ACTIVATION OF MACROPHAGES BY ASTROVIRUS THROUGH A REPLICATION-INDEPENDENT MECHANISM

by

MATTHEW D. KOCI

(Under the Direction of Stacey Schultz-Cherry)

ABSTRACT

Astroviruses are small, non-enveloped, single stranded RNA viruses known to be one of the leading causes of acute gastroenteritis worldwide. 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.

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by

MATTHEW D. KOCI
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MS, The University of Georgia, 1999

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by

MATTHEW D. KOCI

Major Professor: Corrie Brown

Committee:

Zhen Fu
Donald Evans
Terrence Tumpey
Stacey Schultz-Cherry

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

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.
REFERENCES


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 polyproteins 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 poult's 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 kinase (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, dendritic 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 MΦs 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 hemostasis, 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). Vaccinia virus, herpesviruses, adenoviruses, papillomavirus, andenov-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 tropisms, 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.

REFERENCES


CHAPTER 3

REVIEW: AVIAN ASTROVIRUSES

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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 mis-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 poult s 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 poult s (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 (TAvS-1), 80 kDa (TAvS-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-propiolactone, 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

Genomic alignments of the three AAstVs completely sequenced suggest there is far less conservation in nucleotide sequence than that detected among the MAsVs. 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.

ACKNOWLEDGMENTS

The authors wish to acknowledge the efforts of Laura A. Kelley, who’s invaluable work made much of the TAstV-2 work completed in our laboratory possible. We also thank Dr. H. John Barnes at the college of Veterinary Medicine at North Carolina State University for initial tissue samples and Dr. James Guy, also at the college of Veterinary Medicine at North Carolina State University, for supplying monoclonal antibodies.

REFERENCES

   *Lancet, 1*, 1297.

   Cultivation and partial characterization of bovine astrovirus. *Veterinary Microbiology, 19*, 113-25.


   *Veterinary Record, 77*, 1529-30.


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

<table>
<thead>
<tr>
<th>Avian astrovirus</th>
<th>5’ UTR</th>
<th>ORF 1a</th>
<th>ORF 1b</th>
<th>ORF 2</th>
<th>3’ UTR</th>
<th>Totala</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANV</td>
<td>14</td>
<td>3012</td>
<td>1527</td>
<td>2052</td>
<td>305</td>
<td>6927</td>
</tr>
<tr>
<td>TAstV-2</td>
<td>21</td>
<td>3378</td>
<td>1584</td>
<td>2175</td>
<td>196</td>
<td>7325</td>
</tr>
<tr>
<td>TAstV-1</td>
<td>11</td>
<td>3300</td>
<td>1539</td>
<td>2016</td>
<td>130</td>
<td>7003nt</td>
</tr>
</tbody>
</table>

*excluding the poly-A tail*
Table 3.2. Comparison of morphologic and biochemical properties of the small round virus families

<table>
<thead>
<tr>
<th>Family</th>
<th>Diameter (nm)</th>
<th>Surface Structure</th>
<th>Chloroform Resistance</th>
<th>Stable at 60°C for 10 min</th>
<th>Number and size of expected proteins</th>
<th>Bouyant Density (g/ml)</th>
<th>Genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circovirus</td>
<td>12-26</td>
<td>None</td>
<td>Yes</td>
<td>Yes</td>
<td>1 capsid protein in some 50-36kDa 3 proteins in others 15-26kDa</td>
<td>1.33-1.37</td>
<td>Circular –ssDNA 1.7-2.3kb</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>18-26</td>
<td>None</td>
<td>Yes</td>
<td>Yes</td>
<td>2 to 4 major capsid proteins VP1: 96-80kDa VP2: 85-64kDa VP3: 75-60kDa VP4: 52-49kDa</td>
<td>1.39-1.42</td>
<td>Linear ssDNA 4-6kb</td>
</tr>
<tr>
<td>Picornavirus</td>
<td>28-30</td>
<td>None</td>
<td>Yes</td>
<td>Some strains</td>
<td>4 capsid VP1,2,3: 41-24kDa VP4: 13.5-5.5kDa VPg: 2.4kDa</td>
<td>1.33-1.45</td>
<td>Linear +ssRNA 7-8kb</td>
</tr>
<tr>
<td>Calicivirus</td>
<td>30-38</td>
<td>Cup-shaped depressions (Not seen in Norwalk virus)</td>
<td>yes</td>
<td>Some strains</td>
<td>1 major capsid protein 71-59kDa 1 minor protein in some viruses 30-28kDa VPg: 15-10kDa</td>
<td>1.33-1.40</td>
<td>Linear +ssRNA 7.4kb-7.7kb</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>28-30nm</td>
<td>5-6 pointed star (only seen in ~10% of virions)</td>
<td>Yes</td>
<td>Yes</td>
<td>At least 2 major proteins maybe 3 39-29kDa possible smaller proteins 36-13kDa</td>
<td>1.36-1.39</td>
<td>Linear +ssRNA 7.2-7.9kb</td>
</tr>
</tbody>
</table>
Table 3.3: Genus and species described in the family Astroviridae.

<table>
<thead>
<tr>
<th>Astrovirus species</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammalian astrovirus</strong></td>
<td></td>
</tr>
<tr>
<td>Bovine astrovirus</td>
<td>BAstV</td>
</tr>
<tr>
<td>Bovine astrovirus 1</td>
<td>BAstV-1</td>
</tr>
<tr>
<td>Bovine astrovirus 2</td>
<td>BAstV-2</td>
</tr>
<tr>
<td>Feline astrovirus</td>
<td>FAstV</td>
</tr>
<tr>
<td>Feline astrovirus 1</td>
<td>FAstV-1</td>
</tr>
<tr>
<td>Human astrovirus</td>
<td>HAstV</td>
</tr>
<tr>
<td>Human astrovirus 1</td>
<td>HAstV-1</td>
</tr>
<tr>
<td>Human astrovirus 2</td>
<td>HAstV-2</td>
</tr>
<tr>
<td>Human astrovirus 3</td>
<td>HAstV-3</td>
</tr>
<tr>
<td>Human astrovirus 4</td>
<td>HAstV-4</td>
</tr>
<tr>
<td>Human astrovirus 5</td>
<td>HAstV-5</td>
</tr>
<tr>
<td>Human astrovirus 6</td>
<td>HAstV-6</td>
</tr>
<tr>
<td>Human astrovirus 7</td>
<td>HAstV-7</td>
</tr>
<tr>
<td>Human astrovirus 8</td>
<td>HAstV-8</td>
</tr>
<tr>
<td>Ovine astrovirus</td>
<td>OAstV</td>
</tr>
<tr>
<td>Ovine astrovirus 1</td>
<td>OAstV-1</td>
</tr>
<tr>
<td>Porcine astrovirus</td>
<td>PAstV</td>
</tr>
<tr>
<td>Porcine astrovirus 1</td>
<td>PAstV-1</td>
</tr>
<tr>
<td><strong>Avian astrovirus</strong></td>
<td></td>
</tr>
<tr>
<td>Duck astrovirus</td>
<td>DAstV</td>
</tr>
<tr>
<td>Duck astrovirus 1</td>
<td>DAstV-1</td>
</tr>
<tr>
<td>Turkey astrovirus</td>
<td>TAstV</td>
</tr>
<tr>
<td>Turkey astrovirus 1</td>
<td>TAstV-1</td>
</tr>
<tr>
<td>Turkey astrovirus 2</td>
<td>TAstV-2</td>
</tr>
<tr>
<td>Chicken astrovirus</td>
<td>CAstV</td>
</tr>
<tr>
<td>Avian nephritis virus</td>
<td>ANV</td>
</tr>
</tbody>
</table>
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.
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.
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.
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

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, Poul 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 (CCATGATATGCTAGGGGAT) and CQ RT-PCR Rev (GACTCAACATCTGTAGGCT) }. Sample RNA was added at a uniform concentration to each tube of a serial log dilution of cRNA, and amplified under the following conditions; 50° C for 30 min, 95° C for 15 min, 30 cycles of 94° C for 30 s, 55° C for 30 s, 72° C for 30 s, and final 72° 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 poult's were weighed (day 0) and randomly assigned to either a control group or a group infected with astrovirus ($n = 60$ per group). Poult's were orally inoculated with $\sim 10^6$ genomic units of astrovirus in 200 $\mu$l 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 poult's 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 $-70^\circ$ C or placed in 10% phosphate-buffered formalin. Blood was collected in syringes containing heparin, incubated overnight at 4$^\circ$ 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$^\text{TM}$ or inoculation into 20-day-of-age turkey embryos. The animal experiments were repeated five times with different groups of poult's 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 37º C, and hybridized overnight at 42º 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 37º 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 4o 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 37o 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 10³ 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% CO₂, 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-β (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 x 10⁴ 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 18 hr at 37°C in 5% CO₂ in 100 µl/well of DMEM + 0.1% bovine serum albumin (BSA, Invitrogen) containing; recombinant active TGF-β₁ (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\(^\circ\) 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-β 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 \textit{in vivo} activity is that of TGF-\(\beta\) (Fig. 4.7 A). Intestinal filtrates were also tested but were toxic in the NRK assay.

To determine if TGF-\(\beta\) 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 \(\mu\)m filtered. These tissues homogenates where then assayed for active TGF-\(\beta\) 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-\(\beta\) 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-\(\beta\) neutralizing antibody demonstrating that active TGF-\(\beta\) 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-\(\beta\) manuscript in preparation).

DISCUSSION

Very little is known about the pathogenesis of astrovirus infection. Studies \textit{in vivo} in humans and animals are limited (37, 46, 58, 62, 63, 69). In these studies we described the development of an \textit{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 jejunum (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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57. **Snodgrass, D. R., K. W. Angus, E. W. Gray, J. D. Menzies, and G. Paul.**


Table 4.1. Localization of TAstV-2.

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<sup>a</sup>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.

<sup>b</sup>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.

<sup>c</sup>Immunofluorescence using a polyclonal antibody produced to a peptide sequence in the TAstV-2 capsid protein (K<sub>676</sub> – R<sub>691</sub>)

<sup>d</sup><em>In situ</em> hybridization using negative sense riboprobe specific for the 3’ end of the TAstV-2 genome.

<sup>e</sup>Days post inoculation

<sup>f</sup>Not Determined

<sup>g</sup>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°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 poult per group were weighed at days 0, 3, 5, and 12 post-infection. The second Y-axis exhibits the percentage of poult exhibiting diarrhea at the same days of infection. (B) The thymus from 5 random poult 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 poult collected at 2 dpi and stained with hematoxylin and eosin. (A) In TAstV-2-infected poult, 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 poult 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 poult 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 poult 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 poult 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 $\mu$m.
Figure 4.7. TAstV-2 Increases TGF-β Activity. (A). Five-day old turkey poult's were orally inoculated with 0.2 ml PBS or $10^6$ TAstV-2 genomic units, blood was collected from euthanized poult's 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 poult's 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 embryo's 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-β1 (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 ($p<0.05$).
CHAPTER 5

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

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 \textit{in vivo} and \textit{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 CD$^{4+}$:CD$^{8+}$ ratios, production of virus-specific antibodies (Abs), and macrophage activation. We found no changes in the numbers of circulating or splenic lymphocytes, or in CD$^{4+}$:CD$^{8+}$ 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 \textit{ex vivo} stimulation with LPS. \textit{In vitro} analysis demonstrated that TAstV-2 induced macrophage production of inducible nitric oxide synthase (iNOS). Studies using NO donors and inhibitors \textit{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.
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 effector 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 $1 \times 10^{12}$ 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 41º 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 poult (male and female) were obtained from a commercial hatchery. Control and infected poult 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 poult were randomly assigned to either a control group or a group infected with astrovirus (n = 60 per group). Poult 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 cytometry.**

Lymphocytes from peripheral blood and spleen were isolated as described above. Phenotyping of isolated cells was performed as previously described (37). Briefly, cells were washed in cold PBS, then 1 x 10⁶ 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-naphthylethylenediamine 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 ≥10⁵ embryo infectious units/ml (1 x 10⁸ 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/Napthol 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.
ACKNOWLEDGEMENTS

We are very appreciative to Laura Knoll and Yoshi Kawaoka (University of Wisconsin) for the use of their microscopes, the people at British United Turkeys of America for donating turkey poults and eggs, the poultry production staff at Southeast Poultry Research Laboratory (Jerry Hammond, Gerald Damron, and Keith Crawford) for the continual supply of healthy birds, Southern Biotechnology Associates for generous help in identifying Abs specific for turkey lymphocytes, Michael Perdue, James Higgins, Sebastian Botero, and Christina Hohn (Animal Waste Pathogen Laboratory, USDA, ARS) for assistance in developing the Real-Time RT-PCR assay, and Saad Gharaibeh (Jordan University of Science and Technology), Liz Turpin, Dana Mordue, Lindsey Moser (University of Wisconsin), Terry Tumpey (Southeast Poultry Research Laboratory), Donald Evans, Zhen Fu, and Corrie Brown (University of Georgia) for critical review of this manuscript.

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significance of alimentary tract microbes in bone marrow transplant recipients. Diagn Microbiol Infect Dis. 30:75-81.

Table 5.1. CD4/CD8 ratio from infected turkey poults

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<sup>a</sup>Ratio of percentage of single positive CD4<sup>+</sup> cells versus percentage of single positive CD8<sup>+</sup> 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.
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 10⁵ 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.
Figure 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 \textit{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\textsubscript{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) $5 \times 10^6$ VG of TAstV-2, (C) $5 \times 10^6$ VG TAstV-2 + 500 µM SNAP, or (D) $5 \times 10^6$ 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 NITRIC OXIDE

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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% CO2 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 °C 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 41° 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™ 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 (RSRTKTVKIEKKPE, RSR), 194-221 (HPRSALGPRQGWNVDPGD, 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 pp140c-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.

REFERENCES


Table 6.1. Chemical and enzymatic inhibitors of virus binding.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td>Removes sialic acid</td>
</tr>
<tr>
<td>Heparinase I</td>
<td>Removes heparin</td>
</tr>
<tr>
<td>Heparinase III</td>
<td>Removes heparan sulfate</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>Removes chondroitin sulfate A, B, and C</td>
</tr>
<tr>
<td>TPCK-Trypsin</td>
<td>Removes proteins following arginine-lysine residues</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Broadly reactive protease</td>
</tr>
<tr>
<td>EGTA/EDTA</td>
<td>Chelate divalent cations</td>
</tr>
</tbody>
</table>
### Table 6.2. Chemical inhibitors of tyrosine and MAP kinases.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD 98059</td>
<td>MAP kinase kinase (MEK) inhibitor</td>
</tr>
<tr>
<td>SB 203580</td>
<td>p38 kinase inhibitor</td>
</tr>
<tr>
<td>U 0126</td>
<td>MEK1 and MEK2 inhibitor</td>
</tr>
<tr>
<td>SB 202474</td>
<td>MAP kinase inhibitor negative control</td>
</tr>
<tr>
<td>Genistein</td>
<td>Broad tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>AG 18</td>
<td>Broad tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>AG 213</td>
<td>Broad tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>AG 370</td>
<td>Inhibits platelet derived growth factor (PDGF)-induced mitogenesis</td>
</tr>
<tr>
<td>AG 879</td>
<td>Inhibits nerve growth factor (NGF)-dependent pp140C-trk</td>
</tr>
<tr>
<td>AG 1288</td>
<td>Inhibits TNFα-induced cytotoxicity</td>
</tr>
<tr>
<td>AG 43</td>
<td>Tyrosine kinase inhibitor negative control</td>
</tr>
<tr>
<td>Monensin</td>
<td>Inhibits endosomal acidification</td>
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</tbody>
</table>
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 10⁶) 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.
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 cultured 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.
Figure A: Cross-linked Peptides

Figure B: Soluble Peptides

Figure C: Bound Peptide

Figure D: Cross-linked Peptides
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.
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.
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 pouls 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, pouls 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 \textit{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 \textit{in vivo} findings, we infected the avian macrophage (M\(\Phi\)) cell line, HD11, with TAstV-2 and examined M\(\Phi\) 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 M\(\Phi\)s 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 \textit{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 \textit{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, \textit{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.

REFERENCES


