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Infected Peripheral Blood Mononuclear Cells Transmit Latent Varicella Zoster Virus Infection to the Guinea Pig Enteric Nervous System

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Abstract

Latent wild-type (WT) and vaccine (vOka) varicella-zoster virus (VZV) are found in the human enteric nervous system (ENS). VZV also infects guinea pig enteric neurons in vitro, establishes latency and can be reactivated. We therefore determined whether lymphocytes infected in vitro with VZV secrete infectious virions and can transfer infection in vivo to the ENS of recipient guinea pigs. T lymphocytes (CD3-immunoreactive) were preferentially infected following co-culture of guinea pig or human peripheral blood mononuclear cells with VZV-infected HELF. VZV proliferated in the infected T cells and expressed immediate early and late VZV genes. Electron microscopy confirmed that VZV-infected T cells produced encapsulated virions. Extracellular virus, however, was pleomorphic, suggesting degradation occurred prior to release, which was confirmed by the failure of VZV-infected T cells to secrete infectious virions. Intravenous injection of WT- or vOka-infected PBMCs, nevertheless, transmitted VZV to recipient animals (guinea pig > human lymphocytes). Two days post-inoculation, lung and liver, but not gut, contained DNA and transcripts encoding ORFs 4, 40, 66 and 67. Twenty-eight days after infection, gut contained DNA and transcripts encoding ORFs 4 and 66 but neither DNA nor transcripts could any longer be found in lung or liver. In situ hybridization revealed VZV DNA in enteric neurons, which also expressed ORF63p (but not ORF68p) immunoreactivity. Observations suggest that VZV infects T cells, which can transfer VZV to and establish latency in enteric neurons in vivo. Guinea pigs may be useful for studies of VZV pathogenesis in the ENS.

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Conflicts of interest.

The authors declare that they have no conflict of interest: Lin Gan, Mingli Wang, Jason J. Chen, Michael D. Gershon, and Anne A. Gershon

Introduction

Varicella-zoster virus (VZV, *Human herpesvirus 3*) is the causative agent of varicella (chickenpox) and zoster (shingles). Varicella is a primary infection after which VZV establishes latency in neurons of dorsal root (DRG) and cranial nerve ganglia (CNG) (Arvin and Cohen, 2007; Gershon and Silverstein, 2009), as well as in neurons of the enteric nervous system (ENS) (Gershon *et al*, 2012; Gershon *et al*, 2008). Reactivation of VZV from latency produces zoster, which may be followed by the severe neuropathic pain of post-herpetic neuralgia (Gilden *et al*, 2000). Extracutaneous complications of VZV infection include cerebral ataxia, meningoencephalitis, pneumonitis, hepatitis, transverse myelitis, and myositis; however, VZV has also been associated with grave intestinal pseudoobstruction (Edelman *et al*, 2009; Gershon and Silverstein, 2009; Pui *et al*, 2001). The recent observation that VZV establishes latency in the ENS of almost all individuals who have experienced varicella or been vaccinated against it (Chen *et al*, 2011) raises the question of whether the occult reactivation of VZV from latency in enteric neurons disturbs gastrointestinal (GI) function or underlies GI disorders of currently unknown etiology. Investigation of the consequences of VZV latency and reactivation in the ENS would be facilitated if an appropriate animal model were available.

VZV normally provokes disease only in humans and higher nonhuman primates (Arvin and Cohen, 2007). Because attenuation of VZV to prepare varicella vaccine included 12 passages in guinea pig cells in vitro (Takahashi *et al*, 1974), attempts have been made to use guinea pigs as models of VZV infection in vivo. These attempts involved inoculation of cell-associated VZV subcutaneously, intramuscularly, or intranasally into guinea pigs (Lowry *et al*, 1993; Matsunga *et al*, 1982; Myers *et al*, 1980; Myers *et al*, 1985; Myers *et al*, 1991; Sabella *et al*, 1993). Although VZV infected guinea pigs after inoculation with the virus, the infections did not mimic varicella, even in immunosuppressed animals and no evidence of latency was reported. Despite the lack of systemic illness, a transient erythematous papular rash was seen in small numbers of infected hairless and newborn guinea pigs; moreover, viremia and cell-mediated immune responses (CMI) to VZV were also demonstrated (Hayward *et al*, 1991).

There is evidence that expression of a consistent pattern of VZV genes identifies latently infected neurons (Kennedy and Cohrs, 2011; Lungu *et al*, 1998; Mahalingham *et al*, 1996). This pattern consists of expression of a limited number immediate early and early VZV genes without concomitant expression of late genes, such as those encoding glycoproteins (gps). Translation of the proteins encoded by the limited number of expressed transcripts has also been reported in latently infected neurons; however, in contrast to their nuclear location during lytic infection, all of these proteins during latent infection are cytoplasmic. Expression of VZV genes during latency has been questioned, in part because it has been suggested that post-mortem reactivation of VZV occurs, which could confound use of postmortem ganglia to study VZV latency (Ouwendijk *et al*, 2012). Cross-reactivity of antibodies to VZV proteins with antigen of human blood group A has also been reported, suggesting that VZV proteins might be misidentified in subjects with type A blood (Zerboni *et al*, 2012); moreover, it has been alleged that neuromelanin, which is common in DRG neurons, might mistakenly be identified as the diaminobenzidine (DAB) reaction product

that is commonly used to visualize sites of bound antibodies in immunocytochemistry (Zerboni *et al*, 2010b). The VZV genes that have been reported to be expressed during latency in postmortem human DRG (Kennedy and Cohrs, 2011; Lungu *et al*, 1998), however, have also been found to be expressed in enteric ganglia in surgically removed segments of bowel from which RNA was immediately extracted and were also fixed immediately, thereby eliminating the possibility of post-mortem reactivation (Gershon *et al*, 2012). Immunofluorescence, furthermore, was employed to detect VZV proteins in the human gut samples, to avoid confusion with neuromelanin. The latency-associated VZV proteins in the DRG in at least one report (Lungu *et al*, 1998) were subsequently ascertained to have been obtained from a subject with type B blood (Shelanski, M. personal communication). VZV infects and establishes latency in enteric neurons isolated from the guinea pig intestine (Chen *et al*, 2003); the pattern of VZV gene expression is identical to that reported for latently infected human DRG and enteric ganglia (Gershon *et al*, 2012; Kennedy and Cohrs, 2011; Lungu *et al*, 1998). This mimicry occurs only when enteric neurons are exposed to cell-free VZV in the absence of fibroblasts. In contrast, exposure of guinea pig enteric neurons to VZV in the presence of fibroblasts causes lytic infection; gps are expressed, immediate early proteins accumulate in the nucleus and infected neurons die within 48–72 hours. Addition of fibroblasts to latently infected neurons does not cause VZV to reactivate and become lytic; however, if VZV ORF61 or its herpes simplex virus orthologue, ICP0, is expressed in latently infected enteric neurons VZV reactivates; gps appear, immediate early proteins translocate to the nucleus, intact virions can be recognized electron microscopically, infection can be passed to other cells, and neurons die within 48–72 hours (Chen *et al*, 2003; Gershon *et al*, 2008). Guinea pig enteric neurons, therefore, can be utilized as an in vitro neuronal model to examine latency and reactivation of VZV. We therefore decided to use the guinea pig as a model to determine whether viremic spread of VZV can establish latent infection in the ENS. Here we show that intravenous injection of infected T lymphocytes in PBMC preparations of either guinea pig or human origin establishes latent infection of enteric neurons in the guinea pig bowel.

MATERIALS AND METHODS

Cells and viruses

Human embryonic lung fibroblasts (HELFL) were used for propagation of VZV stocks. Guinea pig peripheral blood mononuclear cells (PBMCs) from Hartley guinea pigs were prepared by two successive Ficoll-Paque (Sigma, USA) gradients. VZV-ZW, a wild type varicella strain, was isolated from a patient in China and used after 6 passages in HELFL (Gan *et al*, 2011). Live attenuated varicella vaccine (vOka, Biken strain) was obtained from the Shanghai Institute of Biological Products.

Infection of guinea pig peripheral blood mononuclear cells

VZV-infected PBMCs were prepared by co-culture with VZV-infected HELFL (Soong *et al*, 2000). Briefly, HELFL, in six-well plates were infected with cells containing 5×10^5 PFU/well of VZV. At 24–36 hrs after infection, 3×10^6 of guinea pig PBMCs were added to each well of VZV-infected HELFL. The six-well plates were centrifuged for 45 min at $200 \times g$ at 25°C and incubated at 33°C in an atmosphere containing 5% CO_2 . After 18–20 hrs, PBMCs were

harvested by gently washing them from the monolayer. PBMCs were collected and pelleted, washed with Roswell Park Memorial Institute (RPMI) 1640 medium, re-suspended in RPMI 1640 with 10% fetal bovine serum at 7.5×10^5 cells-ml in cell culture flasks, and incubated at 37°C for the indicated times.

Analyses of VZV-infected PBMC

For immunocytochemistry, approximately $0.5 - 1 \times 10^4$ cells were placed on glass slides and fixed with cold methanol for 5 min. The slides were washed with PBS, blocked with 10% normal rabbit serum, 10% normal mouse serum, and 0.1% Triton X-100. Primary antibodies included polyclonal antibodies against VZV ORF68p (gE; Virusys Corp. Taneytown, MD), ORF62p (Lungu et al, PNAS, 1998), and a monoclonal antibody to CD3 (Millipore, Billerica, MA). The antibodies were applied overnight in a humidified chamber at 4°C. Preparations that were not exposed to primary antibodies were processed simultaneously as controls. Secondary antibodies included goat anti-rabbit IgG (Fab' fragment) conjugated with Alexa 488 and goat anti-mouse IgG (Fab' fragment) labeled with Alexa 594 (Molecular Probes, Eugene, OR). Bisbenzimidazole (Sigma-Aldrich, St. Louis, MO) was used to stain DNA. For transmission electron microscopy (TEM), 3×10^6 PBMCs were fixed with 2% buffered glutaraldehyde, dehydrated with a graded series of ethanols, cleared with propylene oxide, and embedded in an epoxy resin. Thin sections were cut with an ultramicrotome, placed on copper grids, stained with uranyl acetate and lead citrate, and viewed with a JEOLCO (JEM 1200) TEM.

The growth of VZV in guinea pig PBMCs was studied by plaque assay and real-time PCR. At various times after their in vitro infection by co-cultivation with VZV-infected HELF, 2 ml of RPMI 1640 with 10% fetal bovine serum containing 1×10^6 PBMCs were plated into individual wells of a six-well plate containing freshly confluent HELF cell monolayers. The plates were centrifuged for 40 min at $200 \times g$ at 25°C and incubated at 33°C for 4h. PBMCs were removed by gently washing the HELF cells with MEM. Seven days after incubation, HELF cells were fixed and stained with crystal violet. The number of plaques was counted. Total DNA was also extracted from 1×10^6 VZV-infected PBMCs and real-time PCR was used to measure the viral copy numbers by the 5' exonuclease method. Briefly, PCR was carried out in the presence of a Taqman probe (5' FAM reporter fluorophore and 3' TAMRA quencher fluorophore) for VZV ORF29. The reporter fluorophore was detected in real time by using an Applied Biosystems 7500 instrument. A plasmid with an insert encoding the cloned ORF29 PCR fragment was used as a reference standard (Cohrs and Gilden, 2007).

Transwell™ assay

To determine whether VZV-infected guinea pig PBMCs release cell-free virus, the ability of infectious particles to cross filters with a pore diameter of 0.4 µm was measured. VZV-infected PBMCs (1×10^6) were washed, resuspended in 2 ml and cultured in 6-well plates on filters (Transwell™, Millipore, Billerica, MA) over HELF monolayers. Plates were centrifuged at $200 \times g$ for 20 min, twice a day, for the first 2 days of incubation. After 7 to 8 days, CPE was observed in HELF cells, and the monolayers were fixed and stained with a monoclonal antibody to VZV gE (Virusys Corp., Taneytown, MD). Sites of antibody binding were visualized with secondary antibodies (IgG) coupled to alkaline phosphatase

(Millipore, Billerica, MA). Plates were washed with 100mM Tris, pH7.5, 150mM NaCl, and 0.05% Tween 20. Color was developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Roche Applied Bioscience, Indianapolis, IN) in 100mM Tris buffer (pH9.5). Negative controls included uninfected PBMCs or VZV-infected HELF cells (which do not secrete infectious virions (Zhu *et al*, 1995a) grown in Transwell™ assemblies over HELF monolayers. Positive controls, consisting of aliquots of isolated cell-free VZV (Chen *et al*, 2004b; Gabel *et al*, 1989; Zhu *et al*, 1995a) placed over the 0.4 µm filters, were used to verify that infectious cell-free VZV was able to cross the filters in Transwell™ assemblies. A further control was carried out to determine whether the appearance of plaques in HELF underlying the filters in Transwell™ assemblies might be due to the passage through the filters of VZV-infected whole cells or fragments. Mannose 6-phosphate (Man 6-P) is able to compete with cell-free VZV for access to the large cation-independent mannose 6-phosphate receptors (MPRs) on cell surfaces that mediate viral entry (Chen *et al*, 2004b; Gabel *et al*, 1989; Zhu *et al*, 1995a). In contrast, Man 6-P is unable to prevent the infection of HELF by cell-associated VZV, which is independent of MPRs, but dependent on insulin degrading enzyme (IDE) (Ali *et al*, 2009; Li *et al*, 2006; Li *et al*, 2010). In control cultures, therefore, Man 6-P (40 mM) was included in the culture medium in which HELF were grown under Transwell™ assemblies. The Man 6-P in the medium would be expected to inhibit the formation of plaques due to infection with cell-free VZV but be permissive of plaques due to the passage of VZV-infected cells or fragments. The immunostained plaques in HELF monolayers growing under the filters were counted with an inverted microscope.

In vivo infection of guinea pigs

A total of 1×10^6 VZV-infected PBMCs in 50µl was injected into the posterior ocular sinus or saphenous veins of each of 10 female Hartley guinea pigs (4–6 weeks old, Charles River Laboratories). Four control guinea pigs received uninfected PBMCs. At various times after infection, 50 µl of whole blood were obtained from a dorsal pedal or ear vein of the animals and DNA was extracted using a Blood Genome DNA Extraction Kit (Takara, Dalian, China). Viral DNA copy number was determined as described above. Guinea pigs were euthanized 2 days and 4 weeks after infection; liver, lung, and bowel were collected. Tissues were fixed with 4% formaldehyde (from paraformaldehyde) in 0.1M phosphate buffer (pH 7.4).

Nested PCR and RT-PCR

DNA and total RNA were extracted from guinea pig tissues with Trizol™ (Invitrogen) according to the protocols of the manufacturer. Treatment with DNase I was used to remove residual DNA contamination from extracted RNA. Primers used for nested PCR and RT-PCR amplification included those for VZV ORFs 29, 40, 66, 67 and 68. VZV primer sequences are listed in Table 1 and were synthesized and purified by Invitrogen Shanghai Co., Ltd.

In situ hybridization and immunocytochemistry

Guinea pig tissues were fixed overnight at 4°C with 4% formaldehyde (from paraformaldehyde; 0.1 M phosphate buffer, pH 7.4), embedded in paraffin, and sectioned at 3 µm. Sections were deparaffinized with xylene, rehydrated through a graded series of

ethanols and treated for 20 min with proteinase K (100 µg/ml) in PBS. After washing with PBS, the tissues were post-fixed with 4% formaldehyde for 10 min at room temperature, incubated with 0.3 M NaOH for 5 min, and then neutralized with 0.4 M Tris buffer (pH7.4) for 15 min (Zerboni *et al*, 2007). Prehybridization buffer (100 µl; 5xSSC, 1x Denhardt's solution, 10mg/ml of salmon sperm DNA) was applied to each section and incubated at room temp for 2 hrs in a sealed box. Hybridization buffer (100 µl; 5xSSC, 1 x Denhardt's solution, 10.0 mg/ml of salmon sperm DNA, 40 mg/ml of dextran sulfate) containing 40 pmol of VZV probe (Lungu *et al*, 1995) was then applied. Sections on slides were coverslipped and incubated in a sealed box for 10 min at 85°C to denature the target and probe. Following hybridization overnight in a humidified chamber at 37° C, the cover slip was removed and the sections were washed with TNT buffer (100 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH7.5). The sections were then treated at room temp for 1 hr with blocking buffer (100 mM Tris, pH7.5, 150 mM NaCl, 5% goat serum) before antibodies to digoxigenin (1:750) were applied for 2 hrs. The sections were washed with TNT buffer and equilibrated with NTMT buffer (0.1 M Tris buffer [pH9.5], 0.1 M NaCl, 0.05 M MgCl₂, 0.2 mM Levamisole) for 3 min. Color (blue) was developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Roche Diagnostics, Indianapolis, IN). The sections were counterstained with 1% toluidine blue, and mounted with Permount. For immunocytochemistry, tissue sections were simultaneously treated for 90 min in a humidified chamber at 37°C with rabbit antibodies to ORF29p and murine antibodies to ORF68p. Alexa 488- and Alexa 594-conjugated secondary IgG antibodies (against rabbit or mouse) were used to detect sites of antibody binding. Nuclei were stained with bisbenzimidazole.

Statistical analyses

Student's t test was used to compare single pairs of means. One-way ANOVA was employed when the effect of one independent variable was evaluated. To examine the effect of two independent variables on one dependent variable, two-way ANOVA was used.

RESULTS

Cell-associated VZV transfers productive infection to T cells

Co-culture was employed to transfer VZV infection from VZV-infected HELF to guinea pig PBMCs. To determine whether VZV infection was successfully transferred, immunocytochemistry and TEM were used to examine the co-cultured PBMCs. Antigenicity of ORF68p (gE), ORF62p, and ORF29p were used as markers of VZV infectivity. CD3 immunoreactivity was employed to identify T cells. The immunoreactivities of ORF68p (Fig. 1a) and ORF29p (Fig. 1b) were found to be co-localized in co-cultured PBMCs except at the periphery of the cells where there was a corona of gE immunoreactivity that lacked that of ORF29p (Fig. 1a–d). This pattern is consistent with the known insertion of ORF68p, but not ORF29p, into the plasma membranes of VZV-infected cells (Gershon and Gershon, 1999). The inclusion of a late protein, ORF68p, suggests that the infection of guinea pig PBMCs was lytic. The ORF68p-immunoreactive cells, moreover, were also CD3-immunoreactive (Fig. 1e–h); similarly, CD3-immunoreactivity (Fig. 1i–l) co-localized with the immunoreactivity of another immediate early protein, ORF62p (Fig. 1j). All of the cells

that expressed the immunoreactivity of VZV proteins also expressed the immunoreactivity of CD3. These observations imply that T lymphocytes in the PBMC population preferentially become infected with VZV following their co-culture with VZV-infected HELF.

Transmission electron microscopy confirmed that productive VZV infection had been established in PBMCs that were co-cultured with VZV-infected HELF (Fig. 2). Guinea pig PBMCs displayed all of the stages of productive VZV infection that have been described in VZV-infected HELF in vitro (Gershon *et al*, 1994) and in VZV-infected keratinocytes in vivo (Chen *et al*, 2004b). The nuclei of infected cells contained nucleocapsids, most of which contained cores of DNA, although small numbers of incomplete nucleocapsids, lacking DNA cores, were also observed (Fig. 2a). Images were also consistent with the budding of nucleocapsids into the perinuclear cisterna, and with the acquisition of a sharply defined primary envelope from the inner nuclear membrane during the budding process (Fig. 2b, c). The nuclear envelope frequently invaginated and expanded at sites of viral budding to encompass pools of newly enveloped virions (Fig. 2a). Enveloped virions could also be identified within elements of the endoplasmic reticulum (Fig. 2a, c) and naked nucleocapsids were observed in the cytosol. In the Golgi region, C-shaped cisterna wrapped around naked nucleocapsids (Fig. 2c, d), a pattern of partial envelopment that is consistent with the previously been described cytoplasmic re-envelopment of VZV (Gershon *et al*, 1994). Osmiophilic dense material, which resembles tegument, adhered to the concave faces of the enveloping cisternae, suggesting that tegument is incorporated during the re-envelopment process (Fig. 2c, d). In contrast to VZV-infected HELF cells (Gershon *et al*, 1994), accumulations of virions within late endosomes was not observed, although the pleomorphic appearance of both intracellular virions in vacuoles and extracellular virions (Fig. 2e) was consistent with the degradation of VZV prior to exocytosis (Gershon *et al*, 1994). Infected cells tended to adhere to each other and were coated with pleomorphic viral particles (Fig. 2f).

The ability of WT VZV (strain ZW) to infect PBMCs when the PBMCs were co-cultured with VZV-infected HELF was compared quantitatively to that of vOka. PBMCs were incubated in suspension for 12, 24, 48, or 72 hrs following their harvest from WT VZV- or vOka-infected HELF. After each of these times of incubation, one aliquot of cells was transferred to a reporter layer of uninfected HELF (for plaque assay) while DNA was extracted from a second aliquot. The recipient HELF, which received the aliquot of putatively infected PBMCs, were then cultured for a further 72 hrs and the numbers of infectious foci were determined. Real-time PCR was used to quantify genome copy numbers (from the abundance of DNA encoding ORF29) in the aliquot extracted from the putatively infected PBMCs. Both the numbers of infectious foci transferred to HELF (Fig. 3a) and the VZV genome copy number in PBMCs (Fig. 3b) reached a peak 48 hrs after PBMCs were harvested from VZV-infected HELF. By 72 hrs after their infection with VZV, both the number of infectious foci transferred to HELF and genome copy numbers in PBMCs declined. Neither the abundance of infectious foci transferred to HELF (Fig. 3a) nor that of genome copy numbers (Fig. 3b) produced in PBMCs infected with WT VZV differed significantly at any time after infection from those produced in PBMCs infected with vOka. The growth of WT VZV and vOka in PBMC is similarly reflected in curves plotting

numbers of infectious foci transferred to HELF and genome copy numbers in PBMCs as a function of time after infection (Fig. 3a, b).

It is not clear how infected T cells transfer VZV to the neurons in which the virus establishes latency. Exposure of isolated guinea pig enteric neurons to cell-free VZV induces latent infection, whereas lytic infection occurs when similar preparations are exposed to cell-associated VZV (Gershon *et al*, 2008). These observations are compatible with the hypothesis that the establishment of latency requires that cell-free virions infect neurons. Were this idea to be correct, the viremia that occurs during varicella could establish latency if VZV-infected T cells were to secrete cell-free VZV. Most VZV-infected cells, however, fail to release infectious virions; therefore, VZV is highly cell-associated (Weller, 1953). Suprabasal epidermal cells, within which mannose 6-phosphate receptors are downregulated, are exceptionally able to secrete infectious virions, which can transmit VZV to naïve hosts and the cutaneous branches of sensory neurons (Chen *et al*, 2011; Chen *et al*, 2004a). Whether VZV-infected PBMC cells can do the same is unclear. Previous studies have reported that VZV released from infected T cells is filterable (Moffat *et al*, 1995); however, when the pore size of filters is reduced sufficiently to prevent the passage of cells, transmission of infection across a filter no longer occurs (Soong *et al*, 2000). We therefore determined whether VZV-infected guinea pig PBMCs secrete cell-free VZV. To do so, PBMCs that had been infected with WT VZV or vOka (donor cells) were layered over a 0.4 filter in Transwell™ assemblies. HELF, grown in the chamber below the filters (recipient cells), were fixed and immunostained with antibodies to ORF68p after 7–8 days of culture to quantify numbers of infectious foci. Cell-free VZV was also placed directly onto Transwell™ filters over recipient HELF monolayers to verify the ability of cell-free VZV to traverse the filters (Fig. 4a). Twenty $\pm$ 0.8 % of infectious cell-free virions passed through the filters. Recipient cells were cultured $\pm$ Man 6-P (40 mM) to verify that infectious foci, if present, were due to the trans-filter passage of infectious free virions. Man 6-P (40 mM) blocked almost all of the infectivity of CFV traversing the filters (Fig. 4a). Similar comparative experiments were carried out using VZV-infected HELF as donor cells (Fig. 4b). Surprisingly, infectious foci appeared in the recipient HELF monolayer; nevertheless, in contrast to the trans-filter passage of CFV, Man 6-P (40 mM) was unable to prevent infection of recipient monolayers when HELF were used as donors. These observations suggest that intact fragments of VZV-infected HELF are able to traverse filters, even those with a pore size of 0.4 μ m. As was observed with CFV, however, almost no Man 6-P (40 mM)-resistant infectious foci were found in recipient monolayers when VZV-infected PBMCs were used as donor cells (Fig. 4c). These observations suggest that VZV-infected PBMC do not release significant quantities of infectious free virions, and in contrast to HELF, fragments of intact PBMC cytoplasm containing VZV are not able to cross 0.4 μ m filters.

VZV copy numbers in blood following in vivo infection

VZV-infected PBMC were injected intravenously into recipient guinea pigs. Human and guinea pig PBMC were compared as a source of infected PBMC. Human PBMC were freshly prepared from volunteers and treated identically to guinea pig PBMC and were co-cultured with VZV-infected HELF in order to infect them with VZV. Equal numbers of

human and guinea pig VZV-infected PBMC were injected into recipient guinea pigs ($n = 6$ for each type of cell). Blood (50 μ l) was then drawn from the recipient animals and qPCR was used to measure VZV DNA copy number as a function of time after inoculation (Fig. 5). Copy number for human and guinea pig was found (two-way ANOVA) to vary significantly as a function both of time ($p < 0.0001$) and species (guinea pig $>$ human; $p < 0.0001$). Copy numbers for guinea pig PBMC (one-way ANOVA) increased significantly between 24 hrs and 48 hrs and declined significantly thereafter, although copy numbers at 72 hrs were still significantly greater than at 24 hrs. These observations suggest that VZV proliferates in PBMCs following inoculation into a donor animal and does so more efficiently in guinea pig than human PBMCs. VZV-infected cells, moreover, disappear from the circulation. Further studies were thus carried out of the distribution of VZV DNA within organs of the recipient guinea pigs as a function of time to determine whether the infected PBMCs are cleared or whether they transfer VZV to tissues.

VZV DNA in guinea pig lung, liver and gut

Studies were carried out to determine whether VZV-infected PBMC transmit infection to the gut and other guinea pig organs. DNA and transcripts encoding ORFs 29, 40, 66, and 67 were analyzed in lung, liver, colon, and ileum 2 days and 28 days after inoculation with VZV-infected PBMC (Fig. 6a–d). At 2 days, despite the proliferation of VZV in blood cells, neither VZV DNA (Fig. 6a), nor viral transcripts (Fig. 6b) could be detected in colon or ileum. In contrast, viral DNA (Fig. 6a) and transcripts encoding ORFs 29, 40, 66, and 67 (Fig. 6b) were each detected in both lung and liver of 4/4 guinea pigs. Because not only DNA, but also VZV transcripts, were found, it is likely that liver and lung contained proliferating VZV two days after injection of VZV-infected PBMC. At 28 days, however, neither DNA (Fig. 6c) nor transcripts encoding any of the viral genes examined (Fig. 6d) remained either in lung or the liver; nevertheless, by 28 days, the colon and ileum now each contained viral DNA (Fig. 6c) and transcripts (Fig. 6d). DNA encoding all 4 of the ORFs examined was detected at 28 days in both the colon and ileum in each of 4 guinea pigs; nevertheless, transcripts encoding ORFs 40 and 67 could not be detected in 3 of the 4 animals. Transcripts encoding ORFs 29 and 66, however, which encode genes transcribed during latency (Chen *et al*, 2011; Cohen *et al*, 2007; Cohrs *et al*, 2003; Folster *et al*, 2011; Grinfeld and Kennedy, 2004; Sato *et al*, 2003b; Stallings *et al*, 2006), were found in the colon and ileum of all 4 guinea pigs. These observations are consistent with the idea that VZV established enteric latency in 3/4 guinea pigs and that an asymptomatic lytic infection, associated with the expression of ORFs 40 and 67, was present in the bowel of 1 of the 4 animals. Taken together, the observations are compatible with the ideas that VZV proliferates in lung and liver within 2 days after injection of VZV-infected PBMCs and that clearance from those organs is followed within 4 weeks by the establishment of latency in large and small intestines.

Location of VZV-infected cells

To investigate where VZV was harbored following injection of VZV-infected PBMC, *in situ* hybridization was used to locate DNA encoding VZV ORF54 in sections of lung, liver, ileum and colon. Animals were examined at 2 and 28 days following injection of VZV-infected PBMC. No hybridizing DNA was detected at either time interval in control guinea

pigs, which received uninfected PBMC. At 2 days, in guinea pigs that had received VZV-infected PBMC, however, VZV DNA was found in cells within central veins and sinusoids of the liver (Fig. 6e) and in perialveolar capillaries of the lung (Fig. 6f). No hybridizing DNA was observed in parenchymal cells of the liver or the lung. In the ileum (Fig. 6g) and colon (Fig. 6h), hybridizing DNA was detected in cells identified morphologically as neurons (compare with Fig. 7f) within ganglia of the myenteric plexus 28 days after injection of VZV-infected PBMC. The hybridizing DNA was most concentrated in neurons and was not abundant in the ganglionic neuropil, suggesting that little DNA encoding ORF 54 is present in axons and glial cells.

Immunocytochemistry was used to verify the distribution of VZV proteins as a function of time after injection of guinea pigs with VZV-infected PBMC. At 2 days post-injection, ORF29p and ORF68p immunoreactivities were detected in the lung (Fig. 7a–d), liver, and spleen (not illustrated). The immunoreactivities of ORF29p and ORF68p were coincident and their localization in the lung was similar to that of DNA hybridizing with a probe for ORF54 (compare Fig. 7d to 6f). The immunoreactive cells were located in blood vessels. At 28 days, no immunoreactivity of VZV proteins could any longer be found in lung, liver, or spleen (not illustrated); nevertheless, although no ORF68p immunoreactivity was detected in the ileum or colon, the immunoreactivity of ORF63p, which is the VZV protein most commonly expressed during latency (Zerboni *et al*, 2010b), was now found within cells of myenteric ganglia (Fig. 7e, h). All of the ORF63p-immunoreactive cells coincidentally expressed the immunoreactivity of the neuronal marker, HuC/D (Fig. 7f, h) and thus were neurons. Other non-neuronal cells or the muscularis externa (which include smooth muscle, glia, and interstitial cells of Cajal), the positions of which were detected with bisbenzimidazole fluorescence (nuclear DNA), lacked any VZV immunoreactivity. The immunofluorescence of ORF63p was cytoplasmic but restricted to the cell bodies of the neurons. All enteric neurons were ORF63p-immunoreactive.

Discussion

The ENS is the largest and most autonomous region of the peripheral nervous system (Gershon, 2005; Gershon, 2012; Moanna and Rimland, 2013). It is capable of integrative neuronal function and can mediate reflex activity in the absence of input from the brain or spinal cord. The ENS is also essential for life. The congenital or acquired absence of even a small segment of the ENS, as occurs respectively, in Hirschsprung or Chagas disease, causes the gut to become obstructed and is fatal if not corrected (Furness, 2012; Gershon, 2010; Hirano and Pandolfino, 2000; Lake and Heuckeroth, 2013; Langer, 2013). Recent evidence that VZV is latent within the human ENS (Chen *et al*, 2011; Gershon *et al*, 2012; Gershon *et al*, 2008) is therefore significant because of the possibility that latent VZV within enteric neurons might reactivate. Although cultured enteric neurons can survive indefinitely with latent VZV, they invariably die within 48–72 hrs of VZV reactivation (Chen *et al*, 2011; Gershon *et al*, 2008). Reactivation of VZV in human DRG is accompanied by an intense T cell-mediated immune response with associated ganglionic necrosis (Steain *et al*, 2014). It is also followed by an expansion of visual fields, cutaneous deafferentation (Oaklander, 2008), massive loss of myelinated nerve fibers, and ganglionic fibrosis (Watson *et al*, 1991). These effects suggest that although ORF63p protects infected neurons from apoptosis (Hood *et al*,

2006; Hood *et al*, 2003), reactivation of VZV is lethal to many, if not all, of the DRG neurons in which it occurs. VZV reactivation from latency within the human ENS, therefore, would be expected disrupt gut function because of the critical role that the ENS plays in the physiology of the bowel. The manifestations of enteric VZV reactivation, or “enteric zoster”, however, are unknown; moreover, because enteric neurons do not innervate the skin, a VZV-induced disorder of the ENS is likely to occur in the absence of a rash, which is the hallmark of zoster.

In fact, severe abdominal pain, GI bleeding, and intestinal dysfunction have been linked to VZV infection (Sherman *et al*, 1991; Toudic *et al*, 1995; Wyburn-Mason, 1957). Disseminated varicella has been reported to be able to cause a complete secondary destruction of the ENS in immunocompromised patients (Abreu Velez *et al*, 2012; Holland-Cunz *et al*, 2006) and pseudoobstruction due to enteric VZV infection has been found to accompany cutaneous zoster in immunocompromised hosts (Chang *et al*, 1978; Debinski *et al*, 1997; Tanida *et al*, 2013). The cases in which the association of intestinal disease and VZV infection of the bowel has been recognized have all been sufficiently catastrophic to have caused the gut to be examined, most commonly at autopsy. Given that VZV is probably latent in the ENS of most adults (Chen *et al*, 2011; Gershon *et al*, 2012; Gershon *et al*, 2008), “enteric zoster” would either have to be rare or, when it occurs, far less catastrophic than the published case reports. Symptomatic reactivations of VZV that are compatible with at least some function of the ENS, therefore, may be an unsuspected cause of GI disorders. Documentation of VZV infection of the human ENS requires invasive investigation. Justification of invasive studies of the human ENS to determine whether zoster is present, however, is problematic because many GI symptoms, such as abdominal pain, diarrhea, and constipation, are both non-specific and common. It would thus be useful to be able to study VZV latency in the ENS in an animal model.

The restriction of the host range of VZV to humans has handicapped studies of its pathogenesis in animal models. Several of these models have been employed, including the guinea pig (Arvin *et al*, 1987; Hayward *et al*, 1991; Lowry *et al*, 1993; Lowry *et al*, 1992; Matsunga *et al*, 1982; Myers *et al*, 1980; Myers and Stanberry, 1991; Myers *et al*, 1985; Myers *et al*, 1991), rat (Hasnie *et al*, 2007; Kennedy *et al*, 2001), mouse (Wroblewska *et al*, 1993), and cotton rat (Ambagala *et al*, 2010; Sato *et al*, 2003a; Xia *et al*, 2003). Although each of these has yielded information, none has, until now been entirely satisfactory. Injection of cell-associated VZV into the skin or directly into the guinea pig bowel has been shown to lead to VZV latency in enteric neurons (Chen *et al*, 2011; Gershon *et al*, 2012). A viremia, however, occurs during varicella and sometimes zoster (Asano *et al*, 1990; Gershon *et al*, 1978; Ozaki *et al*, 1986), is responsible for transporting VZV to the skin (Moffat *et al*, 1995; Schaap *et al*, 2005), and arguably also to DRG (Mahalingham *et al*, 1991; Zerboni *et al*, 2010a). Infected T lymphocytes carry VZV during the viremia of varicella (Schaap *et al*, 2005; Schaap-Nutt *et al*, 2006). The current study tested the hypotheses that the experimental induction of a viremia similar to that of varicella can establish VZV latency throughout the ENS.

VZV-infected HELF transferred infection to co-cultured PBMC preparations from human or guinea pig. Within the PBMC, VZV preferentially infected CD3-immunoreactive T cells

(Arvin *et al*, 2010). Electron microscopic examination of VZV-infected T cells, moreover, suggested that the intracellular pattern of viral assembly was identical to that described for cultured HELF (Gershon *et al*, 1994) and for cells of the basal layer of the human epidermis in vivo (Chen *et al*, 2004b). In those VZV-infected human cells, nucleocapsids assemble in the nucleus and acquire a temporary envelope from the inner nuclear membrane as they bud from the nucleus into the perinuclear cisterna. From there, the primordial virions travel to the endoplasmic reticulum, with which the perinuclear cisterna is in continuity. The temporary viral envelope then fuses with the membrane of the endoplasmic reticulum, releasing free nucleocapsids to the cytosol. The free nucleocapsids translocate to specialized C-shaped cisterna (“wrapping structures”) derived from the *trans*-Golgi network (TGN). Membrane viral glycoproteins segregate into the concave face of the “wrapping structures” to which tegument proteins adhere (Wang *et al*, 2001). Nucleocapsids enter the concavity that the C-shaped cisternae define. The “wrapping structures” ultimately extend around the nucleocapsids, trapping tegument between the nucleocapsids and the concave membrane. At the end of the process tegument-containing re-enveloped virions are formed. The concave face of the TGN-derived “wrapping structure” becomes the viral envelope while the convex face becomes a transport vesicle. The resemblance of the subcellular pattern of VZV assembly in guinea pig and human T cells to that seen in VZV-infected HELF and basal cells of the epidermis, confirms that VZV proliferated in T cells and that their infection was productive of encapsulated virions. The pleomorphic appearance of extracellular virions adhering to infected T cells, however, was different from the intact extracellular virions, which suprabasal epidermal cells secrete (Chen *et al*, 2004b), suggesting that the infected T cells degrade enveloped VZV before they secrete it.

VZV-infected T cells did not, in fact, appear to secrete appreciable amounts of infectious cell-free VZV, which was defined as infectivity that passed through a filter with a 0.4 μ m pore diameter and could be inhibited by Man 6-P (Chen *et al*, 2004a). Man 6-P competitively interferes with the interaction between plasmalemmal Man 6-P receptors and glycoproteins of the viral envelope (Gabel *et al*, 1989; Zhu *et al*, 1995b). Man 6-P prevents viral entry when target cells are exposed to cell-free VZV but Man 6-P does not interfere with cell-to-cell spread of VZV or infection by cell-associated virus (Li *et al*, 2006; Zhu *et al*, 1995b). When VZV-infected PBMC were injected intravenously into recipient guinea pigs, VZV appeared first to proliferate for at least 2 days in the lung and liver, where in situ hybridization (ORF54) showed that it was associated within and near blood vessels. VZV, however, was cleared from the lung and liver and, by 28 days, both VZV DNA and transcripts were found in the gut. The bowel of 4 recipient guinea pigs, in which DNA encoding ORFs 29, 40, 66, and 67 was found, also contained transcripts encoding ORFs 29 and 66. In contrast, transcripts encoding ORF 67 were detected in the gut of only one of these animals. The protein, gI, that ORF 67 encodes, is expressed during lytic but not latent infection, while ORFs 29 and 66 are also expressed during latency (Cohrs *et al*, 2003; Nagel *et al*, 2011). These observations suggest that VZV established latency in the bowel of 3/4 animals. A low-grade continuing lytic infection might have been present in the one guinea pig in which enteric transcripts encoding ORF 67 were detected.

The cellular locations of both VZV DNA and transcripts suggest that the latent VZV in the gut of recipient guinea pigs was present in enteric neurons. In situ hybridization revealed

that most neurons of the myenteric plexus in these animals contained VZV DNA. Although the immunoreactivities of ORFs 68p and 29p were coincident in cells in the lung and liver 2 days after injection of VZV-infected PBMCs into recipient guinea pigs, ORF68p immunoreactivity was not detected in the bowel at either 2 or 28 days post-injection. In contrast, at 28 days, when transcripts encoding ORFs 29 and 66 were present in the bowel and VZV DNA was detected in enteric neurons by in situ hybridization, virtually all of the neurons of recipient animals displayed the immunoreactivity of ORF63p, which is the VZV gene product that is most highly expressed and most commonly reported in DRG neurons during latency (Nagel *et al*, 2011; Zerboni *et al*, 2010b). No neurons, however, expressed the immunoreactivity of ORF68p, which is the protein that is most highly expressed during lytic VZV infection (Gershon and Gershon, 1999). Despite the remarkable expression of ORF63p immunoreactivity in virtually all of their enteric neurons, recipient guinea pigs remained asymptomatic and showed no adverse effects of their infection; moreover, no immune reactions or morphological alterations of any kind were observed in the infected ganglia. These observations support the ideas that the viremic spread of VZV carried by T lymphocytes can transfer VZV to guinea pig tissue and establish latent infection of the ENS; moreover, the lack of pathological changes in the recipient guinea pig ENS or in GI function, provides further support for the conclusion that enteric neuronal VZV infection is latent.

How T cells transfer the virus to target cells in host guinea pigs is unclear. Because infected T cells were not found to secrete infectious free virions, fusion of infected T cells with their targets would seem, by exclusion, to be the most straightforward mechanism by which host neurons and other cells become infected although other mechanisms are possible. Following the intravenous injection of VZV-infected PBMC, transient infection can be demonstrated in liver, lung, and spleen (not illustrated), which is rapidly cleared. No rash can be demonstrated. Overt infection of the skin, analogous to that which occurs in varicella, therefore, does not occur. Still, it is conceivable that VZV proliferates asymptotically in the skin and releases small numbers of cell-free virions from suprabasal keratinocytes that infect cutaneous sensory nerves. VZV that is injected into guinea pig skin can establish latency in enteric and DRG neurons (Chen *et al*, 2011); however, when the skin is inoculated, the VZV infection of the ENS is limited and segmental, quite different from the near-total infection of the ENS that intravenous injection of VZV-infected T cells produce. If infected T cells are able to fuse with and infect enteric neurons, then it is not clear why infection of the neurons is not lytic. Cell-associated VZV (in infected HELF or MeWo cells) invariably causes lytic infection of guinea pig enteric neurons in vitro (Chen *et al*, 2003; Gershon *et al*, 2008). There may thus be something unique about the means by which infected T cells present VZV to enteric neurons; alternatively, the responses of neurons to cell-associated VZV in vivo and in vitro may be different.

In summary, we find that VZV preferentially infects T cells in isolated preparations of guinea pig PBMC. VZV proliferates and forms encapsulated virions within these cells; nevertheless, both the ultrastructural appearance of extracellular virions, which is pleomorphic, and the failure of infected PBMC to release filterable infectious cell-free VZV, suggest that VZV is degraded intracellularly prior to exiting from infected T cells. When injected intravenously into recipient guinea pigs, VZV-infected PBMC cause a transient

productive infection of lung and liver, which is followed by a long-term latent infection throughout the ENS. The mechanism of transfer of VZV from infected T cells to the enteric neurons within which latency is established remains to be determined. Because VZV is a potential cause of GI disorders and little is known about its life cycle in the ENS, the guinea pig model may help to develop an understanding of the pathogenesis of VZV infections of the gut.

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Fig. 1.

PBMCs acquire the immunoreactivity of VZV-encoded late and immediate early proteins when PBMCs are layered over VZV-infected HELF. PBMCs were layered over VZV-infected HELF cells. After 48 hrs the PBMCs were removed and fixed for immunocytochemical examination with antibodies to ORF68p (gE), ORF62p, ORF29p, and CD3. DNA was stained with bisbenzimidazole. a–d. A single PBMC has been doubly immunostained to visualize ORF68p (a; green) and the early protein ORF29p (b; red). Nuclear DNA displays blue fluorescence (c). The immunofluorescence of ORF68p and ORF29p overlap, except that there is a corona of ORF68p immunoreactivity that is not coincident with that of ORF29p at the periphery of the cell (d; merged image) because ORF68p traffics to the plasma membrane and ORF29p does not. e–h. A field of PBMCs has been immunostained to identify the cells that become infected with VZV in PBMC preparations. The T cell marker CD3 (e; red) is coincident with the VZV late protein ORF68p (f; green). The locations of all cells in the field are indicated by the blue fluorescence of DNA (g). Note in the merged image that all cells that display the immunofluorescence of ORF68p also display the immunofluorescence of CD3 (h). i–l. A field of PBMCs has been immunostained to confirm the identification of cells that become infected with VZV in PBMC preparations. The T cell marker CD3 (i; red) is coincident with the VZV late protein immediate early protein, ORF62p (j; green). The locations of all cells in the field are again indicated by the blue fluorescence of DNA (k). Note that all cells in the merged image that are ORF68p-immunofluorescent are also CD3-immunofluorescent (l). The markers = 10 μ m.

Fig. 2.

Ultrastructure of VZV-infected PBMCs suggests that the infection is productive and lytic. PBMCs were infected by layering over VZV-infected HELF cells. After 48 hrs the PBMCs were removed and fixed for electron microscopy. a. Assembling nucleocapsids (*) can be recognized at the periphery of the nucleus of an infected PBMC. The inner membrane of the perinuclear cisterna (pnc) has invaginated in the nucleoplasm and the lumen of the invagination contains enveloped viral particles that have budded from the nucleus. The marker = 100 nm. b. A viral particle can be seen in the perinuclear cisterna (pnc) just at a point of continuity between the pnc and the endoplasmic reticulum. The marker = 100 nm. c. Multiple forms of productive viral maturation are visible in the field, including naked cytosolic nucleocapsids (*), and TGN-derived viral “wrapping structures” (arrows) in various stages of envelopment. The marker = 100 nm. d. The C-shaped configuration of an early viral “wrapping structure” is illustrated (arrow). Note the osmiophilic coating of dense material with the appearance of tegument on the concave face of the wrapping cisterna opposite a naked nucleocapsid (*) that appears to be entering the cavity define by the C. A nearby vacuole contains pleomorphic, evidently degraded virions (dv). The marker = 100 nm. e. Vacuoles within an infected PBMC are pleomorphic and appear to have been degraded (dv). f. Many pleomorphic degraded virions adhere to the external face of the plasma membrane of a small lymphocyte that itself adheres to a larger VZV-infected PBMC. The lower cell in the field is the cell that is shown at higher magnification in C. The marker = 500 nm.

Fig. 3.

The ability of WT VZV (strain ZW) to infect and proliferate in guinea pig PBMCs does not differ from that of vOka. Guinea pig PBMCs were infected by layering them over monolayers of HELF infected either with WT VZV or with vOka. The PBMCs were harvested after co-culture for 48 hrs and then cultured in suspension for a further 0, 12, 24, 48 or 72 hrs. One aliquot of PBMCs after each time of incubation was used to quantify infectious foci on a recipient HELF monolayer; the VZV genome copy number was quantified in a second aliquot. **a.** Infectious foci. Numbers of plaques increased as a function of incubation time after harvesting from the infected HELF, peaked at 48 hrs, and declined at 72 hrs. Numbers of plaques obtained with WT VZV and vOka did not differ significantly at any time period. **b.** VZV genome copy number. Genome copy numbers increased in PBMCs as a function of time of culture following their removal from an infected HELF monolayer; genome copy numbers reached a maximum at 48 hrs and then decreased at 72 hrs. Copy numbers of the WT VZV and vOka genomes did not differ significantly at any time period from one another.

Fig. 4.

VZV-infected PBMC do not release filterable cell-free VZV. Donor VZV-infected PBMCs were cultured over a filter with a pore diameter of 0.4 μm . The ORF68p immunoreactivity of a recipient layer of HELF cells was examined to quantify infectious foci of VZV. Infectious foci in the recipient HELF monolayer were considered to be due to the trans-filter passage of cell-free if Man 6-P (M6P; 40 mM) was able to block infection. In all figures, the top 2 bars show the effects of viral addition without filtration and the lower two bars depict the effects of filtration. a. Cell-free VZV placed above the filter. The donor preparation was assayed on a reporter layer of HELF cells without filtration. Infectious cell-free virus was present; Man 6-P strongly inhibited infectivity. After filtration, infectious foci were detected in recipient monolayer (M6P-) when Man 6-P was absent, but the recipient layer (M6P+) contained virtually no infectious foci when Man 6-P was present (One of 4 cultures contained a single plaque). b. When HELF cells were used as the donor layer the numbers of infectious foci appearing in the recipient layer did not differ significantly in the presence (M6P+) or absence of (M6P-). This observation suggests that HELF cells or membrane-enclosed cell fragments pass through the filters. c. When the donor layer contained PBMC, very few infectious foci traversed the filter. The few foci that were detected were only found in the absence of Man 6-P (M6P+).

Fig. 5.

VZV can transiently be recovered from the blood of recipient guinea pigs following the intravenous injection of VZV-infected human or guinea pig PBMC. Copy numbers of VZV were measured in PBMC isolated from recipient guinea pigs and plotted as a function of time after injection. Copy numbers increase significantly during the first 48 hours after injection but then decline. Greater copy numbers of VZV are found when guinea pig PBMC are used as donor cells than when the donor cells are human PBMC.

Fig. 6.

Location of VZV in recipient guinea pigs as a function of time after injection of VZV-infected PBMC. a–d. DNA and transcripts encoding VZV ORFs 29, 66, 40, and 67 were assessed in recipient animals 2 and 28 days after injection of VZV-infected PBMC. Lung, liver, ileum, and colon were studied. a. DNA extracted 2 days following injection. DNA encoding each of the ORFs examined is found in 4/4 guinea pigs in lung and liver but not in gut. b. RNA extracted 2 days following injection. Transcripts encoding each of the ORFs examined are found in 4 of 4 guinea pigs in lung and liver but not in gut. c. DNA encoding each of the ORFs examined is found both in ileum and colon of 4 of 4 guinea pigs but no VZV DNA can any longer be detected in lung and liver. d. RNA extracted 28 days following injection. Transcripts encoding ORFs 29 and 66 are found in 4 of 4 guinea pigs in ileum and colon; however, transcripts encoding ORFs 40 and 67 could only be detected in 1 of 4 animals. (*) ORFs 29 and 66 are expressed during latency while ORFs 40 and 67 are probably expressed only when VZV infection is lytic. No transcripts encoding any of the ORFs examined were found in lung or liver. e–h. In situ hybridization with an oligonucleotide probe designed to hybridize with VZV ORF54. The liver (e) and lung (f) were sampled 2 days after infection, while the ileum (g) and colon (h) was 28 days following intravenous injection of VZV-infected PBMC. e. Liver. The field contains a central vein

(cv) surrounded by plates of hepatic parenchymal cells. DNA encoding ORF54 is present in white blood cells within the central vein (arrow) and in scattered cells within sinusoids. No DNA encoding ORF 54 can be detected in hepatic parenchymal cells. f. Lung. The field encompasses a region of lung parenchyma containing an alveolar duct and alveolar sacs. DNA encoding ORF 54 is present in white blood cells within the perialveolar capillaries (arrow). g. Ileum. h. Colon. The inset in g shows a control myenteric ganglion from an uninfected guinea pig. Note that no neurons of the control are labeled. Each field includes longitudinal and circular muscle of the muscularis externa and a ganglion of the myenteric plexus between the muscle layers. DNA encoding ORF 54 is present in neurons (arrows) in each ganglion. The marker = 25 μ m.

Fig. 7.

Cellular location of the immunoreactivities of VZV at 2 and 28 days post-injection. a–d. Lung at 2 days post-injection. a. DNA fluorescence (blue) showing the location of the nuclei of all cells in the field. b. ORF29p immunoreactivity (red). A cluster of immunofluorescent cells can be seen in and/or near blood vessels. One immunofluorescent cell is shown at high magnification in the insert. c. ORF68p (gE) immunoreactivity (green). The same cluster of cells that displayed ORF29p immunoreactivity also contains that of ORF68p. d. Merged image. Note that all cells that contain the immunoreactivity of ORF29p also contain that of ORF68p (yellow). e–h. Gut at 28 days post-infection. e. ORF63p immunoreactivity (red). All of the neurons of a ganglion of the myenteric plexus contain ORF63p immunoreactivity. f. HuC/D immunoreactivity (green). HuC/D is a neuronal marker and immunostains neuronal nuclei and perinuclear cytoplasm. Axons of the ganglionic neuropil are not immunoreactive. g. DNA (blue). Note the presence of many non-neuronal cells, both within the ganglion and in the surrounding connective tissue and smooth muscle. h. Merged image. All of the neurons of the ganglion express ORF63p immunoreactivity (yellow). Note that the red immunoreactivity of ORF63p extends into the processes of the ganglionic neuropil, which lack HuC/D, suggesting that ORF63p is present in neurites as well as in the perikaryal

cytoplasm. No ORF63p immunostaining of non-neuronal cell bodies can be discerned. a–d. The markers = 15 μ m. e–h. The markers = 30 μ m.

Table 1

Sequences of VZV primers and probes

Primer or probe	Sequence (5'-3')	Position in sequence
ORF29 ext-F	ATGGAAAATACTCAGAAGACTGTGACA	50857-50883
ORF29 ext-R	TAAAACGTGTTTGCCTCCGTG	51159-51139
ORF29 int-F	AGAAGACTGTGACAGTGCC	50870-50888
ORF29 int-R	GCCTCCGTGAAAGACAAAG	51147-51129
ORF40 ext-F	GGTTTCATGTCCCGCTAACGT	71548-71568
ORF40 ext-R	ATCTCGCACGTAAGCCAGCTC	71851-71831
ORF40 int-F	GTTTCATGTCCCGCTAACG	71549-71567
ORF40 int-R	AAGCGACAGACAGTCCAAG	71810-71792
ORF66 ext-F	ATGAACGACGTTGATGCAACAG	113037-113058
ORF66 ext-R	TACACGCAAACGCAAACCTT	113367-113347
ORF66 int-F	CCATCTCAACATCACCGTC	113092-113110
ORF66 int-R	CGCAAACGCAAACCTTC	113363-113346
ORF67 ext-F	TTAATCCAATGTTTGATATCGGCC	114502-114525
ORF67 ext-R	AAAAGCGCTCGTTCTAATCCG	114804-114784
ORF67 int-F	ACA TAC AAG TGA CCA ACG C	114468-114486
ORF67 int-R	TTC TAA TCC GGG GAC ATC C	114793-114775
ORF29-F*	GGCGGAACCTTTCGTAACCAA	52952-52971
ORF29-probe*	TCCAACCTGTTTTCGCGCGGC	52973-52993
ORF29-R*	CCCCATTAAACAGGTCAACAAAA	53017-53039

For nested primers, int = internal primer, ext = external primer. F = forward primer. R = reverse primer