Clonogenic Assay of Type A Influenza Viruses Reveals Noninfectious Cell-Killing (Apoptosis-Inducing) Particles[∇][†]

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Clonogenic (single-cell plating) assays were used to define and quantify subpopulations of two genetically closely related variants of influenza virus A/TK/OR/71 that differed primarily in the size of the NS1 gene product; they expressed a full-size (amino acids [aa] 1 to 230) or truncated (aa 1 to 124) NS1 protein. Monolayers of Vero cells were infected with different amounts of virus, monodispersed, and plated. Cell survival curves were generated from the fraction of cells that produced visible colonies as a function of virus multiplicity. The exponential loss of colony-forming capacity at low multiplicities demonstrated that a single virus particle sufficed to kill a cell. The ratios of cell-killing particles (CKP) to plaque-forming particles (PFP) were 1:1 and 7:1 in populations of variants $NS1_{1-124}$ and $NS1_{1-230}$, respectively. This study revealed a new class of particles in influenza virus populations—noninfectious CKP. Both infectious and noninfectious CKP were 6.3 times more resistant to UV radiation than PFP activity. Based on UV target theory, a functional polymerase subunit was implicated in a rate-limiting step in cell killing. Since influenza viruses kill cells by apoptosis (programmed cell death), CKP are functionally apoptosis-inducing particles. Noninfectious CKP are present in excess of PFP in virus populations with full-size NS1 and induce apoptosis that is temporally delayed and morphologically different than that initiated by infectious CKP present in the virus population expressing truncated NS1. The identification and quantification of both infectious and noninfectious CKP defines new phenotypes in influenza virus populations and presents a challenge to determine their role in regulating infectivity, pathogenesis, and vaccine efficacy.

Clonogenic assays have been used to quantify the capacity of virus populations to kill cells. They revealed a phenotypically definable subpopulation of cell-killing particles (CKP) within a virus stock, i.e., poliovirus (46), Newcastle disease virus (23, 24, 28), and vesicular stomatitis virus (VSV) (25, 31, 32, 33, 35). Virus populations analyzed in this manner measure the capacity of a single virus particle to kill a single cell, thereby preventing its growth into a visible colony. CKP activity can be independent of infectivity or the production of progeny virus. By this criterion, most virus populations were shown to contain an excess of CKP over plaque-forming particles (PFP) (23, 24, 25, 28, 31, 32, 33, 35). Rather than measure the lethal action of viruses averaged over the cell population, the clonogenic assay for CKP detects the minimal expression of virion components or products required to kill an individual cell and thus is a very sensitive indicator of cell death.

This report quantifies for the first time the CKP capacity of influenza virus populations. It compares the cell-killing activities of two genetically closely related type A influenza virus variants that differ primarily in the integrity of the encoded NS1 protein (5, 37, 38, 42). Variant A/TK/OR/71-SEPRL (H7N3) encodes full-length NS1 (amino acids [aa] 1 to 230), and variant A/TK/OR/71-delNS1 (H7N3) encodes a truncated version of NS1 (aa 1 to 124); they are hereafter termed variants

$NS1_{1-230}$ and $NS1_{1-124}$, respectively. NS1 acts to regulate both the expression of apoptosis (8, 39, 47, 48) and the temporal appearance of viral mRNAs (36). Significant differences in the ratios of CKP to PFP for the two variants are described, the fraction of the genome that must be expressed to kill a cell is defined by UV target analysis, and the possible role of the integrity of the NS1 protein is considered. Differences are reported between the variants in the temporal development and morphogenesis of the apoptosis that characterizes cell killing by influenza viruses (12, 43). These properties define a new class of particles in influenza virus populations—noninfectious CKP.

MATERIALS AND METHODS

Cells and media. GMK-Vero cells were grown in attachment solution (AS; NCI medium plus 6% calf serum) (4a, 40) and incubated at 37.5°C with a flowing air-CO_2 mixture to maintain pH 7.1. Our line of Vero cells does not produce interferon (IFN) when induced (6, 33) and plates with high efficiency (≥85%). Primary chicken embryo kidney cells (CEK) were prepared from 18-day-old embryos (obtained from Charles River SPAFAS, Inc., North Franklin, CT) and also grown in AS.

Viruses. A/TK/OR/71-SEPRL (H7N3) contains full-length NS1 (aa 1 to 230), and A/TK/OR/71-delNS1 (H7N3) expresses a truncated NS1 protein (aa 1 to 124) (5, 37, 38, 42). Virus stocks were propagated in 9-day-old specific-pathogen-free embryonated chicken eggs (Charles River SPAFAS, Inc., North Franklin, CT). Each egg was injected with 0.1 ml containing ~10^3 infectious particles (as PFP), incubated for 48 to 72 h at 34°C with forced humidified air circulation and egg rotation, and held at 4°C for 24 h before harvesting. The chorioallantoic/amniotic fluid was harvested and stored separately for each egg. Those with high hemagglutinating activity (hemagglutination [HA] titers of ≥1,280) were pooled, aliquoted, and stored at −80°C (30).

CKP assays and generation of cell survival curves. CKP clonogenic assays were carried out as follows. Appropriately diluted egg-derived virus in a final volume of 300 μl AS was attached for 60 min at 37.5°C to just confluent

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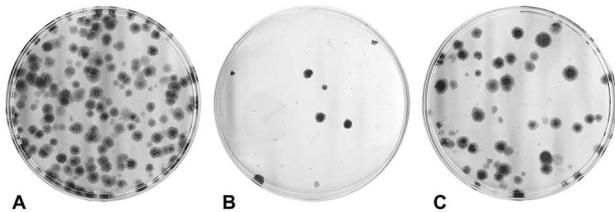
FIG. 1. Representative colonies of GMK-Vero cells that survived exposure to A/TK/OR/71 variants. All 50-mm plates were inoculated with 200 cells, incubated for 10 days, fixed in 10% formaldehyde, and stained with Giemsa. Plates: A, control cells, no virus; B, variant NS1 ($m_{pfp} = 1$, $m_{ckp} = 7$); C, variant NS1$_{1-124}$ ($m_{pfp} = m_{ckp} = 1$).

FIG. 2. Survival curves of Vero cells infected with A/TK/OR/71 variant NS1 (●) or NS1$_{1-124}$ (○) as a function of m_{pfp}. Lower abscissa = m_{pfp} for both variants. Upper abscissas = m_{ckp} calculated separately from the survival curve for each variant as described previously (23, 29) and briefly in Materials and Methods.

monolayers of GMK-Vero cells containing ~2×10^6 cells in 50-mm plastic dishes. Any unattached virus was aspirated, the monolayers were rinsed twice with 2 ml of NCI medium and monodispersed with EDTA-trypsin (40), and the cells were seeded into 50-mm dishes in 5 ml of AS in numbers that allowed the formation of a countable number of visible colonies after 9 to 10 days of undisturbed incubation. To enhance visualization, colonies were fixed with 10% formalin in phosphate-buffered saline (PBS) for 1 h, washed in PBS, and stained with Giemsa. CKP multiplicity (m_{ckp}) was calculated from cell survival curves generated as a function of virus concentration as previously described (23, 24). Briefly, based on a Poisson distribution of CKP in the monolayer of cells, the dilution of virus that produced 0.37 surviving cell was assumed to contain an m_{ckp} of 1. Knowing the number of cells in the infected monolayer, and with attachment of virus virtually 100% (data not shown), it is possible to calculate the titer of CKP per milliliter (23, 24). Since it is established that influenza virus kills cells by inducing apoptosis (references 12 and 43 and data herein), these CKP actually function as apoptosis-inducing particles (AIP), a designation that can be used when the mechanism of cell killing is known to be apoptosis. Use of the term CKP would apply under more general circumstances, where the mode of cell killing may be other than apoptosis—as in death by necrosis. For convenience, the term CKP is used herein.

PFP assays. PFP activity was assayed in primary CEK prepared from 18-day-old embryos and obtained from Charles River SPAFAS, Inc. (North Franklin, CT). Plaque assays of the two variants in CEK cells with a serum-free 0.6% agarose overlay did not require trypsin to activate the fusion peptide required for continued propagation of the virus (16). Countable plaques appeared 2 to 3 days after incubation at 37.5°C. CEK cells are not intrinsically good producers of IFN when stimulated by influenza virus (5), making them ideal hosts for influenza virus plaque assays. As with many types of cells that lack suitable proteases, plaque development in Vero cells required trypsin (Sigma T14263 at 3 μg/ml) in the overlay to activate the fusion peptide (3). This was added in a second agarose overlay 24 h after the first overlay was applied (49) and was effective in counteracting the inhibitor of trypsin that Vero cells produce (13). PFP titers from CEK (without trypsin) and Vero (with trypsin) cells were similar.

UV irradiation and heat inactivation. UV irradiation and heat inactivation were carried out as described previously, with a calibrated source of UV (254 nm) radiation (30); however, heat inactivation was at 55°C.

Microscopy and Hoechst staining. The development of apoptosis in monolayers of Vero cells was recorded microscopically with digital images used to generate the photographs presented. A soft agarose overlay (0.3%) was used to retain the apoptotic cells close to the site of origin. This made it possible to photograph, in a single plane of focus, both the cells attached to the plastic growth substrate and those that had undergone apoptosis and appeared rounded just above the cell monolayer. Hoechst 33342 (Sigma) at 100 ng/ml in PBS was used as a vital stain to visualize DNA fragmentation in the intact cell under fluorescence microscopy.

RESULTS

Typical colony survivors in a CKP assay. Figure 1 shows colonies of Vero cells that developed 10 days after seeding with 200 single cells: mock infection (plate A), TK/OR/71-NS1$_{1-230}$ ($m_{pfp} = 1$) (plate B), or TK/OR/71-NS1$_{1-124}$ ($m_{pfp} = 1$) (plate C). Although plates B and C were infected with equal multiplicities of PFP, the fewer surviving cells or colonies in plate B
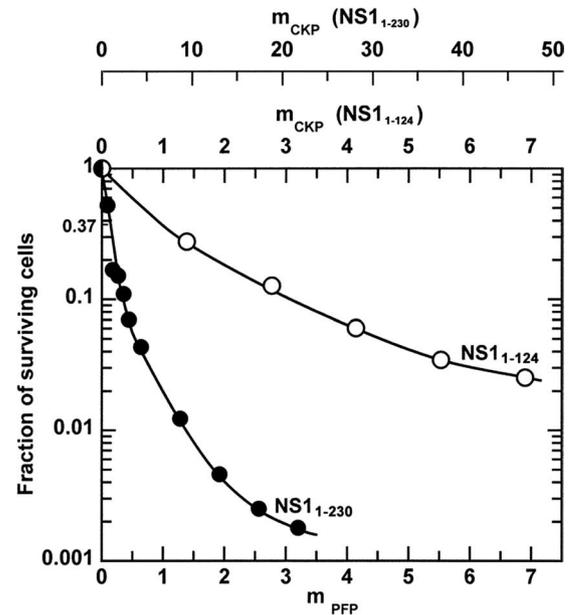
demonstrate that the population of variant NS1$_{1-230}$ contained significantly more CKP than the population of variant NS1$_{1-124}$. Data from Fig. 2 reveal that the cells in plate B received seven times more CKP than did those in plate C; i.e., in plate B, $m_{pfp} = 1$ and $m_{ckp} = 7$ and in plate C, $m_{pfp} = 1$ and $m_{ckp} = 1$. The efficiency of cell plating (plate A) was 85%. Microscopic observations during the first few days after plating of single cells infected at $m_{ckp} = 7$ revealed that >95% did not undergo any cell divisions. These data identify a subpopulation in variant NS1$_{1-230}$ that phenotypically functions as noninfectious CKP. Virus particles with this phenotype are absent in variant NS1$_{1-124}$.

Cell-killing survival curves of Vero cells infected with TK/ OR/71 variant NS1$_{1-230}$ or NS1$_{1-124}$. Figure 2 illustrates typical survival curves generated by Vero cells following infection at the m_{pfp} indicated for each variant. At a low m_{pfp}, both variants reveal an exponential rate of decline in the fraction of cell survivors. Survival curves of human A549 cells show that they responded similarly (data not shown). However, at a higher equivalent m_{pfp}, cell survival curves generated as a function of NS1$_{1-230}$ declined much more rapidly than those produced by the NS1$_{1-124}$ variant, indicating that the population of NS1$_{1-230}$ virus contained more CKP relative to PFP than did populations of NS1$_{1-124}$. Calculated values of m_{ckp} (see Materials and Methods) are presented on the upper abscissas. They reveal that, relative to PFP, the population of variant NS1$_{1-230}$ contained seven times more CKP than did NS1$_{1-124}$. Based on PFP titers obtained from CEK cells, or Vero cells in the presence of trypsin, the ratios of CKP to PFP were 7:1 ($2.2 \times 10^9:3.2 \times 10^8$) and 1:1 ($6.9 \times 10^8:6.9 \times 10^8$) for NS1$_{1-230}$ and NS1$_{1-124}$, respectively. We note that PFP titers of egg-derived variant NS1$_{1-124}$ were invariably higher than those of variant NS1$_{1-230}$
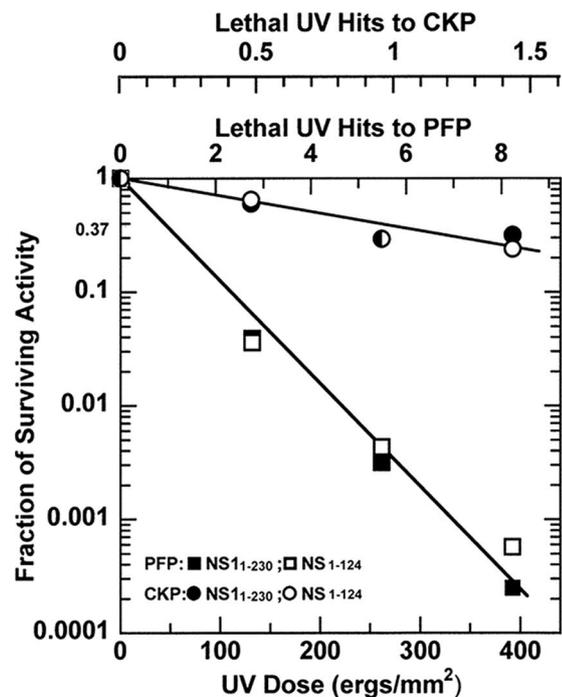
FIG. 3. Comparison of PFP and CKP survival curves of variants NS1 and $NS1_{1-124}$ as a function of the UV (254 nm) dose (lower abscissa). The numbers of lethal UV hits to PFP and CKP shown on the upper abscissas were calculated from the dose of UV that resulted in the survival of 37% of the cells (D_{37}). The key within the graph distinguishes between PFP and CKP for each variant. The ordinate displays the fraction of surviving activity as PFP (squares) or CKP (circles) for the two variants.
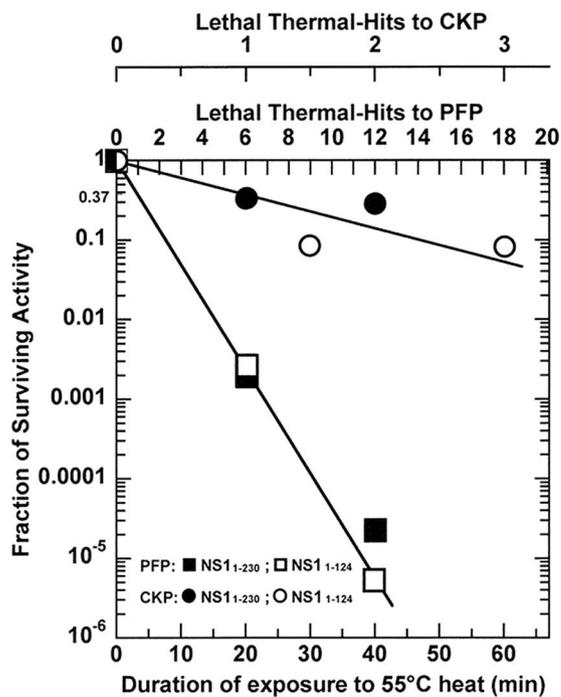

FIG. 4. Comparison of PFP and CKP survival curves of variants NS1 and $NS1_{1-124}$ as a function of time at 55°C. The number of lethal thermal hits to PFP and CKP for each variant is shown in the upper abscissas. The key within the graph distinguishes between PFP and CKP for each variant.

and developed plaques more rapidly. The HA titers of the pooled stocks of variants $NS1_{1-230}$ and $NS1_{1-124}$ were 10,240 and 2,560, respectively.

Both survival curves in Fig. 2 show that, at higher multiplicities, the observed fraction of cells killed is disproportionately less than expected from a Poisson distribution of CKP. Similar results have been observed with neuraminidase-containing Newcastle disease virus (24). We postulate that the simultaneous addition of high multiplicities of these neuraminidase-containing virus particles may contribute to enhanced rates of elution, thereby effectively lowering the actual multiplicity of infection.

Sensitivity of PFP and CKP to UV radiation and heat (55°C). Figure 3 compares the PFP and CKP survival curves of the two TK/OR/71 variants as a function of UV radiation dose. Full CKP survival curves were obtained for each of the UV doses indicated, and the fraction of surviving CKP was calculated as a function of the UV dose (Fig. 3). Based on target theory, a single lethal hit of UV radiation (most likely in the form of a uracil dimer) to the influenza virus genome, i.e., to any one of its eight gene segments, results in the inactivation of infectivity (PFP) (1). Thus, the survival curve for PFP represents that fraction of the PFP population that did not receive a lethal hit. Assuming a Poisson distribution of lethal hits to the virus genome, the dose of UV radiation at which 37% ($1/e = 0.37$) of CKP activity survives is defined by the initial slope of the curve. The larger the size of the UV target (num-

ber of nucleotides), the steeper the slope of the survival curve. If only a fraction of the whole RNA genome is required to express CKP activity, the probability of hitting that RNA decreases in proportion to its size and the slope of the survival curve will be shallower, as shown in Fig. 3, where the rate of inactivation of CKP for both variants is experimentally indistinguishable at 1/6.3 (16%) from that of PFP.

Figure 4 shows that exposure of variants $NS1_{1-230}$ and $NS1_{1-124}$ to heat as a function of time inactivated both PFP and CKP at exponential rates. The rate of inactivation was the same for both variants for a given activity. Thus, a similar rate of loss of CKP to heat is observed whether it is infectious ($NS1_{1-124}$) or noninfectious ($NS1_{1-230}$). However, CKP were about six times more resistant than PFP to thermal inactivation. Thus, a 100-fold decrease in PFP activity is accompanied by only a 2-fold decrease in CKP titer.

Temporal and morphological development of apoptosis differs for noninfectious and infectious CKP. Most influenza virus-host cell interactions result in apoptosis (programmed cell death) (12, 43), and much attention has been devoted to the role of NS1 in the development of apoptosis as recently summarized (47). This prompted a closer look at the development of apoptosis in cells infected by influenza virus populations that differed in the integrity of the expressed NS1 protein. Since the contents of CKP relative to PFP in $NS1_{1-230}$ and $NS1_{1-124}$ variant populations were not equivalent (Fig. 1 and 2), we compared the kinetics of apoptosis under conditions where m_{ckp} were set equal instead of the usual equivalence of m_{pfp}. Figure 5 shows both temporal and qualitative differences in the appearance of the characteristic changes cells undergo
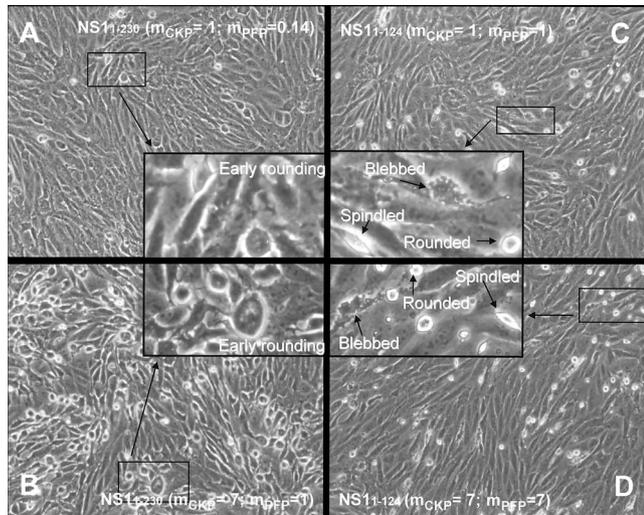
FIG. 5. Appearance of apoptosis in monolayers of Vero cells at 12 hpi with TK/OR/71 variant $NS1_{1-230}$ (A and B) or $NS1_{1-124}$ (C and D). Cells were infected at $m_{ckp} = 1$ (A and C) or $m_{ckp} = 7$ (B and D). The comparable values of m_{pfp} for these values of m_{ckp} are indicated. The higher magnifications displayed in the inserts in panels A and B illustrate the early rounding of cells and paucity of blebbing characteristic of variant $NS1_{1-230}$. The inserts in panels C and D illustrate the smaller-size rounded and spindle-shaped cells and the characteristic blebbing that occurs early in infections with variant $NS1_{1-124}$.

as they progress through apoptosis to cell death. When confluent monolayers of Vero cells were infected separately with an m_{ckp} of 1 of variant $NS1_{1-230}$ (Fig. 5A) or $NS1_{1-124}$ (Fig. 5B), the different ratios of CKP to PFP intrinsic to each population meant that the monolayers also were infected with an m_{pfp} of 0.14 (Fig. 5A) and 1 (Fig. 5B), respectively. The most obvious morphological differences in apoptosis induced by the two variants at 12 h postinfection (hpi) were the abundance of small, rounded, or spindle-shaped, highly refractile, and blebbed cells in Vero cell monolayers infected with variant $NS1_{1-124}$ ($m_{ckp} = 1$, $m_{pfp} = 1$) and their virtual absence in cells exposed to variant $NS1_{1-230}$ at the same m_{ckp} of 1, albeit of noninfectious CKP, where the m_{pfp} was 0.14. Apoptosis induced by noninfectious CKP was slow to accumulate and consisted almost exclusively of large rounded cells beginning to retract from the substrate and accumulate in the soft agarose overlay (Fig. 5B). At an m_{ckp} of 7 for variant $NS1_{1-230}$, the number of rounding cells was, as expected, high compared to that at an m_{ckp} of 1, but blebbing was still a rare event (Fig. 5B). Characteristically, the rounded cells were larger than those induced by variant $NS1_{1-124}$ (Fig. 5C and D). At an m_{ckp} of 7 for variant $NS1_{1-124}$, where the m_{pfp} was 7, the frequency of blebbed cells increased significantly, thereby decreasing the frequency of highly refractile rounded or spindle-shaped cells (Fig. 5D).

Observations made at 20 hpi confirmed that the apoptosis induced by the noninfectious CKP was delayed temporally relative to that induced by the infectious CKP. By 20 hpi, many cells infected with variant $NS1_{1-124}$ had gone beyond the blebbing stage; they had shrunk or disintegrated, leaving some areas of the substrate free of cells, as in a more advanced cytopathic effect. During this time, fewer cells infected with variant $NS1_{1-230}$ had pulled away from the monolayer but

blebbed and fragmented cells had begun to accumulate (data not shown).

Under fluorescence microscopy, Vero cells infected with either of the influenza virus variants and stained vitally with soluble Hoechst 33342 as early as 12 hpi displayed the fragmented DNA in the nucleus characteristic of apoptosis (data not shown).

DISCUSSION

The clonogenic (single-cell plating) assay was used for the first time to determine the content of CKP in populations of two closely related variants of a low-pathogenicity type A influenza virus, TK/OR/71(H7N3), that differed in the size of the NS1 protein expressed. This assay measures the ability of a single virus particle to kill a cell through its capacity to prevent colony formation. The CKP titer is determined from the fraction of surviving cells that form visible colonies (Fig. 1) as a function of multiplicity (Fig. 2). A CKP need not replicate or be infectious to kill a cell. Either the cell is killed, or it survives to form a visible colony. Thus, the assay scores cell killing as an all-or-none effect, rather than averaging the myriad changes that occur in a population of cells undergoing programmed cell death (apoptosis). Since influenza viruses kill cells by inducing apoptosis (12, 43), CKP as defined by the clonogenic assay, whether infectious or noninfectious, also can be termed AIP.

All virus populations were egg derived and therefore contained activated HA fusion peptide (16), ensuring that all of the stages in the initial infection proceeded normally. The Vero cells used as hosts plated with high efficiency but did not activate the fusion peptide of either variant. Furthermore, Vero cells secrete a factor that inactivates trypsin that could otherwise activate the HA fusion peptide (13). Consequently, multiple rounds of infection were precluded (data not shown). This preserves the input multiplicity of virus, ensuring that the fraction of surviving cells is an accurate reflection of the initial multiplicity.

The initial exponential loss of cell viability observed as a function of virus multiplicity (Fig. 2) indicates that infection by a single virus particle suffices to kill a cell (23, 24, 25). Based on a Poisson distribution of virus particles among the cell population, the surviving colonies represent the fraction of cells which, by chance, failed to encounter a CKP. All cells that fail to produce colonies are presumed to have received one or more CKP.

The disproportionately greater-than-expected fraction of cell survivors at higher multiplicities indicates that the effective m_{ckp} is less than expected from a Poisson distribution of particles in the cell population. As a working hypothesis, we attribute this anomaly to the action of large quantities of virion-associated neuraminidase delivered to the cells simultaneously during virus attachment at high multiplicities, the subsequent destruction of sialic acid-terminating receptors, and the ensuing elution of virus. Evidence supporting this view is offered in an earlier study of cell killing by Newcastle disease virus, another neuraminidase-containing virus (24). It showed that when a high multiplicity of virus was attached in three separate low-multiplicity (pulsed) infections, the cell survival curve more closely approached that expected from the total multiplicity delivered. Furthermore, this high-multiplicity anomaly

was not observed with cell killing by VSV, a virus lacking neuraminidase activity (31). The nature of the sialic acid-terminating receptors may also influence virus elution since it was possible to obtain a clone of HeLa cells as hosts to Newcastle disease virus which generated a cell-killing survival curve that more closely fit that expected from the Poisson distribution of particles among the cell population (28).

Although the ratio of CKP to PFP of 1 observed in populations of the NS1$_{1-124}$ variant formally could represent two separate virus subpopulations of equal titers, it seems more likely that only infectious particles are capable of killing cells in the absence of the effector region of NS1. In this context, MDCK cells infected with the truncated NS1 protein expressed ectopically failed to induce apoptosis but did so when NS1$_{1-124}$ was expressed following infection with intact virus particles (39). This suggests that caution should be used in interpreting the role of viral genes in apoptosis when expressed ectopically. The use of intact virus particles mimics more closely the multiple events that regulate apoptosis in virus-infected cells (19, 20, 21).

In contrast to cell killing by the NS1$_{1-124}$ variant, the sevenfold excess of CKP over PFP observed in a pooled population of NS1$_{1-230}$ revealed the presence of a heretofore unrecognized particle in type A influenza virus populations—noninfectious CKP. This meant that infectivity per se was not a prerequisite for cell killing by the variant with fully functional NS1. This also proved to be the case with the NS1$_{1-124}$ variant because noninfectious and infectious CKP were both about six times more resistant to UV radiation (Fig. 3) or heat (Fig. 4) than PFP.

Which influenza virus gene, or genes, if any, need to be expressed to kill a cell, i.e., induce apoptosis? We addressed this question by comparing the observed rate of inactivation of CKP following UV irradiation (Fig. 3), with the rate of inactivation of each of the eight genes, and of infectivity (PFP) as reported by Abraham (1) and verified by others. Since each of the eight genes is transcribed independently of the others, the rate of loss of activity after exposure to UV radiation is related to the size or length of the genome. The larger the gene, the larger the target for inactivation by UV radiation and the steeper the slope of the survival curve. Since all eight genes of influenza virus are required for infectivity (measured by PFP activity), the UV target constitutes the sum of the eight genes, i.e., ~13,750 nucleotides (nt) and hence defines the steepest slope of the UV inactivation curves. If the functions of all three of the polymerase subunit genes were required, inactivation of any one gene would prevent cell killing. The target for the sum of these three genes is ~6,900 nt; i.e., it constitutes about 50% of the target for infectivity. The theoretical slope of inactivation of CKP under these conditions is shown as a dotted line in Fig. 6 (PA+PB1+PB2). If only two of the three polymerase subunit genes were required, the smaller target would be expected to result in a rate of inactivation slightly less steep than the one defined by the summed target of the three polymerase genes and is shown in a second dotted line in Fig. 6 (any two of PA, PB1, and PB2). The expected rate of inactivation of any one of the three polymerase genes, or of the other five genes, is shown by the dashed lines taken from the data of Abraham (1), where the rates of inactivation reflect the size of the gene and its independent expression. Note that a single slope rep-
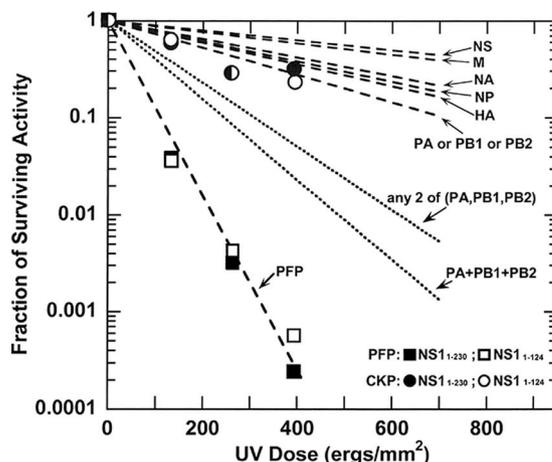

FIG. 6. Comparison of observed CKP and PFP survival curves (data points) with those expected from UV target theory for each independently transcribed gene (upper dashed lines) (1) and for infectivity, the latter represented by a lethal hit in any of the individual eight genes required to express PFP activity (lower dashed line) (1). The effective size of the UV target is taken as proportional to the number of nucleotides in the gene (1). Theoretical curves based on UV target analysis are presented representing the slopes of the survival curves expected if the function of all three, or two of the three, polymerase subunit genes were required for the expression of CKP activity (dotted lines).

resents the rate of inactivation of each of the polymerase subunit genes since their sizes are similar at about 2,300 nt (1). Our data for the rate of inactivation of CKP activity (Fig. 3) are superposed on these observed rates of inactivation and fit best the dashed line which represents inactivation of one of the three polymerase subunit genes. It is not possible to distinguish among them because of the similarity of their sizes.

Based on this analysis, we postulate that one of the polymerase subunit genes must be functional in order to express CKP activity. Since the polymerase subunits of NS1$_{1-230}$ and NS1$_{1-124}$ variants of TK/OR/71 have identical amino acid sequences (5), this requirement appears to be necessary, but not sufficient, to generate noninfectious CKP. Consequently, based on the absence of noninfectious CKP in populations where the integrity of the NS1 gene product is compromised, we further postulate that some NS1 function, not yet identified, may be required for the generation of noninfectious CKP. The reports that a site on the influenza virus NS1 protein can temporally regulate viral RNA synthesis (36) and that apoptosis correlates with the efficiency of viral mRNA synthesis (41) indicate that NS1 may affect a polymerase subunit and thus play yet another role in regulating events in the influenza virus-infected cell (16).

We note with interest that, like noninfectious CKP, defective-interfering particles (DIP) represent another class of noninfectious particles that can be found in populations of influenza virus. Furthermore, the generation of DIP may be regulated by particular polymerase genes (2, 45), if not a single polymerase subunit, PA (10, 11). The possible relationship between noninfectious CKP and DIP is under investigation.

The action spectrum for UV absorption indicates that doses of UV required to inactivate the RNA of polymerase genes do not suffice to inactivate the polymerase proteins extant in the

virion. Consequently, some virion-associated primary transcription may occur and might prove adequate to produce a threshold amount of the cell-killing factor(s) required to initiate apoptosis. Although new polymerase activity appears to be rate limiting for CKP expression as inferred from the UV target for CKP inactivation, left unanswered are which viral gene(s) must be expressed by the input virus particle for it to function as a CKP or AIP. Although virtually all influenza virus gene products have been implicated in the induction of apoptosis, the extent to which they act alone or in concert, possibly along with viral double-stranded RNA (22), is not well understood (19, 20, 21).

The relative resistance of CKP activity to thermal inactivation, whether of an infectious or a noninfectious nature, may reflect the stability of virion-bound polymerase because of its close association with viral RNP (4) to produce the product(s) required to initiate apoptosis. In this context, exposure to heat that inactivates >90% of infectivity leaves >50% of the CKP activity functional (Fig. 4) and actually enhances the IFN-inducing activity of the variant $NS1_{1-230}$ (30).

Yet another viral synthetic event might initiate apoptosis, i.e., the accumulation of influenza virus mRNA and/or its temporal disruption. The onset and rapidity of apoptosis in MDCK cells correlated with the accumulation of viral mRNA transcripts (41). Furthermore, loss of the temporal regulation of viral RNA involves a site on the NS1A protein (36) that is missing in variant $NS1_{1-124}$ (5, 37, 42). This raises an intriguing question. Does a threshold of viral mRNA transcripts per se suffice to initiate apoptosis? Support, but not proof, for this model comes from analyses of clonogenic assay-generated survival curves of Vero cells exposed to another negative-strand RNA virus, VSV (32), that also kills cell by apoptosis (15, 21). The rates of inactivation of CKP and virion-associated transcriptase were similar following UV irradiation, relative to the rate of inactivation of PFP. They indicated that transcription of about 25% of the genome sufficed to initiate cell killing (apoptosis) by VSV (32). Exposure of VSV to heat (50°C) revealed that PFP, CKP, and virion transcriptase activities were inactivated at the same rate, demonstrating that some primary transcription was required and was rate limiting for cell killing (32). Whether the accumulation of primary transcripts per se sufficed to initiate apoptosis or synthesis of N and/or P, products of the two genes closest to the 3′ end, was not determined. This finding does not preclude the possibility of other VSV gene products, like M (14), or double-stranded RNA formation itself, playing a role in the induction of apoptosis. The latter process may be important as a modulator of apoptosis (17, 21).

The delayed expression of apoptosis in the variant producing full-size NS1 (Fig. 5) is in keeping with the report that NS1 expressed by PR/8/34(H1N1) in MDCK cells, chicken embryo cell cultures, or 7-day-old chicken embryos demonstrates antiapoptotic activity relative to that of its variant lacking the NS1 gene (48). This antiapoptotic activity was attributed to the IFN competence of these cell systems since in Vero cells, lacking the type I IFN genes, the two variants showed no differences with respect to the temporal events in apoptosis. However, we observed a delay of apoptosis in Vero cells with the TK/OR/71-$NS1_{1-230}$ variant relative to that expressing truncated $NS1_{1-124}$ (Fig. 5) (6, 33). This difference was most

noticeable early in infection (12 to 15 hpi). In our studies, the multiplicity of infection was set equivalent to *CKP*, not *PFP*, meaning that virtually all of the apoptosis observed with variant $NS1_{1-230}$ was initiated with noninfectious AIP. This may account for the differences reported in the two studies. Nonetheless, even cells showing delayed expression of apoptosis were eventually killed by the noninfectious CKP, as noted in CKP clonogenic assays (Fig. 3), and by the extensive cytopathic effect that developed in infected cell monolayers. Our data suggest that, in the absence of replication, the noninfectious CKP produce sufficient NS1 protein to delay apoptosis.

The apoptosis induced by influenza virus in Vero cells known to lack type IFN-I (α/β) genes (6) demonstrates that IFN is not essential for cell killing by influenza virus. These results contrast with those reported for picornavirus-infected mouse cells (44) and the cell sparing imposed on Vero cells by exogenously added IFN against the lethal action of VSV (33). However, in this context, we recently observed that influenza virus-induced apoptosis is exacerbated in IFN-treated Vero cells following infection with TK/OR/71-$NS1_{1-124}$ and much less so after infection with the $NS1_{1-230}$ variant. Yet, IFN can spare Vero cells from influenza virus-mediated apoptosis (data not shown).

These data demonstrate cell killing through apoptosis. Apoptotic cells gently shaken off from Vero cell monolayers at 12 hpi with either of the variants and stained vitally with Hoechst 33342 revealed nuclear fragmentation typical of apoptosis (data not shown). However, there are clear differences displayed in temporally regulated morphological changes (Fig. 5). Infection with variant $NS1_{1-124}$ at $m_{ckp} = m_{pfp} = 7$ results in a rapid rounding of cells into small, highly refractile, spherical, or sometimes spindle-shaped bodies that lift from the substrate with extensive blebbing at the cell surface and loss of refractility, ending with small, shrunken, and disintegrating cells (Fig. 5D). In contrast, in cells exposed to variant $NS1_{1-230}$ at $m_{ckp} = 7$, where $m_{pfp} = 1$, all facets of the time course of apoptosis were delayed (Fig. 5B). Notably, cells were slow to round up and to reach a refractile state, though they slowly progressed to a stage where the large, less refractile cells retracted further and accumulated above the monolayer, where blebbing finally occurred. This was followed by cell disintegration and, with time, the accumulation of large-size debris. Vero cells infected at $m_{ckp} = 7$ with either variant and examined microscopically after plating as single cells rarely divided, indicating that the onset of apoptosis was rapid enough to preclude cell division.

Analysis of two influenza virus populations that differ primarily in the integrity of the NS1 they express has revealed subpopulations of particles with distinctive, quantifiable biological activities. The most frequently measured phenotype of viruses is infectivity, often quantified as PFP. In influenza virus, this phenotype represents about 5% of the physically identifiable particles observed by electron microscopy (9). When scored as hemagglutinating particles, the numbers more closely approach those associated with physical particles (18). Populations of influenza viruses may contain another phenotype important in pathogenesis, IFN-inducing particles (5, 26, 30), that also approach the numbers of physical particles, as do IFN induction-suppressing particles (30, 34). This report quantifies another heretofore unidentified phenotype in influenza virus populations—cell killing by noninfectious particles.

It is noteworthy that microscopic examination of scores of fields of cells in a monolayer which provide estimates of the fraction of cells that undergo apoptosis initiated with infectious or noninfectious CKP are consistent with the fraction of cells expected to be infected with one or more CKP as scored by the clonogenic assay. The 10-egg pool of variant $NS1_{1-230}$ used most in this study consistently revealed a ratio of noninfectious CKP to PFP of 7. Separate populations obtained from three plaque isolates from this pool and propagated individually in eggs revealed ratios of CKP to PFP of ~4. While this variation may reflect in part the quasispecies nature of RNA viruses (29), it is inconsistent with the constancy of the CKP-to-PFP ratio of 1 observed in populations of the $NS1_{1-124}$ variant from several different egg-derived stocks. The degree of variability in the noninfectious CKP-to-PFP ratio is under study. Work in progress indicates that the ratio of noninfectious CKP to PFP may exceed 100:1.

The definition and quantification in influenza virus populations of phenotypes other than cell killing, e.g., by IFN-inducing particles (30), IFN induction-suppressing particles (30), and DIP (7), that also are often in excess of infectious virus, may contribute to the regulation of pathogenesis or stimulation of the adaptive immune system. Further studies are needed to determine the extent to which this complex mixture of phenotypes in influenza virus populations regulates infectivity, pathogenesis, and vaccine efficacy.

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ADDENDUM IN PROOF

We have found that high-multiplicity passages of avian virus stocks which contain a 7:1 ratio of noninfectious cell-killing particles to plaque-forming particles rapidly generate ratios of noninfectious cell-killing particles to plaque-forming particles of >1,000:1. Thus, under conditions known to generate defective-interfering particles, i.e., von Magnus particles, noninfectious cell-killing particles accumulate in large numbers at the expense of plaque-forming particles. Ongoing experiments indicate that the phenotypes expressed in cells by nonfectious cell-killing particles or by noninfectious defective-interfering particles may result from a common viral genotype (unpublished data).

REFERENCES

1. **Abraham, G.** 1979. The effect of ultraviolet radiation on the primary transcription of influenza virus messenger RNAs. Virology **97:**177–182.
2. **Akkina, R. K., T. M. Chambers, and D. P. Nayak.** 1984. Mechanism of interference by defective-interfering particles of influenza virus: differential reduction of intracellular synthesis of specific polymerase proteins. Virus Res. **1:**687–702.
3. **Appleyard, G., and H. B. Maber.** 1974. Plaque formation by influenza viruses in the presence of trypsin. J. Gen. Virol. **25:**351–357.
4. **Brownlee, G. G., and J. L. Sharps.** 2002. The RNA polymerase of influenza A virus is stabilized by interaction with its viral RNA promoter. J. Virol. **76:**7103–7113.
4a. **Carver, D. H., and P. I. Marcus.** 1967. Enhanced interferon production from chick embryo cells aged in vitro. Virology **32:**247–257.
5. **Cauthen, A. N., D. E. Swayne, M. J. Sekellick, P. I. Marcus, and D. L. Suarez.** 2007. Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J. Virol. **81:**1838–1847.
6. **Diaz, M. O., S. Zeimin, M. M. Le Beau, P. Pitha, S. D. Smoth, R. R. Chilcote, and J. D. Rowley.** 1988. Homozygous deletion of the α- and β_1-interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. USA **85:**5259–5263.
7. **Dimmock, N. J., and A. C. Marriott.** 2006. In vivo antiviral activity: defective interfering virus protects better against virulent influenza A virus than avirulent virus. J. Gen. Virol. **87:**1259–1265.
8. **Ehrhardt, C., T. Wolff, S. Pleschka, O. Planz, W. Beermann, J. G. Bode, M. Schmolke, and S. Ludwig.** 2007. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J. Virol. **81:**3058–3067.
9. **Flint, S. J., L. W. Enquist, V. R. Racaniello, and A. M. Skalka (ed.).** 2004. Principles of virology: molecular biology, pathogenesis, and control of animal viruses, 2nd ed., p. 35. ASM Press, Washington, DC.
10. **Fodor, E., L. J. Mingay, M. Crow, T. Deng, and G. G. Brownlee.** 2003. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase promotes the generation of defective interfering RNAs. J. Virol. **77:**5017–5020.
11. **Fodor, E., and G. G. Brownlee.** 2002. Influenza virus replication, p. 1–29. In C. W. Potter (ed.), Influenza. Elsevier, Amsterdam, The Netherlands.
12. **Hinshaw, V. N., C. W. Olsen, N. Dybdahl-Sissoko, and D. Evans.** 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. **68:**3667–3673.
13. **Kaverin, N. V., and R. G. Webster.** 1995. Impairment of multicycle influenza virus growth in Vero (WHO) cells by loss of trypsin activity. J. Virol. **69:**2700–2703.
14. **Kopecky, S. A., M. C. Willingham, and D. S. Lyles.** 2001. Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus. J. Virol. **75:**12169–12181.
15. **Koyama, A. H.** 1995. Induction of apoptotic DNA fragmentation by the infection of vesicular stomatitis virus. Virus Res. **37:**285–290.
16. **Lamb, R. A., and R. M. Krug.** 2001. Orthomyxoviridae: the viruses and their replication, p. 725–769. In D. M. Knipe and P. M. Howley (ed.), Fundamental virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
17. **Lee, S. B., and M. Esteban.** 1994. The interferon-induced double-stranded RNA-activated-protein kinase induces apoptosis. Virology **199:**491–496.
18. **Levine, S., T. T. Puck, and B. P. Sagik.** 1953. An absolute method for assay of virus hemagglutinins. J. Exp. Med. **98:**521–531.
19. **Lowy, R. J.** 2003. Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms. Int. Rev. Immunol. **22:**425–449.
20. **Ludwig, S.** 2006. Signaling and apoptosis in influenza virus-infected cells, p. 323–340. In Y. Kawaoka (ed.), Influenza virology: current topics. Caister Academic Press, Norfolk, England.
21. **Lyles, D. S.** 2000. Cytopathogenesis and inhibition of host gene expression by RNA viruses. Microbiol. Mol. Biol. Rev. **64:**709–724.
22. **Majde, J. A., N. Guha-Thakurta, Z. Chen, S. Bredow, and J. M. Krueger.** 1998. Spontaneous release of stable viral double-stranded RNA into the extracellular medium of influenza virus-infected MDCK endothelial cells: implications for the viral acute-phase response. Arch. Virol. **143:**2371–2380.
23. **Marcus, P. I.** 1959. Host-cell interaction of animal viruses. II. Cell-killing particle enumeration: survival curves at low multiplicities. Virology **9:**546–563.
24. **Marcus, P. I.** 1959. Symposium on the biology of cells modified by viruses or antigens. IV. Single-cell techniques in tracing virus-host interactions. Bacteriol. Rev. **23:**232–249.
25. **Marcus, P. I.** 1977. Cell killing by viruses: single-cell survival procedures for detecting viral functions required for cell killing, p. 192–213. In C. Boreck, C. M. Fenoglio, and D. W. King (ed.), Differentiation and carcinogenesis: advances in pathobiology no. 6. Stratton Intercontinental Medical Book Corp., New York, NY.
26. **Marcus, P. I.** 1986. Interferon induction dose-response curves. Methods Enzymol. **119:**106–114.
27. Reference deleted.
28. **Marcus, P. I., and T. T. Puck.** 1958. Host-cell interaction of animal viruses. I. titration of cell-killing by viruses. Virology **6:**405–423.
29. **Marcus, P. I., L. L. Rodriguez, and M. J. Sekellick.** 1998. Interferon induction as a quasispecies marker of vesicular stomatitis virus populations. J. Virol. **72:**542–549.
30. **Marcus, P. I., J. M. Rojeck, and M. J. Sekellick.** 2005. Interferon induction and/or production and its suppression by influenza A viruses. J. Virol. **79:**2880–2890.
31. **Marcus, P. I., and M. J. Sekellick.** 1974. Cell killing by viruses. I. Comparison of cell-killing, plaque-forming, and defective-interfering particles of vesicular stomatitis virus. Virology **57:**321–338.
32. **Marcus, P. I., and M. J. Sekellick.** 1975. Cell killing by viruses. II. Cell killing by vesicular stomatitis virus: A requirement for virion-derived transcription. Virology **63:**176–190.
33. **Marcus, P. I., and M. J. Sekellick.** 1976. Cell killing by viruses. III. The interferon system and inhibition of cell killing by vesicular stomatitis virus. Virology **69:**378–393.
34. **Marcus, P. I., and M. J. Sekellick.** 1985. Interferon induction by viruses.

XIII. Detection and assay of interferon induction-suppressing particles. Virology **142:**411–415.

35. **Marvaldi, J. L., J. Lucad-Lenard, M. J. Sekellick, and P. I. Marcus.** 1977. Cell killing by viruses. IV. Cell killing and protein synthesis inhibition by vesicular stomatitis virus require the same gene functions. Virology **79:**267–280.

36. **Min, J.-Y., S. Li, G. C. Sen, and R. M. Krug.** 2007. A site on the influenza virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology **363:**236–243.

37. **Norton, G. P., T. Tanaka, K. Tobita, S. Nakada, D. A. Buonagurio, D. A. Greenspan, M. Krystal, and P. Palese.** 1987. Infectious influenza A virus and B virus variants with long carboxyl terminal deletions in the NS1 polypeptides. Virology **156:**204–213.

38. **Qian, X.-Y., F. Alonso-Caplen, and R. M. Krug.** 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. J. Virol. **68:**2433–2441.

39. **Schultz-Cherry, S., N. Dybdahl-Sisskoko, G. Neumann, Y. Kawaoka, and V. Hinshaw.** 2001. Influenza virus NS1 protein induces apoptosis in cultured cells. J. Virol. **75:**7875–7881.

40. **Sekellick, M. J., and P. I. Marcus.** 1986. Induction of high titer chick interferon. Methods Enzymol. **119:**115–125.

41. **Stray, S. J., and G. M. Air.** 2001. Apoptosis by influenza viruses correlates with efficiency of viral mRNA synthesis. Virus Res. **77:**3–17.

42. **Suarez, D. L., and M. L. Perdue.** 1998. Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Res. **54:**59–69.

43. **Takizawa, T., S. Matsukawa, Y. Higuchi, S. Nakamura, Y. Nakanishi, and R. Fukuda.** 1993. Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. J. Gen. Virol. **74:**2347–2355.

44. **Tanaka, N., M. Sato, M. S. Lamphier, H. Nozawa, E. Oda, S. Noguchi, R. D. Schreiber, Y. Tsujimotot, and T. Taqniguchi.** 1998. Type I interferons are essential mediators of apoptotic death in virally infected cells. Genes Cells **3:**29–37.

45. **Ueda, M., K. Nakajima, and A. Sugiura.** 1980. Extra RNAs of von Magnus particles of influenza virus caused by particular polymerase genes. J. Virol. **34:**1–8.

46. **Vogt, M., and R. Dulbecco.** 1958. Properties of a HeLa cell culture with increased resistance to poliomyelitis virus. Virology **5:**425–434.

47. **Zhirnov, O. P., and H.-D. Klenk.** 2007. Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. Apoptosis **12:**1419–1432.

48. **Zhirnov, O. P., T. E. Konakova, T. Wolff, and H.-D. Klenk.** 2002. NS1 protein of influenza virus A down-regulates apoptosis. J. Virol. **76:**1617–1625.

49. **Zhirnov, O. P., A. V. Ovcharenko, and A. C. Bukrinskaya.** 1982. A modified plaque assay method for accurate analysis of infectivity of influenza viruses with uncleaved hemagglutinin. Arch. Virol. **71:**177–183.