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Herpes simplex virus type 1 ICP4 deletion mutant virus d120 infection failed to induce apoptosis in nerve growth factor–differentiated PC12 cells

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It has been suggested that terminally differentiated neuronal cells and mitotic cells respond differently in many aspects to herpes simplex virus type 1 (HSV-1) infection. The ICP4-deleted, Us3-defective, HSV-1 mutant strain d120 induces classical apoptosis in a variety of mitotic cell lines. Its behavior in postmitotic cells is not known. Here the authors report that mutant d120 virus failed to induce apoptosis in neuronal-like, nerve growth factor (NGF)-differentiated PC12 cells. More strikingly, rather than inducing apoptosis, d120 infection prolonged the life of nondividing NGF-differentiated PC12 cells in the culture flask. The virus genome had a half-life of 30 days. Unlike in other cells, such as Vero, neither wild-type nor d120 infection of NGF-differentiated PC12 cells induced the nuclear factor (NF)-κB p65 pathway, which has been associated with virus-induced apoptosis. Thus, the authors demonstrate, for the first time, that a potent apoptosis inducer mutant d120 failed to induce apoptosis in neuronal-like NGF-differentiated PC12 cells, unlike a number of other cell lines studied. The possible mechanisms involved in the failure of d120 to induce apoptosis in neuronal-like NGF-differentiated PC12 cells are discussed. *Journal of NeuroVirology* (2007) **13,** 305–314.

Keywords: HSV-1 infection in neuron; ICP4 deletion mutant; NF-kappa; viral induced apoptosis

Introduction

Cellular apoptosis has been known to play a crucial role during neuronal development. For example, it was estimated that approximately 50% of total neurons are eliminated by apoptosis during development (Ono *et al*, 2003). After development, neurons are terminally differentiated and any injury causing the death of neurons might result in neuronal degenerative diseases or neuronal disorders. It is reasoned that to protect against neuronal death, mechanisms have evolved to inhibit apoptosis and promote neuronal survival (Ono *et al*, 2003).

Apoptosis is believed to be a mechanism to dispose of cells whose cascades of sequentially ordered events are irreversibly damaged by factors internal or external to the cell, such as infections (Koyama *et al*, 2000). Once apoptosis is triggered, a series of programmed events leads to the death of the cell, manifested by morphological changes, and especially by the degradation of cellular DNA.

PC12 cells are derived from a rat pheochromocytoma. In response to nerve growth factor (NGF), PC12 cells cease division and acquire many biochemical properties of neuronal cells (Greene and Tischler,

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1976). Thus, NGF-differentiated PC12 (NGF-PC12) cells have been used as a model of neuronal cells maintained in tissue culture. Herpes simplex virus type 1 (HSV-1) is a neurotropic virus, with the ability to infect nondividing cells and establish a latent infection in sensory neurons after a primary infection in humans. We have established NGF- PC12 cells as a tissue culture model to understand herpes and neuronal cell interaction (Su *et al*, 1999).

Activation of nuclear factor (NF)-κB is crucial for neuronal survival. NF-κB was activated in PC12 cells in response to NGF treatment, although the activation was transient (Azoitei *et al*, 2005). The activation of NF-κB peaked at day 5 post NGF treatment, but was back to the basal level by day 7. It was also shown that HSV-1 infection can cause activation of the NF-κB pathway in a variety of other cell types, including primary human foreskin fibroblasts (Taddeo *et al*, 2002), murine fibroblast (Taddeo *et al*, 2004), mouse embryo fibroblast (Gregory *et al*, 2004), HEp-2 (Amici *et al*, 2001), C33-A cells (ATCC HTB31) (Patel *et al*, 1998), human epithelial cells (Amici *et al*, 2004), SK-N-SH cells (Taddeo *et al*, 2003), and U937 monocytoid cells (Medici *et al*, 2003). It was therefore suggested that persistent activation (24 h post infection [PI]) of NF-κB might be a general response of host cells to HSV-1 infection (Santoro *et al*, 2003).

Activation of NF-κB was suggested to facilitate viral DNA replication (Amici *et al*, 2001; Gregory *et al*, 2004; Patel *et al*, 1998) and mediate either apoptosis induction or apoptosis inhibition of host cells (Taddeo *et al*, 2003, 2004; Goodkin *et al*, 2003; Yedowitz and Blaho, 2005), depending on the cell types studied and the species of NF-κB dimers studied (Ishige *et al*, 2005; Azoitei *et al*, 2005). Although HSV-1 is a neurotropic virus, the studies of the impact of HSV-1 infection on the regulation of NF-κB and the role of NF-κB involved in HSV-1-host cell interaction were done mostly in mitotic cells, and little is known about neuronal cells.

The interaction of HSV-1 with cells often results in induction of apoptosis pathways. Herpes virus has evolved mechanisms to block or delay the onset of apoptosis to ensure the completion of its life cycle. HSV-1 encodes several antiapoptotic genes such as Us3, Us5, ICP27, ICP4, and gamma 34.5, as well as a family of microRNAs encoded in the region of latency-associated transcript (LAT) that downregulates proapoptotic pathways (Gupta *et al*, 2006; Cui *et al*, 2006). Infection of cells with HSV-1 mutants defective in some of these antiapoptotic genes has been shown to cause apoptosis (Aubert and Blaho, 1999; Leopardi *et al*, 1997; Leopardi and Roizman, 1996; Munger *et al*, 2001; Zhou *et al*, 2000; Zhou and Roizman, 2001; Jerome *et al*, 1999; Galvan *et al*, 1999; Chou and Roizman, 1992).

Among these mutants, HSV-1 mutant strain d120, which lacks both copies of the ICP4 gene and contains a defective Us3 gene, is known to be highly cytotoxic to the mitotic cells it infects. One of the factors responsible for its characteristic cytotoxicity is its potency in inducing apoptosis in infected cells (Leopardi *et al*, 1997; Munger *et al*, 2001). The mutant d120 virus induced apoptosis in all cell lines (Vero, SK-N-SH cells, Hep-2, and HEL cells) tested (Galvan and Roizman, 1998; Galvan *et al*, 1999; Leopardi and Roizman, 1996; Zhou and Roizman, 2000).

The outcome of HSV-1 infection in neuronal cells can be very different from that in mitotic cells. For instance, we have shown that, surprisingly, HSV-1 infection can prevent detachment of the aged NGF-differentiated PC12 cells from the culture flask. The half-life of linear HSV-1 DNA in differentiated PC12 cells was approximately 1 month instead of 2 to 3 days in mitotic cells (Moxley *et al*, 2002). Thus it was of interest to investigate whether the highly cytotoxic mutant d120 virus exerts a different infection pattern in neuronal-like cells as compared to mitotic cells.

In this article we show that mutant d120 not only failed to induce apoptosis in NGF-PC12 cells, but also significantly prevented cell detachment from the culture flasks, which was characteristic of uninfected cultures. Furthermore, HSV-1 infection (either wild-type or mutant d120) of neuronal-like NGF-PC12 cells was not able to activate the NF-κB p65-mediated pathway. The mechanism involved in prevention of apoptosis induced by mutant d120 in NGF-PC12 cells is discussed.

Results

HSV-1 ICP4-deleted, Us3-defective mutant strain d120 infection prevents detachment of NGF-PC12 cells in culture

We have previously reported that HSV-1 infection prevented the detachment of neuronal-like NGF-differentiated PC12 (NGF-PC12) cells maintained for more than 4 weeks in culture (Moxley *et al*, 2002). To study the behavior of a potent apoptotic inducer mutant, HSV-1 KOS-derived strain d120 was used. NGF-PC12 cells were differentiated with 100 ng/ml NGF, as previously described (Su *et al*, 1999), and infected with HSV-1 wild-type parental strain KOS or mutant strain d120 at multiplicity of infection (moi) of 20. d120 is a potent inducer of apoptosis in variety of mitotic cells, and is derived from strain KOS (DeLuca *et al*, 1985). Morphology and cell numbers were followed by serial photomicroscopy, as a function of time after infection. Briefly, areas of approximately 3 mm^2 were marked off on the bottom of flasks prior to seeding, so that photographs of the same area could be taken. Using this method permits cell counting of the same cells over a period of many weeks. Ten separate fields in five culture flasks of each group were counted, and the average numbers of cells were calculated.

The results are shown in Figure 1. To obtain a similar baseline for both uninfected and infected cultures, cell counts were taken prior to viral infection

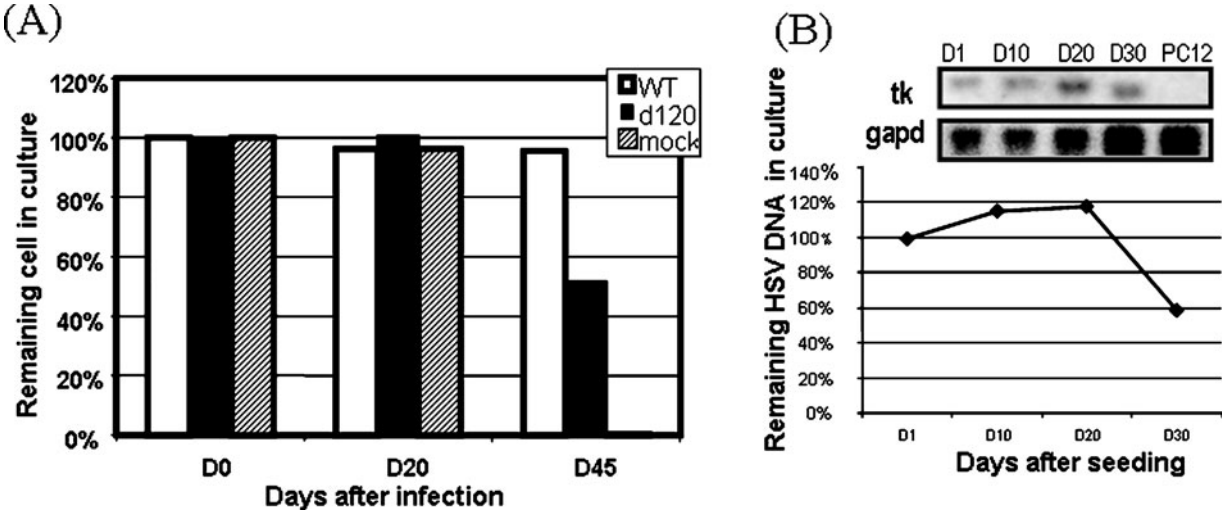

Figure 1 Infection of HSV-1 mutant strain d120 in NGF-PC12 culture. NGF-PC12 cells were prepared as described in Materials and Methods. On day 13 after NGF treatment, cultures were photographed and then infected with 4×10^6 p.f.u./flask of HSV-1 strain KOS, mutant strain d120, or left uninfected. (**A**) Percent of cells remaining in the flask as a function of time after infection. To monitor the percentage of cells remaining in the flask, each flask was marked randomly with two distinct dots. Each frame was photographed and the cells were counted on indicated days after infection. The percentage of cells remaining in the flask was calculated based on the number of cells in the flask on the day of infection (which is set as 100%). There were five flasks per group and the data is the average of the number of cells in ten frames. These data represent two independent experiments. (**B**) The half-life of HSV-1 mutant d120 DNA in NGF-PC12 cultures. To monitor the half-life of d120 DNA in the NGF-PC12 cultures, nuclear DNA of the cultures uninfected (PC12) or infected with mutant d120 was harvested as indicated days after infection. Five micrograms of nuclear DNA was digested with BamHI, resolved on a 2% agarose gel and transferred to a nylon membrane, which was hybridized with the ^{32}P-labeled HSV-1 tk probe or a cellular gene GAPD PCR probe (Su *et al*, 2000). The autoradiographic image was produced (shown) and quantified in a Bio-Rad phosphoimager. The ratio of the intensity of tk to GAPD was calculated and the ratio of the DNA isolated from the D1 after infection was set as 100%. The percent of remaining d120 DNA per cellular DNA of indicated days after infection was plotted.

(day 0 after infection). The number of cells counted on the day of the infection is called "day 0 post infection (PI)," and is taken as "100%." In agreement with our previous studies .(Moxley *et al*, 2002), there was no appreciable cell death observed in cultures infected with HSV1 wild-type, even up to day 45 PI (Figure 1A), whereas almost all the uninfected cells detached from the culture flask by that time. Interestingly, NGF-PC12 cells infected with mutant d120 remained attached in culture flasks significantly longer than the uninfected counterpart (Figure 1A, day 45 PI). Moreover, as with wild-type strain KOS, infection with d120 did not detectably alter the morphology of NGF-PC12 cells. There was no detectable cell loss of d120-infected NGF-PC12 cultures by 20 days after infection. Approximately 50% of the cells in the d120-infected cultures remained at day 45 PI, compared with nearly 100% of cells remaining in wild-type KOS–infected cultures and almost 0% of cells remaining in uninfected cultures.

As mentioned in the Introduction, because infection with d120 resulted in apoptosis in all cell lines previously tested, it was surprising to find d120-infected NGF-PC12 cultures fully viable after 20 days PI. It is possible that the NGF-PC12 cells infected by mutant d120 were eliminated by either apoptosis or other mechanisms and those cultures on day 20 PI had been repopulated by the proliferation of adjacent cells even though the infection was at moi of 20.

To confirm that cells remaining on day 20 PI were infected by mutant d120, the existence of mutant d120 DNA in infected culture as a function of time after infection was examined. NGF-PC12 cells were prepared and infected with mutant d120. Infected cell nuclear DNA was harvested at days 1, 10, 20, and 30 PI and viral and PC12 cellular DNA was analyzed by Southern hybridization with ^{32}P-labeled probes for the HSV-1 tk gene and the cellular gene glyceradehdyde-3-phosphate dehydrogenase (GAPD) as previously described (Su *et al*, 2002) and in Materials and Methods. Band intensity was quantified by PhosphorImaging and the ratio of HSV-1 DNA (HSV-1 tk) and PC12 DNA (GAPD) on day 1 PI infection was set to 1. Note that the intensity of the signal from hybridization with the GAPD probe was used as an internal measurement of the amount of cellular DNA loaded in each lane. It is assumed that all d120 DNA detected in infected cultures was derived from the input HSV DNA, because d120 is a DNA replication–deficient virus. Thus, by comparing the ratio of d120 DNA to cellular DNA, we determined if the d120-infected PC12 cells were lost or died after infection.

As shown in Figure 1B, no hybridization signal was detected in PC12 DNA by the HSV tk probe showing the specificity of the tk probe to HSV DNA. The ratio of HSV-1 DNA to PC12 DNA remained unchanged from days 1 to 20 PI. These data suggest that (1) consistent with the observation in Figure 1A, there was

no detectable cell proliferation, and (2) there was no detectable elimination of initially infected NGF-PC12 cells from days 1 to 20 PI, because no significant loss of input HSV DNA was observed for the first 20 days PI. These data confirmed that d120 infection failed to induce cell death (apoptosis) of NGF-PC12 cells. Furthermore, approximately 60% of input d120 DNA was detected at day 30 PI, suggesting that the half-life of the replication defective mutant d120 DNA was approximately 30 days.

Failure of mutant d120 to induce apoptosis in NGF-PC12 cells

To further confirm that mutant d120 infection did not induce detectable apoptosis in NGF-PC12 cells, a DNA fragmentation assay was performed. To control for apoptosis induction by d120 infection, Vero cells were included in this study. NGF-PC12 cells and Vero cells were infected with HSV-1 wild-type parental strain KOS or mutant strain d120 at a moi of 20 for NGF-PC12 cells, or 5 for Vero cells. Total DNA was isolated 24 h PI and analyzed in a 2% agarose gel. As shown in Figure 2A, mutant d120 infection induced DNA fragmentation in Vero cells, whereas no detectable DNA fragmentation was found for wild-type infected or uninfected Vero cells, as expected. In contrast, when NGF-PC12 cells were infected with mutant d120 (even at a higher moi), no sign of DNA fragmentation was observed at 24 h PI. As expected, DNA fragmentation was not observed in either uninfected NGF-PC12 cells or wild-type infected NGF-PC12 cells. In this experiment, the use of two different concentrations of brefeldin A (BFA) and cycloheximide (CHX) were included to show that NGF-PC12 cells are susceptible to apoptosis induction as demonstrated by the DNA laddering. Thus, in contrast to d120 infection in mitotic cells where apoptosis was evident, no sign of apoptosis was detected in d120-infected NGF-PC12 cells.

HSV-1 infection failed to activate NF-κB in NGF-PC12 cells

Work elsewhere has suggested that the NF-κB pathway mediates stress-induced apoptosis induced by d120 infection, because induction of apoptosis was not observed in NF-κB p50 or p65 knockout mutant cell lines following d120 infection (Taddeo *et al*, 2003, 2004). In addition, NF-κB p65 protein was shown to be involved in oxidative induced neuronal cell death (Ishige *et al*, 2005).

Because d120 infection failed to induce apoptosis in NGF-PC12 cells, we expected that d120 would not be able to activate the p65 protein in these cells, although the activation of p65 protein seems to be a general response to wild-type HSV infection by the host cells, as mentioned in Introduction. To determine whether NF-κB p65 protein was activated, two approaches were taken, based upon established methods. That is, because activation of NF-κB is associated with its translocation from the cytoplasm to the nucleus, as well as its ability to bind specific oligonucleotides, the intracellular localization of NF-κB protein was determined as a function of infection

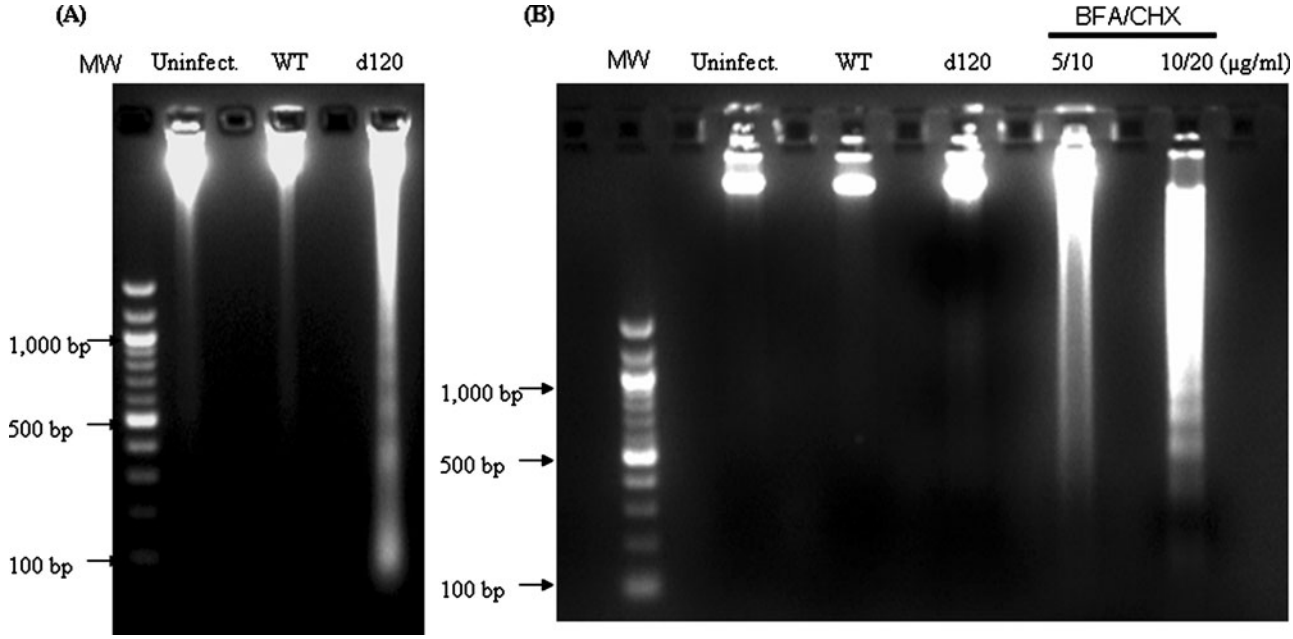

Figure 2 DNA fragmentation in HSV-1–infected cells. HSV-1, strain KOS, and/or d120 were used to infect Vero cells at moi of 5 (**A**) or NGF-PC12 cultures at moi of 20 (**B**) or left uninfected. The BFA (brefeldin A; Sigma-Aldrich, USA) and CHX (cycloheximide; Sigma-Aldrich) cocktail of indicated concentrations were used as an apoptosis inducer in NGF-PC12 cultures. The infected cells were harvested at 24 h PI, followed by DNA extraction as described in Materials and Methods. The DNA ladders were analyzed on a 2% agarose gel, the size of DNA was compared to the 100-bp DNA molecular weight marker (MW).

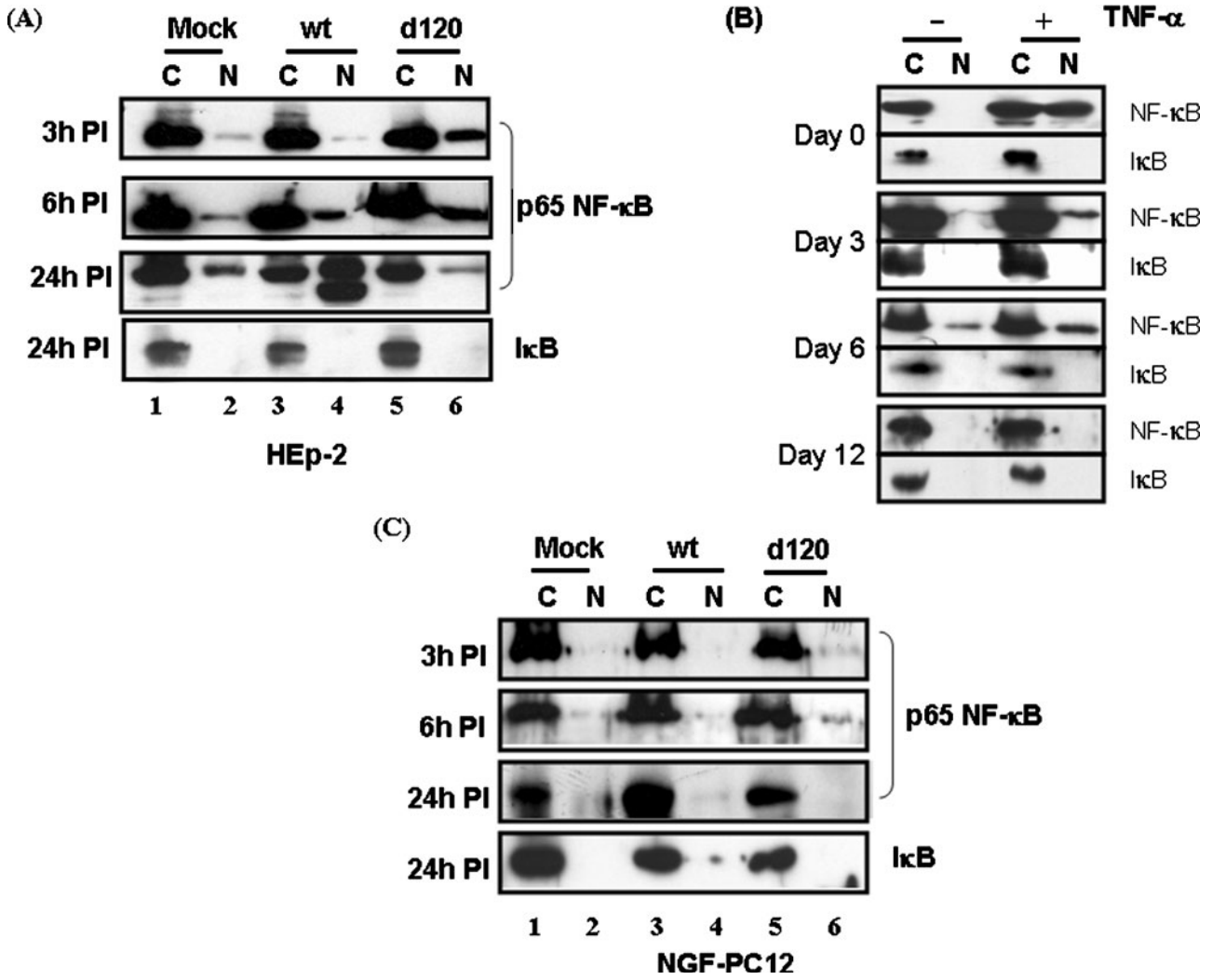

Figure 3 Western Blot analysis of the NF-κB pathway in HSV-1–infected NGF-PC12 cells. HEp-2 cells (**A**) and NGF-PC12 cells (**C**) were infected with HSV-1 strain KOS or mutant strain d120, at moi of 5 for HEp-2 cells and moi of 20 for NGF-PC12 cells, respectively. At 3, 6, and 24 h PI, cytosolic (C) and nuclear (N) lysates were prepared as described in Materials and Methods. As a positive control for NF-κB activation (**B**), PC12 cells at different days of NGF differentiation (days 0, 3, 6, and 12) were treated (+) with TNF-α (100 ng/ml) or left untreated (–). One and a half hours after TNF-α treatment, cytosolic and nuclear lysates were prepared. Approximately 20 μg of nuclear (N) and 50 μg of cytosol (C) lysates were resolved in denaturing gels, transferred to PVDF membranes, and Western blot analyzed using antibodies to NF-κB p65 subunit and IκB, respectively, as described in Materials and Methods.

and electrophoretic mobility shift assay (EMSA) was performed.

The intracellular distribution of NF-κB after HSV-1 infection was determined by Western blotting analysis of nuclear and cytosolic fractions. NGF-PC12 cultures were either left uninfected (mock) or infected with HSV-1 wild-type KOS or mutant strain d120, or for 3, 6, or 24 h PI, as described in Materials and Methods. The cultures were then harvested and fractionated into cytosolic and nuclear fractions (Materials and Methods). Protein lysates from both fractions were prepared and analyzed by Western blot. The results are shown in Figure 3C.

Because a negative result was expected, two positive controls were included. To control for the activation of the p65-mediated pathway by HSV-1 infection, HSV-1–infected HEp-2 cells were included. The amount of p65 protein is increased in the nuclear fraction of HEp-2 cells infected with either wild-type or mutant viruses at 6 and 24 h PI, as compared to mock infected HEp-2 cells (Figure 3A, comparing lane 2 to lane 4). This increase was time dependent, with little to no detectable activation of NF-κB at 3 h PI in wild-type KOS–infected cells.

Interestingly, activation of p65 protein was detected as early as 3 h PI in HEp-2 cells infected with mutant d120. By 24 h PI, the amount of p65 in nuclear fraction of d120-infected HEp-2 cells was back to that of mock infected. Although the kinetics of NF-κB p65 activation between wild-type KOS and mutant d120 infection were different, these data

confirm that HSV-1 infection of HEp-2 cells activated the NF-κB p65-mediated pathway.

Because the nuclear translocation of NF-κB is known to be associated with the reduction of inhibitor of NF-κB (IκB) in the cytosol, and IκB should only be found in the cytosol fraction, the same membrane used in Figure 3A (24 h PI) was analyzed using the antibody against IκB to control for the fractionation. As expected, IκB was detected only in the cytosolic, not in the nuclear fraction (Figure 3A). As compared to the amount of IκB in the mock-infected cytosol fraction, there was slightly reduction of IκB in the wild-type infected cytosol at 24 h PI, but no detectable reduction of IκB in the d120-infected cytosol. This is in agreement with the Western analysis of NF-κB of the same membrane.

Secondly, to control for the intactness of the p65-mediated pathway in NGF-PC12 cells, NGF-PC12 cells were treated with tumor necrosis factor (TNF)-α on different days of NGF differentiation and monitored for the p65 translocation. As expected, and shown in Figure 3B, NF-κB p65 protein was only detected in the cytosol fraction of untreated PC12 cells (day 0). Two hours after TNF-α treatment, the p65 protein was detected in both the nuclear and the cytosol fractions of PC12 cells, suggesting the activation of the NF-κB p65-mediated pathway by TNF-α treatment. The magnitude of NF-κB activation by TNF-α decreased over time with NGF differentiation. After 12 days of differentiation, the p65 protein was no longer detectable in the nuclear fraction of cells after 2 h of TNF-α treatment. Consistent with previous studies (Azoitei *et al*, 2005), NGF differentiation itself induced activation of the NF-κB p65 protein. The p65 protein was detected in the nuclear fraction of cells at days 3 and 6 of differentiation without TNF-α treatment. This activation by NGF differentiation was diminished by day 12. The level of p65 protein in the nuclear fraction on day 12 of NGF differentiation was undetectable. As a control for cytosol-nuclear fractionation, each membrane was also analyzed using antibody against IκB. The data suggest that the p65-mediated pathway was intact in PC12 cells, but attenuated after a long-term treatment of NGF.

Having shown that HSV-1 activates NK-κB p65 subunit in the HEp-2 system and the intactness of p65-mediated pathway in the NGF-PC12 system, it was next of interest to determine the situation in NGF-PC12 cells, which are refractory to d120-induced apoptosis. As shown in Figure 3B, NF-κB p65 protein remained mostly in the cytoplasm in mock (uninfected) cells. This shows that p65 is present but, as expected, is mostly in an inactive state in NGF-PC12 cells. Clearly, as shown in the Western blot experiments in Figure 3C, p65 protein remains predominantly cytoplasmic following infection with either wild-type or mutant d120 virus, over all of the time points studied. The amount of p65 protein detected in the nuclear fractions was very limited, if any. Thus, neither wild-type KOS nor mutant d120 infection caused detectable translocation of p65 protein from cytosol to nucleus of infected NGF-PC12 cells. These data were consistent with the results of the IκB analysis, which showed that IκB was not altered by either wild-type or mutant HSV-1 infections. These data demonstrates that p65 protein localization to the nucleus was not induced by HSV-1 infections, neither parental wild-type strain KOS, nor mutant strain d120.

To further confirm that infection of NGF-PC12 cells with HSV-1 did not activate the NF-κB p65-mediated pathway, EMSA was performed to detect any activated p65 protein. The detailed procedure is described in Materials and Methods. To control for EMSA, nuclear and cytosolic lysates of infected HEp-2 cells (24 h PI) were also included in the EMSA assay as in Figure 4. Because activated p65 protein locates in the nuclear fraction, cytosolic fractions were used as a negative control for the specific activated NF-κB p65 bound species detected in the gel. As shown in Figure 4, mock-infected HEp-2 cell cytoplasm and nuclear fractions contain very minimal, if any (we will call this "baseline"), amounts of material that can "gel shift" NF-κB p65 specific oligonucleitides (Figure 4, lanes M/C and M/N).

On the other hand, and expected, specific DNA gel–shifting species were detected in the nuclear fractions in both KOS- and d120-infected HEp-2 nuclear fractions, suggesting the NF-κB p65-mediated pathway was activated by HSV-1 infection in HEp-2 cells. Although modest amounts of p65-associated species was detected in the cytoplasm of d120-infected cells, this is not entirely surprising given its profound apoptosis inducing ability apparent in these cells at this time (24 h PI) after infection.

The situation in NGF-PC12 cells was quite different. There was a nuclear specific retarded band as indicated, a different species from that of HEp-2 cells, detected in the nuclear lysate prepared from mock-, wild-type–, or d120-infected NGF-PC12 cells. Interestingly, as compared to the mock-infected cells, infection with the wild-type strain, but not the mutant strain, slightly reduced the amount of this complex, which has not been characterized further. Nevertheless, the activation of the NF-κB p65-mediated pathway by the infection with d120 mutant virus in NGF-PC12 cells was not detected. Thus, as expected, the results generated by the EMSA assay agree with the Western blot analysis of the corresponding nuclear fractions (24 h PI) showing that HSV-1 d120 infection was not able to activate the NF-κB p65-mediated activity in neuron-like NGF-PC12 cells.

Discussion

In this study, we demonstrate, surprisingly, that infection of neuronal-like NGF-PC12 cells with a highly potent apoptosis-inducing agent, HSV-1

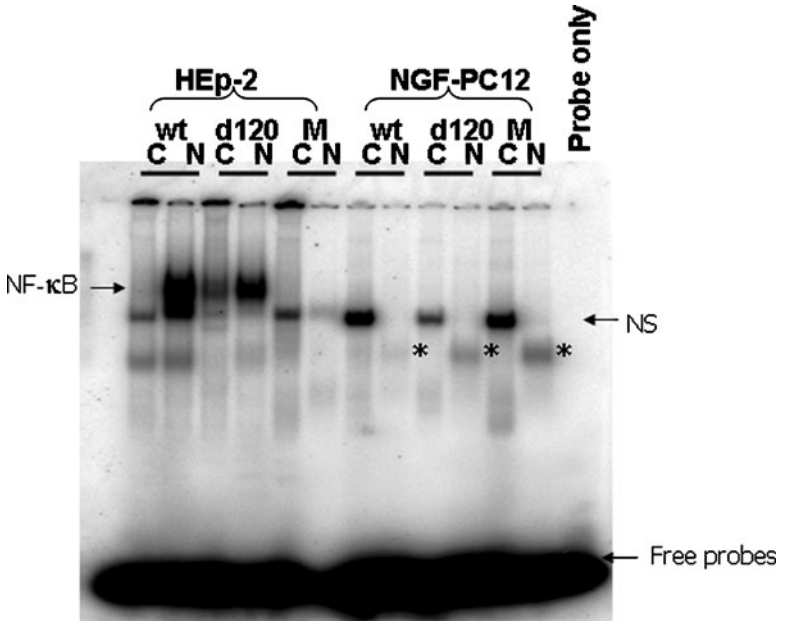

Figure 4 Detection of activated NF-κB by the electrophoretic mobility shift assay (EMSA). Nuclear and cytosol fractions were prepared as described in the legend of Figure 3, approximately 10 μg of protein lysate from either nuclear (N) or cytosol (C) fractions were incubated with ^{32}P-labeled NF-κB oligo probes and then loaded in a nondenaturing polyacrylamide gel, the gel was dried and exposed to PhosphoImage screen as described in Materials and Methods. The NF-κB–bound species (NF-κB), a nuclear-specific oligo-retarded complex (*), and nonspecific bands (NS) are indicated.

mutant strain d120, not only failed to induce detectable apoptosis, but prolonged the life of infected cells in culture, as compared to uninfected cells. To our knowledge this is the first cell line in which d120 infection failed to induce apoptosis within 24 h of infection.

As shown in Figure 1, no significant difference of cell detachment in d120-infected NGF-PC12 cells, as compared to KOS-infected culture, was observed on day 20 PI. However, the infection of mutant d120 only provided approximately 50% of prevention from cell detachment as compared to infection of parental strain KOS on day 45 PI. There was no detectable viral gene expression after 2 weeks of infection except for a low level of LATs, as shown previously (Su *et al*, 2000). One possible explanation for the less potent protection of mutant d120 against cell detachment of NGF-PC12 cells could be due to the left over or a long-term effect from the initial infection. The different outcome of infection between parental strain KOS and mutant d120 was not evident until over 20 days after infection. This implies that neuronal-like cells could be more tolerant of the cytotoxicity of d120 infection than mitotic cells. This is consistent to our previous observations of a longer half-life (more than 20 to 30 days) of linear DNA in neuronal-like NGF-PC12 cells as compared to mitotic cells (2 to 3 days) (Su *et al*, 2002).

The NF-κB/Rel proteins are a family of transcription factors that include five proteins, p50, p52, p65 (RelA), RelB, and c-Rel, which exist as a variety of homo- and heterodimers that are capable of transducing receptor signals to the nucleus (Ghosh *et al*, 1998). Because p65 protein tends to be predominant in the cell, most studies examining the interplay between HSV infection and the NF-κB pathway were done using the tools that are p65 specific. The contribution of NF-κB activation to HSV-1 infection seems to vary with the cells and viral strains used. For instance, a study reported that activation of NF-κB in HEp-2 cells is required to block apoptosis induced by the proapoptotic mutant virus vBSΔ27 (Goodkin *et al*, 2003). This implied an antiapoptotic role for NF-κB in HSV-1–infected cells. The antiapoptotic function of NF-κB in HSV-infected cells has also been suggested by others (Yedowitz and Blaho, 2005; Gregory *et al*, 2004; Medici *et al*, 2003). On the other hand, another set of studies demonstrated that activation of NF-κB was required for stress induced apoptosis that results from mutant d120 infection (Taddeo *et al*, 2003, 2004), suggesting a proapoptotic role in HSV-infected cells.

Interestingly, activation of the p65-mediated pathway was shown to mediate the oxidative stress–induced cell death in neuronal cells (Ishige *et al*, 2005), whereas the c-Rel–mediated pathway involves in the neuronal cell survival as studied using NGF-PC12 cells (Azoitei *et al*, 2005). Studies done by Pizzi and his group (Pizzi *et al*, 2002) also showed the opposing role for NF-κB/Rel factors p65 and c-Rel on the survival of neurons. The authors suggested the prosurvival function of c-Rel–containing dimers in comparison to p65-containing dimers. This clearly demonstrates that activation of different

species of NF-κB dimers could have distinct effects on host cells. In the NF-κB knockout cells, the NF-κB p65-mediated pathway is impaired, resulting in the failure of d120 to induce apoptosis. Interestingly, our studies suggest that although NF-κB p65 protein is present in the NGF-PC12 cells, no activation of the NF-κB p65 was detected following d120 infection. It is possible that the failure of d120 infection to induce apoptosis was due, entirely or in part, to an inability to activate NF-κB p65-mediated pathway. Since HSV-1 induced the prolonged NGF-PC12 cells life in tissue cultures, it is possible that c-Rel mediated pathway was activated in response to HSV-1 infection. Studies are on-going to investigate whether the c-Rel activation is involved in prevention of NGF-PC12 cells detaching from the culture flasks, as shown in Figure 1 and previous studies (Moxley *et al*, 2002).

Collectively, we demonstrated that the response of neuronal-like cells and mitotic cells to viral infection could be very different. Knowledge obtained from studies using mitotic cells to understand how the virus and host interact might not represent what occurs in neuronal cells. Thus, studies using neuronal cells are important to understand how HSV-1 interacts with neurons, and, in particular, establishes latent infections in neurons.

Materials and methods

Cells and viruses

Rat pheochromacytoma (PC12), Vero (African green monkey kidney), and HEp-2 cells were obtained from the American Type Culture Collection. The PC12 cells were maintained in RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum, and 1% antibiotic-antimycotic. Vero cells were grown as monolayer in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. HEp-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HSV-1 KOS is the wild-type strain used in this experiment. HSV-1 d120 mutant is an alpha 4 gene–deleted, Us3-defective HSV-1, kindly provided by P. Schaffer (Harvard University Medical School, Boston, MA).

NGF-differentiated PC12 culture

The culture flasks were coated with poly-l-ornithine hydrobromide (Sigma, USA) at 4°C, overnight. PC12 cells were seeded at 2×10^5 cells per T25 flask with RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum, 1% antibiotic-antimycotic, and 100 ng/ml of NGF (BD biosciences, USA). On day 7 after seeding, the cultures were treated with 20 μM 5'-fluoro-2-deoxyuridine for 3 days and medium then replaced with fresh NGF-containing medium.

Nuclear DNA isolation and HSV DNA analysis by Southern blot hybridization

To isolate nuclear DNA, cells were scraped into medium and collected by centrifugation at 1500 rpm for 10 min at 4°C. The cell pellet was resuspended into nuclei lysis buffer (1 mM $CaCl_2$, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris, pH 7.5, 5% sucrose) containing NP-40, and the nuclei were collected by centrifugation at 1500 rpm for 10 min at 4°C. The nuclear pellet was then resuspended in nuclei lysis buffer containing 0.1% of deoxycholate to strip off nuclear membrane associated proteins. After deoxycholate treatment, nuclear DNA was isolated by sodium dodecyl sulfate (SDS)/proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation.

Southern blot hybridization was performed by digesting the purified DNA with the restriction endonuclease BamHI. The digested DNA was resolved on a 1.5% agarose gel, and transferred to a nylon membrane by capillary transfer. The membrane was hybridized with the selected ^{32}P-radiolabeled HSV tk probe or cellular DNA probe GAPD as described previously (Su *et al*, 2002). The autoradiographic image was generated and quantified by a Bio-Rad phosphoImager analysis.

DNA fragmentation assay

The attached and floating cells were harvested and treated with lysis buffer (10 mM Tris, 0.5% SDS, 100 mM NaCl, and 25 mM EDTA, pH 8.0) containing 0.1 mg/ml proteinase K. After incubation at 50°C for at least 3 hours, phenol/chloroform extraction was performed followed by ethanol precipitation. The DNA pellet was collected by centrifugation at 13,000 rpm at 4°C for 20 min, and then was washed once with 80% ethanol. Total DNA was redissolved with TE buffer at 4°C, overnight. The DNA suspension was treated with RNase A (0.1 mg/ml) at 37°C for 1 hour. One-third of the DNA samples were then analyzed on a 2% agarose gel, the size of DNA fragment was compared with the 100-bp marker (New England BioLabs, Beverly, MA).

Preparation of cytosol and nuclear fractions

The infected cells were harvested at the indicated time points and washed once with ice-cold phosphate-buffered saline (PBS). The cell pellets were resuspended in 100 μl sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 8.0], 3 mM $CaCl_2$, 2 mM MgOAc, 0.1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol [DTT], and 0.5 mM phenylmehtylsulfonyl fluoride [PMSF]). Cytosol fractions were separated by centrifugation at 500 $\times$ *g* for 5 min at 4°C and transferred to new microcentrifuge tubes. The nuclear pellets were washed once with sucrose buffer without NP-40. The pellet was resuspended in 30 μl low-salt buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 20 mM

KCl, 0.2 mM EDTA, 25% glycerol [*v*/*v*], 0.5 mM DTT, and 0.5 mM PMSF) and equal volume high-salt buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 25% glycerol [*v*/*v*], 1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitors) was slowly added and mixed well at 4°C for 45 min on a shaker. The lysates were centrifuged at 14,000 × *g* for 15 min, 4°C, and the supernatants containing the nuclear proteins were transferred to fresh vials. Protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad, USA). The extracts were stored at −70°C until use.

Electrophoretic mobility shift assay (EMSA)
Protein Extracts (10 μg) were incubated for 30 min at room temperature with binding buffer (50 mM Tris HCl [pH 8.0], 750 mM KCl, 2.5 mM EDTA, 0.5% Tritron-X 100, 62.5% glycerol [*v*/*v*], and 1 mM DTT), 1 μg of poly (dI-dC) and 5'-end-^{32}P-labeled double-stranded DNA (dsDNA) oligonucleotide containing an NF-κB binding site corresponded to the sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 5′-GCCTGGGAAAGTCCCCTCAACT-3′. DNA–NF-κB complexes were separated from free labeled DNA by electrophoresis in 5% polyacrylamide gels. Gels were dried at 75°C for 2 h, followed by autoradiography and quantitation by PhosphoImager analysis. For the supershift assay, 2 μg of mouse anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added in the binding reaction before adding the 5′-end-^{32}P-labeled dsDNA oligonucleotides.

Western blot analysis
Approximately 50 μg of infected cell proteins from cytoplasmic fractions and/or 20 μg of nuclear fractions were electrophoretically separated in 10% SDS–polyacrylamide gel electrophoresis (PAGE), transferred to Hybond–PVDF membrane (Amersham Bioscience, USA), and probed overnight with primary antibodies of interest (anti-NF-κB p65 antibody, 1:200 dilution; anti-IκB antibody, 1:500 dilution; both Santa Cruz Biotechnology). Anti-rabbit antibodies conjugated to horseradish peroxidase were used for chemiluminescence detection as recommended by the vendor (Amersham Biosciences). Specific protein bands were visualized by a chemiluminescence detection system (Pierce, Rockford, IL) according to the manufacturer's specifications.

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