Evaluation of the effects of porcine genogroup 1 torque teno virus in gnotobiotic swine

Steven Krakowka, DVM, PhD, and John A. Ellis, DVM, PhD

Objective—To determine whether porcine genogroup 1 torque teno virus (g1-TTV) can infect and cause disease in gnotobiotic swine.

Sample Population—20 conventional baby pigs and 46 gnotobiotic baby pigs.

Procedures—Porcine g1-TTV was transmitted from conventional swine to gnotobiotic pigs via pooled leukocyte-rich plasmas (n = 18) that had positive results for g1-TTV DNA. Bone marrow–liver homogenates that had positive results for torque teno virus (TTV) were used in 4 serial passages in gnotobiotic pigs (2 pigs/ passage). A pathogenesis experiment was conducted with in vivo passages of g1-TTV in various groups of gnotobiotic pigs.

Results—All g1-TTV inoculated pigs had no clinical signs but developed interstitial pneumonia, transient thymic atrophy, membranous glomerulonephropathy, and modest lymphocytic to histiocytic infiltrates in the liver after inoculation with the TTV-containing tissue homogenate; these changes were not detected in uninoculated control pigs or pigs injected with tissue homogenate devoid of TTV DNAs. In situ hybridization was used to identify g1-TTV DNAs in bone marrow mononuclear cells.

Conclusions and Clinical Relevance—Analysis of these data revealed that porcine g1-TTV was readily transmitted to TTV-naive swine and that infection was associated with characteristic pathologic changes in gnotobiotic pigs inoculated with g1-TTV. Thus, g1-TTV could be an unrecognized pathogenic viral infectious agent of swine. This indicated a directly associated induction of lesions attributable to TTV infection in swine for a virus of the genus Anellovirus. (Am J Vet Res 2008;69:1623–1629)

The TTVs or transfusion-transmitted viruses were first identified in the serum of a human transplant patient with idiopathic posttransplantation hepatitis in 1997. Subsequent investigations determined that TTV infections are common among asymptomatic humans, but the incidence of viremia to TTVs typically is increased in patients with a variety of clinically evident diseases, including viral hepatitis, HIV (acquired immunodeficiency syndrome), asthma and related childhood respiratory conditions, and renal disease. However, attempts to link TTV infection with disease in humans are confounded by the high incidence of asymptomatic people with viremia to TTV in control cohort populations, the remarkable genomic diversity within the TTV family, the inability to propagate the agent in vitro, and the lack of established TTV-induced disease in domestic or laboratory animals. Although TTV appears to be acquired by oronasal or fecal-oral transmission, mother-to-infant and in utero transmission have also been reported. Infected people are characterized by a prolonged (months to years) viremia to TTV. Humans may be coinfected with multiple TTV genogroups, and it has been suggested that these genogroups may recombine within infected humans. Although the data are fragmentary, there is also evidence that an antibody response develops against TTV viral nucleocapsid protein during the course of infection and that circulating TTV-antibody immune complexes may develop in infected individuals.

Although amounts of viral DNAs approaching 10^10 copies/mL have been reported, most investigators use the more sensitive nPCR assay to detect TTV DNAs in sera and tissue extracts. Target sequences vary, but primer pairs specific for the untranslated region of

Abbreviations

DAB 3,3′diaminobenzidine
g1-TTV Genogroup 1 torque teno virus
nPCR Nested PCR
PAS Periodic acid–Schiff
PCV2 Porcine circovirus type 2
PPV Porcine parvovirus
PRRSV Porcine reproductive and respiratory syndrome virus
TTV Torque teno virus

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From the Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH, 43210 (Krakowka); and the Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada (Ellis).
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Address correspondence to Dr. Ellis.
TTVs and other members of the family Circoviridae. In contrast to other Circoviridae, TTVs are genomically diverse. On the basis of sequence analyses, at least 5 major genogroups of human TTVs have been identified. In addition to humans and nonhuman primates, domestic animal species (including swine) are also persistently infected with species-specific TTVs.

Similar to the situation in their human counterparts, there are no data that link TTV infection to clinical disease in pigs, except that, in infected herds, the incidence of viremia to TTV is significantly higher in PCV2-associated, PMWS-affected pigs than it is in unaffected clinically normal pigs. For pigs, g1- and g2- TTV have been identified; approximately half of swine sera examined contained g1- and g2- TTVs. Overall, the incidence of viremia to TTV in swine varies from 20% to 100%.

The purpose of the study reported here was to transmit porcine TTV infection to neonatal TTV DNA–negative gnotobiotic swine and to determine whether clinical signs or lesions could be attributable to TTV infection in these pigs.

Materials and Methods

Animals—Litters of gnotobiotic pigs were derived by cesarean section from pregnant sows. Animal husbandry conditions and sampling procedures for periodic collections of blood samples were as reported elsewhere. In vivo experiments were approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Source of infectious TTV and PCR assays—Citrated blood and serum samples were aseptically obtained from 20 healthy young (14- to 16-week-old) pigs from the same source herd where gravid sows were purchased. Blood samples were stored on ice until transported to our laboratory. Leukocyte-rich plasma was obtained by use of low-speed centrifugation and stored separately at –70°C. Companion sera were tested for PCV2 DNAs by use of PCR assays and for g1- and g2- TTV DNAs by use of an nPCR with published primer sequences for swine.

Analysis of amplified sequences—Samples with positive results for nPCR assays were sequenced by use of routine methods. Amplicons obtained were then aligned with porcine g1- and g2- TTV DNA sequences stored in GenBank and identified as porcine g1- or g2- TTV on the basis of sequence identity with the published sequences.

Serial passage in gnotobiotic swine—Eighteen leukocyte-rich plasmas with negative results for PCV2 DNA and positive results for TTV were pooled and inoculated IP into three 3-day-old gnotobiotic pigs (8.5 mL/pig). Twenty-eight days after inoculation, 2 pigs had positive results when tested for viremia to TTV DNA by use of an nPCR assay. These pigs were euthanized, and a 20% (wt/vol) homogenate was made from the liver of 1 pig; the homogenate was frozen at –70°C. The first-passage homogenate was thawed and subjected to 2 rounds of extraction with chloroform to remove infectivity of any extraneous enveloped viruses contained in the first-passage homogenate; the resulting homogenate was designated as cTTVp1. Five milliliters of freshly thawed cTTVp1 homogenate was injected IP into two 2-day-old gnotobiotic pigs; these pigs were then euthanized 10 days after inoculation. The liver and samples of bone marrow were aseptically collected from each of these pigs and tested separately for TTVs by use of an nPCR assay. A 10% (wt/vol) bone marrow–liver homogenate was prepared from the samples of the pig with the most prominent nPCR TTV signal; this homogenate was extracted twice with chloroform, and the aqueous phase was frozen at –70°C. The cTTVp2 homogenate was thawed, and 5 mL was inoculated IP into two 15-day-old gnotobiotic pigs; these pigs were euthanized on day 10 after inoculation, and the liver and samples of bone marrow were collected from each of these pigs. Similar to the preceding procedures, the bone marrow and liver were tested separately for TTV DNAs by use of PCR and nPCR assays, and the paired bone marrow–liver samples with the most prominent TTV band were homogenized in PBS solution as a 10% (wt/vol) suspension (ie, cTTVp3). The cTTVp3 homogenate was inoculated IP into 2 gnotobiotic pigs to create a fourth in vivo passage of infectious TTV. The fourth passage (ie, TTVp4) was not extracted with chloroform; instead, it was divided into 2.0-mL aliquots and frozen at –70°C for use as a stock infectious virus for subsequent in vivo pathogenesis experiments.

Experimental design—Gnotobiotic pigs were inoculated IP with 2.0 mL of cTTVp1 at 2 days of age (day of inoculation was designated as day 0) and euthanized on days 5 (n = 2 pigs), 7 (2), 21 (2), and 34 (3) after inoculation for use in gross and histologic evaluations. Two separately housed pigs from the same litter were euthanized on days 6 and 34, respectively. In a second in vivo challenge-exposure experiment, 11 gnotobiotic pigs from 2 litters were similarly inoculated IP with 2.0 mL of TTVp4 at 2 days of age and euthanized on days 3 (n = 1 pig), 5 (2), 7 (1), 14 (3), and 35 (2) after inoculation. Two separately housed pigs from the same litters served as uninoculated control animals; they were euthanized on day 32. Eight pigs from 3 additional litters were inoculated IP with 4.0 to 10.0 mL of 10% (wt/vol) tissue homogenates negative for g1- TTV; these pigs were euthanized 9 to 17 days after inoculation.

Histologic examination and immunohistochemical analysis—After all TTV-inoculated and control pigs were euthanized, the liver, peripheral blood mononuclear cells, and bone marrow were evaluated for clinical signs of PCV2-associated disease. All pigs were necropsied, and tissue samples were collected from the liver, lung, and bone marrow. Each sample was placed in 10% neutral buffered formalin and processed for histologic examination. The liver, lung, and bone marrow were processed using a standard histologic protocol, and transverse sections were stained with hematoxylin and eosin. Immunohistochemical analysis was performed on the liver and bone marrow to determine the presence and distribution of TTV in the infected pigs.
were euthanized, tissue samples (inguinal, axillary, mesenteric, and bronchial lymph nodes; thymus; bone marrow; spleen; liver; lungs; kidneys; and ileum) were collected into cold (4°C) ethanol, fixed for 24 hours, and then processed for histologic examination with H&E stain by use of routine methods. Tissue section replicates were stained for PCV2 nucleocapsid protein as described elsewhere.38,39 Section replicates of renal tissues were stained with Jones silver and PAS stains for basement membranes and porcine fibrinogen or fibrin by use of a monoclonal antibody against porcine fibrin or fibrinogen followed by biotinylated equine anti-mouse IgG, which were then developed by use of avidin-conjugated horseradish peroxidase and DAB peroxidase substrate. Selected renal section replicates were stained for deposits of porcine IgG by use of goat anti-porcine IgG followed by biotinylated anti-goat IgG, which were then developed by use of avidin-conjugated horseradish and DAB peroxidase.

In situ hybridization—Selected tissues were tested for TTV DNAs by use of in situ hybridization with designed g1-TTV primers and digoxin-labeled nucleotides. Briefly, ethanol-fixed tissue section replicates were deparaffinized with xylene, quenched with hydrogen peroxide in methanol, digested with protease, and hybridized with labeled probe (37°C and 55°C) by use of standard methods. Labeled probe was detected with a monoclonal anti-digoxin antibody coupled to horseradish peroxidase and developed with DAB, essentially as described elsewhere.80 Control samples for in situ hybridization consisted of in situ hybridization with a PCV2-specific probe, in situ hybridization with omission of the monoclonal anti-digoxin antibody, staining of tissues with the monoclonal antibody without prior in situ hybridization, and in situ hybridization of tissue sections with negative results for TTV.

Screening for pathogens—All sera, including samples obtained from conventionally raised pigs with positive results for TTV prior to the experiments and samples from the gnotobiots, and subsequent pooled TTV tissue homogenates were tested to detect PPV, swine influenza virus, and PRRSV; samples were tested for PRRSV by use of a reverse transcriptase–PCR assay and for swine influenza virus RNAs and PCV types 1 and 2 by use of PCR assays. Convalescent sera were also tested for antibodies against these viruses by use of virus neutralization testing (encephalomyocarditis virus and transmissible gastroenteritis virus), agar gel immunoprecipitation (swine influenza virus), hemagglutination inhibition assay (PPV), and an ELISA (PRRSV). Testing was conducted at the Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, Reynoldsburg, Ohio, and used standard diagnostic methods.

Results

Clinical signs and gross necropsy results—No clinical signs were detected in any of the gnotobiots inoculated with cTTVp1 or TTVp4. Interstitial pneumonia was identified by gross examination of tissues in 2 of 4 pigs euthanized on day 5 after inoculation, 1 of 3 pigs euthanized on day 7 after inoculation, and 2 of 3 pigs euthanized on day 14 after inoculation. Mild thymic atrophy was evident in pigs euthanized on days 3 through 7 after inoculation. Two pigs euthanized on day 14 after inoculation had focal edema in the ventral region of the neck and thoracic mediastinum. One pig euthanized on day 21 after inoculation had localized edema in the right axillary lymph node and brachial plexus region. Pigs euthanized on day 34 after inoculation had no grossly detectable abnormalities. Gross or histologic lesions were not detected in the uninoculated control gnotobiots or in the sham-inoculated control pigs.

Direct PCR and nPCR assays—The direct PCR and nPCR assays generated an amplicon of 292 bases from the pooled plasmas (initial source of TTV) and samples of serum, liver, and bone marrow obtained from pigs inoculated with cTTVp1, cTTVp2, cTTVp3, and TTVp4.
PCR products recovered from cTTVp1 and TTVp4 were amplified, sequenced, and compared with reference sequences reported in GenBank for porcine g1-TTV (accession No. DQ229863) and g2-TTV (accession No. DQ229860). Both cTTVp1 and TTVp4 amplicons were matched with the untranslated region of the genome; each had high homology (94.3%) with g1-TTV but < 45% identical sequences with g2-TTV. Both cTTVp1 and TTVp4 amplicons were identical to each other. We did not detect g2-TTV DNAs in any of the pigs inoculated with any of the TTV homogenates. Quantitative PCR assay was used to determine the number of copies of g1-TTV DNA per nanogram of extracted total DNA for inocula of each in vivo passage. Calculated values were as follows: cTTVp1, 1.0 \times 10^4 copies/ng of DNA; cTTVp2, 2.0 \times 10^3 copies/ng of DNA; cTTVp3, 1.6 \times 10^4 copies/ng of DNA; and TTVp4, 1.1 \times 10^4 copies/ng of DNA.

**Histologic findings**—All lymphoid tissues, regardless of infectious status or time after inoculation, were hypoplastic and inactive; a few germinal centers were seen in bronchial and mesenteric lymph nodes of inoculated and control pigs, which was most likely related to antigen stimulation of the mucosa as a result of the diet (sow milk replacement formula). The thymus of each pig euthanized < 7 days after inoculation was variably reduced in size, which was primarily attributable to reduction of the T-lymphocyte–rich thymic cortices. This change was transient (the thymus from each of the pigs euthanized ≥ 14 days after inoculation was indistinguishable from that for each of the control pigs) and mild, and the reduction in thickness of the cortex was not associated with obvious cellular necrosis or inflammatory changes in the cortex. The liver of each of the pigs euthanized ≤ 21 days after inoculation had vacuolar cytoplasmic changes in hepatocytes compatible with mild fatty degeneration or glycogen infiltration. Foci of extramedullary hematopoiesis were evident in control and inoculated pigs through 7 days after inoculation (ie, 10 days of age). Small multiple foci of mixed lymphocytes and histiocytes were evident in the liver of each pig euthanized ≥ 14 days after inoculation.

Time-dependent histologic changes were most dramatic in the lungs of pigs inoculated with g1-TTV. Alveolar septal walls in 2 pigs euthanized on days 5 through 7 after inoculation were thickened by a modest infiltration with mononuclear cells and extracellular protein material. Multifocal areas of fibrinous exudate in alveolar spaces were evident in the pulmonary sections obtained from 1 pig euthanized on day 7 after inoculation (Figure 1). Lungs of the other pig euthanized at that approximate time after inoculation were histologically unremarkable. One of 3 pigs euthanized on day 14 after inoculation and 1 of 4 pigs euthanized on day 34 after inoculation had cellular debris mixed with fibrin in alveolar spaces (Figure 2). By day 21 after inoculation, pulmonary changes represented an obvious diffuse interstitial pneumonia characterized by accumulation of protein material in capillary lumens and diffuse lymphocytic and histiocytic cellular infiltrates into the interstitium (Figure 3). Diffuse and lobular interstitial pneumonia was still evident in 3 of 4 pigs euthanized on day 34 after inoculation. In these pigs, the pulmonary lesions were less severe than those detected in the pigs euthanized on day 21, except for 1 pig with a regionally extensive area of fibrinous alveolitis and interstitial pneumonia associated with activated mononuclear inflammatory cells and syncytiot giant cells. Control pigs did not have histologic changes in pulmonary tissues.

Histologic changes were not detected in the kidneys from pigs inoculated with g1-TTV and euthanized < 7 days after inoculation. However, in the kidneys of pigs inoculated with g1-TTVp4 and euthanized on day 14 after inoculation, renal glomeruli were variably distended with eosinophilic extracellular protein material and a few inflammatory cells entrapped in the glomerular spaces (Figure 4). The renal glomerular lesion pro...
gressed in pigs euthanized on day 21 after inoculation and was still evident in pigs euthanized on day 34 after inoculation, at which time selected glomeruli were scarred with fibrous connective tissue proliferation. The histologic changes in renal glomeruli were compatible with a provisional morphologic diagnosis of membranous glomerulonephropathy. This glomerular lesion was not detected in the control pigs. These glomerular lesions had positive results when stained with PAS stain, irregular nodular thickening of basement membranes with Jones silver stain, and positive results for porcine fibrinogen or fibrin and IgG (Figure 5).

In situ hybridization—Direct observation of g1-TTV DNAs was accomplished in selected tissues by use of in situ hybridization. Bone marrow samples from pigs euthanized on day 5 after inoculation had positive results. The DAB reaction product was confined to the cytoplasm and a few intranuclear locations within large monocytoid-like cells of undetermined lineage (Figure 6). Reaction product was evident as diffuse cytoplasmic staining or as discrete cytoplasmic granules suggestive of viral inclusion bodies. Similar cells containing TTV DNAs were also detected in the spleen of pigs euthanized on day 5 after inoculation.

Other pathogens—All pigs were seronegative for PRRSV as determined by use of an ELISA, and all sera tested by use of reverse transcriptase–PCR assay had negative results for PRRSV RNA. Sera from pigs had negative results for PCV2 DNA as determined by use of a PCR assay, and tissue section replicates had uniformly negative results when stained for PCV2 nucleocapsid protein. Sera had negative results for antibodies against swine influenza virus; sera also had negative results for PPV as determined by use of hemagglutination inhibition assay.

TTV PCR assay—The various TTV tissue homogenates and all of the pigs inoculated with these homogenates had positive results for g1-TTV when tested by use of direct PCR or nPCR assays. Preinoculation sera from all gnotobiots pigs had negative results for TTV DNA. Pigs inoculated with cTTVp2, cTTVp3, or TTVp4 had sufficient TTV DNAs in tissues that a conventional PCR assay was sufficient to detect TTV DNAs. The g1-TTV DNAs were detected in all tissues (sera, lymph nodes, thymus, lungs, liver, kidneys, spleen, and bone marrow) obtained from pigs euthanized > 10 days after inoculation (data not shown).

Discussion

In the experiments reported here, g1-TTV DNAs were identified in the sera of all 20 conventional pigs used as the source material of TTV for inoculation into gnotobiots swine. Use of pooled plasma that had positive results for TTV revealed that TTV was readily transmitted to young gnotobiots pigs that had negative results for TTV DNA. Pigs inoculated with cTTVp2, cTTVp3, or TTVp4 had sufficient TTV DNAs in tissues that a conventional PCR assay was sufficient to detect TTV DNAs. The g1-TTV DNAs were detected in all tissues (sera, lymph nodes, thymus, lungs, liver, kidneys, spleen, and bone marrow) obtained from pigs euthanized > 10 days after inoculation (data not shown).

The spectrum of TTV-associated histologic lesions, although mild, revealed time-dependent increases in severity that were then followed by a reduction during convalescence at > 21 days after inoculation. The lesions were reproducible, and they were not evident in age-matched, uninoculated control littermates and in pigs sham-inoculated with tissue homogenates with negative results for TTV. Histologic changes were not
associated with clinical signs of respiratory distress. In children, TTV infection is associated with respiratory disease and asthma.35–37 The interstitial pneumonia that developed in TTV-infected swine strongly suggested that g1-TTV infection may also be a cause of respiratory disease in swine. Similarly, lymphoid-histiocytic cellular infiltrates in the liver of TTV-infected gnotobiotic pigs suggested that the liver may be a site of TTV replication. Human TTV replicative isoforms (double-stranded DNAs) have been detected in liver homogenates,1,2,6 and TTV DNAs have been identified in the cytoplasm of hepatocytes by use of in situ hybridization.19

The renal glomerular lesions identified in TTV-inoculated gnotobiotic pigs were distinctive. Membranous glomerulonephropathy may be associated with circulating immune complexes. It has been suggested that glomerular lesions may be associated with TTV infection in humans.33 In our series of experiments, renal glomeruli were thickened (Jones silver stain), were variably positive when stained with PAS stain, and contained discontinuous immunoreactive aggregates of porcine fibrinogen or fibrin and IgG. These changes were not evident in pigs euthanized 5 to 7 days after inoculation but were variably identified in pigs euthanized 14 to 21 days after inoculation, which suggested that they were related to viremia attributable to TTV and the developing anti-TTV antibody responses in infected gnotobiotic pigs.

One of the hallmarks of gnotobiology is systemic underdevelopment of lymph nodes and related lymphoid aggregates. The B-cell-associated germinal centers are largely absent, the paracortical T-cell–rich zones are underdeveloped, and subcapsular sinuses are devoid of lymphocytes. As a result, gnotobiotes are lymphopenic and hypogammaglobulinemic.33 However, lymphoid tissues in gnotobiotes are reactive to infectious diseases, such as infection with PCV230,39 and Helicobacter organisms.41–43 Infection with these agents results in prompt immunologic activation that is manifested as lymphoreticular hyperplasia and development of lymphoid follicles; there is a full complement of immune responses in pathogen-infected gnotobiotes. Because histologic changes in lymphoid tissues other than transient thymic atrophy were not evident in TTV-inoculated pigs, the results were surprising and unexplained. Regardless, histologic lesions in the lungs, liver, and kidneys of TTV-inoculated pigs clearly were associated with TTV infection. Importantly, had these changes been detected in conventional swine, they would not be considered diagnostic for TTV (or any other commensal or pathogenic microbe of pigs for that matter) and would likely be dismissed as background histologic changes associated with subclinical responses to a variety of unidentified environmental insults. However, because uninfected gnotobiotic pigs lack these changes, as determined in the study reported here as well as in another report,35 gnotobiotic conditions permit an opportunity to identify subtle histologic changes associated with TTV infection.

The implications of our findings for porcine TTVs may be of interest to the human medical community. Since their discovery in 1997, investigators have repeatedly tried to implicate TTVs in a variety of disease processes in humans. All such studies have been confounded by the high incidence of asymptomatic TTV infections in age-matched cohort control groups and by the fact that the earliest phases of TTV infection cannot be identified in humans, except in those rare instances of mother-to-fetus transmission or perhaps in prospective studies of patients who receive blood and blood products containing TTVs. Furthermore, lack of suitable methods to enable domestic or laboratory animals to be used for the study of TTV infections has hindered investigations into the potential pathogenicity of these agents for humans. Transmission of human-origin TTVs to primates has been accomplished,44 but these experiments are complicated by the fact that nonhuman primates have their own simian TTVs.45

Gnotobiotic techniques represent a novel approach to the study of TTVs. Through the use of gnotobiotic animals, environmental influences can be effectively excluded, including inadvertent transmission of TTVs contained in the environment to experimental subjects. This advantage has been exploited by our laboratory group for in vivo studies of distemper in dogs,45 parainfluenza in dogs,36 canine parvovirus infection,47 Helicobacter pylori–associated gastritis in gnotobiotic swine,15,41–43 and PCV2 infection.38–40 In all of these host-pathogen interactions, gross and histologic changes observed in pathogen-infected gnotobiotes are direct reflections of the interactions between the agent and host and, by definition, are not confounded by external variables such as commensal and concurrent infections, dietary differences, and environmental pressures.48 It appears that infection of gnotobiotic pigs with porcine TTVs can be similarly exploited and that information provided by use of these techniques may be of benefit in the study of TTVs in humans.

The data reported here have broad implications, although it is premature to speculate on the role or roles that TTVs may play in other diseases of pigs. This caveat aside, we believe that we have reported here for the first time in any species that TTVs are pathogenic in the host species of origin when host conditions are appropriately manipulated. Gnotobiotic swine and infection with porcine TTVs may be an excellent method for use in studying TTV infection in humans. Finally, analysis of these data suggests that the commonly held assumption that the TTVs are nonpathogenic orphan viruses should be reexamined.

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