Varicella-Zoster Virus Gene Expression in Latently Infected Rat Dorsal Root Ganglia

Peter G. E. Kennedy,*,1 Esther Grinfeld,* Sebastien Bontems,† and Catherine Sadzot-Delvaux†

*Glasgow University Department of Neurology, Institute of Neurological Sciences, Southern General Hospital, South Glasgow University Hospitals NHS Trust, Glasgow G51 4TF, Scotland, United Kingdom; and †Laboratory of Fundamental Virology, Institute of Pathology, University of Liege, B-4000 Liege, Belgium

Latent infection of human ganglia with Varicella-Zoster virus (VZV) is characterized by a highly restricted pattern of viral gene expression. To enhance understanding of this process we used *in situ* hybridization (ISH) in a rat model of VZV latency to examine expression of RNA corresponding to eight different VZV genes in rat dorsal root ganglia (DRG) at various times after footpad inoculation with wild-type VZV. PCR *in situ* amplification was also used to determine the cell specificity of latent VZV DNA. It was found that the pattern of viral gene expression at 1 week after infection was different from that observed at the later times of 1 and 18 months after infection. Whereas multiple genes were expressed at 1 week after infection, gene expression was restricted at the later time points when latency had been established. At the later time points after infection the RNA transcripts expressed most frequently were those for VZV genes 21, 62, and 63. Gene 63 was expressed more than any other gene studied. While VZV DNA was detected almost exclusively in 5–10% of neurons, VZV RNA was detected in both neurons and nonneuronal cells at an approximate ratio of 3:1. A newly described monoclonal antibody to VZV gene 63-encoded protein was used to detect this protein in neuronal nuclei and cytoplasm in almost half of the DRG studied. These results demonstrate that (1) this rat model of latency has close similarities in terms of viral gene expression to human VZV latency which makes it a useful tool for studying this process and its experimental modulation and (2) expression of VZV gene 63 appears to be the single most consistent feature of VZV latency. © 2001 Academic Press

Key Words: Varicella-Zoster Virus (VZV); latency; dorsal root ganglion.

INTRODUCTION

Varicella-Zoster virus (VZV) is a human herpesvirus which causes varicella (chickenpox) as a primary infection in susceptible individuals, who are usually children. After this acute infection, the virus establishes a latent infection in cells of the trigeminal ganglia and dorsal root ganglion (DRG), following which VZV may reactivate in later life, either spontaneously or following various triggers, to cause herpes zoster (shingles) (Kennedy, 1987; Gilden et al., 2000). The latter may be followed by postherpetic neuralgia and several other neurological complications all of which may be associated with significant morbidity (Johnson, 1998; Gilden et al., 2000). The cellular site of latent VZV in human ganglia is predominantly neuronal with a minority of nonneuronal cells infected (Kennedy et al., 1998; Mahalingam et al., 1999; LaGuardia et al., 1999). Knowledge of viral gene expression during VZV latency is important both to understand the underlying mechanism of latency and to identify viral genes which could be the targets of antiviral therapy. Studies in human ganglia have reported consistent expression of RNA corresponding to VZV genes 21, 29, 62, and 63 (Cohrs et al., 1992, 1994, 1995, 1996; Meier et al., 1993;

Kennedy et al., 1999) and probably gene 4 (Croen et al., 1988; Kennedy et al., 2000). VZV protein expression during latency is also very restricted, with strong evidence for the neuronal expression of VZV gene 63-encoded protein in latently infected human ganglia (Mahalingam et al., 1996; Kennedy et al., 2000), and one report of expression of proteins encoded by several VZV genes (Lungu et al., 1998). The extent to which other viral genes are expressed is unclear at present, and such studies in humans are limited to autopsy tissues.

An additional way of studying VZV latency is to use animal models such as neonatal (Brunnell et al., 1999) and adult (Sadzot-Delvaux et al., 1990; Annunziato et al., 1998) rats as well as guinea pigs (Lowry et al., 1993). Although all of the current animal models have limitations, they serve as useful tools to study VZV latency in a mechanistic way which is clearly not possible in humans. The adult rat VZV model used in this study (Sadzot-Delvaux et al., 1990) has proven useful in defining the expression of VZV gene 63 during latency (Debrus et al., 1995). Here we determined the pattern of viral gene expression during latency using *in situ* hybridization (ISH) for a range of VZV genes on virally infected rat DRG at varying times after footpad inoculation. We hypothesized that viral gene expression in ganglia studied prior to establishment of latency would be different to that observed in ganglia in which a latent infection had

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[1] To whom correspondence and reprint requests should be addressed. Fax: 44-141-201-2993. E-mail: P.G.Kennedy@clinmed.gla.ac.uk.

TABLE 1

Detection by ISH of VZV RNA in Infected Rat DRG[a]

Sample	Region	Gene 4	Gene 18	Gene 21	Gene 28	Gene 29	Gene 40	Gene 62	Gene 63
1) DRG 1wpi	Lumbar	+	+	+	+	+	ND	ND	+
2) DRG 1wpi	Lumbar	−	ND	+	ND	ND	+	ND	+
3) DRG 1wpi	Lumbar	+	ND	−	ND	+	+	ND	+
4) DRG 1wpi	Lumbar	−	ND	ND	ND	−	ND	ND	−
5) DRG 1wpi	Lumbar	−	−	−	−	−	−	−	+
6) DRG 1wpi	Thoracic	−	−	ND	−	−	ND	−	−
7) DRG 1wpi	Lumbar	−	−	−	−	−	ND	−	−
8) DRG 1wpi	Lumbar	−	−	−	ND	−	−	−	−
9) DRG 1wpi	Thoracic	−	−	−	−	ND	ND	ND	−
10) DRG 1wpi	Thoracic	ND	−	ND	−	ND	ND	−	+
11) DRG 1wpi	Lumbar	ND	−	ND	−	ND	−	−	+
12) DRG 1wpi	Thoracic	ND	ND	ND	−	−	−	+	+
13) DRG 1mpi	Lumbar	ND	−	−	ND	−	−	ND	+
14) DRG 1mpi	Lumbar	ND	−	+	−	−	−	ND	−
15) DRG 1mpi	Lumbar	ND	ND	ND	ND	−	+	ND	+
16) DRG 1mpi	Lumbar	ND	ND	+	ND	ND	−	ND	+
17) DRG 1mpi	Lumbar	−	ND	ND	ND	ND	ND	ND	+
18) DRG 18mpi	Lumbar	−	−	ND	−	−	−	+	+
19) DRG 18mpi	Thoracic	ND	−	ND	ND	ND	−	+	−
20) DRG 18mpi	Thoracic	ND	+	−	−	ND	ND	+	+
21) DRG 18mpi	Thoracic	−	−	−	−	−	ND	+	+
22) DRG 18mpi	Lumbar	−	−	ND	−	+	−	+	+
23) DRG 18mpi	Lumbar	ND	−	ND	−	ND	ND	+	−

[a] DRG, dorsal root ganglion; 1wpi, 1 week post inoculation; 1mpi, 1 month post inoculation; 18mpi, 18 months post inoculation; +, positive; −, negative; ND, not determined.

been established. Our findings provide evidence to confirm this postulate and extend our knowledge of gene expression in VZV latency.

RESULTS AND DISCUSSION

We used *in situ* hybridization to detect RNA corresponding to eight viral genes in a total of 23 rat DRG samples obtained from 16 different rats that had been inoculated with wild-type VZV in the footpad. The VZV genes chosen for study spanned representative regions of the viral genome. The DRG were studied at three time points, namely, 1 week postinoculation, 1 month postinoculation, and 18 months postinoculation. The results are shown in detail in Table 1, in which a total of eight rats were studied at the 1-week time point, five rats were studied at the 1-month time point, and three rats at the 18-month time point.

At the1 week postinoculation time point, RNA for VZV genes was detected in 7 of 12 DRG. In three samples RNA corresponding to three to six different viral genes was detected, most frequently gene 63. In the four remaining DRG one to two genes were detected, again gene 63 being the most frequent. No VZV genes were ever detected in uninfected ganglia from four rats or control uninfected rat liver tissue. VZV RNA was located in the neuronal nuclei in most regions of tissue, but in some regions positive signals were also detected in nonneuronal cells with an approximate ratio of neuronal: nonneuronal cells of 3:1. There were also a few regions where only positive nonneuronal cells were seen. When positive, the ISH signal was focal in distribution but very approximately 5–10% of neuronal cells in the DRG sections were positive in most cases.

At the 1-month postinoculation time point fewer tissue samples were available for analysis than at other time points. However, most of the tissues were negative for viral RNA expression with the exception of RNA corresponding to VZV genes 63 and 21. In one sample (sample 15) gene 40 was detected. Where positive, the percentage of cells infected and ratio of neuronal:nonneuronal cells positive were similar to that above and all uninfected control tissues were again consistently negative.

At the 18-month postinoculation time point, which was of particular interest since viral latency would be expected to have been well established by this time, a total of six DRG were studied in detail. It can be seen from Table 1 that RNA corresponding to VZV genes 62 and 63 were detected in the majority of DRG. In addition, gene 18 was detected in one sample and gene 29 in another. The percentage of infected neuronal cells (approximately 5–10%) and their predominant neuronal location (Fig. 1) together with a smaller percentage of nonneuronal cells (ratio of approximately 3:1) were again similar to that described as above.
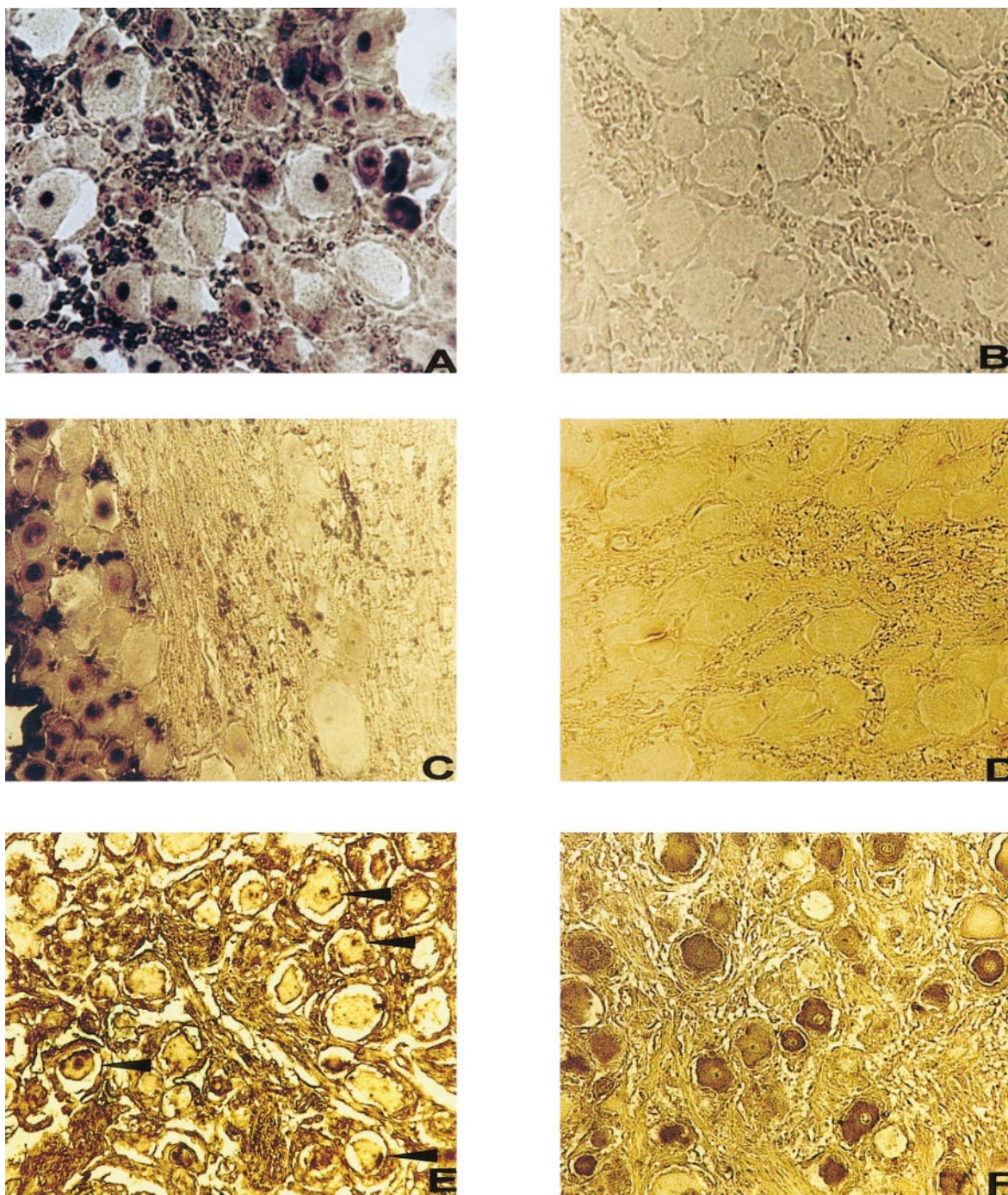
FIG. 1. (A) PCR *in situ* amplification of DNA for VZV gene 62 on rat DRG 1 week after footpad inoculation. Several neuronal cells and nonneuronal cells are labeled. (B) An uninfected control DRG labeled as in (A). No cells are labeled. (C) *In situ* hybridization using a probe to VZV gene 63 RNA on a rat DRG 18 months after footpad inoculation. Several neuronal cells and a few nonneuronal cells contained VZV RNA. (D) ISH on a DRG 18 months after footpad inoculation labeled with a probe to VZV gene 40. No cells are labeled. (E) A DRG 1 week after footpad inoculation labeled with monoclonal antibody to VZV gene 63-encoded protein. Some strongly labeled neuronal cells are shown with arrows. (F) A normal adult human DRG labeled as in (E). Several neuronal cells are labeled. (Magnification, ×450 in all cases.).

Although the main purpose of this study was an analysis of viral gene expression, we also used DNA PCR *in situ* amplification to detect VZV DNA for genes 29 and 62 in nine DRG samples (samples 5, 7, 8, 9, 11, 18, 21, 22, and an uninfected control). VZV DNA was detected in six of eight samples, with samples 7 and 18 being negative.

The localization of VZV DNA was predominantly neuronal with only occasional nonneuronal cells being positive in the latently infected ganglia.

Since VZV gene 63-encoded protein has been previously reported to be present in latently infected neurons in this rat model (Debrus *et al.,* 1995) and human ganglia

(Mahalingam *et al.,* 1996; Kennedy *et al.,* 2000; Cohrs *et al.,* 2000), we also used a newly constructed monoclonal antibody to this protein to detect its presence in the ganglia. Positive staining in neuronal nuclei and cytoplasm was detected with this antibody in 8 of 17 ganglia examined (Fig. 1). The positive DRG were numbers 1, 3, 10, 11, 14, 15, 17, and 21, which represented samples at all three time points. In only one sample (sample 14) was 63 protein staining not associated with expression of RNA for gene 63. Positive staining with this antibody was not detected in any uninfected ganglion specimens.

These results, in a putative animal model of VZV latency, enhance our knowledge of this process in three ways. First, the predominantly neuronal localization of latent VZV DNA, as determined by the PCR *in situ* amplification technique, observed in DRG from these infected rats, is in close accord with the consensus view that the neuron is the predominant site of latent VZV in human DRG and trigeminal ganglia (Mahalingam *et al.,* 1999). The positive ganglia included three from rats which were studied 1 month after footpad inoculation, which is when latency may just have been established, and from one rat 18 months after inoculation, where the presence of VZV very likely represents the establishment of a latent infection.

Second, the pattern of VZV gene expression in the rat DRG, as determined by ISH for RNA corresponding to eight different representative genes, bears close similarities to the restricted nature of latent VZV expression in human ganglia. The genes known to be expressed in the latter include genes 4, 21, 29, 62, and 63 (Cohrs *et al.,* 1996, 2000; Kennedy *et al.,* 2000), which is why they were chosen for study here, and possibly, in some cases, gene 18 (Kennedy *et al.,* 2000). In this study in rat tissues, we observed expression of genes 62 and 63 predominantly as well as some occasional expression of genes 21 and 29 in ganglia which were likely to have been latently infected. The detection of gene 18 in sample 20 at 18-months postinoculation may be significant in view of the infrequent detection of this gene, which codes for the small-unit ribonucleotide reductase (Ostrove, 1990) in a minority of human trigeminal ganglia (Kennedy *et al.,* 2000). While this study was limited by not studying every selected gene in all samples, due to lack of availability of some samples, nevertheless it was clear that RNA for VZV gene 63, which has been reported to function as a transactivator and transrepressor during gene regulation (Jackers *et al.,* 1992), was the most frequently detected in this study, providing further evidence for the notion that expression of gene 63 is the hallmark of VZV latency (Cohrs *et al.,* 2000). It should be noted, however, that a more recent study (Kost *et al.,* 1995) suggested that VZV gene 63 plays only a minor regulatory role in modulating VZV gene expression. At the earlier 1-week-postinoculation time points there was no particular pattern to the genes expressed in the positive samples, which was not

unexpected as latency could not have been established at this time, and the pattern of multiple gene expression in sample 1 is very suggestive of a lytic viral infection. The presence of VZV DNA in sample 7 in the absence of any RNA detection may reflect an early stage of viral infection, differing sensitivities of the DNA and RNA detection assays, or, most likely, the very focal pattern of viral infection in these tissues. While VZV RNA was detected mainly in neurons, we observed more gene expression in nonneuronal cells as well in these rat tissues compared with human ganglia, but the reason(s) for this slight but clear difference is unclear at present. Latent VZV DNA, however, was located predominantly in neurons in these rat ganglia, as had been found previously in human ganglia (Kennedy *et al.,* 1998; LaGuardia *et al.,* 1999).

Third, the detection of the VZV gene 63-encoded protein, with a newly described monoclonal antibody, in neurons in almost half of the DRG studied provides further evidence for the importance of expression of this protein in VZV latency. This is a higher figure than the one of 25% positive which we had reported for this protein in human ganglia (Kennedy *et al.,* 2000), and this may partially reflect the use of the polyvalent antibody used in that study. However, we recently tested this monoclonal antibody on tissue sections of 21 normal human ganglia and found that eight of these, i.e., 38%, were positively stained (Fig. 1 and unpublished observations). The detection of 63 protein in the absence of RNA for gene 63 in sample 14 may reflect a greater abundance of protein than RNA, the focal nature of viral infection, or differences in the assay sensitivities.

The findings reported here indicate that further detailed studies are warranted in this rat model of VZV latency, both to enhance our understanding of the VZV latency process and to assess the modulation of viral latency by various methods such as mutant VZV, drugs, or synthetic peptides.

MATERIALS AND METHODS

Animal inoculations

Animal inoculations were carried out as previously described (Debrus *et al.,* 1995). In brief, 16 six-week-old healthy rats were inoculated in the footpads (both sides) with a suspension of VZV (from a clinical isolate) -infected Vero cells. Each animal received cells corresponding to 25 cm^2 of cells showing approximately an 80% cytopathic effect (CPE) on Vero cells. Virus titrations carried out on Vero cells 8 days after infection for animal inoculations showed that each animal had received about 5×10^5 infected cells. Four uninfected rats acted as controls. Infected rats were killed at 1 week, 1 month, and 18 months after the footpad inoculation, at which points the DRG tissues were removed and fixed in Formalin and wax-embedded for ISH studies and immuno-

cytochemistry. Varying amounts of DRG tissues were available for analysis at each time point.

Oligonucleotide probes

The probes used in this study were the same as previously described (Kennedy *et al.,* 2000), including probes for VZV genes 4, 18, 21, 28, 29, 40, 62, and 63, and were chosen as representing both genes which have previously been reported as being expressed during latency (genes 4, 21, 29, 62, and 63), and those which are not known to be frequently expressed during latency (genes 18, 28, and 40). All oligonucleotides were synthesized by Genosys, UK.

ISH and PCR *in situ* amplification

ISH was used to detect viral RNA, and PCR *in situ* amplification was used to detect viral DNA. These assays were performed as previously described in detail (Kennedy *et al.,* 1998, 1999). In brief, for ISH assays, DRG tissue sections on glass slides were dewaxed, rehydrated, permeabilized with HCl and proteinase K, and acetylated. After treatment with prehybridization buffer, the sections were incubated overnight with the appropriate digoxigenin (DIG)-labeled probe and the resulting hybrids detected using standard DIG agents. After the slides had been dipped in the appropriate buffer, they were exposed to antibody conjugated to alkaline phosphatase, equilibrated in buffer, and treated with the detection agent until the development of the purple color. All sides in both types of assay were read blind. The previously used standard controls were used, e.g., the use of uninfected rat DRG and liver tissues, inappropriate or absent primer sequences, and infected CV-1 cells (Kennedy *et al.,* 1998, 1999).

For PCR *in situ* amplification assays, in brief, the DRG tissue sections were dewaxed, rehydrated, permeabilized with Triton-X and proteinase K, boiled in citric acid, fixed in acetic acid, washed, and dehydrated. Assays were then carried out using the Perkin–Elmer Gene Amp System 1000, with cycling parameters for VZV amplifications as previously detailed (Kennedy *et al.,* 1998), following which the slides were cooled, washed, and processed for color development as above.

Immunocytochemistry with monoclonal antibody to VZV gene 63-encoded protein

The monoclonal antibody to VZV gene 63-encoded protein has not been used previously, as this is the first report using this reagent. The antibody was constructed as follows. BALB/c mice were injected intraperitoneally with 100 μg purified GST-IE63 (Debrus *et al.,* 1995) emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). One month later mice were boosted with the same amount of fusion protein and were then killed, and splenocytes obtained by mechanical dissociation were
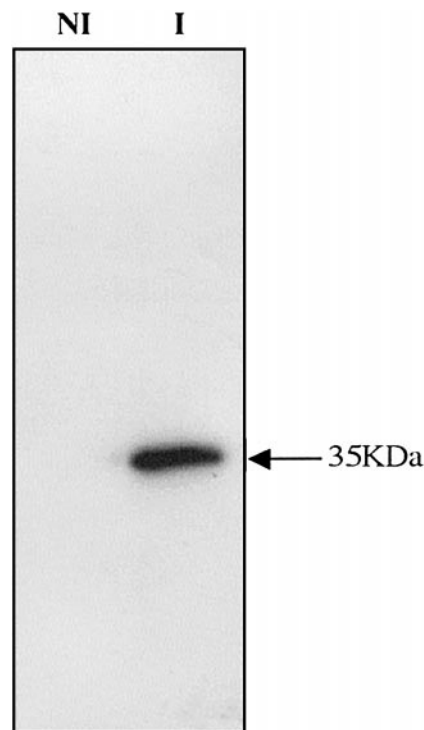

FIG. 2. Western blot analysis of noninfected (NI) or VZV-infected (I) cells, using the anti-IE63 monoclonal antibodies (9A12 at 1/1000) and visualized with peroxidase-conjugated anti-mouse Ig (DAKO at 1/2000).

fused in the presence of 50% polyethylene glycol with Sp-210-Ag14 myeloma cells with a ratio of myeloma cells: spleen cells of 1:2. After a 5-min centrifugation at 200 *g*, cells were resuspended in a selection medium (Iscove's DMEM supplemented with 10% fetal calf serum and hypoxanthine-aminopterin-thymidine). After 1 week cells were plated in a 96-well plate, and supernatants were then screened by EIA for Ig secretion. Screening was performed on 96-well plates coated either with GST alone as a control or with GST-IE63. Cells expressing Ig reacting with the fusion protein but not the GST control were selected and cloned using the limiting dilution procedure. This procedure was repeated three times. Two clones were selected (9A12 and 9D12) for their ability to produce high levels of antibodies and then produced *in vivo* as ascitic fluid. These antibodies can be used for EIA, immunohistochemistry, or Western blotting analysis. In immunohistochemistry this monoclonal antibody recognizes the protein in cell cultures, frozen sections fixed with acetone:ethanol at a ratio of 1:1, or paraffin-embedded tissues. In Western blotting it hybridizes to a band of 35 kDa (Fig. 2).

Immunocytochemistry was carried out as previously described in detail (Kennedy *et al.,* 2000). In brief, the tissue sections on glass slides were dewaxed, rehydrated, and then incubated for approximately 1 h in PBS containing 1% bovine serum albumin and 1% normal sheep serum. Sections were then incubated at 4°C over-

night with mouse monoclonal antibody to VZV gene 63-encoded protein (diluted 1:200). Slides were then washed in PBS and incubated for 1 h with biotinylated anti-mouse antibody in blocking buffer. After washing, the sections were incubated with streptavidin-alkaline phosphatase conjugate (DAKO) in blocking buffer, washed in PBS, and then developed using NBT-BCIP or a Fuchsin kit (DAKO) containing levamisole (0.24 mg/ml) for 10 min. Slides were then washed, mounted in an aqueous mountant, and read blind.

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