JOURNAL OF VIROLOGY, June 1994, p. 3667–3673
0022-538X/94/$04.00+0

Vol. 68, No. 6

Apoptosis: a Mechanism of Cell Killing by Influenza A and B Viruses

VIRGINIA S. HINSHAW,* CHRISTOPHER W. OLSEN, NAOMI DYBDAHL-SISSOKO, AND DAVID EVANS

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received 14 December 1993/Accepted 7 February 1994

In previous studies, we observed that the virulent avian influenza A virus A/Turkey/Ontario/7732/66 (Ty/Ont) induced severe lymphoid depletion in vivo and rapidly killed an avian lymphocyte cell line (RP9) in vitro. In examining the mechanism of cell killing by this virus, we found that Ty/Ont induced fragmentation of the RP9 cellular DNA into a 200-bp ladder and caused ultrastructural changes characteristic of apoptotic cell death by 5 h after infection. We next determined that the ability to induce apoptosis was not unique to Ty/Ont. In fact, a variety of influenza A viruses (avian, equine, swine, and human), as well as human influenza B viruses, induced DNA fragmentation in a permissive mammalian cell line, Madin-Darby canine kidney (MDCK), and this correlated with the development of a cytopathic effect during viral infection. Since the proto-oncogene *bcl-2* is a known inhibitor of apoptosis, we transfected MDCK cells with the human *bcl-2* gene; these stably transfected cells (MDCKbcl-2) did not undergo DNA fragmentation after virus infection. In addition, cytotoxicity assays at 48 to 72 h after virus infection showed a high level of cell viability for MDCKbcl-2 compared with a markedly lower level of viability for MDCK cells. These studies indicate that influenza A and B viruses induce apoptosis in cell cultures; thus, apoptosis may represent a general mechanism of cell death in hosts infected with influenza viruses.

Influenza A viruses continue to cause significant disease problems in humans, other mammals, and birds; however, the actual mechanism(s) by which these viruses kill specific host cells remains undefined. Because the virulent avian influenza A viruses produce devastating disease, infection by these viruses is frequently used as a model system to examine mechanisms of disease. Our own studies have focused on a well-characterized avian strain, A/Turkey/Ontario/7732/66 (H5N9) (Ty/Ont); this highly virulent strain rapidly induces severe lymphoid depletion in birds (18, 32, 33). We hypothesized that this pronounced lymphocyte death is due to apoptosis, or programmed cell death, a well-defined process in lymphocytes (28).

There are two major modes of cell death, i.e., necrosis and apoptosis, and these differ both morphologically and biochemically. Apoptosis is characterized morphologically by cell shrinkage and hyperchromatic nuclear fragments and biochemically by chromatin cleavage into nucleosomal oligomers (28). Apoptosis has now been documented for many cell types in addition to lymphocytes. It can occur in response to stimuli ranging from toxic substances to hormone and cytokine addition or withdrawal (7, 28) and, most importantly from our perspective, viral infections. There is mounting evidence that the induction of apoptosis contributes directly to the pathogenesis of a number of viruses, such as chicken anemia virus (16, 22), feline leukemia virus (27), feline immunodeficiency virus (23), and, most significantly, human immunodeficiency virus type 1 (9). Thus, we initiated studies to determine if Ty/Ont induced apoptosis of an avian lymphocyte cell line, RP9, as evidenced by DNA fragmentation, morphologic changes, and cell death. The results clearly support the theory that Ty/Ont is able to induce apoptotic cell death.

We next extended our studies to mammalian viruses and cells. Since our initial results with RP9 cells indicated that virus infection was required to induce apoptosis, we chose to use MDCK, a cell line permissive for both infection and productive replication of a variety of influenza viruses. Our results clearly indicated that all of the mammalian, as well as all of the avian, influenza viruses tested induce apoptosis in MDCK cells. In agreement with our studies, Takizawa and colleagues very recently published results demonstrating that the human strain A/Udorn/72 (H3N2) induces apoptosis in MDCK and HeLa cells (30).

Recent studies with Sindbis virus (19) showed that the proto-oncogene *bcl-2* blocked apoptosis induced by that virus. *bcl-2* blocks apoptosis in many systems (8, 15), but the mechanism for this is not yet defined. In the studies described in this paper, MDCK cells transfected with *bcl-2* were resistant to influenza virus-induced apoptosis. These studies may indicate that apoptosis is a general mechanism by which influenza viruses kill cells and, therefore, that these viruses can be blocked by cellular inhibitors of apoptosis.

MATERIALS AND METHODS

Virus growth and cell culture. The following viruses were obtained from the influenza virus repository at the University of Wisconsin—Madison: avian strains Ty/Ont, A/Turkey/Oregon/1/71 (H7N3), A/Mallard/Wisconsin/994/82 (H5N2), A/Duck/Ukraine/1/63 (H3N8), A/Chicken/Pennsylvania/10656/86 (H5N2), and A/Tern/South Africa/61 (H5N3); equine strain A/Equine/Miami/1/63 (H3N8); swine strains A/Swine/Nebraska/1/92 (H1N1) (24), A/Swine/Wisconsin/1915/88 (H1N1) (20), A/Swine/Indiana/1726/88 (H1N1), and A/Swine/Iowa/4340/93 (H1N1); and human strains A/PR/8/34 (H1N1) (PR8), A/Japan/305/57 (H2N2), A/Udorn/307/72 (H3N2), A/Maryland/1/91 (H1N1) (a human isolate of swine origin [36]), B/DB/44, and B/Lee/40. Viruses were propagated in the allantoic cavities of 11-day-old embryonated chicken eggs for 48 to 72 h at 35°C; the allantoic fluid was harvested and stored at −70°C. For experiments requiring inactivated virus, allantoic fluid was

* Corresponding author. Mailing address: Dept. of Pathobiological Sciences, University of Wisconsin—Madison, 2015 Linden Dr., Rm. 3174, Madison, WI 35706. Phone: (608) 263-2792. Fax: (608) 263-6573. Electronic mail address: hinshawv@svm.vetmed.wisc.edu.

treated with beta-propionolactone (Sigma Chemical Co., St. Louis, Mo.) as previously described (21).

The RP9 avian lymphocyte cell line, kindly provided b y M. A. Qureshi (North Carolina State University), was grown in equal volumes of L-15 and McCoy's media (GIBCO-BRL Life Sciences, Grand Island, N.Y.) supplemented with 8% fetal bovine serum, 10% chicken serum, 0.5% tryptose phosphate broth, 1% sodium pyruvate, 10 μM 2-mercaptoethanol, and penicillin-streptomycin, as previously described (26). MDCK cells were grown in modified Eagle's medium (MEM) (GIBCO) supplemented with 10% fetal bovine serum and penicillin-streptomycin.

Cytotoxicity assay with XTT. Confluent monolayers of cells were prepared in 96-well cell culture plates; the monolayers were washed once with phosphate-buffered saline (PBS) and then inoculated with 25 μl of allantoic fluid containing Ty/Ont (dilutions of 10^{-3} and 10^{-4}). After 30 min at 37°C, the monolayers were washed three times with PBS, and 100 μl of MEM with 5% bovine serum albumin (BSA) was added. The plates were incubated for 0, 5, 24, 48, and 72 h. At each time point, the supernatants were removed; 50 μl of a solution containing 1.0 mg of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT; sterile filtered) per ml, 0.0075 mg of phenazine methosulfate per ml, and 50 μl of phenol red-free RPMI medium were added to each well (29). After 30 min, the A_{450} was read. Percent viability was calculated by the following formula: [(infected cells − blank)/(uninfected cells − blank)] × 100. We compared the values for percent viability measured by trypan blue exclusion with those measured by the XTT assay and found that the percents viability calculated by the two methods were very similar; however, the XTT assay was more consistent.

Electron microscopy. RP9 avian lymphocytes were pelleted by centrifugation, washed twice with PBS to remove serum, and then fixed in Karnovsky solution. The cells were embedded in Epon araldite; thin sections (70 nm) were stained with lead citrate and uranyl acetate and then examined with a Philips 410 microscope.

DNA fragmentation assay. Fragmentation of cellular DNA into the characteristic apoptotic ladder was assessed as previously described (12), with slight modifications of the lysis conditions. Briefly, approximately 10^7 cells were washed with MEM or RPMI 1640 and then inoculated with virus in media or mock inoculated with media alone and incubated at 37°C for 45 min. Allantoic fluid containing 10^7 to 10^9 for 50% infective doses of inoculated eggs was used for the virus inoculum to provide a multiplicity of infection (MOI) (1 to 100 50% egg infective doses per cell) sufficient to ensure infection of all cells for the short-term (5-h) apoptosis assays. After virus attachment was allowed to occur, the cells were washed in media to remove nonadsorbed virus and incubated for various periods at 37°C in MEM with 5% BSA. To harvest DNA, the cells were washed in PBS, resuspended in 600 μl of ice-cold lysis buffer (10 mM Tris, 0.5% Triton X-100 [Sigma] [pH 7.5]), and incubated on ice for 30 min. After centrifugation of the lysates for 10 min at 13,000 × *g* at 4°C, the supernatants were extracted once with buffered phenol and once with buffered phenol-chloroform, and DNA was ethanol precipitated in the presence of 300 mM NaCl. DNA samples were resuspended in 15 μl of sterile Tris-EDTA buffer (10 mM Tris, 1 mM EDTA [pH 7.5]), treated with RNase A (Sigma) to a final concentration of 1.0 μg/μl, electrophoresed through 2% GTG SeaKem agarose (FMC BioProducts, Rockland, Maine) in Boyer's buffer (500 mM Tris, 200 mM sodium acetate, 20 mM EDTA, 180 mM NaCl [pH 8.05]) at 4°C by using a SuperSub unit (Hoefer Scientific Instruments, San Francisco, Calif.), and stained with ethidium bromide. A 1-kb DNA ladder (GIBCO) was used in the gels for sizing linear fragments ranging in size from 500 bp to 12 kb.

To assess the ability of Zn^{2+} ions to block endonucleolytic DNA cleavage (6), $ZnSO_4$ (Sigma) was added to the cultures to a final concentration of 5 mM at 0, 2, or 4 h after infection. Then, at 5 h postinfection (p.i.), cellular DNA was analyzed as described above.

IFA labelling and Western immunoblotting. Indirect immunofluorescent-antibody (IFA) labelling of cells was conducted on Cytospin (Shandon Inc., Pittsburgh, Pa.) cell preparations. Virus infection was conducted as described above. Mouse monoclonal antibodies to the nonstructural protein (NS) (2) and nucleoprotein (NP) (34) of A/WSN/33 and to the H5 hemagglutinin of A/Chicken/Pennsylvania/83 were kindly provided by R. G. Webster (St. Jude Children's Research Hospital). Rabbit antiserum to amino acids 41 to 56 of the human bcl-2 protein was kindly provided by J. C. Reed (LaJolla Cancer Research Foundation). Cells were reacted with these primary antibodies at a dilution of 1:500 for 45 min at room temperature, washed with PBS, and similarly reacted with a 1:1,000 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G or goat anti-rabbit immunoglobulin G (Organon Teknika, Durham, N.C.)

bcl-2 expression in transfected MDCK cells was also evaluated by Western blotting. Cell lysate preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were conducted by using standard techniques (5). Proteins were electrophoretically transferred to nitrocellulose with a Novablot apparatus (LKB Produkter AB, Bromma, Sweden). Blots were developed by using the rabbit anti-human bcl-2 peptide antisera at a dilution of 1:1,500 and the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, Calif.) with a diaminobenzidine and nickel chloride substrate for horseradish peroxidase (5).

Transfections of cell cultures. MDCK cells were stably transfected with pZIPbcl-2, a vector expressing the coding region (a 910-bp *Eco*RI fragment) from the human *bcl-2* gene (pB4) expressed from a Moloney murine leukemia virus promoter (3, 31); pZIPneo is the parental plasmid lacking the *bcl-2* coding region. The plasmids were kindly supplied by J. C. Reed (LaJolla Cancer Research Foundation) and J. M. Hardwick (Johns Hopkins University). Each plasmid was initially amplified by transformation of DH10B *Escherichia coli* bacteria using standard techniques (1). MDCK cells were transfected by using Lipofectin reagent (GIBCO) according to the manufacturer's protocol. Optimum conditions for transfection of cells in 35-mm-diameter cell culture wells were determined to be 2 to 5 μg of plasmid DNA plus 15 μl of Lipofectin reagent (in 1 ml of MEM) per well of MDCK cells at approximately 30% confluence. After 14 h of incubation at 37°C, MEM with 10% fetal bovine serum was added and the cells were incubated for another 36 h. Thereafter, transfected cells were selected by the addition of 800 μg of geneticin (GIBCO) per ml. Cell lines stably transfected with either pZIPbcl-2 or pZIPneo (MDCKbcl-2 and MDCKneo, respectively) were biologically cloned by colony selection under 0.5% GTG SeaKem agarose (FMC BioProducts) (MDCKbcl-2) or by limiting dilution (MDCKneo). Expression of bcl-2 was assessed by IFA labelling and Western blotting as described above.

RESULTS

Induction of DNA fragmentation in avian lymphocytes by Ty/Ont. Since DNA fragmentation is a well-defined biochem-

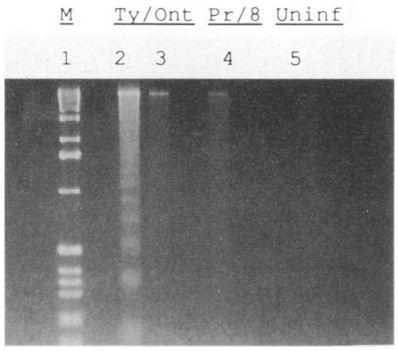

FIG. 1. DNA fragmentation analysis of RP9 avian lymphocytes infected with Ty/Ont and PR8. DNA was isolated (as described in Materials and Methods) from uninfected RP9 cells (lane 5) or RP9 cells infected for 5 h with Ty/Ont (lane 2, MOI = 1.0 [based on the 50% egg-infective dose]; lane 3, MOI = 0.01) or PR8 (lane 4, MOI = 100), electrophoresed through 2% agarose gels, and visualized by ethidium bromide staining. Lane 1 (marked M) contains the molecular weight marker used in all of the gels (1-kb DNA ladder from GIBCO for sizing linear fragments ranging in size from 500 bp to 12 kb).

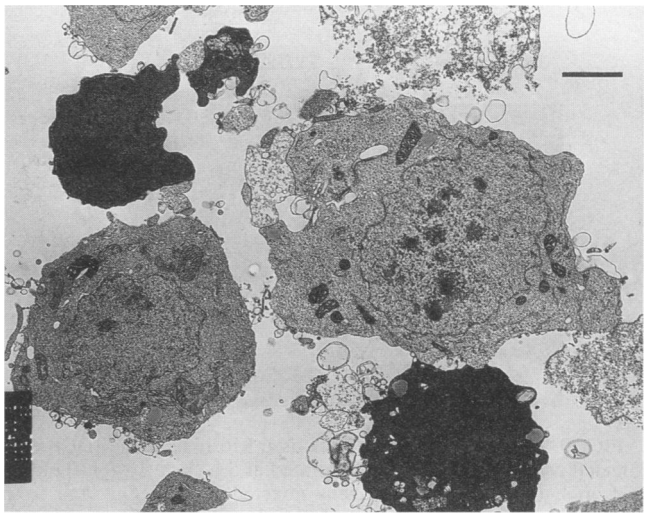

FIG. 2. Electron micrograph of RP9 cells infected with Ty/Ont at 4 h p.i. Note the blebbing and the highly condensed cells resulting from marked cytoplasmic shrinking. Bar, 2 μm.

ical marker of apoptosis (28), we first examined the abilities of Ty/Ont and PR8 to induce DNA fragmentation in the RP9 avian lymphocyte cell line. We chose this cell line on the basis of the in vivo effect of Ty/Ont on chicken lymphocytes. At 5 h p.i., the cells were harvested and their DNA was isolated. In this isolation procedure, the large, intact cellular DNA is removed; therefore, by the end of the process, only low-molecular-weight fragments remain. These fragments are subjected to agarose gel electrophoresis and visualized by staining with ethidium bromide. As shown in Fig. 1, the DNA of cells infected with Ty/Ont was fragmented into the typical 200-bp ladder by 5 h p.i., whereas no fragmentation was observed in uninfected cells or in those cells infected with PR8.

(i) Ty/Ont versus PR8. The results described above suggested a marked difference between the effect of PR8 and the effect of Ty/Ont in these cells. If viral replication were required for this fragmentation, then the difference observed could occur because PR8 didn't infect and replicate in these cells as well as Ty/Ont. To examine this, we stained the cells at 5 h p.i. with a monoclonal antibody to the NS protein, reacted them with fluorescein-conjugated anti-mouse immunoglobulin G, and then visually estimated the number of cells expressing the viral protein. With Ty/Ont, all of the cells were infected, whereas with PR8, only 10% of the cells stained were infected (data not shown). This suggested that there was a difference in the abilities of the two viruses to infect these cells.

(ii) Requirement for infectious Ty/Ont. Since the above-described results suggested that viral replication was required for the induction of DNA fragmentation, we tested the effect of beta-propionolactone-inactivated Ty/Ont on cells. When the virus was inactivated, DNA fragmentation was not detected (data not shown). This also confirms that possible virus-induced cellular mediators in the allantoic fluid inoculum are not mediating the apoptosis observed.

(iii) Effect of zinc. Zinc is a well-defined inhibitor of the endonuclease responsible for the DNA degradation observed in apoptosis (6). Thus, to determine the effect of zinc in Ty/Ont-infected RP9 cells, we added 5 mM $ZnSO_4$ at 0, 2, and 4 h p.i. and then examined the cellular DNA as described above. The addition of Zn at 0 and 2 h p.i. inhibited DNA fragmentation induced by Ty/Ont; addition at 4 h p.i. was too late to inhibit all of the DNA fragmentation, and so faint bands appeared in the gels of these DNA samples (data not shown). This time course was as expected, since Ty/Ont induces DNA fragmentation by 5 h p.i. These results indicate that the fragmentation induced by infection with Ty/Ont is inhibited by zinc in a manner similar to that of other systems.

(iv) Electron microscopic examination of infected cells. The ultrastructural changes observed in apoptotic cells include overall cell shrinkage, plasma membrane blebbing, nuclear condensation, chromatin margination, and ultimately cleavage of the cells into apoptotic bodies (28). We prepared Ty/Ont-infected RP9 cells at 4 h p.i. for electron microscopic examination. As shown in Fig. 2, the changes we noted included cell condensation and membrane blebbing in the infected cells. There was some margination of the chromatin, but this was not dramatic and was difficult to assess in light of the overall condensation of the cells. These morphological changes, in combination with the DNA fragmentation patterns, are indicative of apoptosis.

Induction of DNA fragmentation by influenza A viruses in MDCK. (i) Infection with Ty/Ont. The above-described studies showed that Ty/Ont induced apoptosis in RP9 avian lymphocytes. However, to determine if this extended to mammalian cells, we infected MDCK cells with Ty/Ont and analyzed the cellular DNA at 5, 8, and 16 h p.i. As shown in Fig. 3, DNA fragmentation was quite clear even at 5 h p.i. The fragmentation was still evident at 16 h p.i. but was less clear, presumably because cell degradation had occurred by that time. We also detected no DNA fragmentation when beta-propionolactone-inactivated Ty/Ont was added to MDCK cells; this was in agreement with the results of the RP9 studies described above and suggested that induction of apoptosis by influenza virus was not limited to avian cells or lymphocytes but did require infectious virus.

(ii) Infection with other avian and mammalian influenza viruses. We next tested whether other influenza A viruses could induce apoptosis. In this case, we looked for DNA fragmentation in cells (MDCK and MDCKneo; see below) infected with a variety of avian influenza viruses (A/Turkey/Oregon/1/71 (H7N3), A/Mallard/Wisconsin/994/82 (H5N2), A/Duck/Ukraine/1/63 (H3N8), A/Chicken/Pennsylvania/10656/86

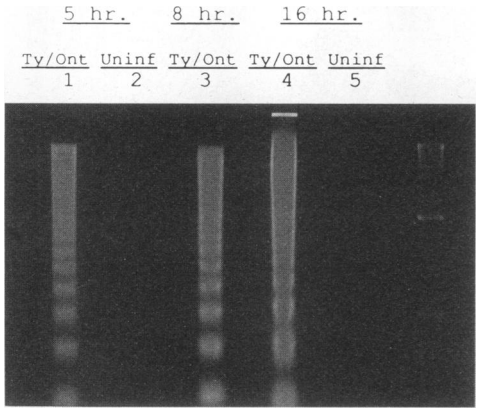

FIG. 3. DNA fragmentation analysis at 5 to 16 h p.i. of MDCK cells infected with Ty/Ont. DNA was isolated at 5 h (lane 1), 8 h (lane 3), and 16 h (lane 4) p.i. from cells infected with Ty/Ont and from uninfected cells (lanes 2 and 5), electrophoresed in 2% agarose gels, and visualized with ethidium bromide. The far right lane contains molecular weight markers.

(H5N2), A/Tern/South Africa/61, and Ty/Ont) and mammalian strains A/Equine/Miami/1/63 (H3N8) and four recent swine influenza isolates, A/Swine/Nebraska/1/92 (H1N1), A/Swine/Wisconsin/1915/88 (H1N1), A/Swine/Indiana/1726/88 (H1N1), and A/Swine/Iowa/4340/93 (H1N1). All viruses tested induced DNA fragmentation (data not shown). This indicated that other influenza viruses, both avian and mammalian, induced apoptosis.

To determine whether human influenza viruses, other than PR8, induce apoptosis, we specifically examined a number of human influenza A (H1N1, H2N2, and H3N2 subtypes) and B strains (Fig. 4). Clearly, all influenza A and B viruses induced DNA fragmentation in the MDCK cells.

Inhibition of apoptosis in MDCK cells transfected with *bcl-2*. Our studies to this point indicated that influenza viruses induce apoptosis in MDCK cells, so we next examined the effect of a known inhibitor of apoptosis, *bcl-2*. To accomplish this, we produced MDCK cell lines stably transfected with pZIPbcl-2 or with pZIPneo as a control. Expression of the bcl-2 protein was monitored by indirect IFA and Western blot

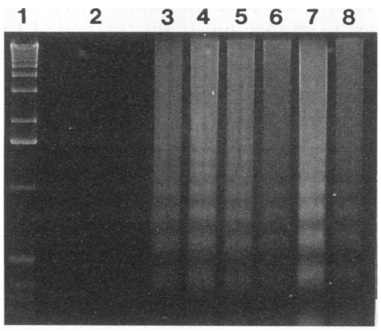

FIG. 4. DNA fragmentation in MDCKneo cells 5 h after infection with human influenza A and B viruses. DNAs were isolated from uninfected MDCKneo cells (lane 2) or MDCKneo cells infected with PR8 (lane 3), A/Udorn/307/72 (lane 4), A/Japan/305/57 (lane 5), A/Maryland/1/91 (lane 6), B/DB/44 (lane 7), or B/Lee/40 (lane 8); electrophoresed in 2% agarose gels; and visualized with ethidium bromide. Lane 1 contains molecular weight markers.

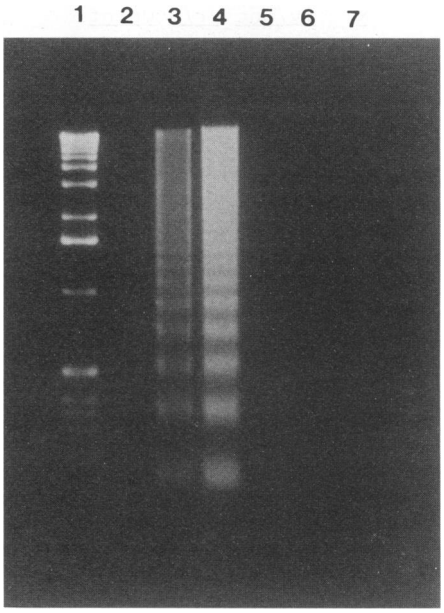

FIG. 5. Inhibition of Ty/Ont- and PR8-induced DNA fragmentation in MDCK cells expressing bcl-2 (MDCKbcl-2). DNAs were isolated from uninfected MDCK cells (lane 2), MDCK cells infected with Ty/Ont (lane 3) or PR8 (lane 4), uninfected MDCKbcl-2 cells (lane 5), or MDCKbcl-2 cells infected with Ty/Ont (lane 6) or PR8 (lane 7). The DNAs were electrophoresed in 2% agarose gels and visualized with ethidium bromide. Lane 1 contains molecular weight markers.

analyses with rabbit antisera to bcl-2. We then infected these cell lines with influenza virus and compared the DNA fragmentation patterns in untransfected MDCK and transfected MDCKbcl-2 cells. An example of these analyses is shown in Fig. 5. As evident in the gel, DNA fragmentation was clearly induced by Ty/Ont (lane 3) and PR8 (lane 4) in MDCK cells, but there was no evidence of fragmentation in the cells expressing bcl-2 (lanes 6 and 7). We have also done these same analyses using cloned MDCKbcl-2 cell lines and using the MDCKneo cells; no fragmentation has been observed in any of the 11 clonal cell lines of MDCKbcl-2 tested, whereas virus-induced apoptosis in MDCKneo cells was identical to that in untransfected MDCK cells. During the DNA fragmentation assays with Ty/Ont and PR/8, we collected cell supernatants at 24 h p.i. and then titrated them in eggs. The supernatants from the MDCKbcl-2 cells contained approximately 10-fold more infectious virus than those from the MDCK cells ($10^{5.5}$ and $10^{4.5}$ virus particles per ml, respectively). We have not examined this aspect in detail, but this result agrees with those of previous studies of Sindbis virus (19), i.e., the cells expressing bcl-2 produce more virus.

We also examined the cytotoxicity induced by Ty/Ont in cells as measured by XTT assay (29). When the virus inoculum that was used for the short-term DNA fragmentation assays was used in the XTT assays, both MDCK and MDCKbcl-2 cells died by 24 h p.i. At that time, however, DNA fragmentation was very dramatic in the MDCK cells, whereas in the MDCKbcl-2 cells only very faint bands were visible (data not shown). This suggested that the MDCKbcl-2 cells were not dying by apoptosis. It is possible that with such a large virus inoculum, the MDCKbcl-2 cells were dying because of high, potentially toxic levels of input viral proteins. We therefore repeated the

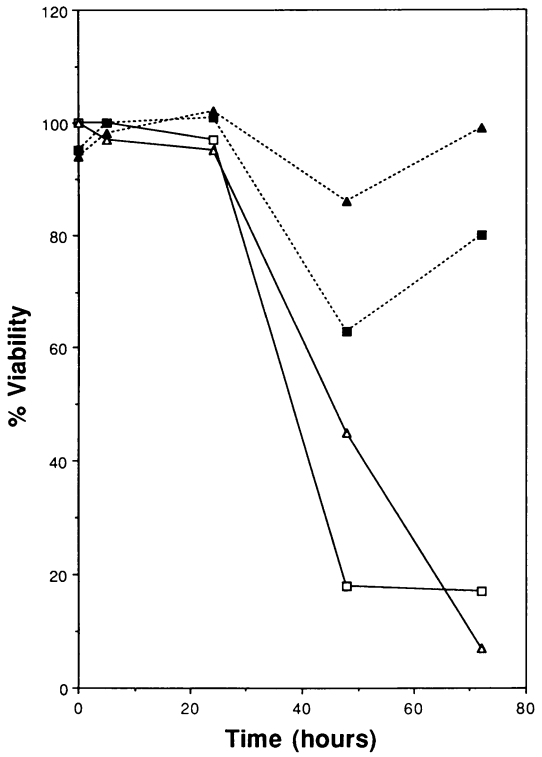

FIG. 6. Viability of MDCK and MDCKbcl-2 cells infected with Ty/Ont. MDCK cells (solid line) and MDCKbcl-2 cells (dotted line) were infected with Ty/Ont (squares, 10^{-3} dilution of allantoic fluid; triangles, 10^{-4} dilution), incubated for 5, 24, 48, and 72 h, and then incubated with XTT for 30 min. The A_{450} was read, and percents viability were calculated as described in Materials and Methods.

XTT assays with lower levels of virus (10^{-3} and 10^{-4} dilutions of allantoic fluid containing Ty/Ont). At these virus concentrations, both MDCK and MDCKbcl-2 cells showed little loss in viability as determined by the cytotoxicity assays at 5 and 24 h p.i. (Fig. 6). By 48 h p.i., the cytotoxicity assays showed a marked difference between the cell lines. At 72 h p.i., 99% of the MDCKbcl-2 cells were viable, in contrast to 7% of the MDCK cells. Although some of this difference may reflect multiplication of the viable MDCKbcl-2 cells by this point, clearly bcl-2 expression promoted an increase in cell viability. Even though MDCKbcl-2 cultures remained viable much longer than MDCKneo cells did, they eventually died (i.e., within 7 to 10 days p.i.). We are currently investigating whether persistently infected cell lines can be established.

Comparison of IFA staining of viral proteins in transfected cells. To detect potential differences in viral protein expression between the MDCK cells and the MDCKbcl-2 cells, we performed IFA staining with monoclonal antibodies to NS, NP, and hemagglutinin. The most notable distinction (Fig. 7) was in the distribution of NS, i.e., there was more intense staining of only the nucleus in MDCK cells (Fig. 7A) versus lighter staining of both the cytoplasm and the nucleus (with nuclear stippling) in MDCKbcl-2 cells (Fig. 7B). The antibody used to detect NS was originally described as reacting with NS1 (2). It is unlikely that this antibody would react with NS2, since the amino acid sequences of the two NS proteins are not similar. The other difference observed involved NP (Fig. 7); NP was distributed throughout the cytoplasm in MDCK cells (Fig. 7C), whereas there was an accumulation of NP around the nuclear membrane in the MDCKbcl-2 cells (Fig. 7D). This suggests that alterations in the localization of viral proteins may occur in the presence of bcl-2, and this may be a factor in the ability of bcl-2 to interfere with apoptosis.

DISCUSSION

In this study, we examined apoptosis as a potential mechanism of cell killing by influenza viruses. Our initial goal was to explain the lymphoid damage produced in birds infected with Ty/Ont by examining the induction of apoptosis in avian lymphocytes in vitro. As we extended our investigation to other viruses in a different cell culture system, we discovered that DNA fragmentation was commonly induced by influenza virus infection. Although we initially envisioned that only selected strains would induce apoptosis, our results clearly indicate that many different strains, including both influenza A and B viruses, induce this cellular response.

In examining DNA fragmentation in virus-infected cells, we noted that cytopathologic effects always accompanied DNA fragmentation, typically within 3 to 6 h after infection. This indicated that the cellular damage and DNA fragmentation occurred very quickly after infection and that they were temporally related. The requirement for infectious virus was also reinforced by the results with beta-propionolactone-inactivated Ty/Ont. At this point, however, we do not know which viral proteins are involved in the induction of apoptosis; our future goal is to identify the roles of specific viral proteins in this event.

After we discovered that many influenza viruses induced this response in cells, we investigated a well-known inhibitor of apoptosis, *bcl-2*. MDCK cells transfected with *bcl-2* differed dramatically in their response to influenza virus from MDCK cells which were not expressing bcl-2 (i.e., those that were untransfected or were transfected with the control vector) in that MDCKbcl-2 cells did not exhibit DNA fragmentation. However, the molecular relationship between these two entities—influenza virus and *bcl-2*—remains to be determined. There is evidence for a relationship between specific viral proteins and an apoptosis pathway involving bcl-2. Suppression of apoptosis may be a mechanism contributing to tumor development by oncogenic viruses. For example, group III B cell lines transformed by Epstein-Barr virus are resistant to apoptosis induced by serum depletion (10); this has been attributed to the expression of Epstein-Barr virus latent membrane protein 1, which upregulates bcl-2 expression (11). In addition, Epstein-Barr virus contains a gene (*BHFR1*) with sequence similarity to *bcl-2* (4). Virus persistence may also involve interaction with bcl-2; Levine et al. (19) recently demonstrated that overexpression of bcl-2 in a rat prostate cell line blocked apoptosis induced by Sindbis virus and, consequently, converted the lytic infection into a persistent one. In the case of influenza virus, we currently do not know what the interaction with bcl-2 might be; however, the extensive antigenic, genetic, and structural data available concerning influenza viral proteins should allow elucidation of this virus-host cell interaction in the future.

With regard to which influenza viral proteins might be involved, we view NS1 as a likely candidate because it is a phosphoprotein of unknown function produced in the nucleus at high levels early in viral infection (25). The fact that it is a phosphoprotein is intriguing, since apoptosis may be controlled by perturbations of protein phosphorylation in other systems (17). In considering cellular proteins with some relationship to NS1, we found several connections with fas (14, 35). fas is a transmembrane cell protein which triggers apoptosis

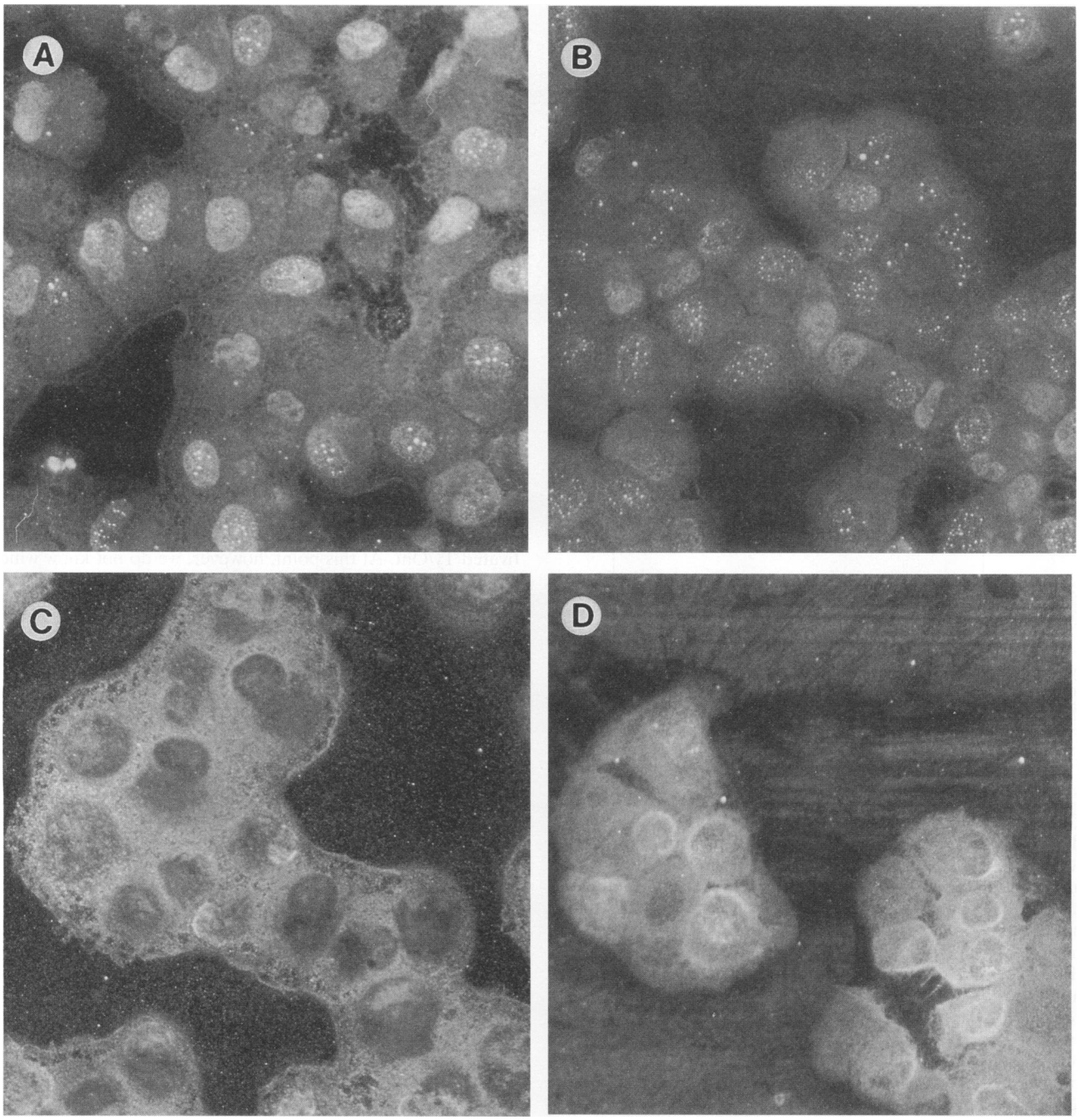

FIG. 7. Indirect IFA staining of MDCK (A and C) and MDCKbcl-2 (B and D) cells infected with Ty/Ont and stained with monoclonal antibody to NS (A and B) or to NP (C and D), as described in Materials and Methods. Magnification, ×93.

when stimulated by anti-fas antibody (14). When we compared the sequences of these genes, we determined that the NS gene product of A/Duck/Alberta/35/76 (H1N1) is 50% similar to the *fas* antigen over a 68-amino-acid region of the large cytoplasmic domain of fas. This region of fas is (i) required for mediating apoptosis and (ii) conserved among human and mouse fas and tumor necrosis factor receptor, another mediator of apoptosis (13). This is particularly interesting, since the recent studies of Takizawa et al. (30) indicate that *fas* expression is induced by influenza virus. We have examined, to a limited extent, the expression and distribution of early proteins, specifically NS1, in cells which did or did not express bcl-2. Our results suggest that coexpression of bcl-2 alters the localization of NS1 and NP. At this point, we don't know whether such changes in viral protein localization are related to protection of the cell from cytopathologic effects.

A current challenge in the field of viral pathogenesis is to understand how virus infection leads to specific tissue damage and disease or death of the host. We initiated the studies reported here because of our in vivo observations with chick-

ens infected with Ty/Ont. On the other hand, the data presented in this paper are clearly based on in vitro studies using cell cultures. At this point, we are currently examining the induction of DNA fragmentation directly in tissues from infected birds to evaluate the role of apoptosis in the intact host. However, our studies extend beyond the avian model system and suggest that apoptosis is an important general mechanism for the induction of cell death by influenza viruses.

ACKNOWLEDGMENTS

We thank J. Reed, M. Hardwick, and R. G. Webster for valuable reagents and R. Bromberg for electron microscopy expertise. We also thank M. McGregor, D. Wentworth, R. Callan, J. A. Lyon, and P. Friesen for many helpful discussions.

This work was supported by Public Health Service research grant AI24902 from the National Institute of Allergy and Infectious Disease and grants 3101 and 3414 from the U.S. Department of Agriculture.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
2. **Brown, L. E., V. S. Hinshaw, and R. G. Webster.** 1983. Antigenic variation in the influenza A virus non-structural protein, NS1. Virology **130:**134–143.
3. **Cepko, C. L., B. E. Roberts, and R. C. Mulligan.** 1984. Construction and application of a highly transmissible murine retrovirus shuttle vector. Cell **37:**1053–1062.
4. **Cleary, M. L., S. D. Smith, and J. Sklar.** 1986. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell **47:**19–28.
5. **Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober.** 1991. Current protocols in immunology. Greene Publishing Associates and Wiley-Interscience, New York.
6. **Compton, M. M.** 1992. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev. **11:**105–119.
7. **Ellis, R. E., J. Yuan, and H. R. Horvitz.** 1991. Mechanisms and functions of cell death. Annu. Rev. Cell Biol. **7:**663–698.
8. **Garcia, I., I. Martinou, Y. Tsujimoto, and J.-C. Martinou.** 1992. Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. Science **258:**302–304.
9. **Gougeon, M., and L. Montagnier.** 1993. Apoptosis in AIDS. Science **260:**1269–1270.
10. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordon, and A. B. Rickinson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature (London) **349:**612–614.
11. **Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. Rickinson.** 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65:** 1107–1114.
12. **Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo.** 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. **11:**3887–3895.
13. **Itoh, N., and S. Nagata.** 1993. A novel protein domain required for apoptosis. J. Biol. Chem. **268:**10932–10937.
14. **Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata.** 1993. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell **66:**233–243.
15. **Jacobson, M. D., J. F. Burne, M. P. King, T. Miyashita, J. C. Reed, and M. C. Raff.** 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature (London) **361:**365–369.
16. **Jeurissen, S. H. M., F. Wagenaar, J. M. A. Pol, A. J. van der Eb, and M. H. M. Noteborn.** 1992. Chicken anemia virus causes apoptosis of thymocytes after in vivo infection and of cell lines after in vitro infection. J. Virol. **66:**7383–7388.
17. **Knox, K. A., M. Finney, A. E. Milner, C. D. Gregory, M. J. O. Wakelam, R. H. Michell, and J. Gordon.** 1992. Second-messenger pathways involved in the regulation of survival in germinal-centre B cells and in Burkitt lymphoma lines. Int. J. Cancer **52:**959–966.
18. **Lang, G., O. Narayan, B. T. Rouse, A. E. Ferguson, and M. C. Connell.** 1968. A new influenza A virus infection in turkeys. II. A highly pathogenic variant, A/Turkey/Ontario/7732/66. Can. Vet. J. **9:**151–160.
19. **Levine, B., Q. Huang, J. T. Issacs, J. C. Reed, D. E. Griffin, and J. M. Hardwick.** 1993. Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. Nature (London) **361:** 739–742.
20. **Luoh, S.-M., M. W. McGregor, and V. S. Hinshaw.** 1992. Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses. J. Virol. **66:**1066–1073.
21. **Lyon, J. A., and V. S. Hinshaw.** 1993. Inhibition of nitric oxide induction from avian macrophage cell lines by influenza virus. Avian Dis. **37:**868–873.
22. **Noteborn, M. H. M., D. Todd, C. A. J. Verschueren, H. W. F. M. de Gauw, W. L. Curran, S. Veldkamp, A. J. Douglas, M. S. McNulty, A. J. van der Eb, and G. Koch.** 1994. A single chicken anemia virus protein induces apoptosis. J. Virol. **68:**346–351.
23. **Ohno, K., T. Nakano, Y. Matsumoto, T. Watari, R. Goitsuka, H. Nakayama, H. Tsujimoto, and A. Hasegawa.** 1993. Apoptosis induced by tumor necrosis factor in cells chronically infected with feline immunodeficiency virus. J. Virol. **67:**2429–2433.
24. **Olsen, C. W., M. W. McGregor, A. J. Cooley, B. Schantz, B. Hotze, and V. S. Hinshaw.** 1993. Antigenic and genetic analysis of a recently isolated H1N1 swine influenza virus. Am. J. Vet. Res. **54:**1630–1636.
25. **Privalsky, M., and E. Penhoet.** 1981. The structure and synthesis of influenza virus phosphoproteins. J. Biol. Chem. **256:**5368–5376.
26. **Qureshi, M. A., L. Miller, H. S. Lillehoj, and M. D. Ficken.** 1990. Establishment and characterization of a chicken mononuclear cell line. Vet. Immunol. Immunopathol. **26:**237–250.
27. **Rojko, J. L., R. M. Fulton, L. J. Rezanka, L. L. Williams, E. Copelan, C. M. Cheney, G. S. Reichel, J. C. Neil, L. E. Mathes, T. G. Fisher, and M. W. Cloyd.** 1992. Lymphocytotoxic strains of feline leukemia virus induced apoptosis in feline T4-thymic lymphoma cells. J. Clin. Invest. **66:**418–426.
28. **Schwartzman, R. A., and J. A. Cidlowski.** 1993. Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocr. Rev. **14:**133–151.
29. **Stevens, M. G., and S. C. Olsen.** 1993. Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. J. Immunol. Methods **157:**225–231.
30. **Takizawa, T., M. Shigeru, 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.
31. **Tsujimoto, Y., and C. M. Croce.** 1986. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc. Natl. Acad. Sci. USA **83:**5214–5218.
32. **Van Campen, H., B. C. Easterday, and V. S. Hinshaw.** 1989. Virulent avian influenza A viruses: their effect on avian lymphocytes and macrophages *in vivo* and *in vitro*. J. Gen. Virol. **70:**2887–2895.
33. **Van Campen, H., B. C. Easterday, and V. S. Hinshaw.** 1989. Destruction of lymphocytes by a virulent avian influenza A virus. J. Gen. Virol. **70:**467–472.
34. **van Wyke, K. L., W. J. Bean, Jr., and R. G. Webster.** 1981. Monoclonal antibodies to the influenza A virus nucleoprotein affecting RNA transcription. J. Virol. **39:**313–317.
35. **Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copland, N. A. Jenkins, and S. Nagata.** 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature (London) **356:**314–317.
36. **Wentworth, D. E., B. L. Thompson, X. Xu, H. L. Regnery, A. J. Cooley, M. W. McGregor, N. J. Cox, and V. S. Hinshaw.** 1994. An influenza A (H1N1) virus, closely related to swine influenza virus, responsible for a fatal case of human influenza. J. Virol. **68:**2051–2058.