JOURNAL OF VIROLOGY, June 1996, p. 4090–4096 Vol. 70, No. 6
0022-538X/96/$04.00+0

Human Isolates of Dengue Type 1 Virus Induce Apoptosis in Mouse Neuroblastoma Cells

PHILIPPE DESPRÈS,[1]* MARIE FLAMAND,[1] PIERRE-EMMANUEL CECCALDI,[2] AND VINCENT DEUBEL[1]

Unité des Arbovirus et Virus des Fièvres Hémorragiques[1] and Unité de la Rage,[2] Institut Pasteur, 75724 Paris, France

Received 26 October 1995/Accepted 7 March 1996

Human isolates of dengue (DEN) type 1 viruses FGA/89 and BR/90 differ in their membrane fusion properties in mosquito cell lines (P. Desprès et al., Virology 196:209–216, 1993). FGA/89 and BR/90 were assayed for their neurovirulence in newborn mice, and neurons were the major target cells for both DEN-1 virus strains within the central nervous system. To study the susceptibility of neurons to DEN virus infection, DEN virus replication was analyzed in the murine neuroblastoma cell line Neuro 2a. Infection of Neuro 2a cells with FGA/89 or BR/90 induced apoptotic DNA degradation after 25 h of infection. Studies of DEN protein synthesis revealed that accumulation of viral proteins leads to apoptotic cell death. The apoptotic process progressed more rapidly following BR/90 infection than it did after FGA/89 infection. The higher cytotoxicity of BR/90 for Neuro 2a cells was linked to an incomplete maturation of the envelope proteins, resulting in abortive virus assembly. Accumulation of viral proteins in the endoplasmic reticulum may induce stress and thereby activate the apoptotic pathway in mouse neuroblastoma cells.

Dengue (DEN) virus, a member of the *Flavivirus* genus (family *Flaviviridae*), is one of the most rapidly spreading mosquito-borne human pathogens in the tropics. DEN viruses are classified into four serotypes (DEN-1, -2, -3, and -4). They cause a spectrum of illnesses ranging from a flu-like disease (DEN fever) to DEN hemorrhagic fever, a fulminating illness which can progress to DEN shock syndrome and death (11). The pathogenesis of DEN hemorrhagic fever-DEN shock syndrome has not been completely elucidated. An abnormal immune response and disturbed hemostasis may be responsible for the severe hemorrhage and shock (13), and the severity of the disease may also be due to the intrinsic biological properties of the infecting strains of DEN virus (14, 21).

We have previously observed biological and molecular differences between DEN-1 virus strains isolated from patients with DEN fever in French Guiana (strain FGA/89) and in Brazil (strain BR/90) (4). The neurovirulences of FGA/89 and BR/90 in mice differ (4). To identify the major target cells of DEN virus infection within the central nervous system, a lethal dose of FGA/89 or BR/90 was administered to newborn Swiss mice by the intracerebral route. Immunohistochemical analysis of the mouse brain sections showed that neurons were positive for DEN viral antigens after 9 days of infection (9). Neurolysis was apparent when DEN virus-infected mice manifested signs of severe encephalitis 12 days after infection (9).

To study the susceptibility of neurons to DEN virus infection, replication rates of FGA/89 and BR/90 in the murine neuroblastoma cell line Neuro 2a were analyzed. Human isolates of DEN-1 virus cloned in the mosquito AP61 cell line were produced in AP61, purified on sucrose gradients, and titrated on AP61 cells (4). In these experiments, Neuro 2a cell monolayers were infected with FGA/89 or BR/90 such that 80% of cells were infected within 1 day as determined by an immunofluorescence assay using anti-DEN virus hyperimmune mouse ascites fluid (HMAF). Mortality of Neuro 2a cells in which FGA/89 or BR/90 was replicating was 5% by 30 h postinfection and 75% after 40 h as determined by Trypan Blue dye exclusion.

Morphological changes in DEN virus-infected Neuro 2a cells were examined by an immunofluorescence assay. Infected cells displayed the characteristics typical of adherent cells undergoing programmed cell death (apoptosis), becoming rounded and condensed. In addition, treatment with the DNA intercalator propidium iodide (PI) revealed extensive condensation of chromatin into several dense masses, characteristic of apoptosis (Fig. 1C and E). Electron microscopy confirmed morphological changes, including chromatin margination along the inner nuclear membrane and membrane blebbing (data not shown). Apoptotic cell death in DEN virus-infected Neuro 2a cells was verified by a DNA fragmentation assay (2). Cell monolayers in 25 cm^2 flasks (10^6 cells) were lysed by incubation for 1 h in 0.5 ml of Tris-EDTA (TE) buffer (pH 7.4) containing 0.2% Triton X-100 and proteinase K (100 μg/ml), and the cell lysates were centrifuged at 15,000 × *g* for 15 min. Total soluble DNA in the supernatant was precipitated by addition of 2.5 M NaCl, 10 μg of glycogen, and 1 volume of isopropyl alcohol and incubation for 1 h at −20°C. The DNA precipitates were recovered by centrifugation, air dried, and resuspended in 50 μl of TE buffer containing RNase A (100 μg/ml). Five microliters of 20% sodium dodecyl sulfate (SDS) and 5 μl of gel-loading solution were added, and samples were heated at 65°C for 10 min. These DNA samples (25 μl) were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. Internucleosomal DNA cleavage into fragments of 180 to 200 bp in DEN virus-infected Neuro 2a cells was clearly observed after 40 h of infection (Fig. 2A).

Addition of 100 μM aurintricarboxylic acid (ATA), an inhibitor of nucleases, inhibits fragmentation of PC12 cell DNA after serum deprivation (1). To test whether ATA prevents the apoptotic DNA fragmentation in DEN virus-infected Neuro 2a cells, cultures were incubated in the presence of 100 μM ATA 20 h after infection. Approximately 95% of infected Neuro 2a cells treated with ATA excluded Trypan Blue at 40 h postinfection. ATA did not interfere with viral protein synthesis since the production of protein E was maintained in ATA-treated

* Corresponding author. Mailing address: Unité des Arbovirus et Virus des Fièvres Hémorragiques, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris, France. Phone: 33-145688723. Fax: 33-145688780. Electronic mail address: pdespres@pasteur.fr.

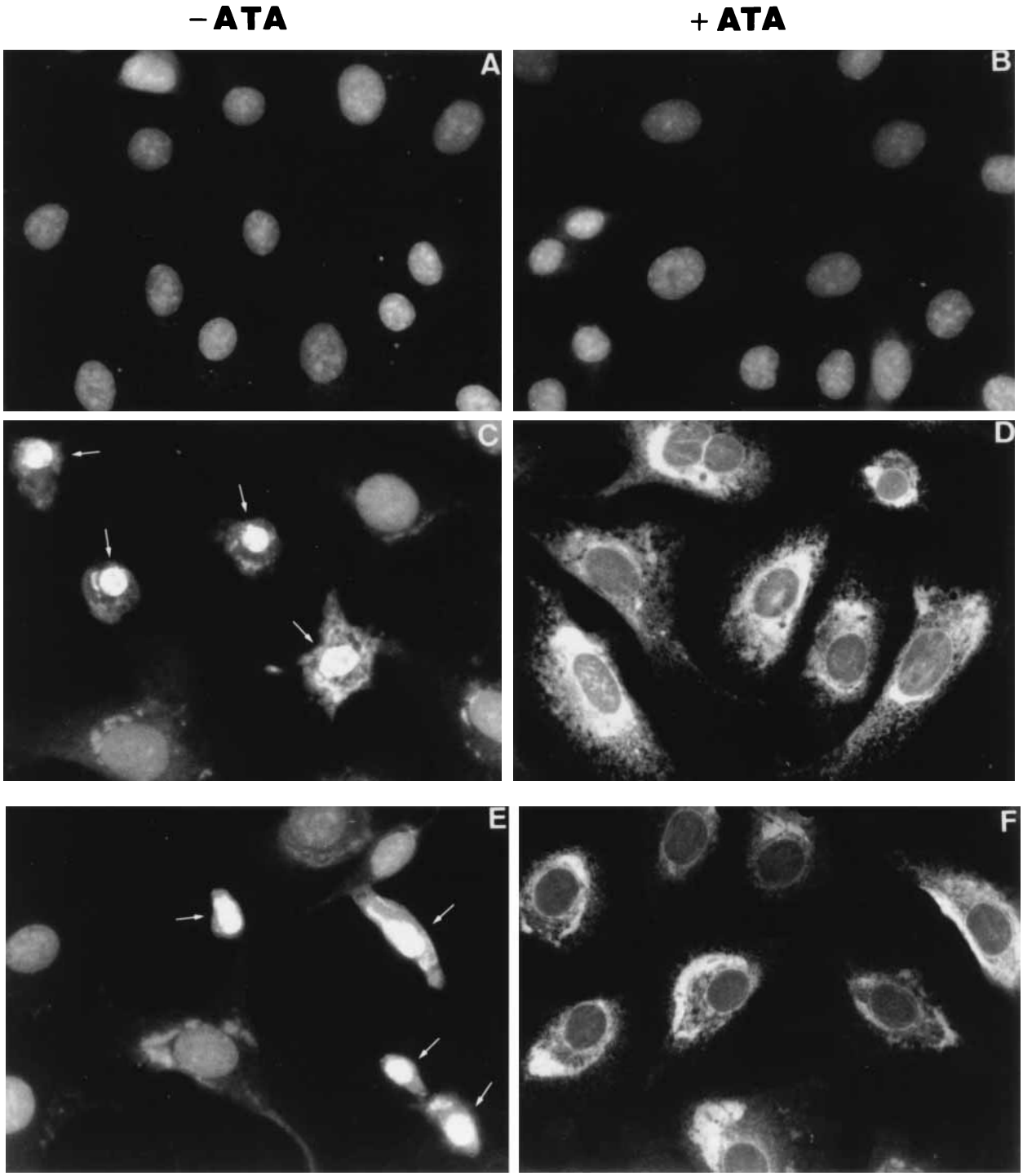

FIG. 1. Morphological changes in DEN virus-infected Neuro 2a cells. Neuro 2a cell monolayers infected with FGA/89 (C and D) or BR/90 (E and F) or mock infected (A and B) were fixed with 3% paraformaldehyde in PBS 40 h postinfection and permeabilized with 0.1% Triton X-100 in PBS (4). Intracellular DEN virus protein E was visualized with MAb 9D12 and nuclei were stained with PI as previously described (4, 5). Infected cells with condensed chromatin are indicated (arrows). The endonuclease inhibitor ATA (100 μM) was added (+ ATA) or was not added (− ATA) 20 h postinfection. Original magnification, ca. ×200.

cells (Fig. 1D and F). Internucleosomal DNA degradation was greatly reduced in ATA-treated Neuro 2a cells infected with FGA/89 or BR/90 40 h postinfection (Fig. 2B). Death of infected Neuro 2a cells treated with ATA 50 h postinfection appears to be due to DEN virus-induced necrosis (data not shown). These results suggest that apoptotic cell death required DEN virus-stimulated endonuclease activity in mouse neuroblastoma cells. Thus, induction of apoptosis by DEN virus infection results in rapid neuronal death.

The VERO cell line, a common substrate for growth of DEN virus, was assayed for susceptibility to DEN virus-induced apoptosis. VERO cells were infected with FGA/89 or

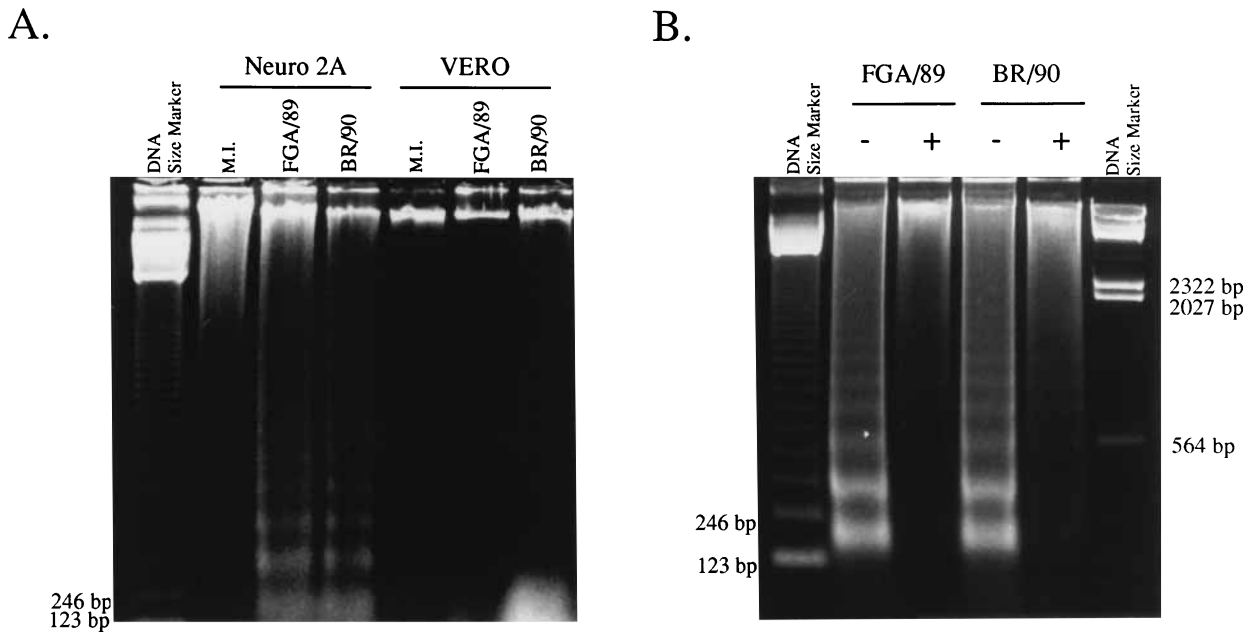

FIG. 2. Effect of DEN virus infection on DNA fragmentation. (A) Soluble DNAs were extracted from Neuro 2a or VERO cell lysates 40 h after mock infection (M.I.) or infection with the indicated DEN virus. (B) ATA (100 μM) was (+) or was not (−) added to DEN virus-infected Neuro 2a cells 20 h postinfection, and DNA fragmentation was observed after 40 h. The sizes of DNA markers are indicated.

BR/90 such that at least 75% of cells were infected within 1 day, as determined by immunofluorescent detection of viral proteins (data not shown). Nucleosome laddering was not observed in DEN virus-infected VERO cells 40 h postinfection (Fig. 2A). The viability of DEN virus-infected VERO cells declined only after 72 h of infection. The slower progression of DEN virus-induced cell lysis and the absence of DNA fragmentation 4 days postinfection suggested that VERO cell death involved necrosis rather than apoptosis (data not shown) (2). These observations indicate that different cell lines exhibit different susceptibilities to DEN virus infection.

To determine the time course of apoptotic DNA degradation in DEN virus-infected Neuro 2a cells, the method of terminal deoxyterminal transferase-mediated dUTP-biotin nick-end labeling (TUNEL) was used (10). Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS)–95% ethanol, and internucleosomal DNA cleavage was detected by specific labeling of free 3′-OH DNA termini with biotinylated dUTP in the presence of terminal deoxyterminal transferase and fluorescein-streptavidin (8). DNA breaks in nuclei of FGA/89- and BR/90-infected Neuro 2a cells were visualized 25 h after infection and were more numerous by 30 h (Fig. 3). Apoptotic cell death was observed to occur in only a small number of DEN virus-infected cells. This may be due to the brevity of morphological changes that contribute to apoptotic cell death (7).

Interestingly, apoptotic DNA degradation was more pronounced in Neuro 2a cells infected with BR/90 than in those infected with FGA/89 (Fig. 3). FGA/89 and BR/90 have different infectivities for Neuro 2a cells (9). To determine whether there is a causal relationship between DEN virus replication and apoptosis, Neuro 2a cells were infected at various multiplicities of infection and apoptotic DNA degradation was monitored by PI staining 30 h postinfection. The proportion of FGA/89-infected Neuro 2a cells in an apoptotic state increased in a virus dose-dependent manner to 15% at the highest multiplicity of infection tested (Table 1). In contrast, more than 20% of BR/90-infected Neuro 2a cells exhibited apoptotic DNA degradation at each multiplicity of infection examined.

We investigated whether the higher cytotoxicity of BR/90 for Neuro 2a cells could be attributed to differences in viral protein production. A radioimmunoprecipitation assay was used to study the synthesis of viral proteins in infected Neuro 2a cells which had been pulse-labeled for 30 min at various times postinfection. The time course of viral protein synthesis was similar in Neuro 2a cells infected with FGA/89 to that in those infected with BR/90 (Fig. 4A). The major DEN proteins were first detected 15 h after infection and peaked by 20 to 25 h. Thus, apoptosis was triggered by DEN virus when viral proteins began to accumulate in the host Neuro 2a cells. No

TABLE 1. Proportion of Neuro 2a cells in the apoptotic state following various multiplicities of DEN virus infection

Virus	MOI[a]	Infected cells[b] (%) (± SD)	Infected cells in apoptotic state[c] (%) (± SD)
FGA/89	50	11 ± 3	<1
	100	27 ± 2	4 ± 2
	200	47 ± 5	9 ± 4
	400	77 ± 1	15 ± 1
BR/90	5	10 ± 2	15 ± 5
	10	26 ± 2	23 ± 2
	20	51 ± 4	23 ± 2
	40	78 ± 4	23 ± 2

[a] MOI, multiplicity of infection. Neuro 2a cell monolayers were infected with various AP61 focus-forming units of DEN virus per cell.

[b] The proportion of cells positive for viral antigens in infected cultures 30 h postinfection was determined by an immunofluorescence assay using anti-DEN virus hyperimmune mouse ascites fluid.

[c] The proportion of infected Neuro 2a cells with chromatin condensation was determined by PI staining. Spontaneous PI uptake in control cultures was less than 3%.

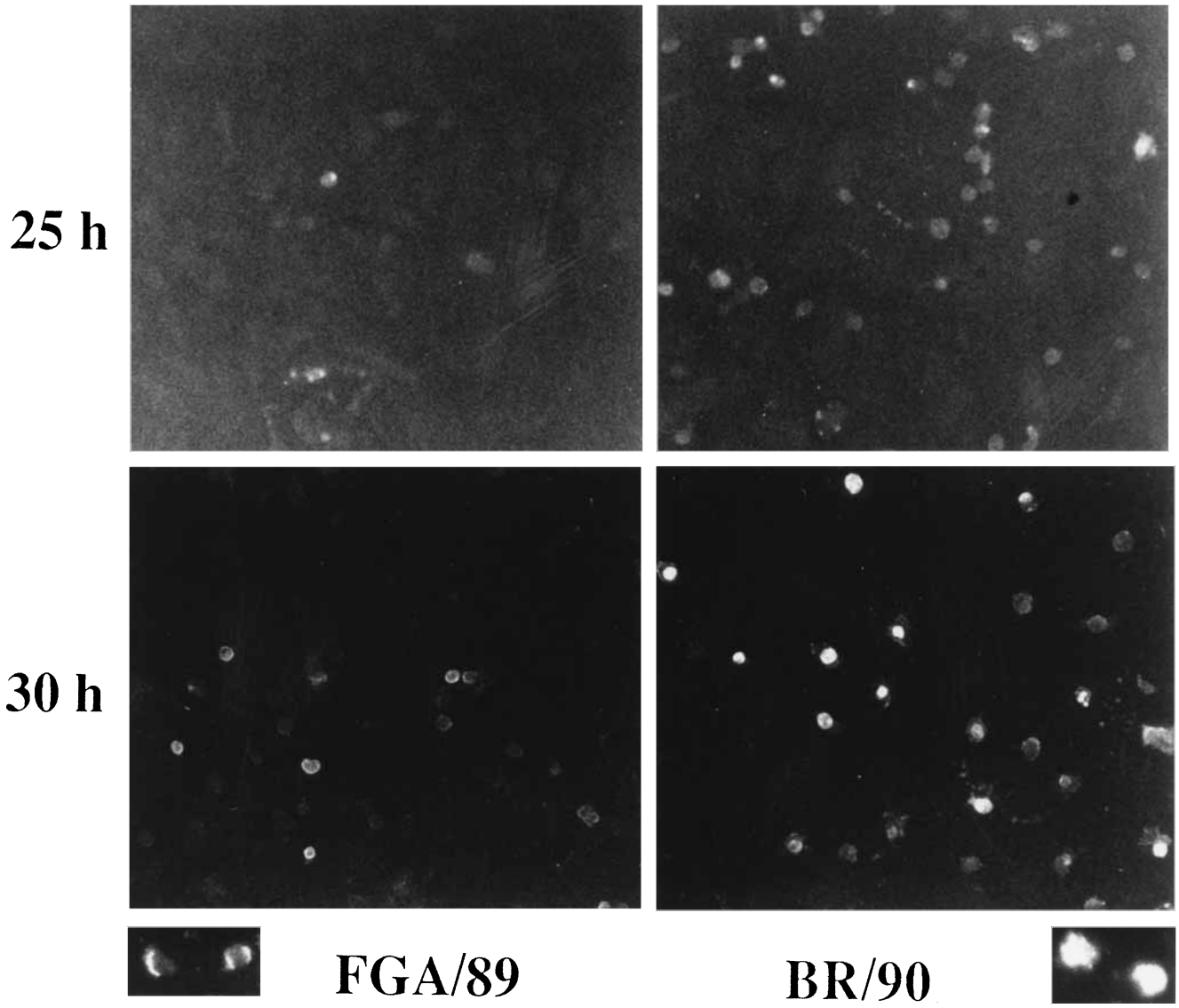

FIG. 3. Time course of DNA fragmentation in Neuro 2a cells infected with DEN viruses. Apoptotic DNA degradation was visualized by TUNEL staining by using fluorescein-conjugated streptavidin (magnification, ca. ×40). The lower panels show the nuclei magnified (magnification, ca. ×200).

obvious differences in viral protein processing between FGA/89 and BR/90 were observed (Fig. 4A). Several observations suggest that secretion of flavivirus envelope (E) protein requires dimerization with the precursor of the membrane protein prM (4, 16, 32). Radiolabeled viral proteins were tested for their reactivity with anti-prM monoclonal antibody (MAb) 15H5, and prM was associated with E in FGA/89-infected Neuro 2a cells (Fig. 4B). In contrast, prM was not associated with E in BR/90-infected Neuro 2a cells (Fig. 4B). As the amino acid sequence differences between the structural proteins of FGA/89 and BR/90 are essentially restricted to protein E (4), we investigated whether BR/90 E protein was misfolded or incorrectly matured in mouse neuroblastoma cells. To study the folding of BR/90 protein E in Neuro 2a cells, radiolabeled proteins were immunoprecipitated with anti-E MAbs 8C2, 4D2, and 2C5. These MAbs are equally reactive with E proteins of either DEN-1 virus particles produced in AP61 cells (4). MAb 8C2 binds a linear epitope in domain A (19), whereas MAb 4D2 binds to a conformational epitope on the mature virion; MAb 2C5 is most reactive with the intracellular form of protein E (4). MAb 8C2 recognized newly synthesized FGA/89 and BR/90 E proteins equally well (Fig. 4C). In contrast, the immunoreactivities of MAbs 4D2 and 2C5 with BR/90 E protein were significantly lower than they were with FGA/89 E protein, suggesting that BR/90 E protein does not mature properly (Fig. 4C).

We investigated whether a defect in the processing of envelope proteins alters BR/90 morphogenesis. No infectious BR/90 particles were detected in the culture media of infected Neuro 2a cells, whereas FGA/89 was released at a rate of about 1 AP61 focus-forming unit per h per cell 25 h postinfection. By electron microscopy, we found a large number of FGA/89 particles within the endoplasmic reticulum (ER) but no BR/90 particles (data not shown). As DEN virus assembly takes place within the ER (11), the absence of virion formation may be associated with greater accumulation of BR/90 proteins than FGA/89 proteins in cell membranes. Thus, DEN virus-induced apoptosis appears to be due to the accumulation of viral pro-

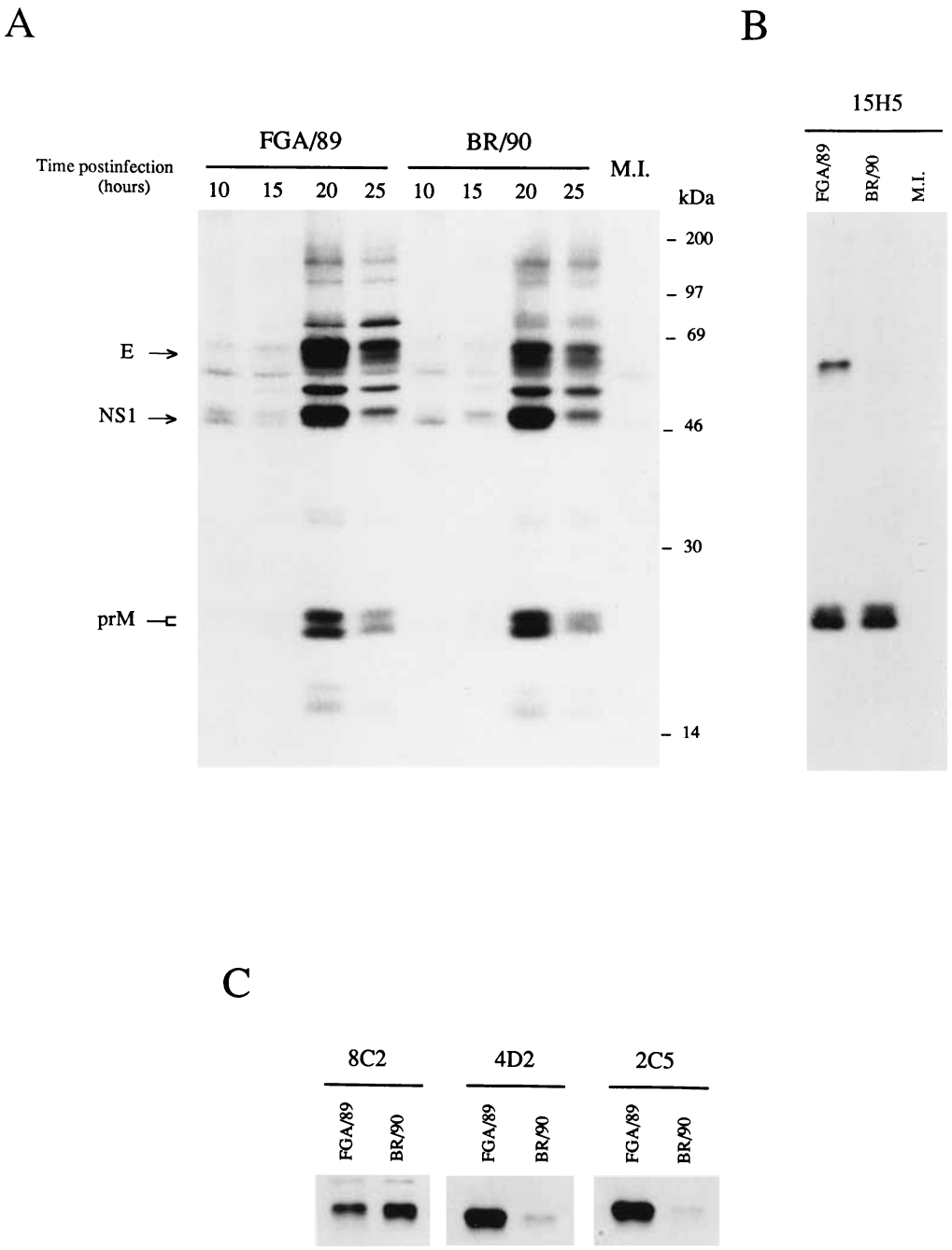

FIG. 4. Analysis of viral protein synthesis in Neuro 2a cells. Neuro 2a cells were infected with FGA/89 (multiplicity of infection [MOI] of 400) or BR/90 (MOI of 40) or mock infected (M.I.). Cells were labeled for 30 min at various times postinfection (A) or for 180 min 20 h postinfection (B and C) with 250 μCi of [^{35}S]Translabel (ICN) as previously described (4, 5). Cell lysates in radioimmunoprecipitation assay buffer were analyzed by radioimmunoprecipitation with anti-DEN virus hyperimmune mouse ascites fluid (HMAF) (A) or MAbs (B and C) and electrophoresis in SDS–15% polyacrylamide gels as previously described (4–6). (A) Pulse-labeled proteins were immunoprecipitated with anti-DEN-1 virus HMAF. Viral proteins E, prM, and NS1 are indicated. The positions of protein markers and their sizes are indicated. (B) Protein prM was tested with anti-prM MAb 15H5. (C) Protein E was tested with anti-E MAbs 8C2, 4D2, and 2C5.

teins in cell membranes rather than to virus assembly and release.

To exclude the possibility that apoptosis is triggered by DEN virus-induced cell membrane injury, we determined whether the plasma membrane Na^+-K^+-ATPase (Na^+ pump) activity was maintained in infected Neuro 2a cells. Cells were labeled with K^+-free PBS containing 5 mM RbCl and 0.5 μCi of ^{86}Rb per μmol of RbCl for 20 min (5, 6). The Na^+ pump activity was defined as the difference between the uptakes of ^{86}Rb in the presence and absence of 10 mM ouabain, a specific Na^+-K^+-ATPase activity inhibitor. The Na^+ pump activity in DEN virus-infected Neuro 2a cells 24 h postinfection was similar to that in mock-infected cells (30 μmol of K^+ uptake per min per mg of protein). Thus, DEN virus-induced apoptosis is not initiated by damage to the plasma membrane. This is consistent with the finding that apoptosis was initiated by an intracellular stress triggered by DEN virus replication.

We sought to identify the signals by which DEN virus infec-

tion induces apoptosis in Neuro 2a cells. Many biochemical pathways may be involved in apoptotic cell death, and apoptosis often requires the action of numerous proteases, including lamina protease, interleukin-1 β-converting enzyme-like proteases (prICE), apopain, and calpain (8, 12, 17, 18, 22, 29). To determine whether proteases are involved in the DEN virus-induced apoptosis, Neuro 2a cells infected with FGA/89 or BR/90 were incubated 20 h postinfection with a variety of cysteine and serine protease inhibitors (29). For protease inhibitor peptide analysis, cells were permeabilized by a short glycerol shock. At concentrations having a minimal effect on the viability of mock-infected Neuro 2a cells, acetyl-leucyl-leucyl-normethional leupeptin (100 μM), epoxysuccinyl compound E-64 (500 μM), calpain inhibitor peptide, phenylmethylsulfonyl fluoride (500 μM), and 3,4-dichloroisocoumarin (20 μM) all failed to prevent DEN virus-induced apoptotic cell death 40 h postinfection (data not shown). This suggests that protease-dependent pathways are not necessary for apoptosis triggered by DEN virus infection.

To determine whether cellular RNA transcription is required for DEN virus-induced apoptosis, DEN virus-infected Neuro 2a cells were incubated with actinomycin D 15 h postinfection. Actinomycin D at 80 nM, a concentration having no significant effect on the viability of mock-infected Neuro 2a cells, inhibited cell RNA synthesis by only 75% and did not prevent apoptotic cell death 40 h postinfection as monitored by PI staining (data not shown).

Apoptotic pathways may include continuous production of Fos-related antigen proteins, the components of DNA binding activity that recognize AP-1 sites in gene promoters (20, 26, 31). AP-1 DNA binding activity in nuclear extracts from DEN virus- and mock-infected Neuro 2a cells was tested by an electrophoretic mobility shift assay using a ^{32}P-labeled oligonucleotide with a consensus AP-1 binding site (23). There was no detectable AP-1–DNA activity in lysates of Neuro 2a cells infected with DEN virus 25 h postinfection (data not shown).

Oxidative stress induces apoptosis in neuroblastoma cells (27). Oxygen radicals are involved as messengers in the activation of transcription factor NF-κB in the cytoplasm, which may in turn activate genes involved in programmed cell death (15, 20, 30). We investigated whether the apoptotic pathway involved radical oxygen intermediates and thus whether NF-κB was activated in Neuro 2a cells infected with FGA/89 or BR/90. Antioxidant *N*-acetyl-L-cysteine (15 mM) did not prevent apoptotic cell death in infected Neuro 2a cells (data not shown) (15). However, DNA binding activities of NF-κB were similar in mock- and DEN virus-infected Neuro 2a cells 25 h postinfection (Fig. 5). Thus, there was no evidence for an association among apoptotic cell death in DEN virus-infected Neuro 2a cells, oxidative stress, and direct activation of NF-κB.

The ability of the ER-resident viral proteins to activate NF-κB (24) led us to test the possibility that DEN virus replication could prevent NF-κB activation in Neuro 2a cells. The ER stress-inducing agent thapsigargin, which activates NF-κB (23), was added to infected cells after 20 h of infection. As shown by an electrophoretic mobility shift assay with an NF-κB probe, a new, more slowly migrating DNA-protein complex was observed 25 h postinfection in nuclear extracts prepared from DEN virus-infected Neuro 2a cells treated with thapsigargin (Fig. 5). This suggests that NF-κB expression is not down-regulated by DEN virus infection. However, DEN virus replication in Neuro 2a cells enhanced the thapsigargin-induced NF-κB activity (Fig. 5). Thus, the ER-resident DEN proteins may act synergistically with thapsigargin to enhance the ER stress-mediated NF-κB induction. Interestingly, thapsigargin, which is also an inhibitor of the ER Ca^{2+} ATPase,

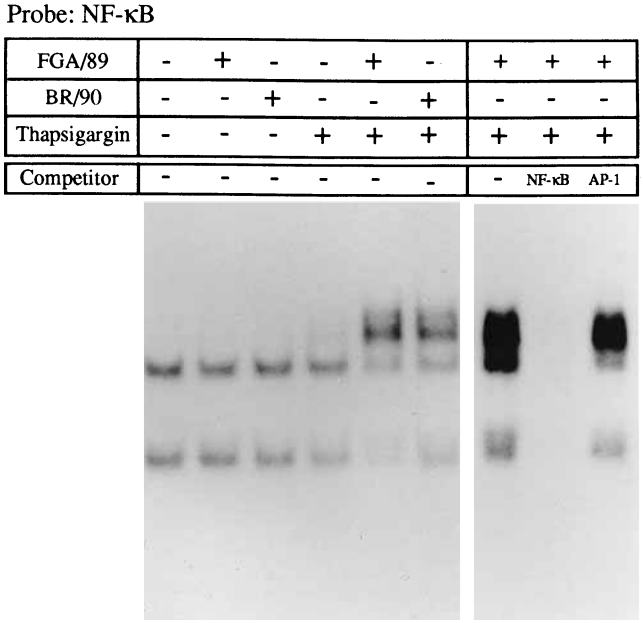

FIG. 5. DEN virus replication does not activate NF-κB in Neuro 2a cells. Nuclear extracts in Totex buffer from DEN virus- or mock-infected Neuro 2a cells 25 h postinfection were prepared as previously described (23). As a positive control of NF-κB activation, mock- or DEN virus-infected cells were treated 20 h postinfection with 1 μM thapsigargin for 5 h. Equal amounts of protein (4 μg) were tested in an electrophoretic mobility shift assay with ^{32}P-5′-end-radiolabeled oligonucleotide containing a consensus NF-κB site. The specificity of the retarded complexes was assessed by preincubating extract with a 50-fold excess of unlabeled NF-κB or an unrelated AP-1 probe.

increased the proportion of DEN virus-infected Neuro 2a cells in an apoptotic state threefold by 28 h of infection (data not shown). Moreover, the levels of ER-resident 78-kDa glucose-regulated protein (GRP78/BiP) were increased in DEN virus-infected Neuro 2a cells (data not shown). The overexpression of GRP78/BiP may be a response to the accumulation of viral proteins within the ER. GRP78/BiP is implicated in calcium sequestration (28). These observations suggest that DEN virus-induced ER stress may contribute to apoptotic cell death by acting on the calcium signalling pathways (3). A high intracellular concentration of Ca^{2+} promotes DNA degradation by direct stimulation of Ca^{2+}- and Mg^{2+}-dependent endonucleases and also affects the dynamic organization of myosin (2, 3, 25, 33). In a preliminary study, we observed progressive disruption of the actin cytoskeleton but not of microtubules in DEN virus-infected Neuro 2a cells. We are currently investigating whether calcium signalling pathways are involved in triggering apoptosis in mouse neuroblastoma cells infected with DEN virus.

We thank Pierre Gounon and Wlodzimierz Topilko for electron microscopy. We thank Marie-Pierre Courageot for her help with electrophoretic mobility shift assays and Marie-Pascale Frenkiel for technical expertise. We are indebted to Mary K. Gentry and Robert J. Putnak (Walter Reed Army Institute of Research, Washington, D.C.), Andrew Falconar (London School of Hygiene and Tropical Medicine, London, United Kingdom), and David M. Morens (University of Hawaii, Hononulu) for providing MAbs.

This work was supported by grants from INSERM (no. 683-CSS13) and CNRS (no. 95N82/0134).

REFERENCES

1. **Bastitatou, A., and L. A. Greene.** 1991. Aurintricarboxylic acid rescues PC12 cells and sympathetic neurons from cell death caused by nerve growth factor

deprivation: correlation with suppression of endonuclease activity. J. Cell. Biol. **115:**461–471.
2. **Bortner, C. D., N. B. E. Oldenburg, and J. A. Cidlowski.** 1995. The role of DNA fragmentation in apoptosis. Trends Cell Biol. **5:**21–26.
3. **Clapham, D. E.** 1995. Calcium signalling. Cell **80:**259–268.
4. **Desprès, P., M.-P. Frenkiel, and V. Deubel.** 1993. Differences between cell membrane fusion activities of two dengue type-1 isolates reflect modifications of viral structure. Virology **196:**209–216.
5. **Desprès, P., J. W. Griffin, and D. E. Griffin.** 1995. Effects of anti-E2 monoclonal antibody on Sindbis virus replication in AT3 cells expressing *bcl-2*. J. Virol. **69:**7006–7014.
6. **Desprès, P., J. W. Griffin, and D. E. Griffin.** 1995. Antiviral activity of alpha interferon in Sindbis virus-infected cells is restored by anti-E2 monoclonal antibody treatment. J. Virol. **69:**7345–7348.
7. **Earnshaw, W. C.** 1995. Apoptosis: lessons from *in vitro* systems. Trends Cell Biol. **5:**217–219.
8. **Faucheu, C., A. Diu, A. W. E. Chan, A.-M. Blanchet, C. Miossec, F. Hervé, V. Collard Dutilleul, Y. Gu, R. A. Aldape, J. A. Lippke, C. Rocher, M. S. S. Su, D. J. Levingston, T. Hercend, and J. L. Lalanne.** 1995. A novel human protease similar to the interleukin-1β converting enzyme induces apoptosis in transfected cells. EMBO J. **14:**1914–1922.
9. **Flamand, M., M. Huerre, M.-P. Frenkiel, P. Avé, V. Deubel, and P. Desprès.** Unpublished data.
10. **Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson.** 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. **119:**493–501.
11. **Henchal, E. A., and R. J. Putnak.** 1990. The dengue viruses. Clin. Microbiol. Rev. **3:**376–396.
12. **Kumar, S.** 1995. ICE-like protease in apoptosis. Trends Biochem. Sci. **20:** 198–202.
13. **Kurane, I., and F. A. Ennis.** 1994. Cytokines in dengue virus infections: role of cytokines in the pathogenesis of dengue hemorrhagic fever. Semin. Virol. **5:**443–448.
14. **Lee, E., D. J. Gubler, R. C. Weir, and L. Dalgarno.** 1993. Genetic and biological differentiation of dengue 3 isolates obtained from clinical cases in Java, Indonesia, 1976–1978. Arch. Virol. **133:**113–125.
15. **Lin, K.-I., S.-H. Lee, R. Narayanan, J. M. Baraban, J. M. Hardwick, and R. Ratan.** 1995. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. J. Cell Biol. **131:**1149–1161.
16. **Lobigs, M.** 1993. Flavivirus premembrane protein cleavage and speake heterodimer secretion require the function of the viral proteinase NS3. Proc. Natl. Acad. Sci. USA **90:**6218–6222.
17. **Martin, S. J., and D. R. Green.** 1995. Protease activation during apoptosis: death by a thousand cuts. Cell **82:**349–352.
18. **Martin, S. J., G. A. O'Brien, W. K. Nishioka, A. J. McGahon, A. Mahboubi, T. Saido, and D. Green.** 1995. Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J. Biol. Chem. **270:**6425–6428.
19. **Mason, P. W., M. U. Zügel, A. R. Semproni, M. J. Fournier, and T. L. Mason.** 1990. The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*. J. Gen. Virol. **71:**2107–2114.
20. **Meyer, M., R. Schreck, and P. A. Baeuerle.** 1993. H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary anti-oxidant-responsive factor. EMBO J. **12:**2005–2015.
21. **Morens, D. M., N. J. Marchett, M. C. Chu, and S. B. Halstead.** 1991. Growth of dengue type 2 virus isolates in human peripheral blood leukocytes correlates with severe and mild dengue disease. Am. J. Trop. Med. Hyg. **45:**644–651.
22. **Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, N. A. Munday, S. M. Raju, M. E. Smulson, T.-T. Yamin, V. Yu, and D. K. Miller.** 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature (London) **376:**37–43.
23. **Pahl, H., and P. A. Baeuerle.** 1995. A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF-κB. EMBO J. **14:**2580–2588.
24. **Pahl, H., and P. A. Baeuerle.** 1995. Expression of influenza virus hemagglutinin activates transcription factor NF-κB. J. Virol. **69:**1480–1484.
25. **Peitsch, M. C., B. Polzar, H. Stephan, T. Crompton, H. R. McDonald, H. G. Mannherz, and J. Tschopp.** 1993. Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J. **12:**371–377.
26. **Pennypacker, K. R., J.-S. Hong, and M. K. McMillan.** 1995. Implications of prolonged expression of Fos-related antigens. Trends Pharmacol. Sci. **16:** 317–321.
27. **Ratan, R. R., T. H. Murphy, and J. M. Baraban.** 1994. Oxidative stress induces apoptosis in embryonic cortical neurons. J. Neurochem. **62:**376–379.
28. **Sambrook, J. F.** 1990. The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum. Cell **61:**197–199.
29. **Sarin, A., D. H. Adams, and P. A. Henkart.** 1993. Protease inhibitors selectively block T cell receptor-triggered programmed cell death in a murine T cell hybridoma and activated peripheral T cells. J. Exp. Med. **178:**1693–1700.
30. **Schreck, R., P. Riever, and P. A. Baeuerle.** 1991. Reactive oxygen intermediate as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. **10:**2247–2258.
31. **Smeyne, R. J., M. Vendrell, M. Hayward, S. J. Baker, G. G. Miao, K. Schilling, L. M. Robertson, T. Curran, and J. I. Morgan.** 1995. Continuous c-*fos* expression precedes programmed cell death *in vivo*. Nature (London) **363:**166–169.
32. **Stocks, C. E., and M. Lobigs.** 1995. Posttranslational signal peptidase cleavage at the flavivirus C-prM junction in vitro. J. Virol. **69:**8123–8126.
33. **Wolenski, J. S.** 1995. Regulation of calmodulin-binding myosins. Trends Cell Biol. **5:**310–316.