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Coronavirus MHV-3-Induced Apoptosis in Macrophages

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Infection with mouse hepatitis virus strain 3 (MHV-3) results in lethal fulminant hepatic necrosis in fully susceptible BALB/c mice compared to the minimal disease observed in resistant strain A/J mice. Macrophages play a central role in the pathogenesis of MHV-3-induced hepatitis. In the present study we have shown that MHV-3 infection of macrophages induces these cells to undergo apoptosis. Three methods to detect apoptosis were applied: flow cytometry analysis of nuclear DNA content, fluorescence microscopic visualization of apoptotic cells labeled by the TUNEL assay, and gel electrophoresis to detect DNA laddering. Apoptosis in A/J and BALB/c macrophages was first detected at 8 h postinfection (p.i.) and reached a maximum by 12 h p.i. The degree of MHV-3-induced apoptosis was much greater in A/J-derived macrophages than in BALB/c-derived cells. Apoptosis was inversely correlated with the development of typical MHV cytopathology, namely syncytia formation. Infected macrophages from A/J mice did not form syncytia in contrast to the extensive syncytia formation observed in BALB/c-derived macrophages. In MHV-3-infected BALB/c macrophage cultures, apoptotic cells were not incorporated into syncyta. Apoptosis was also inversely correlated with the expression of MHV-3-induced fgl2 prothrombinase in macrophages. These results add the murine coronavirus MHV-3 to the list of RNA-containing viruses capable of inducing apoptosis.

INTRODUCTION

During the past few years many viruses have been found to induce apoptosis, and this effect is thought to contribute to the cytopathogenic effects of these viruses (Cuff and Ruby, 1996; Teodoro and Branton, 1997). DNA-containing viruses that induce apoptosis include adenoviruses (Rao et al., 1992; Chiou et al., 1994; Antoni et al., 1995), Epstein-Barr virus (Gregory et al., 1994), and poxviruses (Ray et al., 1992; Hu et al., 1994). Several RNA-containing viruses have also been reported to induce apoptosis: these include polioviruses (Tolskaya et al., 1995b), Sindbis virus (Levine et al., 1993; Esolen et al., 1995), reovirus (Tyler et al., 1995), and influenza viruses (Takizawa et al., 1993; Fesq et al., 1994). Apoptosis is characterized by distinctive morphological and biochemical features. Typical signs of apoptosis are nucleolytic degradation of chromosomal DNA, compaction and fragmentation of chromatin, cellular shrinkage, cytoplasmic blebbing, and fragmentation. These changes ultimately result in the formation of apoptotic bodies (Wyllie et al., 1980; Duvall and Wyllie, 1986; Bowen, 1993).

The ability of viruses to replicate efficiently in macrophages is an important determinant in the pathogenesis of many diseases. For example, enhanced replication of dengue virus in macrophages in patients with heterotypic antibody may result in dengue hemorrhagic fever rather than the less severe disease, which develops in a naive host (Gollins and Porterfield, 1984). Another well-established example is the hepatitis induced in mice during infection with the murine coronavirus, mouse hepatitis virus (MHV) (Bang and Warwick, 1960; Piazza, 1969; Virelizier and Allison, 1976). In fully susceptible strains of mice, such as BALB/c, MHV-3 infection produced a fulminant hepatitis, characterized by abnormalities of the hepatic microcirculation (Levy et al., 1983). Strain A mice (A/J) are resistant to the development of disease even though MHV-3 replicates in their livers (Dindzans et al., 1985). The induction of a unique macrophage prothrombinase (fgl2 prothrombinase) in response to MHV-3 infection correlates with the severity of infection. This unregulated elaboration of fgl2 prothrombinase during infection appears to be a major contributor to the pathogenesis of the lethal hepatitis observed in susceptible strains of mice (Dindzans et al., 1986; Pope et al., 1995).

In the present work we showed that coronavirus MHV-3 was capable of inducing apoptosis in primary macrophage cultures. Though the timing of MHV-3-
induced apoptosis was similar for macrophages isolated from A/J and BALB/c mice, the percentage of apoptotic cells was five times higher in A/J macrophages.

RESULTS

TUNEL analysis of MHV-3-induced apoptosis

MHV-3 infection of cultures of BALB/c macrophages produced pronounced cytopathic effects in the majority of the cells, namely cell fusion with syncytial giant cell formation, after which the cells die. This contrasts with the effect of MHV-3 on cultures of A/J macrophages, in which there was no detectable syncytia formation up to 12 h p.i. Rather MHV-3-infected A/J macrophages developed a rounded and shrunken appearance that was consistent with the development of apoptosis during the course of infection.

To determine whether MHV-3 infection induced apoptosis, virus infected A/J and BALB/c macrophages were assayed for the presence of cellular DNA fragmentation by in situ TUNEL assay (Gavrieli, 1992). A/J and BALB/c macrophages, growing on Lab-Tek Chamber Slides were mock or MHV-3 infected. Nuclear TUNEL staining was performed at various time points post infection and visualized directly by fluorescence microscopy. Representative photomicrographs of the same fields obtained by phase-contrast and fluorescence microscopy at 10 h p.i. are shown in Fig. 1. Mock-infected A/J- or BALB/c-derived cells had the typical morphology of intact macrophages and contained few TUNEL-positive apoptotic cells (not shown). The majority of MHV-infected BALB/c macrophages underwent cell fusion forming large syncytia (Fig. 1D). TUNEL-positive cells were observed in a small fraction of the macrophages, and these cells were not incorporated into syncytia (Fig. 1B). Quite different results were observed when A/J macrophages were infected with MHV-3. There was no visible syncytia formation during the first 10 h p.i. (Fig. 1C), and in fact syncytia formation was not observed in infected A/J macrophages during a 12-h period of observation. A large number of TUNEL positive cells were observed in MHV-infected A/J macrophages by 10 h p.i. (Fig. 1A). There was only a slight increase in the percentage of TUNEL-positive cells at 12 h p.i. compared to assays performed at 10 h p.i. Cell counts at 12 h p.i. indicated that TUNEL-positive cells made up 55% of the total cell population (Table 1). Based

FIG. 1. Detection of apoptosis by TUNEL assay. Macrophages from A/J and BALB/c mice were infected with MHV-3. At 10 h after infection samples were collected and processed for TUNEL assay as indicated under Materials and Methods. Phase-contrast (C and D) and fluorescence (A and B) photomicrographs of the same fields are shown for A/J (A and C) and BALB/c (B and D) macrophages.
on these data we conclude that MHV-3 induces apoptosis in both A/J and BALB/c macrophages and that TUNEL-positive apoptotic cells were not incorporated into or form syncytia.

To investigate the possibility that preventing MHV-3-induced syncytia formation would result in greater numbers of BALB/c macrophages undergoing apoptosis, we infected macrophages seeded at the normal density and at 10- and 30-fold lower densities than normal with MHV-3. At 10 and 12 h p.i. the percentage of cells that were TUNEL positive and the percentage of cells that had formed syncytia were determined. Lower cell density effectively prevented syncytia formation (decreased from 95% to 5% of cells) and doubled the percentage of cells which were TUNEL positive (increased from 5 to 10% of cells), although the percentage of apoptotic cells was still much lower than that observed with A/J macrophages.

To confirm that MHV-3-infected A/J macrophages were undergoing apoptosis, DNA was extracted from mock and MHV-3-infected A/J macrophages and examined for DNA fragmentation by agarose gel electrophoresis. As shown in Fig. 2, DNA fragmentation into a ladder characteristic of apoptosis was only observed in the sample obtained from MHV-3-infected cells (lane 3); no laddering was observed in samples from uninfected cultures (lane 2).

**Flow cytometric analysis of MHV-3-induced apoptosis**

To further address the possibility that only single cells which were not incorporated into syncytia underwent apoptosis we used flow cytometric analysis. Sample preparation and gating parameters for the flow cytometer (see Materials and Methods) exclude syncytial giant cells from the analysis. Macrophages were infected with MHV-3 and at various times post infection analyzed for DNA content by flow cytometry. Cell cycle phase distribution analysis revealed that at 2 and 4 h p.i. the majority of infected A/J and BALB/c macrophages show a typical G0+G1 DNA profile, similar to that of uninfected cells (Fig. 3, A/J macrophages; Fig. 3, BALB/c macrophages). At 8 h p.i. a subpopulation of cells with less than G0+G1 DNA content appeared that subsequently increased at 12 h p.i. The timing of this change in DNA profile was closely correlated with the timing of an increase in the proportion of shrunken cells detected by 90° light scatter (data not shown). DNA fragmentation with a resulting decrease in cellular DNA content and a decrease in cell size are the hallmarks of apoptosis (Wyllie et al., 1980; Bowen, 1993; Yuan, 1995; Teodoro and Branton, 1997).

To demonstrate that apoptotic cells were also MHV-3 infected, double staining with an anti-MHV nucleocapsid antibody and propidium iodide (PI) was performed. The apoptotic subpopulation was identified based on cellular DNA content, and their GFI was also determined, as described in Materials and Methods (Table 2). The cell subpopulation with a DNA content less than G1 also had a high level of green fluorescence. Based on these data we concluded that the great majority of apoptotic cells were also virus infected.

**MHV-3 replication in A/J and BALB/c macrophages**

The two channel flow cytometry results presented above (Table 2) indicated that viral genes were expressed in A/J and BALB/c macrophages undergoing apoptosis. To determine whether there was any correlation between production of infectious virus and virus-induced apoptosis, we compared the kinetics of MHV-3 growth in A/J and BALB/c macrophages. As shown in Fig. 4, MHV-3 yields at 12 h p.i. were 6.7-fold lower in A/J macrophages compared to the yields achieved in BALB/c macrophages. In addition to A/J macrophages producing somewhat lesser amounts of infectious virus than BALB/c macrophages, the growth curves suggested

### Table 1

| Time (h p.i.) | A/J macrophages | BALB/c macrophages |
|--------------|-----------------|--------------------|
| 0            | 0.52 ± 0.15     | 0.75 ± 0.25        |
| 4            | 0.65 ± 0.12     | 0.93 ± 0.16        |
| 12           | 55.5 ± 10.4     | 10.2 ± 1.5         |

*The percentage of TUNEL-positive cells was determined as described under Materials and Methods. Representative fields were photographed under both fluorescence and phase microscopy to count the number of fluorescently labeled TUNEL positive cells and the total number of cells. Ten fields at ×100 magnification with about 500 cells/field were counted for each time point. The mean values for the percentage of apoptotic cells are shown.*
FIG. 3. DNA histograms of MHV-3-infected A/J and BALB/c macrophages. Macrophages from A/J and BALB/c mice were infected with MHV-3 (0 time point represents uninfected cells). At indicated time points samples were collected and processed for flow cytometry as indicated under Materials and Methods. Cells' numbers (y axis) were plotted against red fluorescence (x axis). The apoptotic subpopulation (light gray) with less than G0/G1 DNA content is indicated by the bars. The G0/G1 subpopulation is represented by the prominent dark gray peak. The G2 subpopulation is indicated by the minimal dark gray peak. The black region indicates a population of cells with a DNA content consistent with S phase.
that virus replication in A/J macrophages initiated more slowly than in BALB/c macrophages and plateaued between 10 and 12 h p.i., while BALB/c macrophages continued to support MHV-3 growth at that time.

Fgl2 prothrombinase expression

We have previously shown (Parr et al., 1995) that the appearance of fgl2 mRNA after MHV-3 infection was delayed by about 2 h in A/J macrophages (5–6 h p.i.) relative to its appearance in BALB/c macrophages (3–4 h p.i.). To determine whether the timing of MHV-3 induction of syncytia formation in BALB/c macrophages as well as higher levels of apoptosis in A/J macrophages could be correlated with blunting the expression of the fgl2 prothrombinase, we investigated the time of appearance of the fgl2 protein. At various times after MHV-3 infection cell lysates were prepared from A/J and BALB/c macrophages and analyzed for the presence of fgl2 protein by Western blotting (Fig. 5A). Fgl2 protein was not detected in A/J macrophages at any time, while in BALB/c macrophages fgl2 protein was first detected at 8 h p.i. and had increased further at 12 h p.i. To verify that the A/J macrophages were infected with MHV-3, the same samples were probed with an anti-MHV nucleocapsid antibody (Fig. 5B). The nucleocapsid protein was detected in both A/J and BALB/c macrophages.

DISCUSSION

In the present study, we have shown by three different methods that MHV-3 infection induced apoptosis in primary macrophage cultures. The shift in DNA content demonstrated by flow cytometry was observed in macrophages isolated from both A/J and BALB/c mice. However, in situ TUNEL assays demonstrated that MHV-3

![MHV-3 titer](image_url)

FIG. 4. Virus growth in A/J- and BALB/c-infected macrophages. A/J and BALB/c macrophages were infected with MHV-3 and at the indicated times p.i. samples were prepared and processed for virus titration as described under Materials and Methods. Results are the mean titers of triplicate samples. Error bars indicate the standard errors of the mean.

|                  | A/J macrophages | BALB/c macrophages |
|------------------|-----------------|--------------------|
| Uninfected       | 42              | 53                 |
| 8 h p.i.         | 300             | 390                |
| 12 h p.i.        | 550             | 420                |

a Cultures of macrophages from A/J and BALB/c mice were infected with MHV-3 at a MOI of 1 or mock-infected and at the times indicated, stained with propidium iodide and the anti-MHV nucleocapsid monoclonal antibody 1161, and analyzed by flow cytometry as described under Materials and Methods.
infection of cultures of A/J macrophages resulted in five-fold more apoptotic cells than infection of BALB/c-derived macrophages. The source of this discrepancy is likely due to the exclusion of syncytial giant cells from the flow cytometric analysis. Large giant cells cannot pass the aperture, and smaller giant cells and fragmented syncytia will be excluded by the gating parameters, which exclude particles that are not the correct size for intact cells. MHV-3 infection of BALB/c macrophages resulted in cell fusion and syncytial giant cell formation. These cells were excluded from the flow cytometric DNA content analysis. Thus this analysis only represents the single cell fraction. For A/J macrophages, almost no syncytia were formed, thus the DNA content analysis reflects events in virtually all of the macrophages in the culture. The in situ TUNEL assay is free of the bias against cells undergoing fusion in response to MHV infection. This method also revealed that only single cells that were not incorporated into syncytia in response to MHV-3 infection underwent apoptosis.

Measles virus also produces cell fusion and syncytia formation. In larger measles virus-induced syncytia, TUNEL-positive cells are observed only in the center of the multinuclear giant cells, while nuclei at the periphery are not labeled (Esolen et al., 1995) These data suggest that during measles virus infection the nuclei of the cells recruited into the syncytia were initially normal, and they subsequently undergo DNA fragmentation. The apparent differences in the development of apoptosis in measles-infected cells (Vero cells and monocyte-like cells) and MHV-3-infected macrophages may reside in differences in the cellular pathways to apoptosis triggered by these two viruses.

A large number of RNA viruses, including influenza viruses (Fesq et al., 1994), picornaviruses (Jelachich and Lipton, 1996), and alphaviruses (Lewis et al., 1996) have been shown to induce apoptosis under certain conditions. Perhaps the most studied of these viruses is Sindbis virus, which induces apoptosis in both cultured cell lines and in neurons in 2-week-old and younger mice (Levine et al., 1993, 1996; Ubol et al., 1996). Although the mechanism by which Sindbis virus infection triggers apoptosis has not yet been elucidated, it is known that the inductions of apoptosis by Sindbis virus can be blocked by the overexpression of bcl-2. Recently, using TUNEL assays and flow cytometry analysis, it was demonstrated that mosquito cell cultures, unlike vertebrate cell cultures, do not undergo apoptosis in response to Sindbis virus infection (Karpf and Brown, 1998). These authors suggest that the mechanism for cytopathology induced in mosquito cell cultures by Sindbis virus may be distinct from vertebrate cell cultures.

The difference in the outcome of MHV-3 infection of macrophages from BALB/c and A/J mice could be due to a somewhat more rapid replication cycle of MHV-3 in BALB/c macrophages (Fig. 4; Macnaughton and Patterson, 1980). This could commit infected BALB/c macrophages to high levels of S-protein expression, with resulting cell fusion prior to the cells acting on the apoptotic signal. In BALB/c macrophages, apoptosis is only slightly increased by infecting cells under conditions where syncytia formation does not occur (low cell density). Thus it appears that cell fusion by itself is not a major variable in preventing apoptosis in BALB/c macrophages. In this scenario, in BALB/c macrophages MHV-3 simply outruns the apoptotic response, in A/J macrophages the response is effective. This would serve to limit spread by cell-to-cell fusion of MHV-3 in cultures of A/J-derived macrophages and thus limit virus yields. Other possible explanations for the different outcomes of MHV-3 infection of BALB/c and A/J macrophages might be variations in the levels of bcl-2, TNF-α or other cytokines important in decreasing programmed cell death in infected cells. In this regard, it was recently shown that overexpression of bcl-2 can block or delay apoptosis induced by Sindbis virus (Levine et al., 1996), La Crosse virus (Pekosz et al., 1996), adenovirus (Chiou et al., 1994), and influenza virus (Hinshaw et al., 1994).

The induction of apoptosis during virus infection can be viewed as a nonspecific cellular defense mechanism to limit virus replication. Indeed, induction of apoptosis restricts replication and infectivity of baculovirus (Clem and Miller, 1993). Although the extent of viral replication in A/J mice is considerable, the titer achieved is less and the kinetics are slower compared to BALB/c mice (Dindzans et al., 1986), paralleling the results, which have been obtained in cultured macrophages (Macnaughton and Patterson, 1980; this work). In addition to restricting viral yield (see Fig. 4) and slowing spread of the virus throughout the host, the induction of apoptosis in macrophages during MHV-3 infection may have an additional protective effect in this system. BALB/c macrophages...
elaborate the fgl2 prothrombinase in response to MHV-3 infection. This prothrombinase response has been shown to be an important determinant in the pathogenesis of the MHV-3-induced fulminant hepatitis that develops in this strain of mice (Li et al., 1992). Macrophages from A/J mice do not elaborate detectable fgl2 prothrombinase in response to MHV-3 infection (Fig. 5), although there is a slow (compared to that seen in BALB/c macrophages) induction of prothrombinase mRNA (Parr et al., 1995). In A/J macrophages, the possibility that the development of apoptosis in the majority of MHV-3-infected cells prevents the expression of the fgl2 prothrombinase protein needs further investigation.

Regardless of variations between A/J and BALB/c macrophages in other responses to MHV-3 infection, the fact that apoptosis was observed in both cell lines adds coronavirus MHV-3 to the list of RNA-containing viruses capable of inducing apoptosis.

**MATERIALS AND METHODS**

**Virus and cells**

Stocks of MHV-3 were grown as described by Levy et al. (1981). Peritoneal macrophages were harvested from female 8- to 10-week-old A/J and BALB/c mice 4 days after injection of 2 ml of 5% thioglycolate broth (Difco). Macrophages were washed twice in DMEM with 10% fetal bovine serum supplemented with 2 mM L-glutamine (DME-10), plated in 24-well tissue culture plates (5 × 10⁵ cells/well) and maintained at 37°C in a humidified atmosphere of 5% CO₂. Four hours after seeding, the cells were washed twice with DME-10 to separate adherent macrophages from nonadherent cells. Macrophages were infected with 5 × 10⁵ PFU of MHV-3 (m.o.i. = 1) for 30 min at 37°C in a humidified atmosphere of 5% CO₂, washed twice in DME-10, and subsequently incubated at 37°C with 0.5 ml of DME-10 per well. At various times post infection (p.i.) cells were harvested with a rubber policeman, resuspended in PBS containing 4% paraformaldehyde, incubated at 4°C for 10 min, washed with PBS, and fixed in ice cold 70% ethanol (EtOH). The samples were stored at −20°C before they were analyzed by flow cytometry.

**Virus titration**

At various times postinfection macrophages from A/J and BALB/c mice were scraped into the incubation medium, transferred into 1.5 ml tubes, frozen in liquid nitrogen, and stored at −80°C. The virus titer in each sample was determined by plaque assay, as described previously (Levy et al., 1981).

**Antibodies**

The anti-MHV nucleocapsid monoclonal antibody 1161 has been described previously (Leibowitz et al., 1987). An anti-fgl2 antiserum was prepared by immunizing rabbits with a KLH-peptide conjugate. A 13-amino-acid synthetic peptide containing an amino terminal cysteine followed by 12 amino acids (LQADDHDRDPGGN) corresponding to residues 99±110 of the fgl2 gene (Parr et al., 1995) was synthesized and coupled to maleimide activated KLH (Pierce Chemicals) according to the manufacturer’s directions. A New Zealand white rabbit was immunized by subcutaneous injection with 1 mg of KLH-peptide conjugate in complete Freund’s adjuvant, followed by three subsequent booster immunizations with 0.5 mg of antigen in incomplete Freund’s adjuvant at 14-day intervals. The development of anti-peptide IgG antibodies was monitored by ELISA using plates coated with BSA-coupled peptide.

**TUNEL assay**

Macrophages from A/J and BALB/c mice were plated on Lab-Tek Chamber Slides (10⁵ cells/well), mock or MHV-3-infected, at various times after infection, and DNA free ends labeled using the apoptosis detection system, fluorescein, TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling) assay (Promega, USA), according to the manufacturer’s protocol. This assay detects the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12dUTP at the 3’-OH DNA ends. TUNEL-positive cells were then visualized by fluorescence microscopy and counted, thus allowing the screening of large numbers of cells over the course of infection. For the data shown in Table 1, 10 fields at ×100 magnification with about 500 cells/field were counted for each time point. The mean values for the percentage of apoptotic cells are shown.

**Flow cytometry analysis**

Fluorescence measurements were performed on a Becton Dickinson FACS flow cytometer as described previously with some modifications (Belyavskyi, 1994). Briefly, before analysis cells were passed through a 23-gauge needle to disperse clusters of cells. Fixed macrophages were washed twice in PBS (pH = 7.0) and resuspended in 0.5 ml of PI solution (50 μg/ml propidium iodide, 100 μg/ml RNase A in PBS) for 20 min at 37°C. Red fluorescence (the value for DNA content from PI staining) was collected above 630 nm. The data were gated to eliminate any particles that were not the correct size for intact cells and analyzed using a two-parameter histogram, plotting light scatter vs red fluorescence. At least 5 × 10⁴ cells were counted in each assay.

To correlate individual infected and apoptotic cells double staining with the anti-MHV nucleocapsid monoclonal antibody 1161 (Leibowitz et al., 1987) and PI was done. Fixed macrophages were washed twice in PBS (pH = 7.0), resuspended in 0.5 ml of a 1:50 dilution of antibody 1161 in PBS±BSA buffer (PBS with 0.5% BSA;
0.1% sodium aside; 0.05% Triton X-100), and incubated 90 min at 37°C. The cells were washed twice with PBS±BSA, resuspended in 0.5 ml of FITC-labeled secondary anti-mouse antibodies (1:40 dilution in PBS-BSA), and incubated 60 min at 4°C. The cells were washed two times with PBS±BSA and finally resuspended in 0.5 ml of PI solution for 20 min at 37°C. The cells were analyzed using two-parameter histogram, plotting green fluorescence vs red fluorescence. The mean green fluorescence intensity (GFI) of subpopulation of cells with less than G1+G0 DNA content (<G1+G0) was calculated using CellQuest software (Becton Dickinson Inc.) At least 5 × 10⁴ cells were counted in each assay.

DNA laddering

At 12 h p.i. DNA was extracted from 1 × 10⁶ mock-infected and MHV-3-infected macrophages using Triazol reagent (Life Technologies) according to the directions provided by the vendor. The extracted DNA was precipitated with ethanol dissolved in TE and 1 μg of DNA was digested at 37°C for 15 min with 1 U of DNase-free RNase (Promega). DNA fragments were resolved by agarose gel electrophoresis and stained with Syber Gold (Molecular Probes) to visualize fragmented DNA.

Western blotting

At various times post infection macrophages from A/J and BALB/c mice were harvested with a rubber policeman, washed in PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5) 150 mM sodium chloride; and 0.5% NP-40. Lysates were microcentrifuged at 4°C for 10 min, and the supernatants were stored at −20°C. Protein concentration was determined by the Bio-Rad protein assay. Equal amounts of total protein (20 μg) were resolved by 10% SDS-PAGE and then transferred from the gel to an Immobilon membrane by semidry blotting. Membranes were probed with antibodies to the fg2 prothrombinase or MHV nucleocapsid protein and detected by ECL according to the manufacturer's (Amer sham) instructions.

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Note added in proof. Evidence for apoptosis-inducing activity of coronaviruses has been obtained by Eleouet et al. (1998). J. Virol. 4918±4924.

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