A Model of Lytic, Latent, and Reactivating Varicella-Zoster Virus Infections in Isolated Enteric Neurons

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Because human primary afferent neurons are not readily obtained, we sought to develop a model in which the lytic, latent, and reactivating phases of varicella-zoster virus (VZV) infection were recapitulated in neurons from an animal source. Enteric neurons were obtained from the small intestine of adult guinea pigs and from the bowel of fetal mice. Latency was established when these neurons were infected by cell-free VZV in the absence of fibroblasts or other cells of mesodermal origin. In contrast, lytic infection ensued when fibroblasts were present or when the enteric neurons were infected by cell-associated VZV. Latency was associated with the expression of a limited subset of viral genes, the products of which were restricted to the cytoplasm. Lysis was associated with the expression of viral glycoproteins, nuclear translocation of latency-associated gene products, and rapid cell death. Reactivation was accomplished by expressing VZV open reading frame (ORF) 61p or herpes simplex virus ICP0 in latently infected neurons. Isolated enteric neurons from guinea pigs and mice recapitulate latent gene expression in human cranial nerve and dorsal root ganglia. Expression of latency-associated VZV gene products was detected in 88% of samples of adult human intestine, suggesting that VZV not only infects enteric neurons but also is latent in the human enteric nervous system. This in vitro model should facilitate further understanding of latency and reactivation of VZV.

Varicella-zoster virus (VZV) is the etiologic agent of varicella and herpes zoster (HZ); in nature, it is entirely a human pathogen. Although VZV is extremely infectious and spreads readily from person to person [1], it is highly cell associated when propagated in vitro and is virtually unable to emerge from cells in an infectious form [2]. VZV establishes latent infection during its primary systemic infection, varicella, and it can subsequently reactivate to cause a secondary infection, HZ, which is often followed by the severe pathological pain of postherpetic neuralgia [2]. The preference of VZV for human hosts has greatly complicated the development of animal models and has made viral latency and reactivation difficult to study. Human neurons can be obtained for study, but not readily.

VZV establishes latency in the primary afferent neurons of dorsal root and cranial nerve ganglia [3]. Analogous primary afferent neurons are also intrinsic to the mammalian enteric nervous system (ENS), which, uniquely, can mediate reflex activity in the absence of input from the brain or spinal cord [4]. Because enteric ganglia, containing intrinsic primary afferent neurons (IPANs), can be isolated from guinea pig and mouse gut, we tested the hypothesis that VZV could be induced to abandon its preference for human cells if presented with the opportunity to infect purified, IPAN-containing populations of enteric neurons from guinea pigs and/or mice. Like the central nervous system, enteric ganglia contain glia, but, unlike dorsal root and cranial nerve ganglia, they lack fibroblasts or other cells of mesodermal origin [4]. Neurons isolated from guinea pig or mouse ganglia can be maintained in culture for many weeks and used for experimentation. We also obtained DNA and RNA from adult human gut

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removed during surgery for reasons unrelated to varicella or HZ.

METHODS

To isolate ganglia from the guinea pig intestine, the outer longitudinal muscle layer with adherent myenteric plexus was dissected from the bowel wall and dissociated with collagenase [4]. Individual ganglia were selected with a micropipette and plated in 2-well chamber slides that had previously been coated with poly-D-lysine (10 μg/mL) and mouse laminin (10 μg/mL). Ganglia were cultured in a medium consisting of DMEM-F12 (Gibco) supplemented with 2% fetal bovine serum (FBS; Gibco), penicillin-streptomycin (1%), gentamycin (100 μg/mL; Sigma), and fungizone (5.25 μg/mL; Gibco). Mitotic inhibitors (5-fluoro-2′-deoxyuridine [10 μmol/L], uridine [10 μmol/L], and cytosine β-D-arabinofuranoside [1 μmol/L]; Sigma) were added to prevent the growth of nonneuronal cells. Ganglia were maintained in vitro for ~5 days, after which mitotic inhibitors were removed and cell-free VZV (10–100 pfu/culture; Ellen, Jones, or Oka strain) was adsorbed for 4 h.

No differences were consistently observed between different strains of VZV. After inoculation with VZV, cultures were subsequently maintained in the absence of mitotic inhibitors. Enteric ganglia were also obtained from mice. In this case, the fetal (E14) mouse gut was removed and dissociated with collagenase. The neural crest–derived precursors of neurons and glia were then immunoselected by using antibodies to $p75^{NTR}$ [5, 6], the common neurotrophin receptor, which is a marker for crest-derived cells in the mammalian gut [7–9]. The immunoselected cells were then maintained in culture as described above for guinea pig enteric ganglia. Neurons and glia arise in the cultures within 2 days, but the cultures contain few fibroblasts or other cells of mesodermal origin. Portions of human colon and small intestine were taken from surgical specimens removed for the treatment of cancer. The tissue examined was distant from that involved with disease. RNA was extracted from the tissue, converted to cDNA, and analyzed by polymerase chain reaction. Specimens were also examined immunocytochemically with antibodies to detect VZV open reading frame (ORF) 29p, ORF62p, and gE. Antibodies to β3-tubulin were employed to identify neurons.

RESULTS

When neurons from isolated guinea pig or mouse ganglia were exposed to cell-free VZV, the neurons survived for up to 6 months without evident cytopathic effect; nevertheless, transcripts encoding ORFs 4, 21, 29, 40, 62, 63, and 66, but not 14 (gC), 67 (gI), or 68 (gE), were detected in the cultures (table 1). The immunoreactivities of ORFs 4p, 21p, 29p, 62p, and 63p, but not those of gB, gI, or gE, were also observed in the cytoplasm of neurons. These observations suggest that infection with cell-free VZV established latent infection in guinea pig and mouse enteric ganglia, because only immediate early and early proteins and their corresponding transcripts were found in the cultures, and these proteins were excluded from the nuclei of infected cells. Neurons survived, moreover, and the late glycoproteins, gB, gI, and gE, were not detected. These findings are typical of latent VZV infection. In contrast, when isolated guinea pig or mouse enteric ganglia were exposed to cell-associated VZV or when the dissociated whole wall of the guinea pig gut, which contains fibroblasts, was exposed to cell-free VZV, results were different. Neurons now expressed gB and gE, exhibited nuclear translocation of ORFs 29p and 62p, and died within 48–72 h. These findings are typical of lytic VZV infection. Thus, latent infection appeared to be induced when isolated ganglia were exposed to cell-free VZV, but lytic infection resulted when enteric neurons were exposed to VZV in the presence of fibroblasts and other nonneuronal cells [10–13].

Table 1. Varicella-zoster virus (VZV) gene expression and protein localization in guinea pig intrinsic enteric neurons exposed to cell-free VZV (latent infection).

ORF	DNA	mRNA	Protein	Protein location	Type of protein
4	+	+	+	Cytoplasm	IE
21	+	+	+	Cytoplasm	E
29	+	+	+	Cytoplasm	E
40	+	+	+	ND	E
62	+	+	+	Cytoplasm	IE
63	+	+	+	Cytoplasm	IE
66	+	+	+	ND	E
14	+	−	−	…	L
67	+	−	−	…	L
68	+	−	−	…	L

NOTE. +, present; −, not present; E, early (regulatory and structural); IE, immediate early (regulatory); L, late (structural); ND, not determined; ORF, open reading frame.

In an attempt to reactivate latent VZV in enteric neurons, latency was first established by infecting isolated guinea pig or mouse neurons with cell-free VZV. The latently infected neurons were then maintained for at least 5–7 days. After that period, the latently infected neurons were superinfected with a lentiviral or adenoviral vector expressing the nonstructural VZV protein ORF61 or its herpes simplex virus (HSV) orthologue ICP0. Superinfection with either an ORF61- or ICP0-expressing virus resulted in the apparent reactivation of VZV [13]. Reactivation was made evident by the sudden appearance of the manifestations of lytic infection. These included the neuronal expression of viral glycoproteins gB and gE; the nuclear translocation of ORFs 29p, 62p, and 63p; and the death of the neurons within 48–72 h. Even more strikingly, varicella virions could be visualized within infected cells by electron microscopy, and infection could be transferred to cocultured MeWo cells

[12]. VZV was also reactivated by exposing neurons to products secreted by activated human intestinal mucosal mast cells [10]. These data suggest that the ORF61 protein may act as a switch that activates the lytic cascade of VZV. Although the evidence thus implies that the ORF61 protein or its HSV orthologue are sufficient to unleash the lytic cascade in a neuron harboring latent VZV, further studies are needed to determine whether ORF61p is also necessary for VZV to manifest lytic infection.

The human gut was studied to establish the relevance of VZV infection of rodent enteric neurons to human disease. Human specimens were obtained from surgical specimens that would normally be discarded. RNA encoding at least 1 latency-associated VZV gene product was detected in 88% of 30 human specimens of large and small intestines. Not all of the proteins were found in each sample of bowel. The immunoreactivities of ORF29p and ORF62p were also detected in scattered enteric neurons in both myenteric and submucosal plexuses; however, no gE immunoreactivity was observed. In 2 of 2 patients studied, immunoreactive cells were present in 1 of 4 ganglia, on average, usually in 2 or 3 randomly distributed neurons. These observations are consistent with the idea that latent VZV is present in the ENS of the majority of human adults who presumably experienced varicella during childhood [12, 13].

DISCUSSION

Although the development of a varicella vaccine has ameliorated many of the problems caused by VZV infections, the persistence of latent wild-type virus and the failure to use vaccination in many countries outside of the United States ensure that VZV will continue to circulate for the foreseeable future [14]. Understanding the details of how VZV behaves in vitro has helped to explain its behavior in vivo. Although VZV is highly cell associated both in vitro and in vivo, there is one in vivo location in which enveloped, cell-free VZV can emerge. This is the upper layer of the epidermis, where vesicular lesions characteristic of varicella and HZ appear [15]. Enveloped virions free of cells are seen when fluid from vesicular lesions is examined electron microscopically [15]. The extracellular virions are obviously well formed and intact. These virions are also abundant in the intercellular space between keratinocytes, allowing virions to slough from skin when keratinocytes do. Because the human epidermis constitutively desquamates and disseminates as dust, one can envision that infectious virions similarly aerosolize from desquamating infected skin and transmit VZV to susceptible individuals through the air. Obviously, virions that remain cell associated cannot be aerosolized. Although isolation of VZV from the respiratory tract has, on occasion, been accomplished during early varicella, a recent study showed that extracellular VZV could not be demonstrated in the oral pharynx or in exhaled breath [16]; nevertheless, other herpesviruses, such as cytomegalovirus and herpes simplex virus, were readily demonstrated in the oral pharynx in this study. It is, thus, relatively much easier to isolate VZV from skin lesions early in varicella and HZ [14] than from the respiratory tract.

Although the superficial epidermis continually sloughs even without disease, the pruritic nature of the rash of chickenpox is likely to enhance the spread of VZV through the air and facilitate its transmission. Studies of the transmission of both wild-type and Oka strains of VZV have shown that the presence of skin lesions is directly related to the occurrence of transmission [17–19]. The number of skin lesions, moreover, is a good predictor of the chances of viral spread [18, 19]. A recent report of VZV transmission during autopsy indicates that respiratory transmission is not required to spread VZV from an infected host to a susceptible individual [20].

The presence of VZV in skin lesions not only is vital for transmission of infection but also appears to be involved in acquisition of latent infection in dorsal root and/or cranial nerve ganglia. The presence of epidermal vesicles has been shown in studies of vaccinees to be an excellent predictor of the later development of HZ [21–23]. Finally, it has long been recognized that the areas of the body where HZ most often occurs, the trunk and the face, are the very same areas where the rash is most highly concentrated during varicella [24]. Thus, available data suggest that, by minimizing infection of the skin during varicella and by reducing vaccine-associated rashes, transmission of VZV to susceptible hosts will be reduced, and latent infection will be mitigated.

We developed an enteric ganglionic model of VZV infection to identify parameters that contribute to the establishment of latency and reactivation. This is, to our knowledge, the first in vitro model that enables VZV latency and reactivation to be studied in neurons. Although a model must be understood to be artificial, in that VZV does not normally infect rodents, it is striking that VZV gene expression in rodent enteric neurons recapitulates that demonstrated in neurons from human cranial nerve and dorsal root ganglia obtained from autopsy specimens [25–36]; thus, it appears that guinea pig and mouse enteric neurons provide a faithful model of VZV latency. It is also of interest that the Oka strain of VZV is capable of establishing latent infection in rodent enteric neurons in vitro, just as it is capable of establishing latency in human neurons in vivo.

Expression of as many as 8 genes of the 71 of VZV during *latent* infection have been identified by a number of investigators studying human ganglia, although transcripts have been detected with far greater frequency than the proteins themselves [25–36]. The VZV genes most often expressed during latency as transcripts and/or proteins are ORFs 4, 21, 29, 62, 63, and 66. Expression of ORFs 18 and 40 has been described less frequently. The first VZV protein to be identified in latently infected neurons was ORF63p, which evidently is the major

VZV protein expressed during latent infection [36, 37]. Thus far, each of the VZV proteins that have been identified by immunocytochemistry in latently infected neurons, including ORFs 4p, 21p, 29p, 62p, and 63p, have been observed to be restricted the cytoplasm [30, 35–37]. During reactivation of VZV, these VZV proteins are translocated to the nucleus and can be located both in the cytoplasm and the nucleus, or they can be entirely intranuclear [35]. By reason of the fact that ORF61p is not a structural protein, we and others have proposed that expression of ORF61p may act a switch that converts a latent to a lytic infection [38]. This hypothesis is consistent with our observations of latent and lytic VZV infection in enteric neurons. Cell-free virions lack ORF61p because it is nonstructural. Virions, thus, cause latent VZV infection. In contrast, cells that are infected with VZV contain ORF61p, which they acquire from the VZV-infected cells with which they fuse. Of course, they pass ORF61p, as well as viral DNA, to the target cells with which they, in turn, fuse when they transmit infection by cell-to-cell contact. Therefore, it appears that, when ORF61p is present at the time of infection, the infection is lytic. ORF61p can also reactivate VZV and initiate the lytic cascade if, as a result of superinfection with an appropriate vector, it is expressed in latently infected neurons. Because latently infected neurons all contain DNA encoding ORF61p, an environmental or stochastic event could intervene to initiate ORF61 expression and reactivate VZV infection in situ.

As noted, latent infection of isolated enteric neurons could be established only by using cell-free VZV. If cell-associated VZV was used for infection, lytic infection developed, and neurons did not survive for >2–3 days. Latency-associated proteins were not confined to the cytoplasm, and glycoproteins were demonstrated. These observations suggest a mechanism for the establishment of VZV latency in vivo. The only location where cell-free VZV is produced in the body in abundance is in the superficial epidermis, where natural down-regulation as a function of squamous cell differentiation causes the mannose-6-phosphate receptor not to be expressed [15]. As a result, VZV is not diverted in superficial epidermal cells to late endosomes and thus is not inactivated before it is released from cells. Sensory nerves from dorsal root and cranial nerve ganglia innervate the epidermis. They lose their sheaths at the dermal-epidermal junction and wander between keratinocytes as naked nerve fibers. In this position, nerve fibers in VZV-infected regions of skin are bathed in the infectious virions that are released within the epidermis. The primary afferent neurons of the dorsal root and cranial nerve ganglia, thus, can acquire latent infection because they become infected by cell-free VZV within the epidermis. In contrast, if they were to be infected during viremia by cell-associated VZV, lytic infection would be anticipated. We suggest, therefore, that retrograde spread from the epidermis and infection by cell-free VZV is how latency is acquired. We also propose that HZ is a reversal of this process, in which the now-reactivated lytic VZV kills the neuron in which it proliferates and also spreads in the anterograde direction to invade the epidermis in a dermatomal distribution. These hypotheses account for the segmental distribution of HZ, which is only rarely disseminated and, when it is so, it usually occurs in an immunocompromised host. Production of enveloped virions in the epidermis, thus, is probably critical both for the spread of VZV to additional hosts and for the establishment of latent VZV infection in the single host [15]. Although the Oka strain of VZV can cause latent infection, there may be a lower load of latent virus in vaccinees than in patients who experience varicella, because there is much less proliferation of VZV in the epidermis after vaccination [12, 22, 39].

In the future, isolated enteric neurons should be a useful model to use to identify the VZV genes that are required for establishment and maintenance of latent infection. It should also be possible to test the efficacy of the immune response in preventing reactivation or recognizing it when it occurs. Enteric neurons should also prove useful in evaluating antiviral drugs as agents for the control of reactivation. Although various strains of VZV can also be compared, it will be necessary to validate animal enteric ganglia as a faithful mimic of their human counterparts; nevertheless, that said, primary afferent neurons from guinea pigs and mice are far easier to obtain and infect with VZV under controlled experimental conditions than are those of humans.

The demonstration of latent VZV in human intestinal specimens is of potential biological importance and warrants further investigation. Intestinal HZ has been linked to pseudo-obstruction of the intestine in several case reports [40–43]. Studies to determine whether the reactivation of VZV occurs in the bowel and gives rise to other gastrointestinal diseases, such as appendicitis, inflammatory bowel disease, and chronic constipation are needed. It is also possible that periodic reactivation in the immunologically rich environment of the gut contributes to the long-term persistence of immunity to VZV. Again, this possibility requires further investigation.

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