4.5 A). A similar staining pattern for TAstV-2 capsid protein was observed, with antigen detected in the cytoplasmic portion of specific enterocytes at the mid-region of the villi (Fig. 4.4 E).

TAstV-2 Infection Does Not Increase Cell Death.

The lack of histologic lesions in the intestines of TAstV-2-infected animals was surprising given the levels of viral replication and diarrhea. To determine if TAstV-2-

infected cells undergo cell death, intestinal sections from control and infected poults were double-labeled for TAstV-2 capsid protein and cell death using TUNEL analysis. Not surprisingly, there was a great deal of TUNEL staining in both control and TAstV-2-infected intestines (Fig. 4.6 A – 4.6 D). In contrast astrovirus staining was found only in the cytoplasm of enterocytes of infected (Fig. 4.6 F) but not control (Fig. 4.6 E) intestines. Double-labeling the tissues resulted in no overlap of TUNEL-positive cells with TAstV-2-infected cells, suggesting that astrovirus replication does not result in an increase in cell death (Fig. 4.6 G and H). Identical results were observed in TAstV-2-infected embryos (data not shown). These experiments suggest that TAstV-2 does not increase cell death, which supports the histopathology observations (Fig. 4.3).

Increased Activation of TGF-B During TAstV-2 Infection.

Given the severity of the clinical disease, the length of diarrhea and virus shedding (> 9 dpi), one would expect that an inflammatory response would be initiated. One possible explanation for the apparent lack of inflammation would be the increased expression of an immunosuppressive cytokine during TAstV-2 infection. One of the most potent immunosuppressive cytokines is TGF-β (26) To determine if TGF-B activity increased during TAstV-2 infection, serum from inoculated turkeys was collected and tested using the NRK colony-forming soft agar assay, a highly specific and sensitive biological assay for TGF-β activity (51). We observed substantial increases in serum TGF-β bioactivity after infection with TAstV-2 (Fig. 4.7 A). TGF-β activity was elevated within 1 dpi and remained increased even at day 12 compared with the control turkeys. A neutralizing antibody against TGF-β inhibited the increased colony formation observed in the infected serum samples, suggesting that the *in vivo* activity is that of TGF-β (Fig. 4.7 A). Intestinal filtrates were also tested but were toxic in the NRK assay.

To determine if TGF-β activity also increases in inoculated embryos, 20-day old turkey embryos were inoculated with 10^9 genome units of TAstV-2 or PBS and incubated for 5 days at which time the intestines were removed, homogenized, and 0.22 μm filtered. These tissues homogenates where then assayed for active TGF-β using the Mv1Lu-PAI (PAI, plasminogen activator inhibitor) luciferase assay. Intestines from TAstV-2 inoculated embryos had an 11-fold greater amount of active TGF-β as compared to an equal amount of total protein from PBS inoculated embryos (Fig. 4.7 B). The increase in luciferase activity was inhibited with a TGF-β neutralizing antibody demonstrating that active TGF-β in the intestinal homogenates is driving the luciferase expression off the PAI promoter (Fig. 4.7 B). Finally, the intestinal fluid isolated from TAstV-2-infected SPF embryos contained elevated levels of active TGF-β manuscript in preparation).

DISCUSSION

Very little is known about the pathogenesis of astrovirus infection. Studies i*n vivo* in humans and animals are limited (37, 46, 58, 62, 63, 69). In these studies we described the development of an *in ovo* method to propagate high titers of infectious virus and a small animal model that will be useful to further understand astrovirus pathogenesis and the host response to infection. The present studies are the first, to our knowledge, to examine the pathogenesis of astrovirus infection including the kinetics of astrovirus replication, the location of the virus and its ability to localize to extra-intestinal sites, and, most surprisingly, the induction of diarrhea in the absence of either cellular damage or an increased inflammatory response.

In vitro all of the human astrovirus (HAstV) strains were adapted to replicate in cell lines (7, 25, 60). To date, we have been unable to propagate TAstV-2 in primary turkey or chicken cells, or the cell lines that support HAstV replication. Fortunately, we were successful at propagating TAstV-2 in turkey embryos. Inoculation of TAstV-2 in the yolk sac of 20-day-of-age turkey embryos resulted in productive viral replication, accompanied by an accumulation of fluid in the intestines of infected embryos. Routine testing of this fluid indicates that it typically contains 10^{11} viral genomic units/ml as determined by CQ RT-PCR. Limiting dilutions in embryos followed by immunofluorescent staining for the viral capsid protein suggested that the fluid contained at least 10^{9} infectious viral particles/ml (data not shown).

Although previous animal studies yielded some information about astrovirus pathogenesis, none were fully developed into animal models. Therefore, we set out to determine if we could use the turkey poult model to understand viral pathogenesis. TAstV-2 was highly infectious and extremely stable in the environment (54); therefore, control birds had to be housed in separate rooms to avoid cross contamination. Additionally, placing naïve poults in contact with infected birds or in cages that previously housed TAstV-2 infected birds resulted in immediate infection and diarrhea. Similar to mammalian astroviruses, younger animals are more susceptible to TAstV-2 infection. Infecting older naïve birds with TAstV-2 induced diarrhea; however, the duration of viral replication and the clinical signs were reduced in older animals (data not shown). Infecting naïve poults with TAstV-2 resulted in diarrhea in 100% of the birds within 24 hr post-infection. Infected poults had a reduced growth rate, and remained significantly smaller than controls throughout the experiment. In addition to the growth depression, infected poults also had significantly reduced thymus weights, although this difference had resolved by the end of the experiment. The mechanism for the reduced growth rate and undersized thymus is not understood; however, both are likely directly related to the diarrhea. Infected birds likely suffer some nutritional deficiencies. Infected birds consumed the same amount of feed as the age matched controls, but did not gain weight at the same rate. In additional studies, birds given nutritional additives did not have as severe weight loss or changes to the thymus.

TAstV-2 RNA and infectious virus were detected in every tissue examined, including the blood. To confirm that TAstV-2 RNA and infectious virus present in non-intestinal tissues was independent of contaminating blood, tissues were washed extensively in PBS or incubated overnight in large volumes of formalin followed by a second 48 hr incubation in PBS prior to processing. Thus, it is unlikely the TAstV-2 is due to contaminating blood. Additionally, we confirmed the presence of TAstV-2 in non-intestinal organs by immunofluorescent staining for the capsid protein. The distribution of viral antigen and RNA throughout non-intestinal organs peaked at 5 dpi then waned. By 12 dpi, only the intestine contained virus (Table 4.1). There was limited capsid staining in lymphoid areas of the thymus and bursa and in the kidney epithelia. However, most of the TAstV-2 capsid staining in the extra-intestinal tissues was associated with vasculature. Previously it was unknown if astroviruses induced viremia. In this study, TAstV-2 RNA and low titers of virus were detected in plasma samples from infected poults. Many viruses induce viremia during which the viruses circulate in the blood, serum, or white blood cells (WBCs) and are spread to target organs to initiate infection (38). The mechanism by which TAstV-2 enters the blood stream and spreads to extra-intestinal organs is unknown. Studies with astrovirus in lambs and calves suggested a possible role for macrophages, Peyer's patches, and M cells in infected animals (57, 68). We demonstrated that macrophages isolated from the spleens of TAstV-2-infected poults did not contain infectious virus (Koci et al manuscript submitted). Unfortunately, at the current time, markers for turkey-specific APCs are not available. Collectively, these results suggest that viremia occurs following TAstV-2 infection and that the TAstV-2-positive sera contain infectious virus.

Although, extra-intestinal tissues contained TAstV-2 antigen and RNA, only the intestine appeared to support viral replication as determined by *in situ* hybridization. Limited replication was observed in the cecal tonsils and distal small intestine within 1 dpi. By 3 dpi, replication was pronounced in the cells of the mid-villus of the cecal tonsils and distal small intestine (duodenum) with expansion to the epithelium of the large intestine and small intestine. By 9 dpi, only minimal viral replication was observed (4).

Many enteric pathogens induce diarrhea by destroying enterocytes in the villous epithelium ultimately leading to cell death and villous atrophy (29). This does not appear to be the case with TAstV-2. In spite of the diarrhea, there were only minimal to mild histologic changes in the intestines during TAstV-2 infection. The lack of substantial histologic changes noted in the intestines was supported by TUNEL analysis. TUNEL staining demonstrated that cell death was not increased during infection, either in general or specifically in TAstV-2 infected cells. Similar results were obtained using the apoptosis-specific antibody, caspase 3 (data not shown).

Another common mechanism to induce diarrhea is through an increased inflammatory response. However, there is no increase in inflammatory cell infiltrates in response to TAstV-2 infection. This could be due to a number of factors including the lack of cell death and/or to the upregulation of an anti-inflammatory cytokine by TAstV-2 infection. One such factor is transforming growth factor-β (TGF-β). TGF-β is a potent immunomodulatory factor that is important in intestinal homeostasis. In the intestine, TGF-β is produced by enterocytes and localizes primarily at the villus tip in the jejuneum (61) and occasionally lymphocytes in the lamina propria are immunopositive (3). Once activated, TGF-β mediates epithelial restitution (11, 45, 61) plays a major role in the development of regulatory T cells (56) potentially leading to generation of a TH3-type response eliciting oral tolerance (65) modulates the severity of inflammatory diseases (6, 30) activates neutrotrophic factors (21, 42, 59) and preserves the epithelial barrier function (43, 44). There is no information in the literature on the role of activated TGF-β during viral gastroenteritis. However, based on other models of intestinal injury, we hypothesize that the increased TGF-β may be important in preserving or maintaining the epithelial barrier (3, 11, 20, 49). Our work demonstrated that systemic TGF-β activity was increased during TAstV-2 infection. Increased TGF-B activity occurred within 1 dpi and remained elevated throughout the course of the experiment. This pattern was similar to that seen with the activation of TGF-β by influenza virus (52). Similar experiments using infected embryos demonstrated that TAstV-2-infected intestines contain 11-fold more active TGF-β as compared to control intestines. This is a significant increase in TGF-β activity. Additionally, the fluid that accumulated in infected embryo intestines following 5 days of incubation contained high levels of active TGF-β (data not shown).

These results are unique to TAstV-2. Embryos or poults infected with other enteric pathogens that induce diarrhea including turkey coronavirus virus and reovirus failed to activate TGF-β (data not shown). Studies are underway to determine the mechanism of activation and role of TGF-β in astrovirus pathogenesis.

Currently, we do not know how TAstV-2 induces diarrhea. However, it is through a mechanism that does not involve either an inflammatory response or extensive cellular damage. Studies are underway to determine the mechanism of astrovirus-induced diarrhea including the possibility that astrovirus, like rotavirus and SIV, encodes a viral toxin.

In summary, these studies developed a procedure to cultivate large quantities of infectious astrovirus, and established the kinetics and distribution of astrovirus replication using an avian astrovirus in a young turkey model. We also showed, for the first time, that an enteric astrovirus induced viremia and extra-intestinal distribution of virus. Finally, we demonstrated that astrovirus caused diarrhea without inducing cell damage or cell death, and began exploring the cellular response to infection. At this time we can not compare our results to those observed in a mammalian model since those studies have not been performed. It will be interesting to determine if viremia is a general feature of astrovirus infection. The presence of astrovirus RNA in serum may aid in the rapid diagnosis of infection. Additionally, experiments examining the induction of diarrhea by heterologous strains of astrovirus in the turkey model are necessary to fully explore the potential use of turkey poults or embryos as an animal model for astrovirus infection. TAstV-2 is phylogenetically distinct from the mammalian astroviruses; however, given the similarities in disease and age distribution this difference is likely due to the evolutionary distance between mammals and birds (28). Further studies are warranted to determine if the findings in turkeys are generalizable to mammals. Astroviruses are one of the leading causes of viral gastroenteritis worldwide. This animal model will be useful in increasing our knowledge of the mechanisms involved in inducing astrovirus diarrhea, defining important features of the host response to infection, and possibly lead to improved therapeutics.

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Table 4.1. Localization of TAstV-2.

	RT-PCR[a]	Virus Isolation[b]	IFA[c]	*In situ* Hybridization[d]
Feces	2-9[e]	1-12	ND[f]	ND
Intestines	1-12	1-12	1-12	1–9
Bursa	5	5	3-5	Neg[g]
Thymus	3-7	3-7	3-5	Neg
Spleen	3-7	3-7	3-5	Neg
Kidney	3	3-12	5	Neg
Liver	3-5	3	5	Neg
Skeletal Musc	5	5	ND	Neg
Marrow	3-5	3-5	3	Neg
Pancreas	3	3	3-5	Neg
Plasma	3-5	3-5	ND	ND

[a]Tissues from 5 random control or infected animals were collected at different days post-infection, pooled at each time point, and RNA isolated for TAstV-2-specific RT-PCR.

[b]Tissues collected as described above, were homogenized, filtered and diluted 1:100 then inoculated into 20-day of age embryonated turkey eggs. Five-day post-inoculation, embryos were monitored for clinical signs of infection and intestines isolated for RT-PCR.

[c]Immunofluorescence using a polyclonal antibody produced to a peptide sequence in the TAstV-2 capsid protein (K_{676} – R_{691})

[d]*In situ* hybridization using negative sense riboprobe specific for the 3' end of the TAstV-2 genome.

[e]Days post inoculation

[f]Not Determined

[g]Negative at all times tested

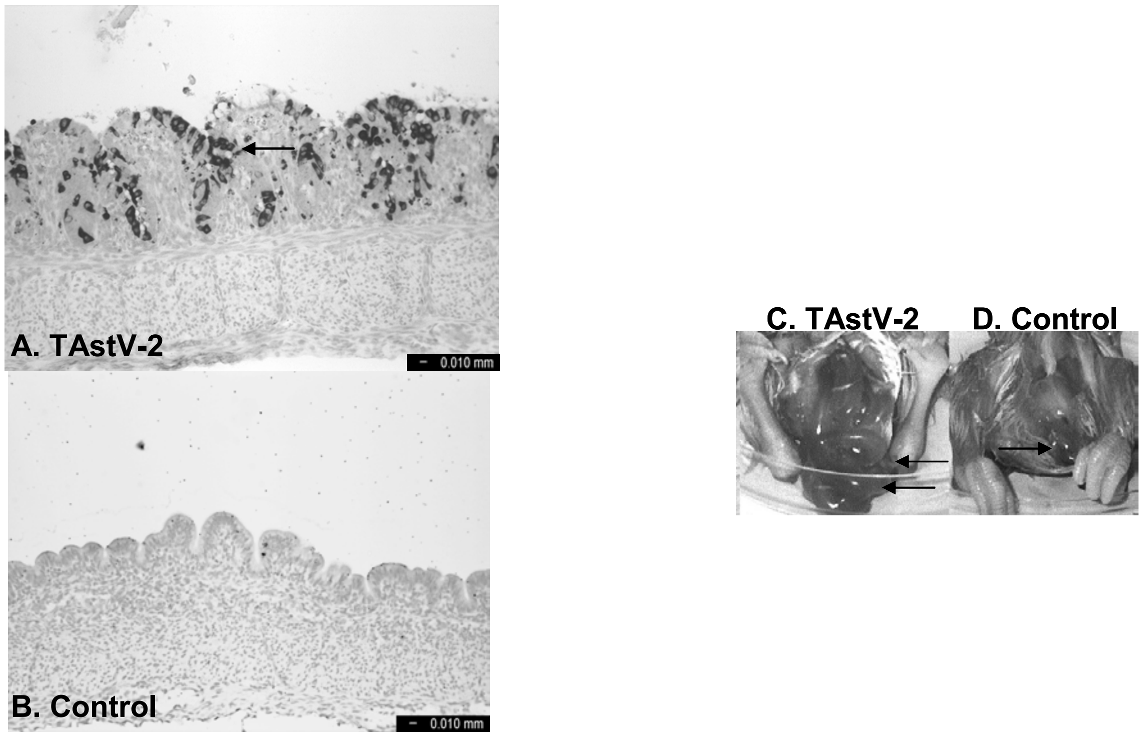


Figure 4.1 Propagation of TAstV-2 in Embryos. Twenty-day-old specific pathogen-free turkey embryos were inoculated with a tissue filtrate from healthy or TAstV-2 inoculated turkey poults and incubated for 5 days at 37^{o}C. (A) TAstV-2 replication was detected in inoculated embryo intestines via *in situ* hybridization. (B) No TAstV-2 *in situ* hybridization staining was detected in PBS inoculated embryos. (C) At 5 dpi, TAstV-2-infected embryo intestines were enlarged, thin-walled, fluid-filled and distended as compared to controls (D).

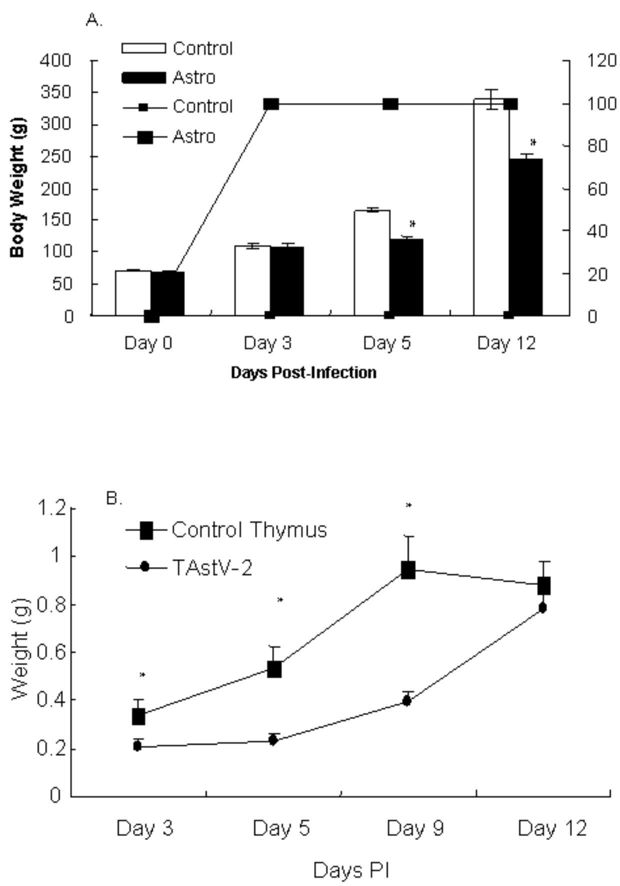


Figure 4.2**.** TAstV-2 Infection Results in Growth Depression and Decreased Thymic Size. Five-day old turkey poults were orally inoculated with 10^6 TAstV-2 particles or with PBS (0.2 ml) and (A) twenty to forty-five random poults per group were weighed at days 0, 3, 5, and 12 post-infection. The second Y-axis exhibits the percentage of poults exhibiting diarrhea at the same days of infection. (B) The thymus from 5 random poults per group were weighed on days 3, 5, 9, 12 post-infection and the average weight per group determined. Stars represent statistically significant difference in weight as determined by one-way analysis of variance $P \leq 0.05$.

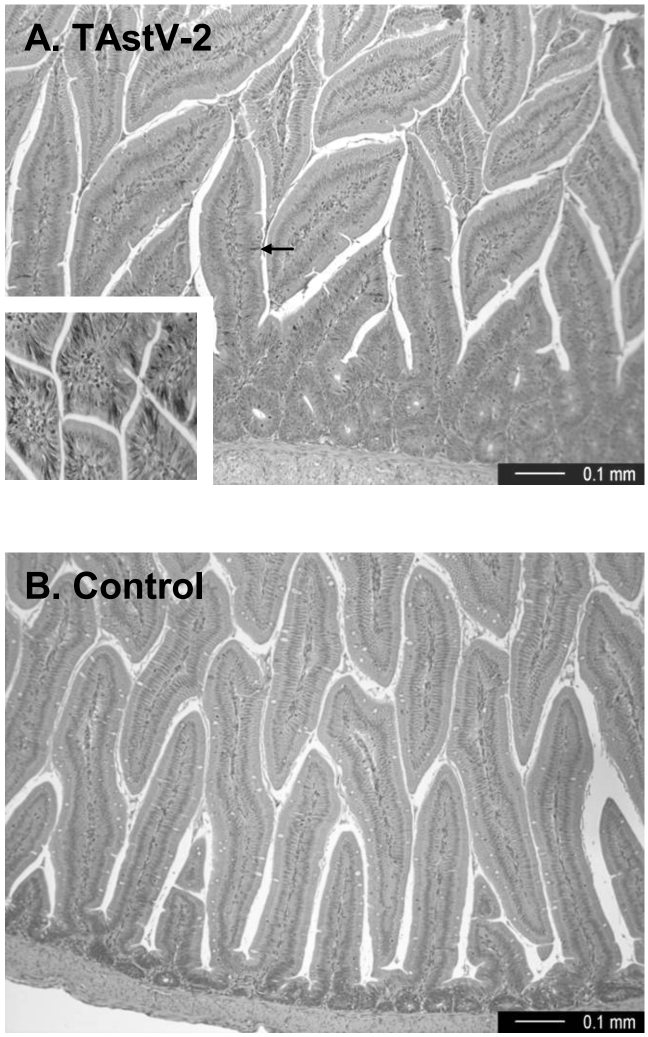


Figure 4.3. TAstV-2 Infection Results in Minor Histopathologic Changes. Histologic lesions in the duodenum of (A) TAstV-2-infected or (B) control poults collected at 2 dpi and stained with hematoxylin and eosin. (A) In TAstV-2-infected poults, histopathological lesions are limited to scattered single degenerating villous epithelial cells predominantly in the basal portions of the villi (arrows). Original magnification shown in figure. Inset image is a higher magnification of affected area.

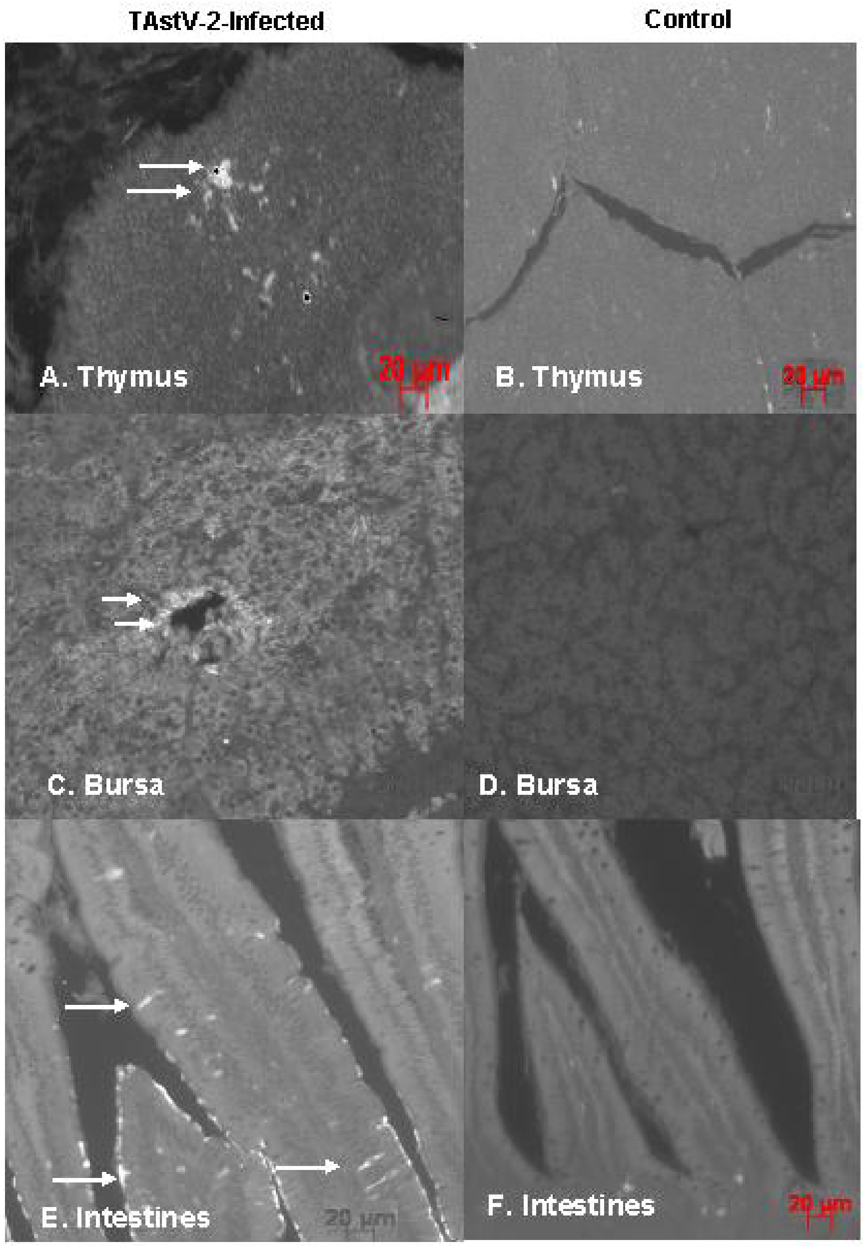


Figure 4.4. TAstV-2 is Present in Extra-Intestinal Tissues. Photomicrograph of the distribution of specific immunofluorescence staining against astrovirus capsid antigen of (A), thymus (C), bursa (E), intestine of 10-day old TAstV-2-infected poults at 5 dpi and (B), thymus (D), bursa and (F), intestine of 10-day old PBS mock-infected control birds. Original magnification shown in figure.

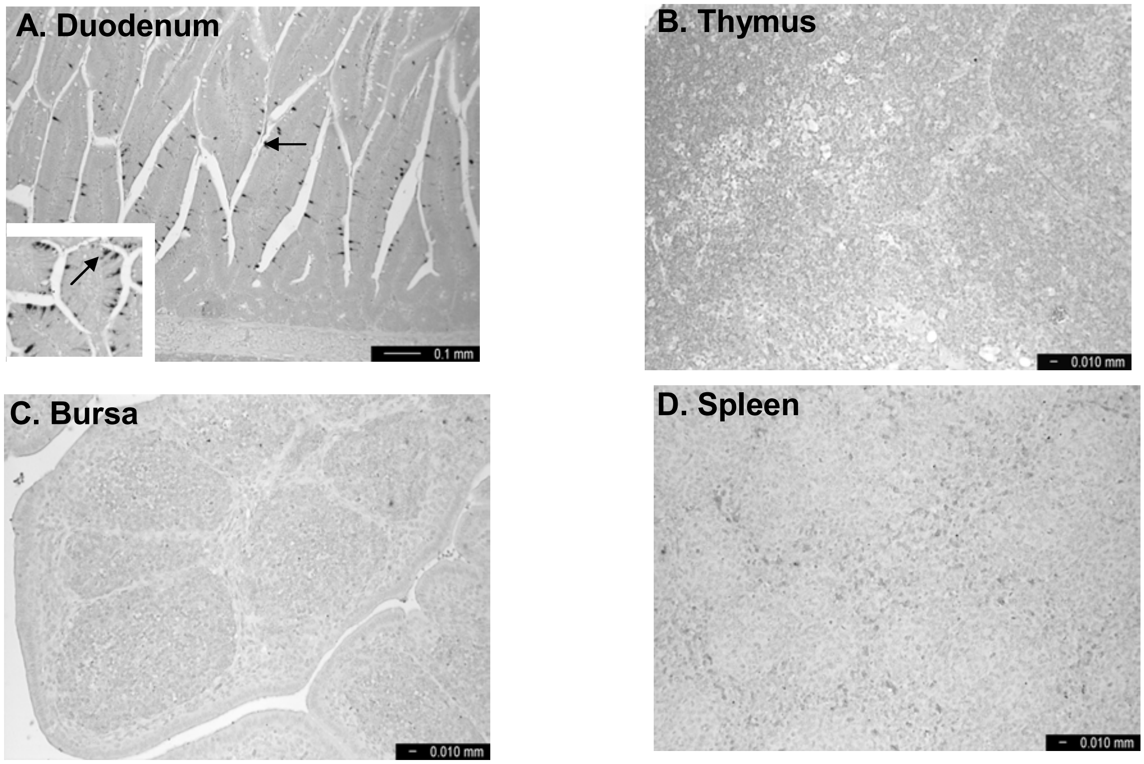


Figure 4.5. TAstV-2 Replication is limited to the intestines. Photomicrograph of the distribution of specific *in-situ* hybridization staining for TAstV-2 in the (A), duodenum (B), thymus (C), bursa and (D), spleen of 10-day old TAstV-2-infected poults at 5 dpi. (A) Arrows denote *in situ* positive cells; inset image is a higher magnification of TAstV-2 *in situ* positive cells. No staining detected in non-intestinal tissues, or control tissues. Original magnification shown in figure.

Figure 4.6. TAstV-2 Infection does not increase apoptosis. Five-day old turkey poults were orally inoculated with 0.2 ml PBS or 10^6 TAstV-2 particles and formalin-fixed intestines from 10-day old PBS mock-infected control birds (A, C, E, G) or 10-day old TAstV-2-infected poults at 5 dpi (B, D, F, H) were sequentially stained for cell death using TUNEL-conjugated with rhodamine (A – D) and then with anti-TAstV-2 peptide antibody, followed by biotin, then Alexa488-conjugated avidin (E-F). Panels A-D represent TUNEL alone, Panels E and F represent staining for TAstV-2, and Panels G and H are merged differential interference contrast (DIC), rhodamine, and Alexa488 images. Panels A and B, original magnification shown on figure, white bar on panels C-H represents 20 μm.

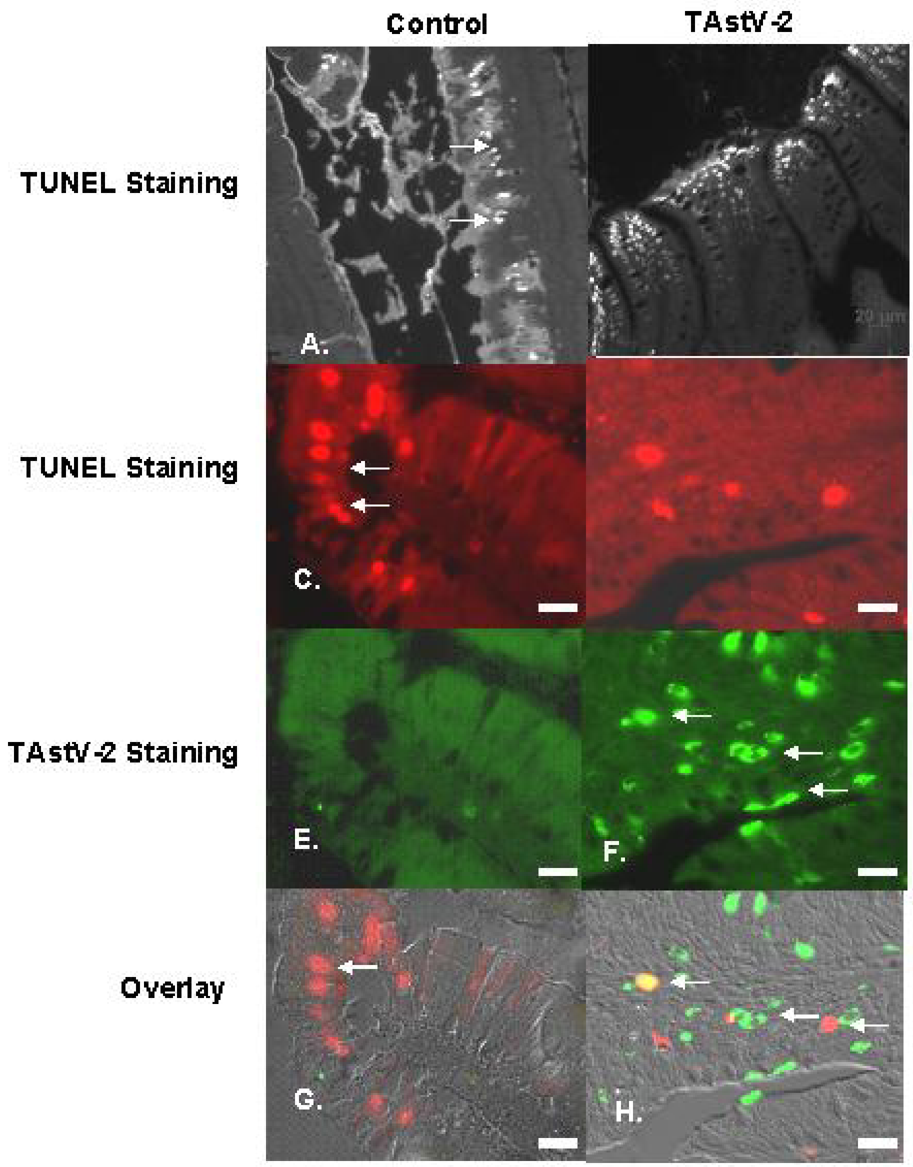
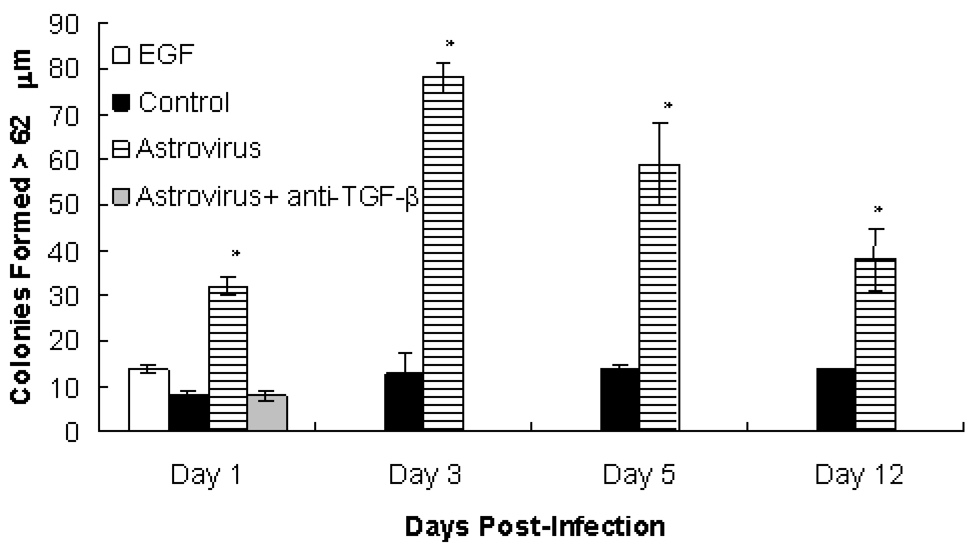

Control
TAstV-2
TUNEL Staining
A.
TUNEL Staining
C.
TAstV-2 Staining
E.
F.
Overlay
G.
H.

Figure 4.7. TAstV-2 Increases TGF-β Activity. (A). Five-day old turkey poults were orally inoculated with 0.2 ml PBS or 10^6 TAstV-2 genomic units, blood was collected from euthanized poults at 1, 3, 5, and 12 dpi and sera isolated. Aliquots of sera (100 l) were tested for TGF-β activity by the NRK colony forming soft agar assay. EGF was the negative control for the soft agar assay. Five poults per condition were tested and the results are expressed as the means of triplicate determinations; error bars indicate standard deviations, stars represent statistical significance ($p< 0.05$). To demonstrate specificity, the day 1 sample was pre-incubated with 2.5 μg of anti-TGF-β clone 1D11 (R&D Systems) for 40 min at RT. These results are representative of at least three experiments.

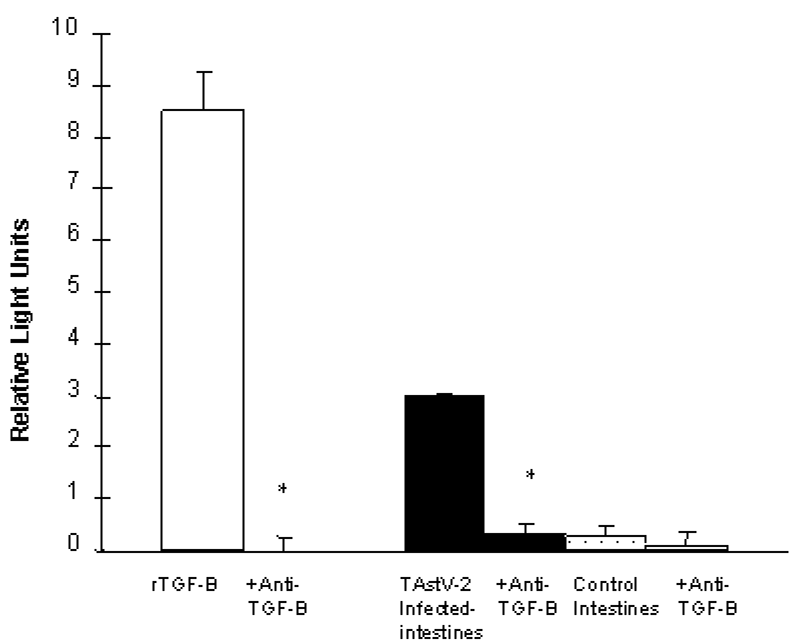
(B). Twenty day-old turkey embryos were infected with TastV-2 (10^9 genome units) or PBS, then five days post-infection, intestines were removed and homogenized and equal protein concentrations from infected and uninfected embryos were brought to a final volume of 100 μl in DMEM containing 0.1% BSA. Duplicate samples were pre-incubated with 2.5 μg of anti-TGF-β clone 1D11 for 40 min at RT to show specificity. Recombinant TGF-β_1 (6.25 pM) was used as a positive control. MV1Lu-PAI cells were incubated with samples for 16 hours before luciferase levels were determined. Results are reported as relative light units and the results are expressed as the means of triplicate determinations; error bars indicate standard deviations, stars represent statistical significance ($p< 0.05$).

A.



EGF
Control
Astrovirus
Astrovirus+ anti-TGF-β
Colonies Formed > 62 µm
90
80
70
60
50
40
30
20
10
0
Day 1
Day 3
Day 5
Day 12
Days Post-Infection

B.



Relative Light Units
10
9
8
7
6
5
4
3
2
1
0
rTGF-B
+Anti-TGF-B
TAstV-2 Infected-intestines
+Anti-TGF-B
Control Intestines
+Anti-TGF-B

CHAPTER 5

ASTROVIRUS-INDUCED EXPRESSION OF NITRIC OXIDE CONTRIBUTES TO VIRUS CONTROL DURING INFECTION[1]

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[1]Koci, M. D., L. A. Kelley, D. L. Larsen, and S. Schultz-Cherry. 2003.*Journal of Virology*. Submitted

ABSTRACT

Astrovirus is one of the major causes of infant and childhood diarrhea worldwide. However, our understanding of astrovirus pathogenesis trails behind our knowledge of its molecular and epidemiologic properties. This short-coming is mostly due to the lack of a small animal model for in-depth examination of pathologic mechanisms. We recently isolated and characterized an astrovirus which causes significant disease in young turkeys, and have characterized the kinetics of viral replication both *in vivo* and *in ovo*. Using this small animal model, we are investigating the mechanisms by which astrovirus induces diarrhea and the role of both the adaptive and innate immune response to turkey astrovirus type-2 (TAstV-2) infection. Astrovirus infected animals were analyzed for changes in total lymphocyte populations, alterations in $CD4^+$:$CD8^+$ ratios, production of virus-specific antibodies (Abs), and macrophage activation. We found no changes in the numbers of circulating or splenic lymphocytes, or in $CD4^+$:$CD8^+$ ratios as compared with controls. In addition we found only modest production of virus specific Abs. However, adherent spleen cells from infected animals produced more nitric oxide in response to *ex vivo* stimulation with LPS. *In vitro* analysis demonstrated that TAstV-2 induced macrophage production of inducible nitric oxide synthase (iNOS). Studies using NO donors and inhibitors *in vivo* clearly demonstrated, for the first time, that NO inhibits astrovirus replication. The studies suggest that NO is important in limiting astrovirus replication and are the first, to our knowledge, to describe the potential role of innate immunity in astrovirus infections.

INTRODUCTION

Astroviruses were first identified and associated with enteritis in infants in 1975 by Madeley and Cossgrove (26). Since then astroviruses have been recognized as one of the leading causes of childhood diarrhea worldwide. By the age of five, 90% of children have antibodies against astroviruses (19, 28). In addition to its endemic nature, astroviruses also cause outbreaks of enteritis in schools, geriatric care facilities, children's hospitals, and in immune compromised individuals (28). In fact, the elderly and the immuno-compromised, such as AIDS patients, represent an expanding demographic of astrovirus disease (33).

Astroviruses are believed to be transmitted mainly through a fecal-oral route (27). The virus typically has an incubation period of 1-4 days and causes an acute gastroenteritis which lasts approximately 4 days (9). Diarrhea is the most common symptom; however, vomiting, abdominal distention, and dehydration can occur (28). Much of what is known about astrovirus-mediated disease comes from epidemiological studies involving routine surveillance for enteric disease agents, following outbreaks, and serologic studies. Observational data from human samples and serological surveys have suggested that antibodies are the key mediators of protection. However, experimental evidence describing the contributions to resistance by each effecter arm of the immune system has not been studied. This is due to the lack of a small animal model for astrovirus infection.

We have recently developed a small animal model using turkey astrovirus type-2 (turkey astrovirus type 2/North Carolina/034/1999, TAstV-2) to study the mechanisms of viral pathogenesis and immune protection (3, 16, 18). Using young turkeys, we defined the replication, kinetics, and pathogenesis of astrovirus infection. We found that astrovirus replicates in the intestines with viral loads peaking between days 3 and 5, and dissipating by day 9 post inoculation. We also detected viral antigen in non-intestinal tissues and isolated infectious virus from these tissues and blood, primarily between 3 to 5 days post infection (dpi) indicating viral spread was systemic, although viral replication was only detected in the intestine (3, 16).

To begin to understand the host response to astrovirus, we examined the role of the adaptive and innate immune response in the control and clearance of astrovirus infections using our turkey model. Our results demonstrate T cell populations and viral-specific antibodies (Abs) were not substantially altered in response to TAstV-2 infection. However, these data indicate virus infection induced macrophage (MΦ) production of nitric oxide (NO), and NO suppressed viral replication during infection. This is the first experimental evidence of an interaction between astrovirus and MΦs, and demonstrates a potentially significant role for innate immunity in primary astrovirus infection.

METHODS AND MATERIALS

Viral propagation.

TAstV-2 was isolated and propagated as described (18, 35). Briefly, fluid isolated from the intestines of TAstV-2 infected turkey poults were clarified by centrifugation (500 x g for 10 min), 0.2 μm filtered, diluted 10^{-3} in PBS, and inoculated into the yolk sac of 20-day-old specific pathogen-free (SPF) turkey embryos. Five days post-inoculation, embryo intestines and intestinal fluid were isolated separately. Infected and control intestines were homogenized in 1 ml of DMEM (Cellgro), clarified by centrifugation, filtered as above, and total protein determined by the Bradford colorimetric assay (BCA kit, Pierce). Embryo intestinal fluid (EIF) was collected from infected embryos (negative embryos do not contain fluid in their intestines), clarified by centrifugation, filtered as above, and tested for viral load by Real Time RT-PCR and limiting dilutions in eggs. EIF was determined to contain 1 x 10^{12} viral genomes (VG)/ml, and roughly 10^{9} infectious particles/ml as determined by limiting dilutions and subsequent detection using fluorescent antibody (Ab) staining of inoculated embryo intestines.

Virus purification.

TAstV-2 containing EIF was purified by size fractionation using a 1.5 x 50 cm column (BioRad) with 80 ml of Sephacryl CL-6B (Sigma) gel pre-equilibrated in tris-buffered saline (TBS, pH 7.4). The column was run at a flow rate of 0.5 ml/min and 2 min fractions collected, with a total run time of 180 min. Fractions were analyzed for total protein concentration using BCA analysis, and tested for TAstV-2 using RT-PCR, SDS-PAGE, and western blot analysis. SDS-PAGE and western blot analysis were performed using a 5-20% gradient gel, separated by electrophoresis at 100 V for 60 min and then either stained for total protein using GelCode Blue Stain Reagent (Pierce) or transferred to nitrocellulose (BioRad) and viral antigen detected using convalescent Abs from TAstV-2 infected turkeys. Column fractions were also assayed for infectious virus by inoculation into eggs and examined for the accumulation of intestinal fluid, as well as viral replication by RT-PCR.

Cell culture.

The chicken MΦ cell line (HD11) was kindly provided by Dr Kurt Klasing (University of California, Davis). Cells were cultured with RPMI 1640 (Cellgro) supplemented with 5% heat inactivated fetal bovine serum (FBS, Cellgro) and L-glutamine (2mM, Cellgro), in a humidified incubator with 5.5% CO_2 at 41° C.

Chemicals.

The following chemicals were used at various stages of these experiments. N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin (Pierce) was used at final concentrations of 1 μg/ml, 5 μg/ml or 10 μg/ml. Lipopolysaccharides (LPS) from *Escherichia coli* 0127:B8 (Sigma) was used at a concentration of 10 ng/ml as a positive control for stimulation of inducible NO synthase (iNOS) activity *in vitro*. The endotoxin inhibitor polymyxin B sulfate (PMB, Fluka) was used at a final concentration of 1.5 μg/ml. Actinomycin D (ActD, Sigma), which inhibits RNA transcription from a DNA template, was used at a final concentration of 1 μg/ml. NOS activity was blocked using N^G-Monomethyl-L-arginine (L-NMMA, Calbiochem) at 4mM, a 4-fold molar excess of normal L-arginine. The NO donor compound (±)-S-Nitroso-N-acetylpenicillamine (SNAP, Calbiochem) was used at 500 μM. The potent inhibitor of iNOS, N-[3-(aminomethyl)benzyl]acetamidine, dihydrochloride (1400W, Calbiochem) was used 10 mg/kg body weight. Each compound at the above concentration was demonstrated have no effect on HD11 cell or viability, as determined by trypan blue exclusion. Likewise, diluents used for each chemical were tested on HD11 cells at analogous concentrations and shown to have no affect on activity or viability.

RT-PCR.

Total RNA was isolated from EIF (100 µl), tissues (~100 mg), cells (10^5-10^6), or purified virus (100 µl) using TRIzol Reagent™ (Invitrogen) following the manufacturer's instructions. Routine detection of TAstV-2 was done by RT-PCR as described (17).

TAstV-2 Real-Time RT-PCR.

TAstV-2 replication and viral load were determined using Real Time RT-PCR. Total RNA isolated from cells or tissue was quantitated using spectrophotometry and an equal amount of total RNA from each sample added to a 25 µl reaction using the one-step RT-PCR kit, QuantiTect Probe RT-PCR Kit (Qiagen). The presence of TAstV-2 in experimental samples was detected using primers and probe specific to the polymerase gene (TAV2TMpolFWD: 5'*GAC TGA AAT AAG GTC TGC ACA GGT* 3', TAV2TMpolREV: 5' *AAC CTG CGA ACC CTG CG* 3', TAV2TMpolPRB: 5'-6-carboxyfluorescein/ *ATG GAC CCC CTT TTT CGG CGG*/ black hole quencher-1/-3") and quantitated by comparing the samples to a TAstV-2pol RNA standard curve, as previously described (16). Primers and probe were designed using the Primer Express v1.5 (Applied Biosystems) and constructed by Integrated DNA Technologies, Inc. Reactions were performed using the ABI Prism 7700 Sequence Detector and analyzed using Sequence Detector v 1.7 (Applied Biosystems). All samples and standards were amplified in duplicate. All experiments were performed at least 3 times.

iNOS RT-PCR.

Increased expression of iNOS RNA was detected by RT-PCR using primers specific to the chicken iNOS gene (accession number: U46504). Primers; (MKChiNOSFwd: 5'-*CTG TGC TTC ATA GCT TCC AG*-3' and MKChiNOSRev: 5'-*AGG CAC AGA ACT*

CAG GAT AC-3') were designed using PRIMER Designer Version 2.01 (Scientific and Educational Software). Briefly, total RNA was isolated from HD11 cells treated with media alone, LPS (10 ng/ml), or 2 x 10^5 VG/well of purified TAstV-2 following 1, 2, 4, 8, 12, 24, or 48 hrs of incubation. Equal amounts of isolated RNA were treated with 1 unit of DNase I, Amplification Grade (Invitrogen) for 15 min at room temperature (RT). The enzyme was inactivated by the addition of 1 µl EDTA (25 mM) and the reaction heated to 65º C for 10 min. Treated RNA was then brought to a final volume of 20 µl with the addition of 1st Strand Buffer, dithiothreitol (DTT), reverse primer, and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. RNA was incubated at 45º C for 60 min, at which time 2 µl were removed and used as template in a 50 µl PCR reaction using Platinum Taq (Invitrogen), as instructed by the manufacturer. Following amplification, products were separated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide staining. Results are representative of 3 experiments.

Animals.

Two-day-old unvaccinated British United Turkey of America poults (male and female) were obtained from a commercial hatchery. Control and infected poults were housed in separate BL2 containment facilities in individual Horsfall units with HEPA filtered inlet and exhaust air valves. Birds were fed routine turkey starter from the University of Georgia and given free access to clean water. After a brief acclimation period, five-day-old poults were randomly assigned to either a control group or a group infected with astrovirus (n = 60 per group). Poults were orally inoculated with ~10^6 genomic units of astrovirus in 200 µl total volume, or phosphate buffered saline (PBS) alone, as previously described (16). Birds were monitored daily for signs of clinical disease. On days 5, 9, 11, 16 and 21 pi, five random poults per group were euthanized by cervical dislocation and the spleens, intestines, gall balder and blood were collected. Spleens from 3-5 poults were pooled and placed in cold RPMI and homogenized by physical disruption as previously described (22). Heparinized blood from 3-5 turkeys was pooled and diluted 1:1 in PBS. Leukocytes were then isolated from single cell suspensions of blood and spleens using Histopaque 1077 (Sigma), following the manufacturer's instructions. Cells from buffy coat were isolated and subsequently tested for TAstV-2 by RT-PCR and egg culture as previously described. Additional cells were seeded in 96-well plates at 1 x 10^6 cell/well and treated with complete RPMI 1640 with and without LPS (10 ng/ml). Following 48 hrs of stimulation, nitrite levels present in the supernatants were assayed using the Griess assay (38). The animal experiments were repeated 3 times with different groups of poults with similar results. All animal experiments were approved by the USDA Animal Care and Use Committee and complied with all federal guidelines.

TAstV-2-specific ELISA.

To detect TAstV-2-specific Abs, ELISAs were performed by coating Immunlon 4 microtiter plates (Dynex) with 2.5 μg of recombinant capsid protein or 1% bovine serum albumin (BSA, Invitrogen) and incubated with decreasing concentrations of serum or bile from infected and control animals. Recombinant protein was generated using the single tube protein-system 3 (STP-3) (Novagen), a plasmid containing open reading frame 2 of TAstV-2 (pcDNA3.1$^-$/TAstVcap10), and purified using anion exchange column chromatography (BioRad). ELISA was performed as previously described (22). Briefly, serum IgG, or bile IgA were detected by diluting samples in phosphate-buffered saline

(PBS) containing 0.5% Tween-20 (PBST) and incubated for 1 hr at RT. Plates were washed with PBST, incubated with either alkaline phosphatase conjugated rabbit anti-chicken/turkey IgG (Zymed) or anti-chicken IgA (Bethyl Laboratories, Inc), and incubated for 1 hr at RT. Plates were washed with PBST and detected using 100 µl of SIGMA FAST p-Nitrophenyl Phosphate Tablets (Sigma) according to the manufacturer's instructions and incubated for 30 min at RT in the dark. The presence of TAstV-2 capsid specific IgG or IgA was then measured at 450 nm on a microplate spectrophotometer. Samples were determined to positive if their optical density was at least twice that of the negative controls.

Flow cytometery.

Lymphocytes from peripheral blood and spleen were isolated as described above. Phenotyping of isolated cells was preformed as previously described (37). Briefly, cells were washed in cold PBS, then 1 x 10^{6} cells were incubated with 0.5 µg of mouse anti-chicken CD4 directly conjugated to fluorescein (clone CT-4), 0.25 µg of mouse anti-chicken CD8α directly conjugated to phycoerytherin (clone 3-298, a generous gift from Southern Biotechnology Associates, Inc), or corresponding amount of mouse IgG1 isotype controls directly conjugated to fluorescein or phycoerytherin for 1 hr on ice. The cells were then washed with 1 ml of cold PBS, fixed using cold 1% PBS buffered formalin (Invitrogen) and analyzed using an Epics XL flow cytometer (Beckman Coulter). Lymphocytes were identified by their forward and side scatter properties. Gated lymphocytes were analyzed for $CD4^{+}$ and $CD8^{+}$ cells using both single color and two color analysis to ensure proper compensation. Percentage of each phenotype was determined based on cells positive for only one of the two markers. For each sample

10,000 total cells were analyzed. The CD4:CD8 ratios reported are the average of three separate experiments.

Nitrite assay.

Up-regulation and expression of iNOS in HD11 cells was measured indirectly by determining the levels of nitrite in cell culture supernatants using the Griess assay (38). Briefly, 1 x10^5 cells/well were treated with RPMI alone, RPMI containing LPS, or TAstV-2 infectious material (EIF, homogenized embryo intestines, or column purified virus), and incubated in a final volume of 100 µl at 41º C for 48 hr in a 96-well tissue culture plate (Corning Incorporated). Following incubation, 50 µl of cell free supernatant was assayed for the presence of nitrite by mixing with equal volumes of 1% sulfanilamide (in 5% phosphoric acid, Sigma) and 0.1% *N*-1-napthylethylenediamine dihydrochloride (Sigma). Plates were incubated for 15 min in the dark, absorbance measured at 550 nm using a spectrophotometer, and the nitrite concentration determined by comparing to a nitrite standard curve. All treatments were done in triplicate, and each experiment performed at least three times. Media, EIF, and column purified TAstV-2 were tested for contaminating endotoxins using the Limulus Amebocyte Lysate QCL-1000 Kit (BioWhittaker). All reagents tested were found to have less than 1 endotoxin unit (EU)/ml. LPS treatment added to HD11 cells contained at least 6 EU/ml.

HD11 cell infection with TAstV-2.

To determine if HD11 cells support TAstV-2 replication, 5 x 10^5 cells were seeded into each well of a 24-well plate and incubated overnight to allow cells to attach. Media was removed and cells inoculated with 5 µl of EIF, or 5 µl of PBS in a final volume of 250 µl of serum-free media (SFM). Cells were incubated for 1 hr at 41º C at which time the inoculum was removed and replaced with either fresh complete media, or SFM containing increasing concentrations of TPCK-treated trypsin (1-10 μg/ml). To control for the detection of input virus, replicate cells were fixed by drying at RT for 1 h and then inoculated with TAstV-2 as above and incubated with SFM containing 10 μg/ml trypsin. Cells were monitored for cytopathic effect and viability by trypan blue exclusion. Cells were collected by vigorous pipetting at 24, 48, and 72 hr post inoculation, pelleted by brief centrifugation, and supernatants removed. Whole cell pellets were lysed in 1 ml of TRIzol Reagent™. RNA concentrations were determined using spectrophotometry and 500 ng of each sample RNA was used to detect TAstV-2 genomes using Real Time RT-PCR.

Role of NO in ovo.

To examine the role of NO in viral replication, TAstV-2 was inoculated into SPF turkey embryos with either the NO donor compound SNAP or the iNOS inhibitor 1400W and viral titers measured by real time RT-PCR. Briefly, 0.2 μm filtered EIF was diluted in PBS to contain $\geq 10^5$ embryo infectious units/ml (1×10^8 VG/ml) and 50 ul was incubated with either SNAP (500 μM) or 1400W (1.91 mg/ml), for 45 min at RT in a final volume of 100 μl. Three eggs were each inoculated with 100 μl of either SNAP+TAstV-2, 1400W+TAstV-2, or TAstV-2+PBS (positive control). Two eggs were inoculated with 100 μl of SNAP+PBS, 1400W+PBS or PBS alone (negative controls). Eggs were monitored daily for viability, and opened at 5 dpi and intestines collected. Sections of caecum and duodenal loop were collected from each inoculated embryo, pooled, and either preserved in 10% formalin solution (Fisher) for histological examination, or placed into 500 μl of RNAlater (Ambion) for RNA isolation. Pooled tissues for RNA isolation were weighed to ensure that between 80 and 100 mg of tissue was per manufacturer's instructions. Tissues were homogenized in 1 ml of TRIzol Reagent™, RNA was resuspended in RNase-free water and quantitated by spectrophotometry. From each sample 10 ng of total RNA was used to determine the amount of TAstV-2 present by Real Time RT-PCR.

In ovo Immunostaining.

Tissues from control and infected embryo intestines were fixed in 10% phosphate-buffered formalin overnight, then processed, embedded, and sectioned (0.3 μm). Sections were deparaffinized with Citrisolv (Fisher), and antigenic sites exposed by microwaving for 5 min in a citrate buffer, as previously described (16). The ability of TAstV-2 to replicate *in ovo* was detected using a rabbit polyclonal Ab generated to a peptide sequence in the TAstV-2 capsid protein followed by a universal biotin conjugated Ab from the Vectastain Universal ABC-AP Kit (Vector), and then detected using a streptavidin-labeled-Alexa488 (Molecular Probes) as previously described (16). In addition to viral antigen, serial sections were stained for the presence of nitrated tyrosine residues, an *in situ* indicator of peroxynitrite formation, using a rabbit polyclonal anti-nitrotyrosine Ab (Molecular Probes), the Vectastain Universal ABC-AP Kit and detected using Fast Red TR/Naphthol AS-MX Tablets (Sigma) following the manufacture's instructions. Tissues were counterstained with Harris Modified Hematoxylin (Fisher).

RESULTS

No evidence of adaptive immune response to TAstV-2 infection.

To begin to understand the effects of astrovirus infection on the host response we examined leukocytes isolated from infected poults. Leukocytes from peripheral blood and spleens were analyzed for TAstV-2 by RT-PCR and virus isolation using egg culture. Repeated attempts to demonstrate TAstV-2 was associated with the leukocyte fraction were unsuccessful, suggesting TAstV-2 is not spread to extra-intestinal tissues by white blood cells. To evaluate the adaptive immune response to astrovirus, we examined the numbers of leukocytes, induction of astrovirus-specific antibodies, and alternations in T cell populations during infection. There was no difference in peripheral blood and splenic lymphocyte counts between infected and control animals (data not shown). To determine the concentration and type of Abs produced in response to TAstV-2-infection, we assayed serum and bile for virus-specific IgG and IgA respectively (Fig 5.1). Although virus-specific Abs were detected, titers were very low. Specific IgG was undetectable at 11 dpi, with a titer of only 8 at 21 dpi (Fig 5.1). The levels detected for IgA were also undetectable at 11 dpi however these titers increased 4-fold over those of IgG by 21 dpi (Fig 5.1). Subsequent experiments using these sera failed to demonstrate the presence of neutralizing Abs; furthermore, these animals were not protected when re-challenged with TAstV-2. These low Ab titers were not due to an inability to mount a specific Ab response, as age matched controls infected with Newcastle Disease Virus produced protective Abs titers (data not shown).

In addition to viral-specific Abs, the levels of $CD4^+$ and $CD8^+$ T cells were measured using the limited tools available for the turkey model. Experiments examining the ratios of $CD4^+$-to-$CD8^+$ cells in the spleen and peripheral blood showed no significant alteration in T cell populations relative to controls (Table 5.1). Together these experiments suggest that the adaptive immune response is not critical for viral clearance in primary infected poults.

TAstV-2 infection primes MΦs in vivo.

To investigate the role of MΦs in astrovirus disease, we examined adherent splenocytes isolated from infected and control poults. Adherent splenocytes from infected poults produced more NO than mock infected controls when cultured *ex vivo* and stimulated with LPS. This increase in NO activity over controls was measured between days 8 and 11 dpi (Fig 5.2). This data suggests that astrovirus infection primes MΦ *in vivo* making them more readily activated upon *ex vivo* stimulation with LPS.

TAstV-2 infected intestines activate MΦ in vitro.

To specifically study the interaction between TAstV-2 and avian MΦs we examined the ability of TAstV-2 to stimulate the well characterized chicken MΦ cell line, HD11 (8). HD11 cells have been used extensively to examine *in vitro* interactions between avian MΦs and pathogenic organisms including several different viruses (8, 24, 32). We first asked if EIF could stimulate HD11 cells to release NO. HD11 cells were treated with various dilutions of EIF, and assayed for activation as determined by the Griess assay. EIF increased NO production in a dose dependent manner (Fig 5.3 A). To determine if the NO production was due to TAstV-2 we treated HD11 cells with 10 µg of homogenized intestines from infected or mock infected embryos (5 dpi) and assayed for nitrite in the supernatants. Only the infected intestine homogenate stimulated HD11 cells to produce NO, while the mock infected intestinal homogenate did not (Fig 5.3 B). These results suggest the virus, or a host factor up-regulated by infection, stimulates MΦ activation.

Astrovirus directly activates MΦ production of NO.

The NO activity stimulated by EIF and infected homogenized embryo intestines does not directly implicate TAstV-2, as both of these samples contain other proteins and lipid products. Therefore we developed a low pressure chromatography method to purify TAstV-2 from EIF by size exclusion. EIF separated into three major protein peaks when applied to a Sephacryl column (Fig 5.4). Molecular weight markers indicated that the first peak is slightly after the void volume of the column and contains proteins from ~158 kDa to 670 kDa, the second peak contains proteins from ~17 kDa to ~100 kDa, and the third peak is comprised of proteins <17 kDa in size. Representative fractions from all three peaks were tested for TAstV-2 by SDS PAGE, western blot, and RT-PCR. All three assays demonstrated that TAstV-2 eluted in the first peak, specifically from fractions #43-114 (Fig 5.4). Inoculation of these fractions into embryonated eggs demonstrated that the column-purified TAstV-2 was infectious. Similar results were obtained using TAstV-2-infected intestinal homogenates. Uninfected intestinal homogenates do not have a corresponding peak (data not shown).

To demonstrate that purified TAstV-2 stimulated HD11 cells, 3.5 μg of total protein from each of the column fractions were added to HD11 cells and assayed for NO (Fig 5.4 A). NO activity was stimulated by two groups of fractions, one corresponding to the elution of TAstV-2, and another by fractions at the end of the profile containing proteins < 17 kDa (Fig 5.4 A). Preliminary data suggests that the second group (#200-

262, Fig 5.4 A) contains interferons, which may account for their NO inducing activity (data not shown).

To demonstrate that the induction of NO by EIF and column-purified TAstV-2 is not a result of contaminating endotoxin, samples were tested in the presence of the endotoxin inhibitor PMB. PMB inhibited the stimulation of MΦ by LPS but had no effect on purified TAstV-2 (Fig 5.5) suggesting that TAstV-2 directly stimulates HD11 cells. There was minimal inhibition of EIF in the presence of PMB. Endotoxins present in the EIF were likely introduced during sample collection as embryos are bacterial-free. To control for the effects of exogenous endotoxin in TAstV-2 samples, PMB was added to all samples (except LPS positive controls) prior to their addition to HD11 cell cultures.

TAstV-2 stimulates iNOS up-regulation.

To confirm that TAstV-2-increased NO activity was due to elevated expression of iNOS, we incubated TAstV-2-treated MΦs with inhibitors of NOS activity. Cells treated with the RNA transcription blocker, ActD, demonstrated a 10-fold inhibition of NO, indicating NOS activity required gene transcription (Fig 5.6 A). These findings were supported by experiments using the NOS inhibitor, L-NMMA. L-NMMA blocked NO release by LPS, EIF and purified TAstV-2, suggesting that induction of nitrite by TAstV-2 is due to increased NOS enzymatic activity (Fig 5.6 A). Finally, RT-PCR confirmed an increase in iNOS message following TAstV-2 stimulation. iNOS RNA was elevated within 4 hrs post-stimulation in both the LPS and TAstV-2 treated cells, and remained elevated at 12 hrs post stimulation in the virus treated cells (Fig 5.6 B).

MΦ activation is independent of productive TAstV-2 replication.

To determine if TAstV-2-induced iNOS was due to viral replication, cells were inoculated with TAstV-2 in complete and serum free media in the presence of increasing concentrations of trypsin and monitored for cytopathic effect, viability, and viral replication as determined by Real-Time RT-PCR. Throughout the course of these experiments no cytopathic effect was observed. Similarly, no changes in cellular proliferation or viability were detected by 5-bromo-2'-deoxyuridine incorporation or trypan blue exclusion. Examination of the cell pellets for TAstV-2 viral genome over time, as determined by Real Time RT-PCR, showed no significant differences in viral load as compared to that of fixed cells (Fig 5.7). Although it is not possible to rule out abortive replication, it is clear that there is no productive replication. Additional experiments to detect the presence of viral negative strand by RT-PCR, and viral message using *in situ* hybridization in inoculated HD11 cells were all negative, further evidence against productive viral replication.

Astrovirus infection induces NO in vivo.

TAstV-2 induced NO activity in stimulated macrophages *in vitro* in a replication-independent manner. To determine if TAstV-2 increased NO activity *in vivo,* embryos were inoculated with EIF and intestinal sections stained for the presence of nitrotyrosine. Increased nitrated tyrosine residues is directly correlated to increased concentrations of reactive oxygen species (13). Embryos infected with TAstV-2 showed a substantial increase in staining for nitrotyrosine as compared with mock infected embryos (Fig 5.8). There is intense staining of the lamina propria of infected tissues as compared to that of the controls. These results suggest that the host responds to astrovirus infection in part through increased NO activity.

NO inhibits TAstV-2 replication.

To determine the role of NO in astrovirus pathogenesis, embryos were inoculated with EIF in the presence of either the NO donor SNAP (41), or the iNOS enzyme specific inhibitor 1400W (11). Following 5 days of incubation the embryos were examined for the accumulation of fluid in their intestines. There was a slight reduction in the amount of fluid in the SNAP treated infected embryos as compared to the positive controls, and a substantial increase in the amount of fluid in the intestines of 1400W treated infected embryos as compared to the positive controls. To evaluate viral titers, total RNA was isolated from intestines and viral titers determined using Real Time RT-PCR. Analysis of 10 ng of total embryo intestinal RNA showed a greater than 5-log reduction in TAstV-2 viral RNA in SNAP treated embryos as compared with the positive controls, and a 3-log increase in TAstV-2 in the 1400W treated embryos (Fig 5.9). These results were further supported by immunofluorescence data. Staining for viral antigen in these embryo intestines showed a significant increase in fluorescence in the 1400W treated embryos as compared with positive controls, and almost no detectable viral staining in the SNAP treated infected tissues (Fig 5.10). These results represent the first description of a potential role for NO in the host response to astrovirus infection and suggest its importance in limiting or preventing viral replication.

DISCUSSION

Astroviruses have a major impact on human health, and are one of the leading causes of infant and early childhood diarrhea (12). Their distribution is known to be worldwide, with 7% of children developing Abs against astrovirus by the age of one:

however, by the age of five, 90% of the population has seroconverted (19, 20). Serologic studies of adults indicate around 60% of people have Abs against astrovirus (19), suggesting routine exposure throughout life. In spite its vast distribution, very little is known about the host response to infection or the mechanisms that lead to astrovirus disease. Human volunteer studies have led many to speculate that neutralizing Abs are key to protection against astrovirus (21). Because of the biphasic age distribution of astrovirus, and its growing importance in immunocompromised patients, a better understanding of the basic pathogenesis of astrovirus, and the innate host response is needed to better understand the disease and develop new therapies.

Until recently these questions could not be addressed, due to the lack of a small animal model. Our turkey model provides us with a system to ask basic questions about the host-pathogen interactions of astrovirus and young animals, both *in vitro* and *in vivo*. We have previously described the kinetics and sites of replication in an effort to understand the histological changes associated with astrovirus infection (3, 16). In this study we examined the effects of astrovirus infection on both the innate and adaptive immune responses. In our system, infected animals had no differences in the numbers of circulating or splenic lymphocytes, the ratios of $CD4^+$-to-$CD8^+$ cells, and minimal production of astrovirus-specific Abs. These data indicate a nominal role for adaptive immunity during primary infection. However, we did notice an increase in MΦ activation and release of NO in response to astrovirus infection. Increases in NO activity were measured both *in vivo* and *in vitro*, and shown to be involved in limiting viral replication. These data suggest that the innate immune system, specifically MΦs and iNOS, play a key role in controlling astrovirus replication.

We were surprised to find little evidence of an adaptive immune response following astrovirus infection in turkeys (Fig 5.1, Table 5.1). Both B cells and T cells respond to human astrovirus infection. Virus-neutralizing Abs are considered key to astrovirus resistance. Human volunteer studies demonstrated that those with pre-existing Ab titers, did not show signs of astrovirus disease (21). The protective role of virus-specific Abs has also been demonstrated therapeutically. Intravenous immunoglobulin therapy has been used to treat persistent astrovirus infections in immune compromised patients (4, 42). Molberg *et al*, have also demonstrated astrovirus-specific Th-1 $CD4^+$ T cells in the intestines of healthy adults (29). However, these previous reports primarily involve healthy adults, and demonstrate factors involved in protecting the host from repeated infection. Astrovirus infections are typically associated with immature or infirmed immune systems. In these hosts, the role of humoral and cellular immunity is hindered or non-existent however, astroviruses seldom establish persistent infections. As a result we were interested in studying the host response to primary infection in a young animal. Understanding the mechanisms involved in viral clearance and disease resolution under these circumstances would greatly advance our understanding of viral enteritis and potential general therapies. The lack of acquired immunity to TAstV-2 infection suggests the turkey model may reflect the host response in the non-competent immune host.

Our results suggest that in the absence of an adaptive immune response, the innate immune system may be critical in controlling the disease. We observed that adherent splenocytes from the infected animals produced more NO when stimulated with LPS than mock infected controls (Fig 5.2). This response has also been noted to a lesser extent with LPS treated peripheral blood leukocytes (data not shown). These results indicate that adherent splenocytes are effectively primed by the astrovirus insult, making them more susceptible to secondary stimulation with LPS (1). To determine the role of macrophages in astrovirus pathogenesis, we initially used a less complex, well-defined avian macrophage cell line, HD11 cells. HD11 cells released NO following *in vitro* inoculation with viruses (32) and bacterial products, and are more easily activated than primary avian cells (8).

TAstV-2 increased iNOS activity using EIF, homogenized intestines, and purified virus *in vitro* (Fig 5.2 and 5.4), through a replication-independent mechanism (Fig 5.6). NO is important in several viral diseases, and its effects can range from pro-pathogen to pro-host (25). Production of NO enhanced HIV replication (5), and increased the inflammation and pneumonia associated with influenza infection in mice (2). Inversely, the production of NO inhibited viral replication and delayed death in rabies and coxsackievirus infected mice (10, 39, 40, 43). The increased expression of iNOS following stimulation with TAstV-2 led us to speculate that NO may play a role in astrovirus pathogenesis.

To determine the effect of NO on astrovirus infection we initially assayed for increased NO activity in infected tissues. Examination of infected and control embryo intestines demonstrated increased staining for nitrotyrosine indicating reactive oxygen species were generated during infection (Fig 5.7). The differences in nitrotyrosine staining, between controls and infected embryos was most notable at 5 dpi. This observation was significant since fluid accumulation in infected embryos is not detected until 5 dpi, suggesting NO may be involved in fluid accumulation. NO is known to affect enterocyte barrier function and ion transport (6, 31, 34). The increased nitrotyrosine detected in infected embryo intestines support our *in vitro* evidence that TAstV-2 induced NO expression in macrophages. It is possible responding cells such as MΦ may be responsible, however enterocytes also produce iNOS in response to stimuli (30), and express many of the pattern recognition receptors of the innate immune system (7). However, given the lack of phenotypic markers available for turkeys, we are not able to identify the cell type responsible for the increased NO activity.

Regardless of the source of NO, it is important in controlling viral replication and viral clearance. This is supported by our finding that exogenous NO dramatically inhibited the replication of TAstV-2 *in ovo*, and inhibition of the iNOS response led to increased viral titers (Fig 5.8 and 5.9). We are currently examining the specific mechanisms involved in iNOS expression, and the mechanisms by which NO inhibits astrovirus replication. The current studies indicate iNOS activity can be increased independent of viral replication. Studies are underway to determine if NO inactivates TAstV-2 directly, or if increased NO levels lead to increases in interferon or other cytokines expression which may establish an anti-viral state within the tissue. Preliminary studies suggest EIF contains interferon activity, and that exogenous interferon can limit viral replication. In addition, we are examining the role of NO in astrovirus pathogenesis. Previously we demonstrated levels of active transforming growth factor-β (TGF-β) increase significantly in infected poults and embryos. TGF-β is typically considered anti-inflammatory, and has been described to down-regulate iNOS expression (23). Determining the relationship between these powerful immune-modulating compounds, in context of astrovirus infection, will increase our understanding of astrovirus disease, and viral enteritis in general.

Acute viral gastroenteritis is second only to acute respiratory diseases in terms of impact on human health (12). The intestinal mucosal surface represents both the largest point of entry as well as largest organ of the immune system (14, 36). Through a greater understanding of how viruses interact with and are recognized by the intestinal system it will be possible to manipulate these mechanisms in order to cure or prevent disease. Astrovirus is an endemic intestinal pathogen which has the greatest impact on infants and the elderly (28). In any primary infection it is the responsibility of the innate immune system to recognize the foreign antigen and initiate and direct the body's response (15). It is this line of defense which we hope to understand. These data represent the first report, to our knowledge, of an astrovirus-mediated activation of macrophages, as well as description of an important role of macrophages and innate immunity in the host response to astrovirus infection. The development of effective vaccines has been and remains the most sought after therapeutic or prevention measure to viral diseases. However, given the acute nature of the disease and the immune status of those most affected therapies based on an understanding of the innate immune response may be more efficacious.

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Table 5.1. CD4/CD8 ratio from infected turkey poults

	5 dpi	9 dpi	16 dpi
PBLs			
Control	13[a]	7	21
TAstV-2	11	8	21
Spleen			
Control	14	7	10
TAstV-2	9	11	9

[a]Ratio of percentage of single positive $CD4^+$ cells versus percentage of single positive $CD8^+$ cells isolated from peripheral blood or spleens of infected or non-infected poults. Value reported is average of three experiments.

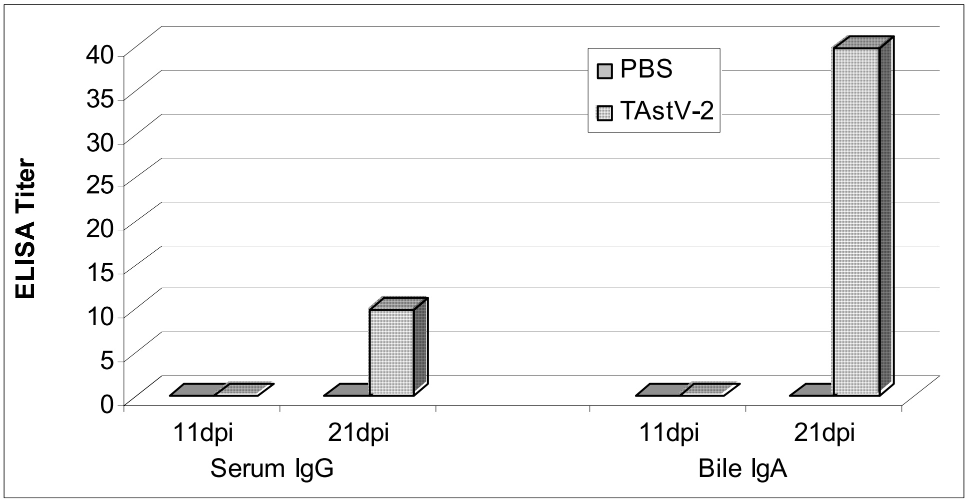


Fig 5.1. TAstV-2 specific IgG and IgA responses following infection. Serum and bile were isolated from TAstV-2-infected or mock infected turkey poults at 11 and 21 days post-infection. Serial-dilutions were incubated with recombinant TAstV-2 capsid protein complexed to microtiter plates and IgG and IgA detected using alkaline phosphatase conjugated goat-anti-chicken IgG or IgA. ELISA titers are reported as the reciprocal of the dilution factor for each sample. Results are representative of 3 experiments.

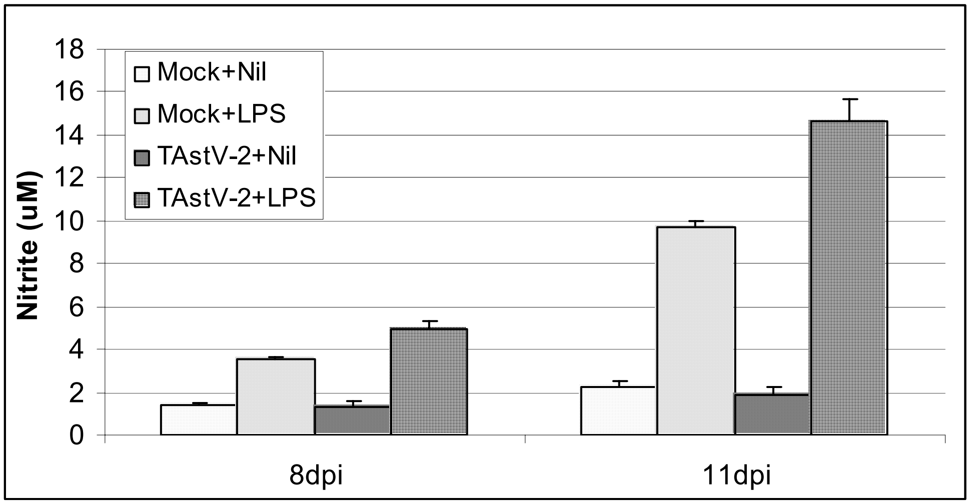


Fig 5.2. Adherent splenocytes from TAstV-2 infected poults are more responsive to LPS stimulation. Commercial turkey poults were inoculated at 5 days of age with TAstV-2 or sham inoculated with PBS. At days 8 and 11 post inoculation, 5 birds from both infected and non-infected groups were sacrificed and spleens harvested. Adherent cells were selected by culturing overnight on glass plates, collected and cultured in a 96-well plate at 10^6 cells/well. Cells were incubated with and without LPS (10 ng/ml) for 48 hrs, then 50 µl supernatants removed and Griess assay performed. Results are expressed as the average µM concentration of nitrite present in the supernatant of triplicate wells and error bars represent standard deviation of the mean. This data is representative of at least 3 experiments.

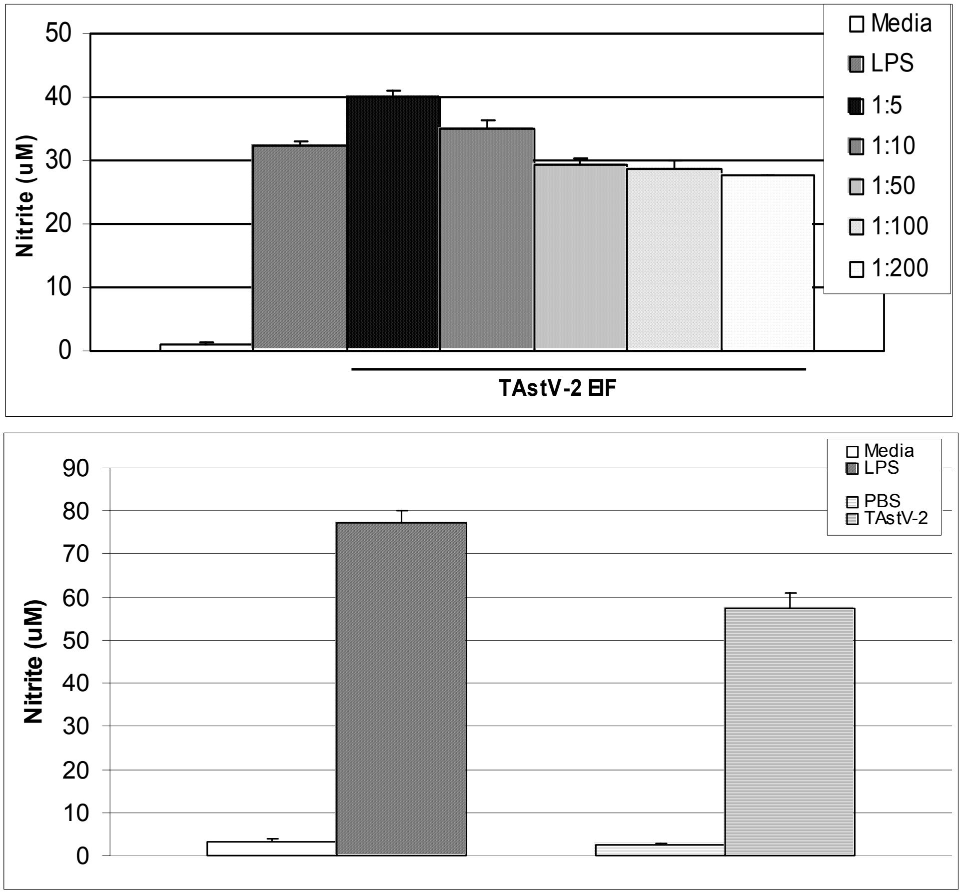
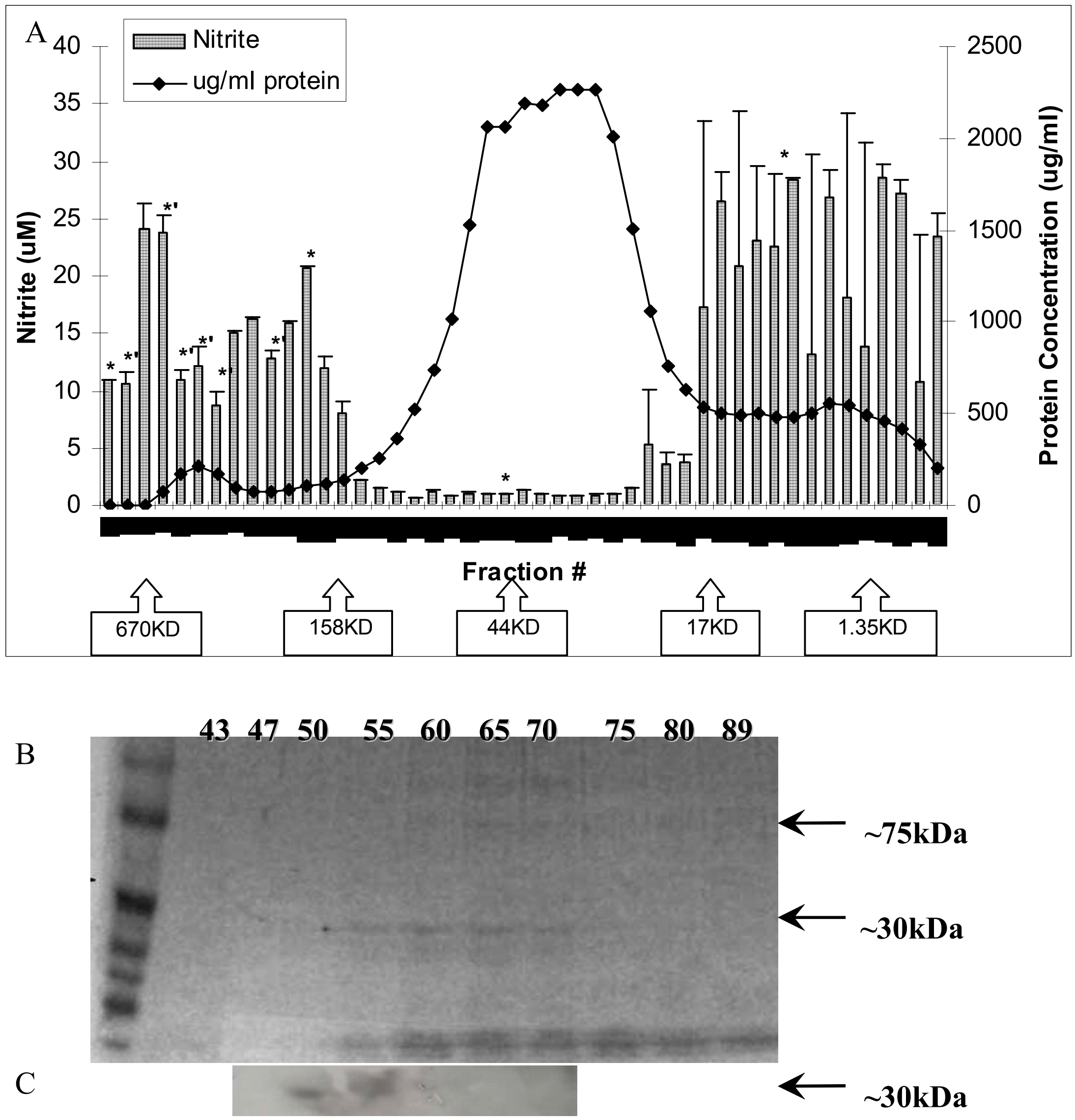


Fig 5.3. Stimulation of avian macrophages by crude TAstV-2 infectious material. HD11 cells (1 x 10^5) were incubated with media alone, 10 ng/ml LPS, and EIF added to a final dilution of 1:5, 1:10, 1:50, 1:100, 1:200 (A), or with 10 µg of total intestinal protein from PBS controls or TAstV-2 inoculated embryos (B) for 48 hrs. All wells were brought to final volume of 100 µl and nitrite measured using the Griess assay. Results are expressed as the average µM concentration of nitrite present in the supernatant of triplicate wells and error bars represent standard deviation of the mean. This data is representative of at least 3 experiments.

Fig 5.4. Purification of TAstV-2 by size exclusion low pressure liquid chromatography. TAstV-2 containing fluid was fractionated using a Sephacryl CL-6B gel filtration column pre-equilibrated in TBS pH 7.4. The column was run at a flow rate of 0.5 ml/min and 2 min fractions collected, with a total run time of 180 min. A) Bars represent the μM concentration of nitrite present in the supernatants of HD11 cells following 48hr incubation with 3.5 μg of total protein from each column fraction as determined by Griess assay. Line represents the total protein in each column fraction as determined by BCA assay. Column fraction with * represent those which were negative for TAstV-2 by RT-PCR, while *' represent those which were positive by RT-PCR. Arrowed boxes at the bottom of the panel represent relative size cut offs as previously determined by prestained markers and related back based on time. B) Total protein stain for TAstV-2 of column fractions found to be positive for virus by RT-PCR. Predicted astrovirus proteins seen at approx. 75 kDa and 30 kDa size. C) Detection of 30 kDa TAstV-2 surface protein by convalescent sera.


A
Nitrite
ug/ml protein
Nitrite (uM)
Protein Concentration (ug/ml)
Fraction #
670KD
158KD
44KD
17KD
1.35KD
B
43 47 50 55 60 65 70 75 80 89
~75kDa
~30kDa
C
~30kDa

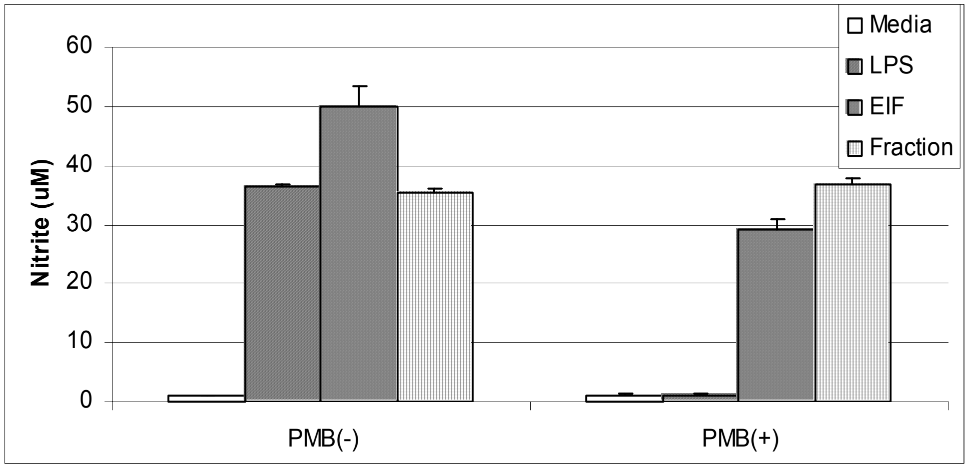


Fig 5.5. TAstV-2 mediated expression of NO is not inhibited by PMB. HD11 cells were incubated for 48 hrs with media alone, LPS (10 ng/ml), 20 µl of EIF or 2 x 10^5 VG/well of column purified TAstV-2 fraction with or without the endotoxin inhibitor PMB (1.5 µg/ml). Results are expressed as the average µM concentration of nitrite present in the supernatant of triplicate wells and error bars represent standard deviation of the mean. This data is representative of at least 3 experiments.

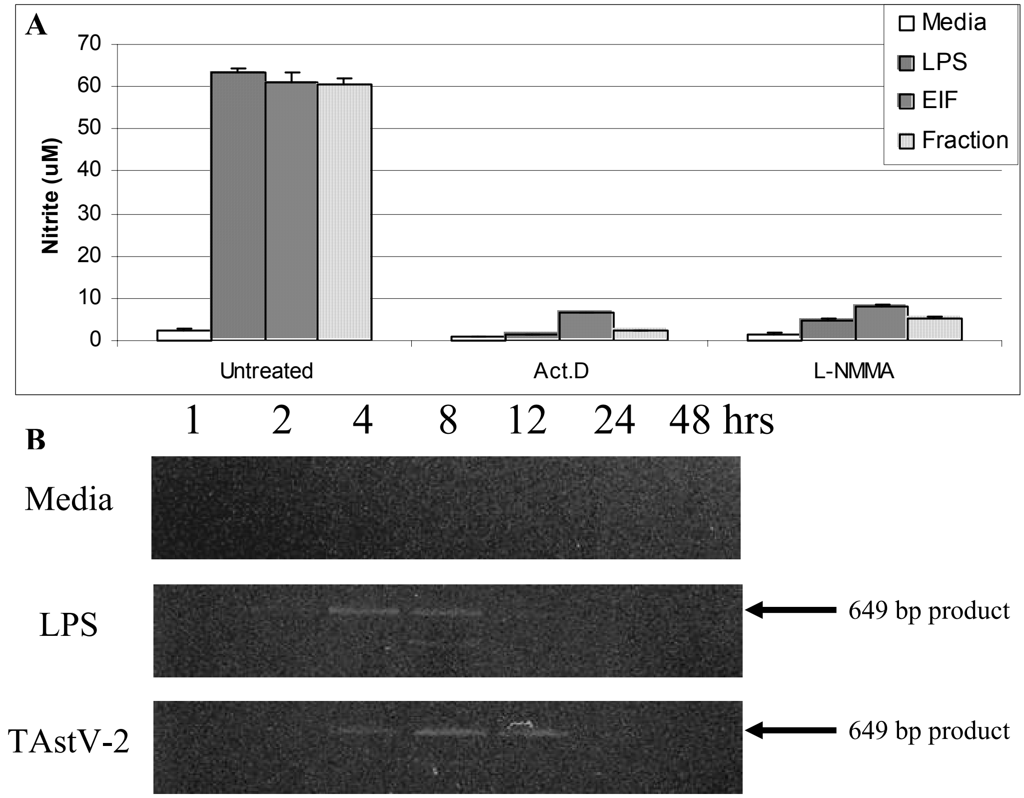


Fig 5.6. TAstV-2 mediated expression of NO requires gene transcription and NOS activity. A) HD11 cells were incubated for 48 hrs in the presence of media alone, LPS (10 ng/ml), 20 µl of EIF or 2 x 10^5 VG/well of column purified TAstV-2 fraction with or without the RNA transcription inhibitor actinomycin D (1 µg/ml) or the nitric oxide synthase inhibitor L-NMMA (4 mM). Results are expressed as the average µM concentration of nitrite present in the supernatant of triplicate wells. B) TAstV-2 induces expression of iNOS RNA. HD11 cells stimulated with media alone, LPS (10 ng/ml), or TAstV-2 (2 x 10^5 VG/well) for 1, 2, 4, 8, 12, 24, or 48 hrs. At each time point, total RNA was isolated and treated with DNase I. Samples were then analyzed by RT-PCR for the presence of iNOS RNA. Detection of expected 649 bp product was observed. These data are representative of at least 3 experiments. Error bars represent the standard error of the mean.

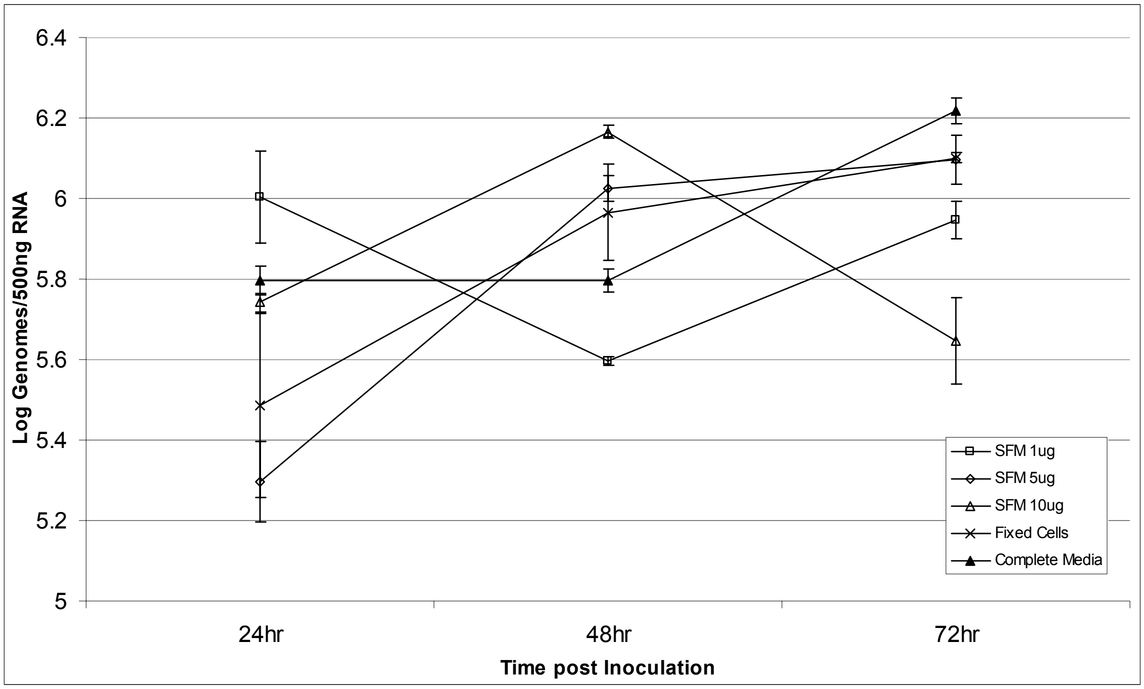


Fig 5.7. TAstV-2 RNA detected in HD11 cells following 24, 48, and 72 hrs of culture. HD11 cells (5 x 10^5) were incubated overnight, infected with TAstV-2 and cells cultured in 500 µl of SFM media containing 1 µg/ml (open square), 5 µg/ml (open diamond), 10 µg/ml trypsin (open triangle), or 500 µl of complete media (closed triangle). As a control for input virus, cells were fixed by drying and then infected as above. These cells were incubated in SFM containing 10 µg/ml trypsin (x's). All samples were amplified in duplicate; data are representative of at least 3 experiments. Results are reported as the $\log_{10}$ of the number of viral genomes detected in 500 ng of total RNA. Cells mock infected with PBS and cultured in complete media were used as a negative control, and assayed to have no detectable virus (not shown). Error bars represent the standard error of the mean.

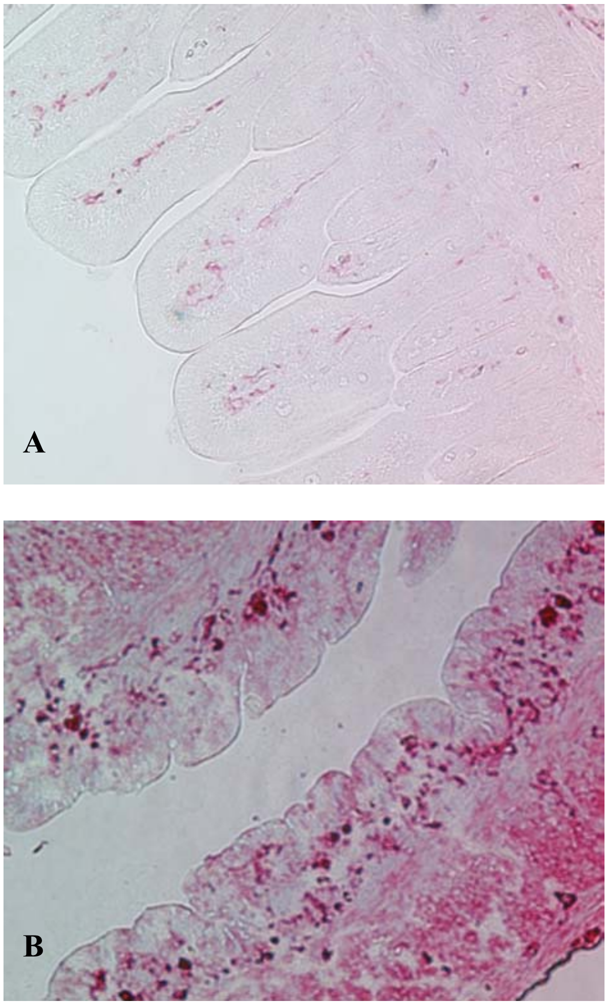


Fig 5.8. Increased nitrotyrosine staining in TAstV-2-infected intestines. Embryos were inoculated with 100 µl PBS (A) or 100 µl of TAstV-2 containing 1 x 10^8 VG (B), incubated for 5 days, then the duodenum isolated and stained for nitrotyrosine residues followed by FastRed detection. Panels are representative of 3 separate groups of infected animals. Original magnification is 20X.

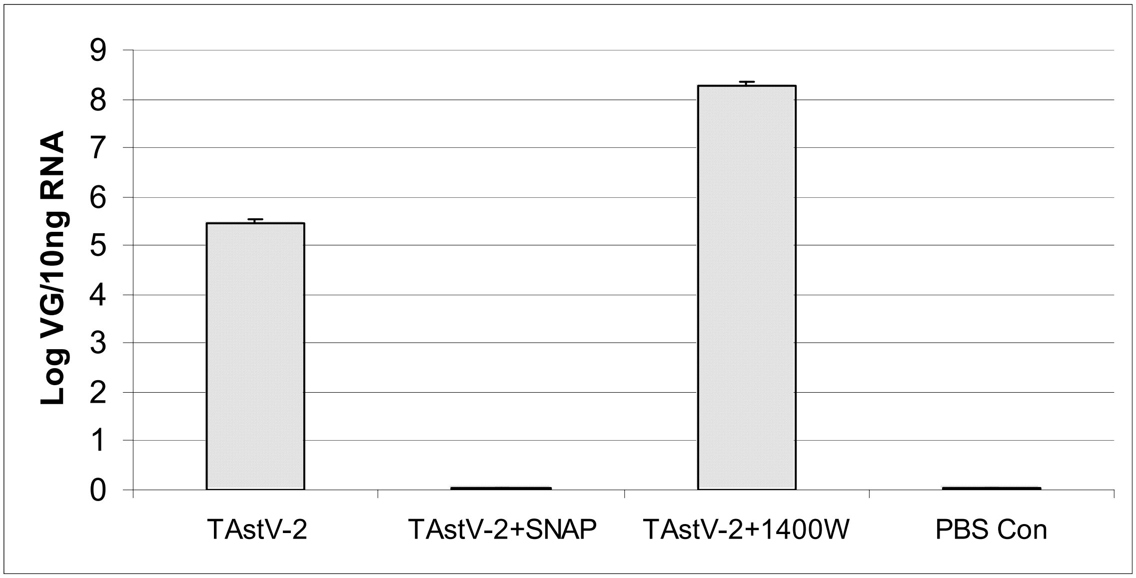


Fig 5.9. NO inhibits TAstV-2 replication *in ovo*. Total RNA isolated from control and TAstV-2 infected embryo intestines incubated with PBS, SNAP (500 μM) or 1400W (10 mg/kg) and analyzed for TAstV-2 genome levels by Real Time RT-PCR. All samples were amplified in duplicate; data is representative of at least 3 experiments. Results are reported as the log_{10} of the number of viral genomes detected in 10 ng of total RNA. Error bars represent the standard error of the mean.

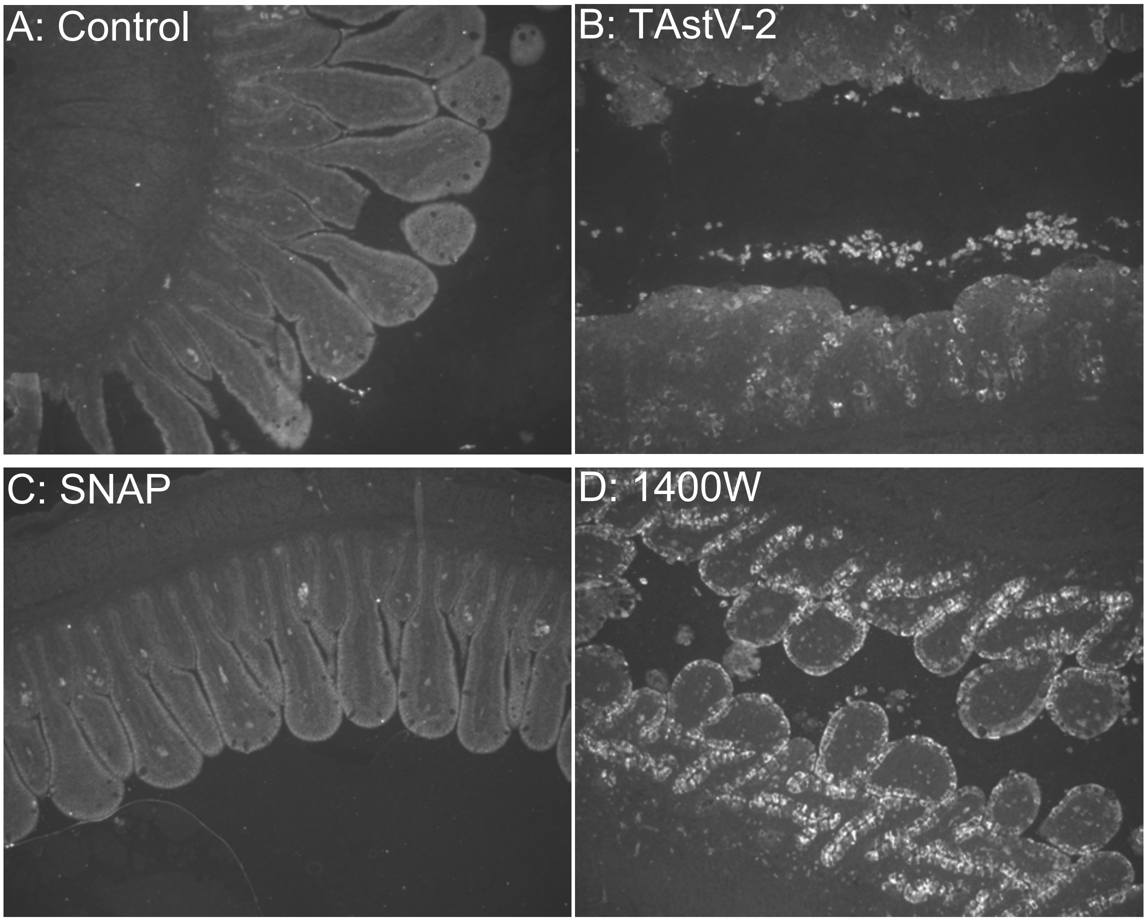


Fig 5.10. NO affects levels of TAstV-2 antigen staining *in ovo*. Photomicrograph of the distribution of specific immunofluorescence staining against astrovirus capsid antigen of (A) 100 μl of PBS, (B) 5 x 10^6 VG of TAstV-2, (C) 5 x 10^6 VG TAstV-2 + 500 μM SNAP, or (D) 5 x10^6 VG TAstV-2 + 10 mg/kg 1400W intestines of embryo intestines at 5 dpi. Panels are representative of intestines of 3 embryos. Original magnification is 10X.

CHAPTER 6

ASTROVIRUS BINDS AVIAN MACROPHAGES TO STIMULATE NITIRIC OXIDE[1]

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[1]Koci, M. D. and S.Schultz-Cherry. 2003. *Journal of General Virology*. To be Submitted

ABSTRACT

Macrophages (MΦs) play a key role in the immune response to a number of viral infections. They function as innate responders to infection, antigen presenting cells and initiators of the inflammatory and adaptive immune responses. The anti-viral response mediated by MΦs involves the release of compounds which act directly to inactivate the virus itself, killing and removal of infected cells, and recruitment of other responding cells. We recently described the activation of avian MΦs by turkey astrovirus type-2 (TAstV-2) in the absence of viral replication, and the ability of nitric oxide (NO) to inhibit TAstV-2 replication *in vivo*. In the current study we investigated the nature of the interaction between the avian MΦ cell line, HD11, and TAstV-2. We demonstrated that TAstV-2 specifically bound to HD11 cells by an unidentified surface protein. This binding interaction was partially inhibited by EDTA-mediated removal of divalent cations. However, removal of sialic acid, heparin, heparan sulfate, or chondroitin sulfate, did not inhibit TAstV-2 binding to HD11 cells. Following binding, TAstV-2-induced NO activity through a pathway involving endocytosis and tyrosine kinase activity. To confirm that NO activation is independent of low levels of viral gene expression or abortive replication, HD11 cells were treated with baculovirus-expressed recombinant TAstV-2 capsid protein. These experiments demonstrated that the capsid protein stimulated avian MΦs. Collectively these data demonstrated that avian MΦs specifically recognized and responded to TastV-2 in a manner which led to an up-regulation of inducible NO synthase expression, and suggested that this interaction was a key aspect of the host response to primary astrovirus infection.

INTRODUCTION

Macrophages (MΦs) are a subset of leukocytes found in every tissue of the body, and function both as one of the first lines of defense and initiators/regulators of adaptive immunity (Gordon, 1998). MΦs play a vital role in most infectious diseases. The beneficial aspects of the MΦ response to bacterial agents are well documented; however, only recently have we begun to appreciate their role in the response to viral infections (Guidotti & Chisari, 2001). Activated MΦs and their products are important in controlling replication of poxviruses virus (Karupiah *et al*., 1993), rhabdoviruses (Bi & Reiss, 1995), herpesviruses (Komatsu *et al*., 1996), picornaviruses (Zaragoza *et al*., 1999), and hepadnaviruses (Guidotti *et al*., 1996). The role of MΦs in viral immunity is to detect the presence of the offending agent and respond by producing compounds which both directly combat the virus as well as recruiting additional cell populations. Activated MΦs release products such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), and nitric oxide (NO) which limit the replication of many viruses (Janeway & Medzhitov, 2002, Reiss & Komatsu, 1998). Additionally, a variety of other cytokine and chemokines released by activated MΦs recruit neutrophils, T cells, and B cells to the sites of infection to initiate and direct the inflammatory response and acquired immunity (Gordon, 1998).

We recently demonstrated that MΦs respond to astrovirus infection by increasing inducible NO synthase (iNOS) expression. NO then inhibits astrovirus replication in vivo (Koci *et al*., 2003a). In humans, astroviruses are one of the leading causes of childhood diarrhea, and are of increasing importance in the elderly and immunocompromised (Mitchell, 2002). Very little is known about the host factors involved in disease resolution and viral clearance during primary infection (Matsui & Greenberg, 2001). Our previous studies demonstrated that *in vitro*, a turkey astrovirus (turkey astrovirus type 2/North Carolina/034/1999, TAstV-2) stimulated avian MΦs through a replication-independent mechanism, while the addition of exogenous NO significantly inhibited viral replication *in vivo* (Koci *et al*., 2003a). In the current study we investigated the nature of this interaction. These data demonstrated that TAstV-2 bound to the avian MΦ cell line, HD11, through an as yet unidentified surface protein, and increased the expression of NO through a mechanism requiring tyrosine kinases and endocytosis. Finally, our data demonstrated that the TAstV-2 capsid protein was sufficient for increased iNOS expression. These data increase our understanding of the interaction between astroviruses, and the immune system, specifically MΦs, and suggest that these cells are vital in the host response to primary astrovirus infection.

METHODS AND MATERIALS

TAstV-2 isolation.

TAstV-2 was isolated and propagated as described (Koci *et al*., 2003b, Koci *et al*., 2000b). Briefly, fluid isolated from the intestines of TAstV-2 infected turkey poults were clarified by centrifugation (500 x g for 10 min), 0.2 μm filtered, diluted 10^{-3} in PBS, and inoculated into the yolk sac of 20-day-old specific pathogen-free (SPF) turkey embryos. Five days post-inoculation, embryo intestines and intestinal fluid were collected separately. TAstV-2 was purified by size fractionation as previously described (Koci *et al*., 2003a). Briefly, a 1.5 x 50 cm column (BioRad) with 80 ml of Sephacryl CL-6B (Sigma) gel pre-equilibrated in tris-buffered saline (TBS, pH 7.4) was run at a flow rate of 0.5 ml/min and 2 min fractions collected, with a total run time of 180 min. Fractions were analyzed for total protein concentration using BCA analysis (Pierce), and tested for TAstV-2 using RT-PCR, SDS-PAGE, and western blot analysis. Routine detection of TAstV-2 by RT-PCR was performed using TRIzol Reagent™ (Invitrogen) to isolate total RNA from embryo intestinal fluid (100 μl), or purified virus (100 μl) as previously described (Koci *et al*., 2000a).

Cell culture.

The chicken macrophage cell line (HD11) was kindly provided by Dr Kirk Klasing (University of California, Davis). Cells were cultured with RPMI 1640 (Cellgro) supplemented with 5% heat inactivated fetal bovine serum (FBS, Cellgro) and L-glutamine (2 mM, Cellgro), in a humidified incubator with 5.5% CO_2 at 41º C.

Binding Assay.

The ability of TAstV-2 to bind to HD11 cells was determined using a flow cytometry based binding assay as described (Stewart & Stewart, 1997) with modifications. Briefly, 1 x 10^6 cells were washed with 5 volumes of cold PBS, incubated on ice with or without 7.5 μg of purified TAstV-2 for 1 hr, washed with 1 ml of cold PBS then incubated for 1 hr on ice with 5 μl of a polyclonal mouse ascites generated against purified TAstV-2 capsid protein. Finally, after further washing cells were incubated with fluorescein conjugated goat anti-mouse IgG (Sigma) for 1 hr then resuspended in 1 ml of cold PBS. TAstV-2 binding was analyzed using a BD FACScan flow cytometer, and Cell Quest software (BD Biosciences). Viable cells were identified by forward and side scatter properties and exclusion of propidium iodide (Calbiochem) staining and gated. A total of

10,000 events were analyzed. Each assay was repeated a minimum of three times. Results were analyzed using WinMDI version 2.8 (Joseph Trotter, Scripps Institute).

Chemical inhibition of TAstV-2 binding.

To determine the cellular protein(s) binding to TAstV-2, cells were treated with various chemicals and enzymes and their effects on virus binding determined by flow cytometry as described above or inhibition of NO activity. HD11 cells (1 x 10^6) were resuspended in 200 µl of serum free RPMI and incubated at 37 ºC for 1 hr with each of the following enzymes. N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Pierce) was used at final concentrations of 0.25-10 µg/ml. Proteinase K (BioRad) was used at final concentrations of 1 or 5 µg/ml. Recombinant α2-3,6,8,9-Neuraminidase (Calbiochem) was used at final concentrations between 30-120 mU/ml. Recombinant chondroitinase ABC (Calbiochem), heparinase I and heparinase III (Sigma) were each used at final concentrations between 0.25-5 U/ml. EDTA (Fisher) or EGTA (Sigma) were added to a final concentration of 100 mM, and cells incubated with virus on ice in the presence of chelating agent. Each compound at the above concentrations was demonstrated to have no effect on HD11 cell viability, as determined by trypan blue exclusion.

Nitrite assay.

Up-regulation and expression of iNOS in HD11 cells was measured indirectly by determining the levels of nitrite in cell culture supernatants using the Griess assay (Mullins *et al*., 1999). Briefly, 1 x10^5 cells/well were treated with RPMI alone, RPMI containing Lipopolysaccharides (LPS) from *Escherichia coli* 0127:B8 (Sigma), or column purified TAstV-2, and incubated in a final volume of 100 µl at 41º C for 48 hr.

Following incubation, 50 μl of cell free supernatant was assayed for the presence of nitrite by mixing with equal volumes of 1% sulfanilamide (in 5% phosphoric acid, Sigma) and 0.1% *N*-1-napthylethylenediamine dihydrochloride (Sigma). Plates were incubated for 15 min in the dark, absorbance measured at 550 nm using a spectrophotometer, and the nitrite concentration determined by comparing to a nitrite standard curve. All treatments were done in triplicate, and each experiment performed at least three times. Media and column purified TAstV-2 were tested for contaminating endotoxins using the Limulus Amebocyte Lysate QCL-1000 Kit (BioWhittaker). All reagents tested were found to have less than 1 endotoxin unit (EU)/ml. LPS treatment added to HD11 cells contained at least 6 EU/ml. The endotoxin inhibitor polymyxin B sulfate (PMB, Fluka) was added at a final concentration of 1.5 μg/ml to samples to eliminate any affects of LPS.

Inhibition of iNOS signaling.

The role of tyrosine kinase and Mitogen-activated protein kinases (MAP kinase) activity in TAstV-2 induced iNOS expression was determined using the Tyrphostin Inhibitor Set II, the MAP Kinase Inhibitor Set II (Calbiochem), or the broad spectrum tyrosine kinase inhibitor, Genistein (Spectrum Laboratory Products). The role of the endocytic pathway in TAstV-2-induced NO activity was determined using monensin (Calbiochem), a chemical which blocks endosomal acidification. HD11 cells (1 x 10^5/well) were pretreated with each compound for 1 hr at 41º C and then stimulated with LPS or TAstV-2 and the levels of nitrite measured at 48 hrs as described above. Each compound at its corresponding concentration was demonstrated to have no effect on viability using trypan blue exclusion, likewise diluents used for each chemical were tested on HD11 cells at analogous concentrations and shown to have no affect on activity or viability (data not shown).

Expression of baculovirus-expressed recombinant TAstV-2 capsid protein.

Recombinantly-expressed TAstV-2 capsid protein was generated utilizing the Bac-To-Bac Baculovirus Expression System (Invitrogen) following manufacturer's instructions. Briefly, the TAstV-2 capsid gene was sub-cloned from pcDNA3.1^{-}/TAstVcap10 into the pFastBac^{TM} HTa expression vectors (Invitrogen) to generate pFastBacHT/TAstV-2capsid. The resultant plasmid was screened by sequence analysis to ensure generation of the fusion protein, and to confirm the integrity of the TAstV-2 gene. The construct was recombined into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome via DH10Bac cells. The recombinant baculovirus (rAcNPV/TAstV-2capsidHis) was propagated in serum-free media (SFM)-adapted Sf9 insect cells and used to express TAstV-2 capsid protein. Baculovirus infected insect cells were lysed with 50 mM Tris (pH 8) containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 1% NP40. Cells were frozen and thawed twice and cell debris removed by centrifugation. His-tagged rTAstV-2 capsid protein was purified using Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. Affinity purified His-tagged protein was purified over a D-Salt Excellulose GF-5 Desalting Column (Pierce) to remove the imidazole elusion buffer, and samples were checked for protein by SDS PAGE and western blot using Penta-His Ab (Qiagen) or anti-KHL IgG as previously described (Koci *et al.*, 2003b).

TAstV-2 capsid peptides.

Three peptides derived from predicted amino acid antigenicity and surface probability analysis were synthesized commercially (Invitrogen) corresponding to amino acid positions 32-47 (RSRTKKTVKIIEKKPE, RSR), 194-221 (HPRSALGPRQGWWNVDPGD, HPR) and 676-691 (KHLEEEKNYWKNQCER, KHL). These peptides were used to stimulate HD11 cell, in soluble form, immobilized on microtiter plates, or cross-linked using disuccinimidyl suberate (DSS, Pierce). HD11 cells (1 x 10^5/ well) were stimulated with 1-25 μg of peptides or bovine serum albumin (BSA) in each of the above forms.

RESULTS

TAstV-2 binds to HD11 cells.

We previously demonstrated that TAstV-2 induced NO activity in HD11 cells in the absence of productive replication (Koci *et al*., 2003a). Based on this observation, we hypothesized TAstV-2 activated macrophages through binding. To determine if binding alone was sufficient for NO stimulation, we first asked whether TAstV-2 specifically bound to HD11 cells using flow cytometry. TAstV-2 bound to 60% of the HD11 cells (Fig. 6.1). Bovine serum albumin (BSA) had no effect on binding, suggesting that the binding is specific. We were unable to demonstrate binding saturation using purified virus.

To determine the general class and/or mechanism of TAstV-2-receptor interaction, HD11 cells were pretreated with a panel of compounds and enzymes to remove or block specific cellular moieties typically utilized as viral receptors (Table 6.1). Since saturable binding was not obtained, cells were incubated under conditions in which 50-60% of the total cells were positive for virus binding, and the effect of each compound on binding measured as a change in the percentage of positive cells. The positive control was set to

100% and each treatment group was reported as percent positive cells relative to the positive controls. To ensure that the reduction in binding observed for each compound was specific, a minimum of 25% reduction in binding was required to be considered an inhibitory treatment (Martinez & Melero, 2000). Using these criteria we found minimal effects on binding by the majority of these compounds. Pre-treatment with recombinant neuraminidase, chondroitinase ABC, and heparinase I and III, had only minimal affect on virus binding (Fig. 6.2 A and B), suggesting that TAstV-2 does not utilize sialic acid, chondroitin sulfate, heparin or heparan sulfate glycosaminoglycans for binding to macrophages. Incubating cells with trypsin reduced binding by 34% suggesting that a surface protein may be important for binding. Increasing concentrations of trypsin did not inhibit viral binding (Fig. 6.2 B). Digestion of surface proteins with the broadly reactive proteinase K inhibited binding in a dose-dependent manner (Fig. 6.2 C). TAstV-2 binding to cells treated with 5 μg/ml of proteinase K was reduced by 75%. These results suggest TAstV-2 specifically binds to an unidentified protein on the surface of HD11 cells, through a mechanism which is not dependent on sialic acid, heparan sulfate or chondroitin sulfate residues.

To determine if metal ions were important for TAstV-2 binding to HD11 cells, binding was measured in the presence of the chelating agents EDTA and EGTA. EDTA (100 mM) reduced binding by ~30% while 100 mM EGTA had no effect on binding (Fig 6.2 C). Together these results suggested that the binding interaction of TAstV-2 and HD11 cells involves a surface protein, but sialic acid, chondroitin sulfate, or heparan sulfate carbohydrate structures are not involved. The inhibition detected in the presence of EDTA suggests that divalent cations may be important in virus binding.

Recombinant TAstV-2 capsid protein stimulates NO.

TAstV-2 bound to HD11 cells. To demonstrate that binding alone is sufficient for NO activation, recombinant TAstV-2 capsid protein (rTAstV-2cap) was produced in the baculovirus expression system. Western blot analysis and electron microscopy confirmed that recombinant capsid protein was expressed in infected insect cells (data not shown). His-tagged TAstV-2 capsid protein was affinity purified (Fig 6.3) and added to HD11 cells. The addition of 1 μg of affinity purified rTAstV-2cap to HD11s stimulated NO production (Fig 6.4). NO levels were similar in cells treated with purified TAstV-2. These data demonstrated that the TAstV-2 capsid protein was sufficient to stimulate MΦ expression of NO.

TAstV-2 capsid peptides do not stimulate iNOS.

To begin defining the region(s) of the capsid important in activation of iNOS, cells were treated with peptides derived from the TAstV-2 capsid sequence. These peptides were selected based on surface probability and antigenicity index analysis, as well as sequence conservation (Fig 6.5 A). HD11 cells were treated with the peptides in soluble, bound, and cross-linked forms (Fig 6.5 B-D). None of the peptides stimulated NO production regardless of form (Fig 6.5). In addition, pre-incubating TAstV-2 with purified IgG specific to these peptide sequences failed to inhibit NO activity when added to HD11 cells, and pre-incubating the cells with the peptides did not inhibit binding (data not shown). These results suggested that these peptides did not represent the cellular binding regions.

TAstV-2-induced iNOS signaling requires tyrosine kinases and endocytosis.

To determine the key intracellular signaling events involved in TAstV-2-mediated-iNOS stimulation, we pre-treated HD11 cells with a panel of tyrosine kinase and MAP kinase inhibitors (Table 6.2) and determined which inhibitors affected NO activity following TAstV-2 stimulation. Cells were treated with TAstV-2 in the presence of the broadly reactive inhibitor of tyrosine kinase activity, Genistein (GEN) (Kogut *et al*., 2001). Treatment with GEN resulted in an 18-fold reduction on the amount of NO activity following TAst-2 stimulation (Fig 6.6). As compared to the 11-fold reduction in LPS induced NO activity. These results suggest that tyrosine kinase activity is involved in TAstV-2-mediated iNOS expression.

To further examine the role of intracellular kinase activity in the HD11 cell response to TAstV-2, cells were treated with a panel of chemical inhibitors of tyrosine kinases and the serine/threonine kinases MAP kinases. Treatment of HD11 cells with 5 different tyrosine kinase inhibitors each demonstrated partial inhibition of NO activity ranging from 20% to 48% (Fig 6.7 A). The effects of AG879, described to inhibit NGF-dependent pp140$^{c\text{-}trk}$ tyrosine phosphorylation, and AG1288 (Ohmichi *et al*., 1993), described to block TNF-α induced cytotoxicity (Novogrodsky *et al*., 1994), had the greatest effect on TAstV-2 stimulated NO activity. AG879 demonstrated a 48% reduction in TAstV-2 NO activity, while it decreased LPS stimulated NO activity by 88%. AG1288 inhibited TAstV-2 stimulated NO by 38%, while having only modest effects LPS stimulation (10%) (Fig. 6.7 A). Conversely, inhibition of MAP kinase had very little effect on activation. Only one of the MAP kinase inhibitors, SB 203580 demonstrated a 20% reduction in TAstV-2-mediated NO activity (Fig 6.7 B). SB 203580 is a specific inhibitor of p38 kinase, and blocked IL-1 and TNFα production in response to LPS.

Together, these data further suggest that tyrosine kinase activity was important in TAstV-2-induced signaling events; however, these signaling events may also involve p38 kinase.

To further understand the mechanisms which lead from virus binding to the release of NO, we examined the role of endocytosis. Previous studies with human astrovirus demonstrated that viral entry utilized the endosomal machinery (Donelli *et al*., 1992). We examined the possibility that similar pathways were involved in HD11 iNOS signaling by pre-incubating cells with monensin and then stimulated with TAstV-2. These results demonstrated a 58% reduction in the amount of NO activity measured (Fig 6.8) suggesting that TAstV-2 mediated signaling requires endosomal acidification. These results imply a need for virus internalization for iNOS signaling.

DISCUSSION

The role of MΦs in the immune system is that of surveillance, antigen presentation, direct engagement of pathogens, as well as recruitment, activation, and direction of other responding cells (Gordon, 1998, Janeway & Medzhitov, 2002). This makes MΦs an important part of both the innate and adaptive immune response to viral agents (MacMicking *et al*., 1997). The understanding of the immune response to an agent is incomplete without an adequate appreciation for the contributions made by these cells. We demonstrated that TAstV-2 stimulated MΦs to express NO, and that NO inhibited viral replication *in vivo* (Koci *et al*., 2003a). In the current study, we demonstrated that TAstV-2 specifically bound to avian MΦs and activated NO by a pathway dependent on tyrosine kinase activity and endocytosis. Additionally, TAstV-2 capsid protein was activated HD11 cells demonstrating that binding alone was sufficient for activation.

Collectively these results suggest avian macrophages are important in the response to astrovirus infection as they are capable of specifically recognizing TAstV-2 and responding with increased expression of NO.

Viral receptors are defined as cell surface structures that bind directly to the native virion; however, many viruses bind to multiple cellular factors through a series of secondary and tertiary co-receptors (Young, 2001). The use of co-receptors confers species barriers and tissue tropisms, and can also allow virion binding to cells which do not support replication (Schneider-Schaulies, 2000). These binding events lead to intracellular signaling and cellular activation (Gern *et al*., 1996). The astrovirus receptor is unknown. Astroviruses encode one structural precursor protein; however, our understanding of how this one protein is folded and modified to create the icosahedral infectious virion is incomplete. To begin to understand how TAstV-2 binds to cells, we examined the ability of the virus to bind to HD11 cells following removal of sialic acid, glycosaminoglycans, surface proteins, or metal ions, all factors known to be utilized as viral receptors (Greber, 2002, Martinez & Melero, 2000, Schneider-Schaulies, 2000). These results demonstrated that TAstV-2 binding to HD11 cells did not involve sialic acid, heparin, heparan sulfate, or chondroitin sulfate. However, removal of metal ions by EDTA reduced viral binding to HD11s, suggesting that metal ions were involved in the cellular receptor-virus interaction. Surface receptors such as integrins and C-type lectins require metal ions to stabilize their receptor functions (Leitinger *et al*., 2000, Weis *et al*., 1998). Conversely metal ions are also important in stabilizing the capsid structure of some viruses (Harrison, 2001). Experiments are currently underway to determine the role of divalent cations in the TAstV-2-HD11 cells interaction. The most significant reduction of TAstV-2-HD11 cell binding was through removal of cellular surface proteins using trypsin and proteinase K, suggesting that the virus bound to a surface protein. However, attempts to isolate and characterize this as yet unidentified surface protein through membrane overlay and receptor cross-linking experiments have been unsuccessful.

To further characterize the effects of TAstV-2 on HD11 cell iNOS expression, we examined the intracellular events following stimulation using a panel of cell signaling inhibitors. These experiments demonstrated that the stimulation of iNOS by TAstV-2 required tyrosine kinases, similar to LPS-induced iNOS expression. The effects of AG879 and AG1288 demonstrated different levels of inhibition between that of TAstV-2 stimulated NO and LPS, suggesting differences in some signaling components. These experiments also showed that the MAP kinase, p38, is also involved in iNOS signaling by TAstV-2. These results suggested that the intracellular events following TAstV-2 stimulation used similar pathways as that of LPS stimulated signaling. This includes the role for endocytosis. The stimulation of NO by both LPS and TAstV-2 were inhibited by inhibition of the endosomal pathway using monensin. This implies that endocytosis and receptor recycling are important in TAstV-2 stimulated NO as it is in LPS activation (Lichtman *et al*., 1998). Finally, we demonstrated that the astrovirus capsid protein alone was stimulated MΦs.

Initial experiments to understand the regions of the capsid protein important in HD11 cell stimulation using capsid protein derived peptides suggested that the three regions examined are either not involved, or require a tertiary structure that was not achieved with these peptides. However, experiments demonstrating that polyclonal sera against the peptides did not inhibit TAstV-2-mediated NO activity, or neutralize the virus (data not shown) indicated that these regions were not involved in binding. Additionally, the addition of peptides to cells did not inhibit virus binding to HD11 cells.

These results are the first, to our knowledge, to demonstrate that astrovirus specifically bound to MΦs. We show that TAstV-2 binding, internalization, and intracellular signaling resulted in increased expression of NO. These events may play a critical role in the control of viral replication, viral clearance, and disease resolution *in vivo*, and may represent new strategies for non-vaccine therapies to astrovirus infection through modulation of innate mucosal immunity.

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Table 6.1. Chemical and enzymatic inhibitors of virus binding.

Compound	Effect
Neuraminidase	Removes sialic acid
Heparinase I	Removes heparin
Heparinase III	Removes heparan sulfate
Chondroitinase ABC	Removes chondroitin sulfate A, B, and C
TPCK-Trypsin	Removes proteins following arginine-lysine residues
Proteinase K	Broadly reactive protease
EGTA/EDTA	Chelate divalent cations

Table 6.2. Chemical inhibitors of tyrosine and MAP kinases.

Compound	Effect
PD 98059	MAP kinase kinase (MEK) inhibitor
SB 203580	p38 kinase inhibitor
U 0126	MEK1 and MEK2 inhibitor
SB 202474	MAP kinase inhibitor negative control
Genistein	Broad tyrosine kinase inhibitor
AG 18	Broad tyrosine kinase inhibitor
AG 213	Broad tyrosine kinase inhibitor
AG 370	Inhibits platelet derived growth factor (PDGF)-induced mitogenesis
AG 879	Inhibits nerve growth factor (NGF)-dependent $pp140^{C\text{-}trk}$
AG 1288	Inhibits TNFα-induced cytotoxicity
AG 43	Tyrosine kinase inhibitor negative control
Monensin	Inhibits endosomal acidification

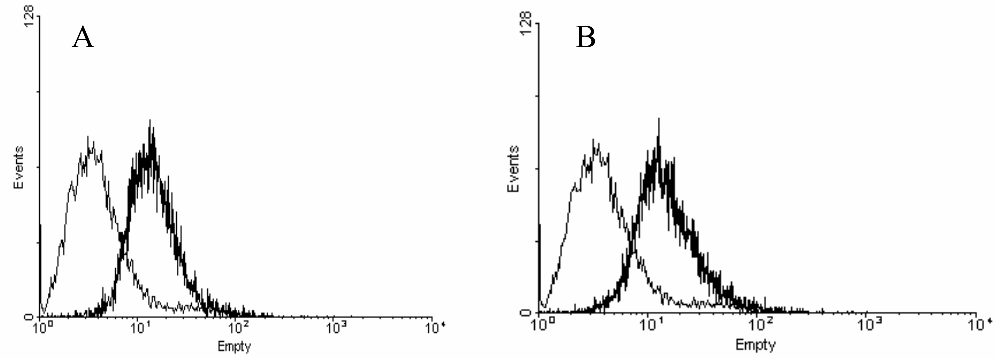
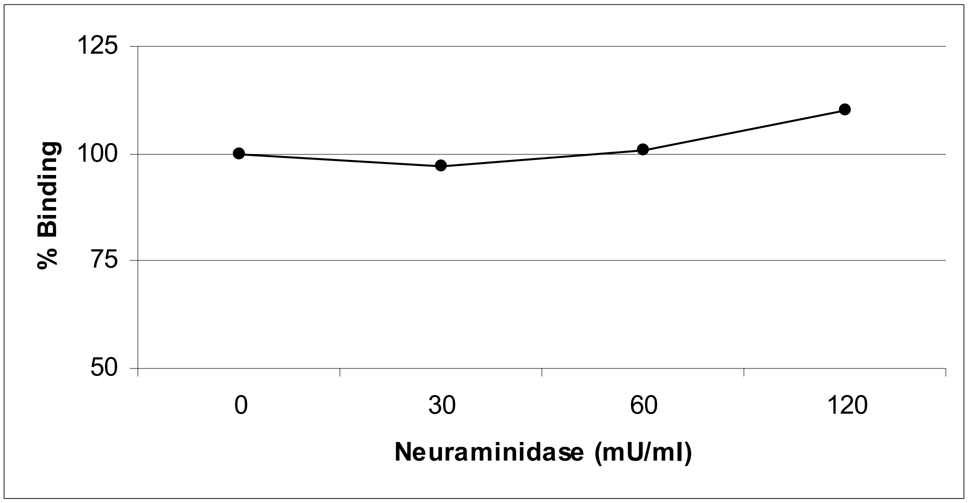
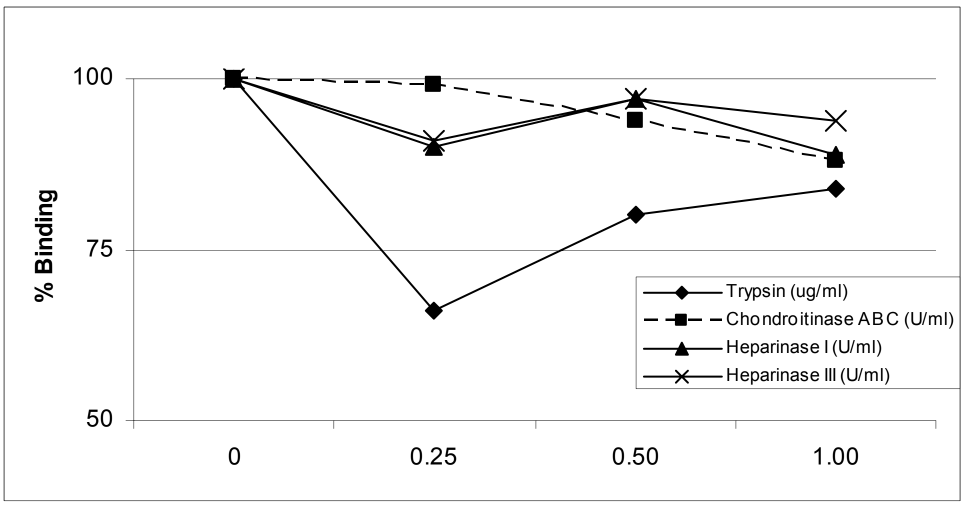


Fig 6.1. TAstV-2 bound to HD11 cells. HD11 cells (1 x 10^6 cells) were washed in cold PBS and allowed to bind to TAstV-2 at 4º C for 1 hr in PBS alone (A)or in PBS+1% BSA (B). Cells were then washed and virus bound to the cell surface detected utilizing a polyclonal mouse anti-TAstV-2 capsid IgG and a FITC conjugated goat-anti-mouse IgG. Cells incubated in PBS+1%BSA without TAstV-2 were used a negative control. Cells were resuspended in 1 ml of cold PBS and analyzed by flow cytometry. HD11 cells incubated with TAstV-2 or TAstV-2 + 1% BSA were found to be 60% and 65% positive for virus binding, respectively.

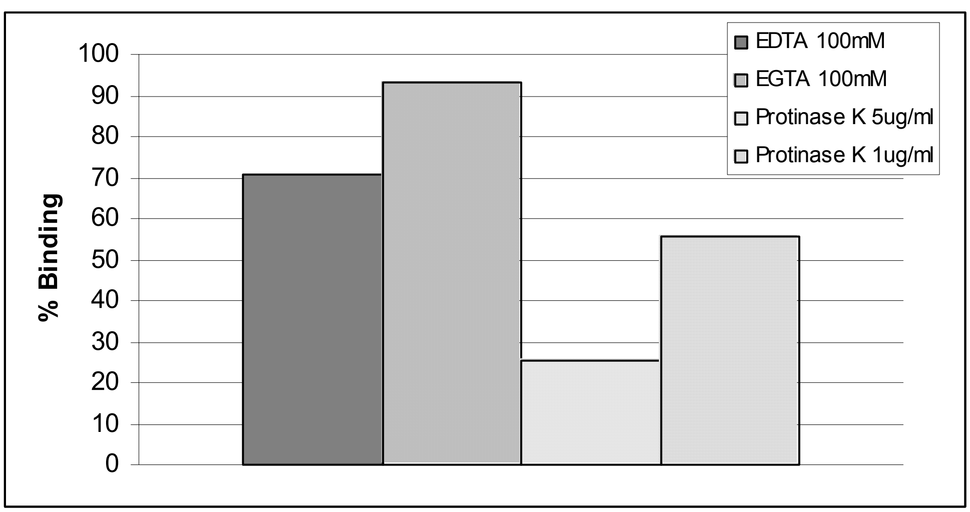
Fig 6.2. Inhibition of TAstV-2 binding. HD11 cells (1 x 10^6) were treated with A) neuraminidase (0, 30, 60, 120 mU/ml), B) trypsin (0, 0.25, 0.5, 1 μg/ml), chondroitinase ABC (0, 0.25, 0.5, 1 U/ml), heparinase I and III (0, 0.25, 0.5, 1 U/ml), or C) proteinase K (0, 1, 5 μg/ml) for 1 hr at 37º C, and then washed with cold PBS. Additional cells C) were also incubated with 100 mM EDTA or EGTA for 1 hr on ice. All cells were then assayed for virus binding as determined by flow cytometry. TAstV-2 bound to untreated cells were used to maximum binding (100%) and values for each treatment group are percentage of maximum binding. Experiments are representative of at least 3 separate experiments.


125
100
75
50
% Binding
0
30
60
120
Neuraminidase (mU/ml)

A


100
75
50
% Binding
0
0.25
0.50
1.00
Trypsin (ug/ml)
Chondroitinase ABC (U/ml)
Heparinase I (U/ml)
Heparinase III (U/ml)

B


100
90
80
70
60
50
40
30
20
10
0
% Binding
EDTA 100mM
EGTA 100mM
Protinase K 5ug/ml
Protinase K 1ug/ml

C

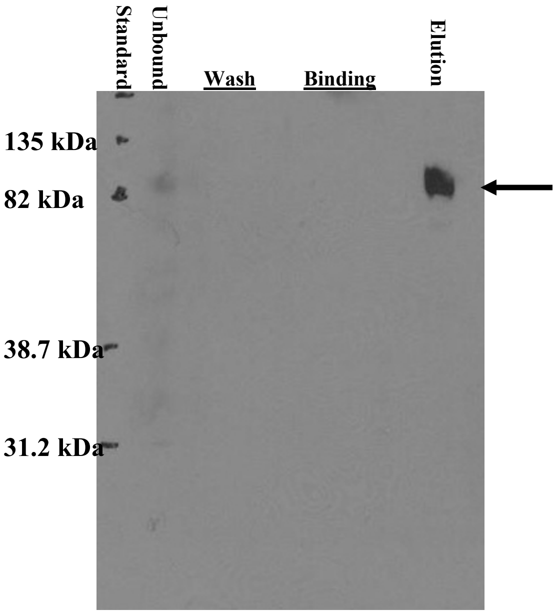


Fig 6.3. Purification of His-tagged rTAstV-2 capsid protein. rTAstV-2cap was purified from rAcNPV/TAstV-2capsidHis infected Sf9 cell lysates using Ni-NTA resin. 1ml of cell lysates were incubated with 1 ml of resin at room temp of 2 hrs with agitation. Resin-protein complex was added to column support and unbound protein collected. Column was washed with 20 mls of wash buffer (50 mM Tris, pH 8+ 500 mM NaCl), then washed with 20 mls of binding buffer (50 mM Tris, pH 8+ 500 mM NaCl + 5 mM imidazole). rTAstV-2cap was then eluted using elution buffer (50 mM Tris, pH 8+ 500 mM NaCl + 500 mM imidazole). The purification was monitored using SDS-PAGE and western blot analysis. Samples were probed for rTAstV-2cap using a rabbit anti-KHL peptide IgG. Purified rTAstV-2cap of the expected size was detected in affinity column elution buffer.

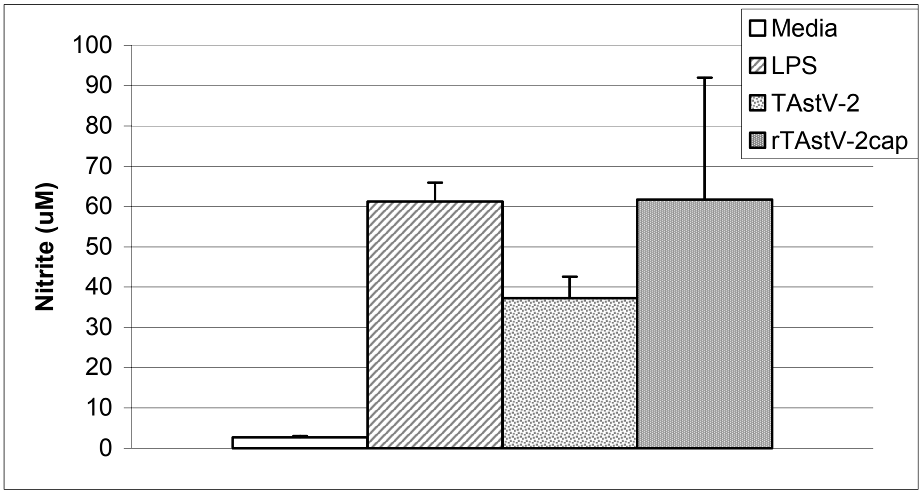
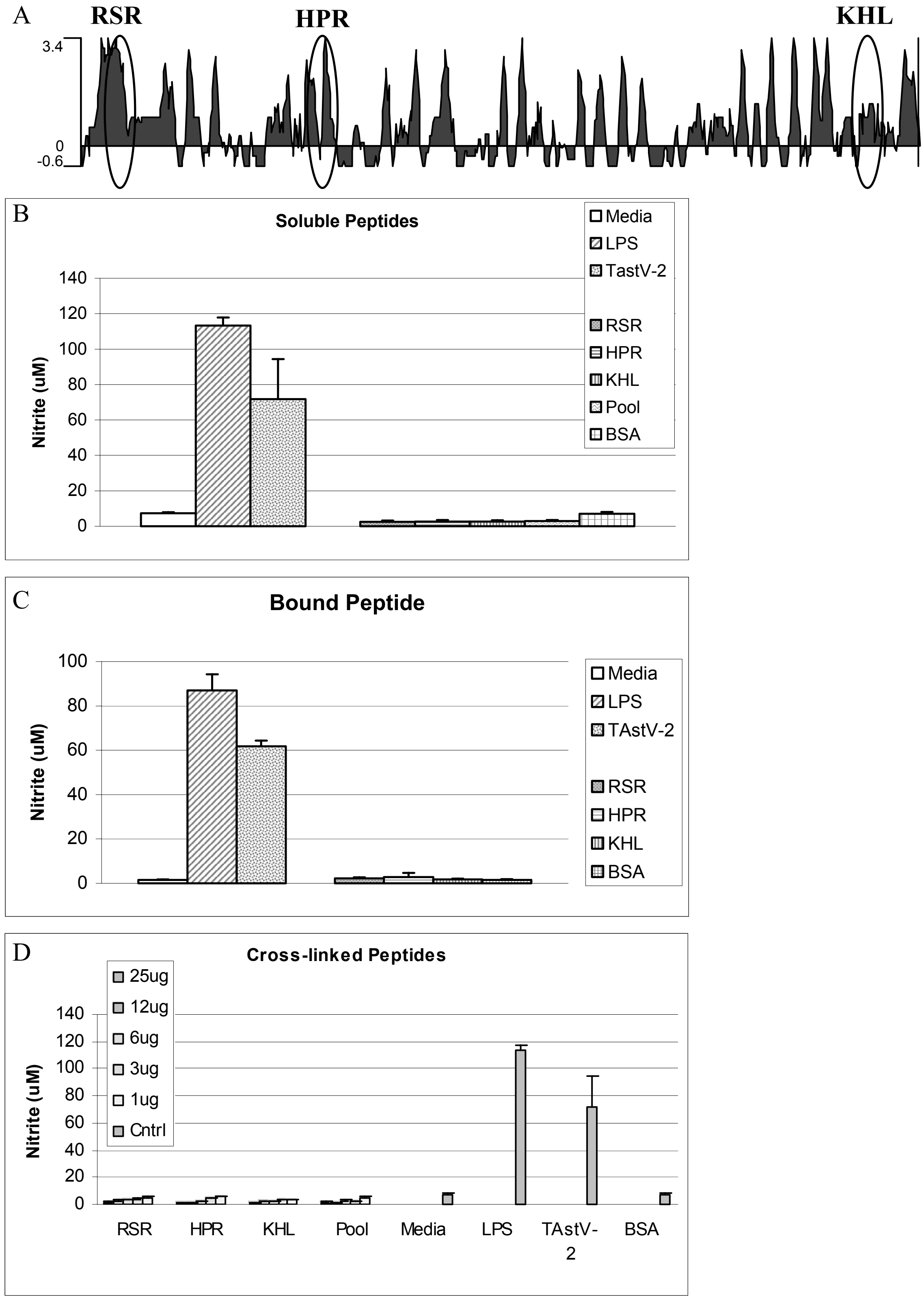


Fig 6.4. Recombinant His-tagged TAstV-2 stimulates HD11 cell production of NO. HD11 cells were stimulated with complete media alone, LPS (10 ng/ml), TAstV-2 (7 μg), or rTAstV-2cap (1 μg). Following 48 hr of incubation supernatants were tested for the presence of nitrite using the Griess assay. Error bars represent the standard deviation of the mean of three wells. Results are representative of at least 3 experiments.

Fig 6.5. TAstV-2 derived peptides do not stimulate NO. Three peptides were synthesized based on predicted amino acid surface probability, sequence conservations, and antigenic index, as determined by Protean Software, DNA Star. A) Antigenic index plot of TAstV-2 capsid amino acid sequence. The three peptides RSR, HPR, and KHL are shown by the circled regions. Peptides were used to stimulate 1 x 10^5 HD11 cells. B) RSR (25 μg/well), HPR (25 μg/well), KHL (25 μg/well), peptide pool (8.3 μg RSR + 8.3 μg HPR + 8.3 μg KHL/well) or BSA control (25 μg/well) were added in soluble form to HD11 cultures. C) Peptide or BSA control were bound to microtiter plates (15 μg/well) using 0.1 Bicarbonate binding buffer (pH 9.6). Plates were then washed with PBS. D) RSR, HPR, KHL, equal molar peptide pool, or BSA was cross-linked using DSS. Cross-linked peptides were then added to HD11 cells to a final concentration of 1, 3, 6, 12, or 25 μg/well, or BSA at 25 μg/well. Cells were culture for 48 hrs and stimulation measured by testing for nitrite using the Griess assay. For each assay cells treated with media alone, LPS (10 ng/ml) or TAstV-2 were used as negative and positive controls. Error bars represent the standard deviation of the mean of three wells. Results are representative of at least 3 experiments.


A
RSR
HPR
KHL
3.4
0
-0.6
B
Soluble Peptides
Media
LPS
TastV-2
RSR
HPR
KHL
Pool
BSA
140
120
100
80
60
40
20
0
Nitrite (uM)
C
Bound Peptide
100
80
60
40
20
0
Nitrite (uM)
Media
LPS
TAstV-2
RSR
HPR
KHL
BSA
D
Cross-linked Peptides
25ug
12ug
6ug
3ug
1ug
Cntrl
140
120
100
80
60
40
20
0
Nitrite (uM)
RSR
HPR
KHL
Pool
Media
LPS
TAstV-2
BSA

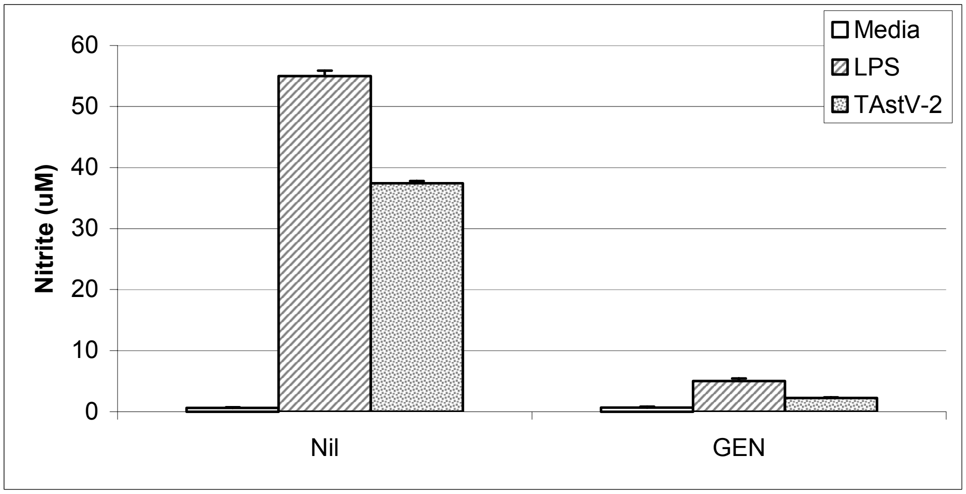
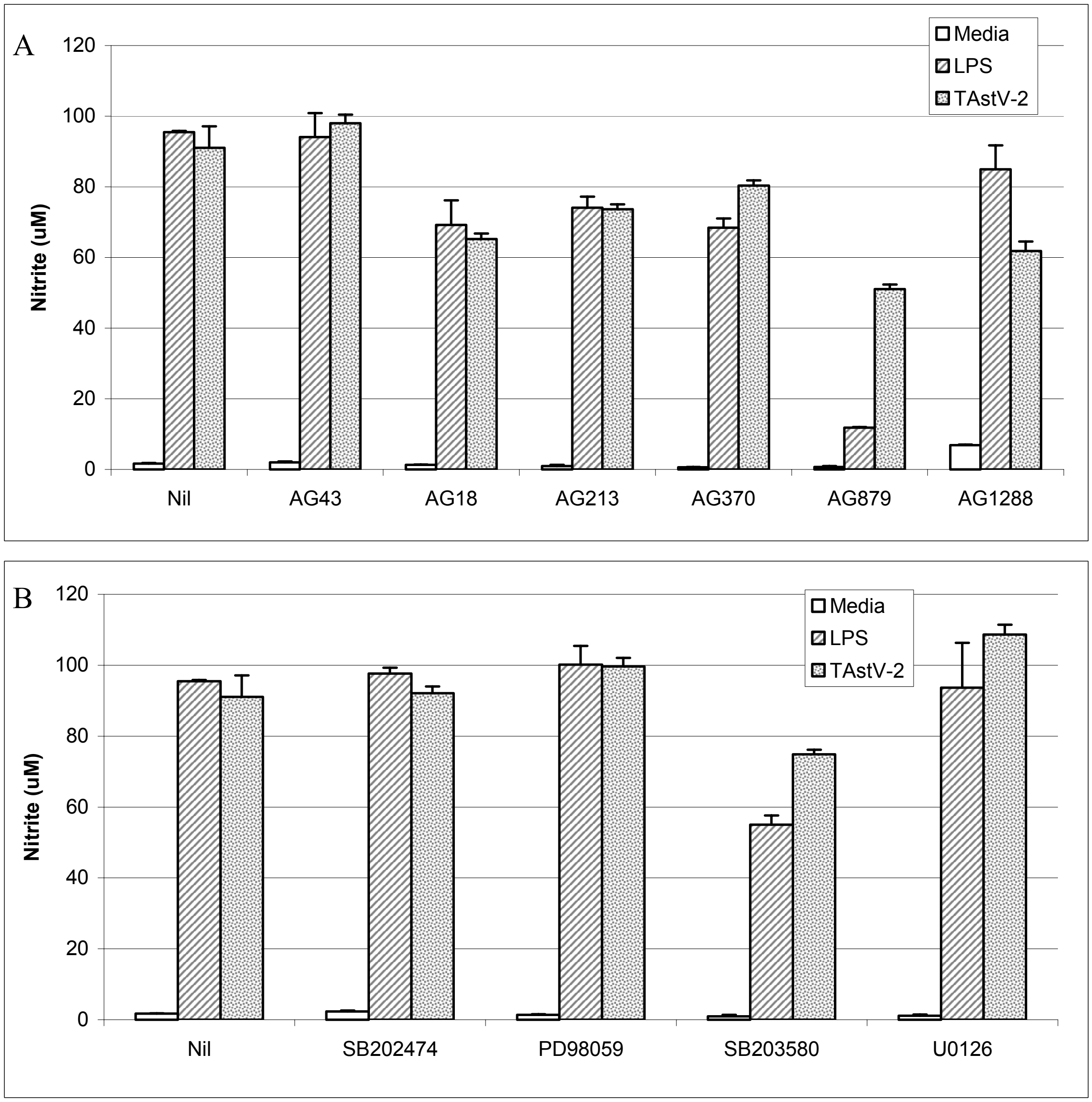


Fig 6.6. TAstV-2-induced iNOS expression is dependent on tyrosine kinase activity. HD11 cells were stimulated with complete media alone, LPS (10 ng/ml), or TAstV-2 with or without 100 μM genistein. Following 48 hr of incubation supernatants were tested for the presence of nitrite using the Griess assay. Error bars represent the standard deviation of the mean of three wells. Results are representative of at least 3 experiments.

Fig 6.7. Effects of tyrosine and MAP kinase inhibitors. HD11 cells were stimulated with complete media alone, LPS (10 ng/ml), or TAstV-2 with or without A) tyrosine kinase inhibitors AG43 (6.5 mM), AG18 (40 μM), AG213 (60 μM), AG307 (50μM), AG879 (10 μM), AG1288 (50 μM) or B) MAP kinase inhibitors SB202474 (2 μM), PD98059 (2 μM), SB203580 (2 μM), U0126 (202 nM). Following 48 hr of incubation supernatants were tested for the presence of nitrite using the Griess assay. Error bars represent the standard deviation of the mean of three wells. Results are representative of at least 3 experiments.


A
B
Media
LPS
TAstV-2
Nitrite (uM)
0
20
40
60
80
100
120
Nil
AG43
AG18
AG213
AG370
AG879
AG1288
SB202474
PD98059
SB203580
U0126

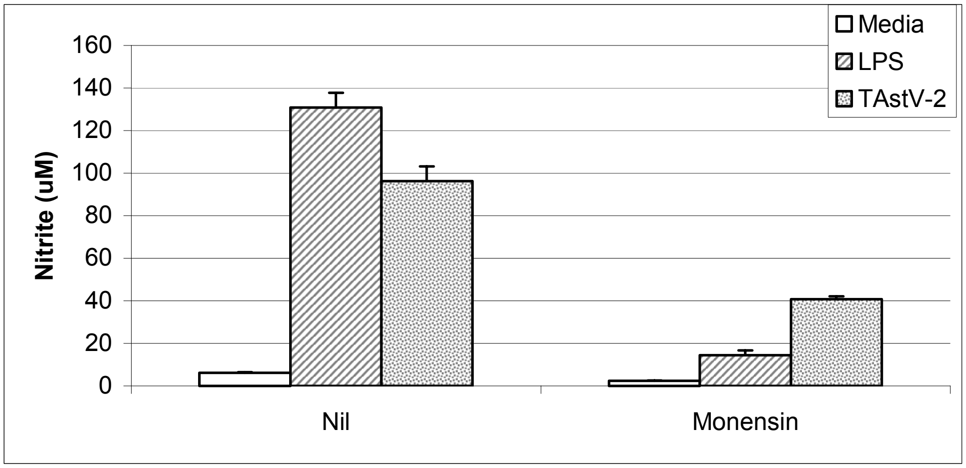


Fig 6.8. TAstV-2-mediated NO activity requires internalization. HD11 cells were stimulated with complete media alone, LPS (10 ng/ml), or TAstV-2 with or without monensin (20 μM). Following 48 hr of incubation supernatants were tested for the presence of nitrite using the Griess assay. Error bars represent the standard deviation of the mean of three wells. Results are representative of at least 3 experiments.

CHAPTER 7

DISCUSSION

Astroviruses were first described in 1975 associated with acute diarrhea of infants (4). Since that initial discovery, astroviruses are now recognized as an endemic cause of enteric disease in humans, and is arguably the number one cause of acute gastroenteritis in children under one year of age (5). Astroviruses are also recognized as an emerging cause of enteritis in the elderly and the immunocompromised, and the burden of astrovirus disease in the developing world may be exceptionally high (6). Several reports suggest that greater than 90% of the human population have been exposed to astrovirus (1, 3). In spite of its world-wide distribution and prevalence, our collective understanding of astrovirus pathogenesis has lagged behind that of other viral diseases. This has been due to a lack of a small animal model.

To directly address this problem we have developed a small animal model using young turkeys and a novel strain of turkey astrovirus isolated in our laboratory. This virus was originally isolated from the thymus of young turkeys suffering from an emerging infectious disease known as poult enteritis mortality syndrome (PEMS), and was the first non-human astrovirus to be completely sequenced (2, 7). We demonstrated that TAstV-2–induced clinical disease is similar to that seen in infants. Therefore we set out to establish TAstV-2 as a laboratory model for the study of astrovirus pathogenesis.

To begin to understand how astroviruses cause disease we studied the gross and histopathologic changes associated with TAstV-2 infection. These experiments demonstrated that infected animals suffered severe diarrhea, significant weight loss, and elevated mortality as compared with mock infected controls. Upon closer examination, we also noted that the thymus of infected birds were significantly undersized relative to body weight. Microscopic inspection of the intestines, demonstrated only mild pathologic changes. Studies investigating sites of viral replication, demonstrated that TAstV-2 only replicates in the intestines as determined by *in situ* hybridization. However, virus was found in non-intestinal tissues including the blood. We were most surprised to see very little evidence of cell death or an inflammatory response given the levels of viral replication and diarrhea.

These observations led us to study the host response to infection. Infection of poults demonstrated that replication in the intestines waned by 9 days post inoculation; however, there was no histologic evidence of an immune response. Therefore, we were interested in determining the factors contributing to viral clearance. Initially we examined aspects of both cellular and humoral immunity, both of which have been suggested as important in protecting healthy adults from human astrovirus infection. We did not find any evidence of lymphocyte proliferation in response to infection or changes in $CD4^+$ to $CD8^+$ ratios. Likewise, we found only modest production of TAstV-2-specific antibodies later in infection (primarily 21 days). No neutralizing antibodies were detected. Furthermore, poults re-challenged with TAstV-2 were not protected against disease, suggesting no acquired immune response had developed. These studies suggested that viral clearance during primary infection in turkeys is independent of the cellular and humoral immune responses. Therefore, to examined the role of the innate immune response in clearance of TAstV-2 infection. Initial experiments demonstrated adherent splenocytes from infected poults were more responsive to *ex vivo* stimulation with LPS than that of age matched controls, suggesting that these cells were activated as a result of infection. To more completely define our *in vivo* findings, we infected the avian macrophage (MΦ) cell line, HD11, with TAstV-2 and examined MΦ activation by examining the production of nitric oxide (NO).

These experiments demonstrated that HD11 cells bound TAstV-2, and binding led to the upregulation of inducible NO synthase (iNOS). The activation of avian MΦs was independent of viral replication. This was most clearly demonstrated by the stimulation of NO by purified recombinant TAstV-2 capsid protein. NO is an important aspect of the innate response to a variety of viral infections. Therefore, we examined the role of NO in TAstV-2 infection *in vivo*. Examination of infected embryo intestines for 3-nitrotyrosine, a by-product of NO, showed that infection led to increased NO activity. Finally, we demonstrated that NO is capable of modulating viral replication in infected embryo intestines. The addition of a NO donor compound led to a significant reduction in TAstV-2 titers, while the *in vivo* inhibition of the iNOS enzyme resulted in increased replication.

These results detail the creation, development, and implementation of the TAstV-2 model for study of astrovirus pathogenesis. They are the first studies to define the kinetics of viral replication and disease using virus isolation, RT-PCR, *in situ* hybridization, and immunofluorescent antibodies throughout multiple tissues. Additionally, these results were the first experimental examination of host factors involved in viral clearance during a primary astrovirus infection. Likewise, these studies were the first to demonstrate a specific interaction between astroviruses and MΦs, and to describe the anti-viral effects of NO on astrovirus replication. Collectively these experiments greatly enhance our understanding of basic astrovirus pathology, and suggest that the innate immune system is a critical component to viral control.

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