JOURNAL OF VIROLOGY, Oct. 2009, p. 9940–9951 Vol. 83, No. 19
0022-538X/09/$08.00+0 doi:10.1128/JVI.01167-09

Interactions between Viral and Prokaryotic Pathogens in a Mixed Infection with Cardiovirus and Mycoplasma▽

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Received 8 June 2009/Accepted 6 July 2009

In the natural environment, animal and plant viruses often share ecological niches with microorganisms, but the interactions between these pathogens, although potentially having important implications, are poorly investigated. The present report demonstrates, in a model system, profound mutual effects of mycoplasma and cardioviruses in animal cell cultures. In contrast to mycoplasma-free cells, cultures contaminated with *Mycoplasma hyorhinis* responded to infection with encephalomyocarditis virus (EMCV), a picornavirus, but not with poliovirus (also a picornavirus), with a strong activation of a DNase(s), as evidenced by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) immunofluorescence assay and electrophoretic analysis of host DNA. This degradation was reminiscent of that observed upon apoptosis but was caspase independent, judging by the failure of the specific pan-caspase inhibitor Q-VD-OPh to prevent it. The electrophoretic mobility of the enzyme responsible for DNA degradation and dependence of its activity on ionic conditions strongly suggested that it was represented by a DNase(s) of mycoplasma origin. In cells not infected with EMCV, the relevant DNase was dormant. The possibility is discussed that activation of the mycoplasma DNase might be linked to a relatively early increase in permeability of plasma membrane of the infected cells caused by EMCV. This type of unanticipated virus-mycoplasma "cooperation" may exemplify the complexity of pathogen-host interactions under conditions when viruses and microorganisms are infecting the same host. In the course of the present study, it was also demonstrated that pan-caspase inhibitor zVAD(OMe).fmk strongly suppressed cardiovirus polyprotein processing, illustrating an additional pitfall in investigations of viral effects on the apoptotic system of host cells.

Traditionally, virologists are predominantly studying properties of individual viruses and much rarely interactions between different viruses. Even less attention is paid to interactions between viruses and microbes, although animal and plant viruses quite often share, in the natural environment, their ecological niches with microorganisms. Interactions of microbes with viruses other than phages are essentially a virgin soil of either virology or microbiology. The scarcity of relevant information may be illustrated by a recent review focused on the interaction between enteric viruses and enteric bacteria (23). There is ground to believe, however, that the impact of microbial flora on viral growth and pathogenicity (and vice versa) is much broader and deeper than is currently thought. One of the reasons for such a belief is the well-known effect of bacteria (and of their metabolic products) on diverse signaling systems of animal cells, in particular those involved in innate immunity (14, 41, 85, 89). A recent relevant example is demonstration that a prokaryotic infection may protect drosophila from a variety of RNA viruses (39). On the other hand, viral infection may significantly alter the outcome of concomitant bacterial infections. For example, it was reported that herpesvirus latency in mice may confer significant resistance to *Listeria* and *Yersinia* pathogens (8).

The aim of the present report is to demonstrate, in a model system, profound mutual effects of mycoplasma and cardioviruses during their interaction with animal cells. Cardioviruses, a genus in the picornavirus family, are represented by such widely investigated viruses as encephalomyocarditis virus (EMCV) and its strain mengovirus (MV), as well as Theiler's murine encephalomyelitis virus (TMEV). Cardioviruses are pathogens naturally affecting many animal species, in particular, rodents, pigs, and some primates (49). Recently, TMEV-related viruses have been implicated in human pathology (1, 12, 21, 29, 45, 46, 51, 96). Picornaviruses, which also include such viruses as poliovirus, hepatitis A virus, foot-and-mouth disease virus, and many others, possess a 7.2–8 kb-long single-stranded RNA genome of positive polarity and share general features of their reproduction mechanism (2). The genomic RNA contains a large single reading frame (certain strains of TMEV, but not EMCV, encode also a protein, L*, in an alternative frame; see reference 47) translated into a polyprotein, which is processed into a dozen "mature" proteins by a series of proteolytic events.

Interaction of picornaviruses with susceptible cells may have different outcomes: it may result in cytopathic (necrotic) effect (CPE) or apoptotic death, or it may lead to persistent infection. The character of these outcomes has obvious implications for the pathogenesis of the viral disease. Necrotic death is a

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▽ Published ahead of print on 15 July 2009.

strong trigger of inflammatory reactions. Apoptosis, which leads to degradation of host DNA and fragmentation of cells into membrane-coated so-called apoptotic bodies and their eventual "consumption" by macrophages and other scavenger cells, could limit not only the spread of the viral progeny but also prevent potential damages to the neighboring cells caused by inflammation. Therefore, apoptosis is generally considered to represent a defensive host reaction. The fate of the picornavirus-infected cell depends on the genetic properties of the both partners of virus-host interaction, as well as on the status of cell differentiation and environmental conditions (3, 13, 69, 70, 79, 80, 86). In particular, cardioviruses may evoke apoptotic reaction in certain, especially partially restrictive, cells (42, 43, 75, 76, 92).

Mycoplasmas are the smallest prokaryotic obligate parasites devoid of cell wall and of many synthetic capabilities and able to trigger a variety of diseases, such as pneumonia, uretritis and many others; they also frequently contaminate cultured cells. Mycoplasmas could be attached to plasma membrane of eukaryotic cells but in some cases they are able to invade the cells as well. They may exert a variety of effects on signaling systems of animal cells affecting, in particular, their innate immunity (67, 71).

We will demonstrate here that, in several mycoplasma-contaminated cell cultures, infection with EMCV (but not with poliovirus) results in activation of the microorganism-derived DNase(s), which accomplishes degradation of host cell DNA resembling that occurring during apoptosis but which was caspase independent. The same but mycoplasma-free cells respond to EMCV infection with a canonical CPE (70). This type of unanticipated virus-mycoplasma "cooperation" may illustrate the complexity of pathogen-host interactions under conditions when both viruses and microorganisms are present.

MATERIALS AND METHODS

Cells and viruses. HeLa-B cells (80), HeLa-3E cells constitutively expressing 3× EGFP-NLS, i.e., three copies of the enhanced green fluorescent protein fused to the simian virus 40 nuclear localization signal (9), and RD (human rhabdomyoblastoma) cells were grown on petri dishes in Dulbecco modified Eagle medium with 10% fetal bovine serum at 37°C under 5% CO_2. HeLa-3E cells expressing baculovirus p35 protein (HeLa-p35) were obtained by lentiviral transduction as described previously (30) using MarxIV-p35-puro as a vector. MarxIV-p35-puro was generated by cloning of BamHI/XbaI fragment from a p35-encoding plasmid into MarxIV–puro vector (both constructs were kindly donated by Y. Lazebnik). As control, cells, HeLa-MIV, similarly transfected with the empty MarxIV-puro vector (31) were used. Both types of transfected cells were grown in the presence of 1.5 μg of puromycin/ml.

To eliminate mycoplasma from contaminated cells, they were grown in Eagle medium with 10% fetal bovine serum in the presence of 10 μg of ciprofloxacin hydrochloride (ICN Biomedicals)/ml. To check the efficiency of decontamination, the cured cells were subjected to a passage without the drug, followed by the application of the cellular suspension onto FTA MicroCard (Whatman), three washes with FTA purification reagent, two washes with TE buffer (10 mM Tris-HCl and 0.1 mM EDTA [pH 8]), and subsequent PCR assay with the primers GPO-3 and MGSO described previously (82).

EMCV, its strain MV, and poliovirus type 1 Mahoney strain were used. To eliminate mycoplasma from possibly contaminated viruses, viral stocks were subjected to 3 passages in mycoplasma-free RD cells in the presence of 10 μg of ciprofloxacin hydrochloride/ml.

Mycoplasma identification. Cells were detached from the substrate, pelleted at 1,500 × *g* for 10 min at 4°C, suspended in 20 mM Tris-HCl (pH 7.4)–10 mM EDTA–0.5% Triton X-100, and incubated for 20 min at 4°C. The supernatant obtained after centrifugation in Eppendorf centrifuge at 2,000 rpm for 5 min at 4°C was subjected to the sodium dodecyl sulfate (SDS)-phenol extraction, nucleic acids were ethanol precipitated, dissolved in water placed onto Whatman paper, and air dried. The material from the paper was eluted with 75 μl of water and the 16S rRNA-based mycoplasma group-specific PCR was performed with 1-μl eluates essentially according to the protocol previously described (82) but using a different pair of primers: GPO-3 and 5′-ACTCCTACGGGAGGCAGCAGTA-3′. The PCR products were sequenced.

Infection and single-cycle growth experiments. Cells were detached by EDTA treatment and plated onto 35-mm petri dishes (Corning-Costar) at a density of ~10^5 cells/cm^2 and cultivated overnight under 5% CO_2 at 37°C in Eagle medium with 10% fetal bovine serum. The growth medium was discarded, and the virus was added in a volume of 1 ml to provide an input multiplicity of infection (MOI) of ~100 PFU/cell. After a 30-min incubation at room temperature, the cells were washed off with 2.5 ml of Eagle medium, and 1 ml of serum-free Eagle medium was added. After incubation at 37°C for the indicated time intervals, the cells were detached from the substrate by EDTA treatment, combined with the supernatant medium, subjected to three cycles of freezing-thawing, and frozen at −80°C until virus titer determination by plaque assay on RD cells.

EMCV replicon and luciferase assay. An EMCV-based luciferase-expressing replicon (pE-luc) (4) was kindly donated by Ann Palmenberg. In this replicon, the region encoding proteins 1C-1D (VP3-VP4) was replaced by the firefly luciferase gene. Translation of the replicon RNA transcript generated active luciferase. HeLa cells were transfected with in vitro-synthesized runoff transcripts of the replicon using DEAE dextran as described previously (81). At the indicated times, the cells were lysed and firefly luciferase levels were determined by using the luciferase assay system (Promega) according to the recommendations of the manufacturer by using a Modulus luminometer (Turner Biosystems).

Apoptosis induction. Protein synthesis inhibitor cycloheximide (CHI; 100 μg/ml) or transcription inhibitor actinomycin D (ActD; 3 μg/ml) was used as a nonviral inducer of apoptosis. Restrictive poliovirus infection was used as a viral inducer (80). To this end, guanidine-HCl (100 μg/ml) was added to the infected cells at an early step, 1.5 h postinfection (p.i.), and the cells were fixed for microscopic analysis several hours thereafter. The broad-spectrum caspase inhibitors zVAD(OMe).fmk, zVAD.fmk, and Q-VD-OPh (MP Biomedicals, Inc.) were used to suppress apoptosis.

TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay. Infected and mock-infected cells were grown on coverslips for the time intervals indicated, stained with Hoechst-33342, and fixed at room temperature with Safe Fix for 30 min. The cells were treated with 96 and 70% ethanol and stored at −20°C. The assay was performed by using the apoptosis detection system, Fluorescein kit (Promega) according to the manufacturer's instructions. The filter cube I3 was used for the registration of TUNEL-positive cells.

DNA electrophoresis. DNA fragmentation was assayed essentially as described previously (80). Briefly, the cells were detached from the plastic by EDTA treatment, suspended in a buffer containing 20 mM EDTA and 10 mM Tris-HCl (pH 7.4), and lysed with 0.5% Triton X-100 for 20 min on an ice bath. The suspension was subjected to centrifugation in an Eppendorf Minifuge (12,000 rpm, 15 min, 4°C), and the nuclei-free supernatant was treated with phenol-SDS. The nucleic acids were precipitated with ethanol, dissolved in 10 μl of H_2O, and treated with RNase A (10 μg/ml, 37°C, 30 min). The samples were subjected to electrophoresis on 1.5% agarose gels.

DNase assay. Cells treated as indicated were collected by the EDTA treatment and suspended in lysis buffer (137 mM NaCl, 10% glycerol, 1% NP-40, 20 mM Tris-HCl [pH 7.8]). Samples were equalized according to their protein concentration, supplemented with 2× sample buffer (2% SDS, 0.1% bromophenol blue, 10% glycerol, 350 mM β-mercaptoethanol, 50 mM Tris-HCl [pH 6.8]), heated for 10 min at 60°C, and applied to SDS-free polyacrylamide gels containing calf thymus DNA (50 μg/ml). After electrophoresis, the gels were fixed with 25% isopropanol in 10 mM Tris-HCl (pH 7.5), for 30 min at room temperature. Isopropanol was removed by two washes with 10 mM Tris-HCl (pH 7.5) for 30 min each; the gels were soaked in the appropriate reaction buffer at 37°C for 12 h and stained with ethidium bromide for visualization. If not otherwise indicated, the reaction buffer contained 3 mM concentrations (each) of $CaCl_2$, $MgCl_2$ and $MnCl_2$ and 10 mM Tris-HCl (pH 7.5).

In vitro translation. Translation of viral RNAs was performed in micrococcal nuclease-treated S10 Krebs-2 ascites carcinoma cell lysates as described previously (50, 78). Briefly, viral RNA, 10 to 20 μg/ml, was incubated in reaction mixtures containing 50% (vol/vol) S10 lysates and the following compounds (the final concentrations are indicated): 75 mM potassium acetate, 1 mM $MgCl_2$, 12 mM ATP, 8 mM GTP, 5 mM CTP, 5 mM UTP, 200 mM creatine phosphate, 1 μg of creatine kinase/ml, 5 μCi of [^{35}S]methionine (200 Ci/mmol), and 0.2 mM concentrations of 19 unlabeled L-amino acids. When appropriate, the mixtures were supplemented with caspase inhibitors at the indicated concentrations. The reactions were carried out for 3 h at 32°C and terminated by addition of sample

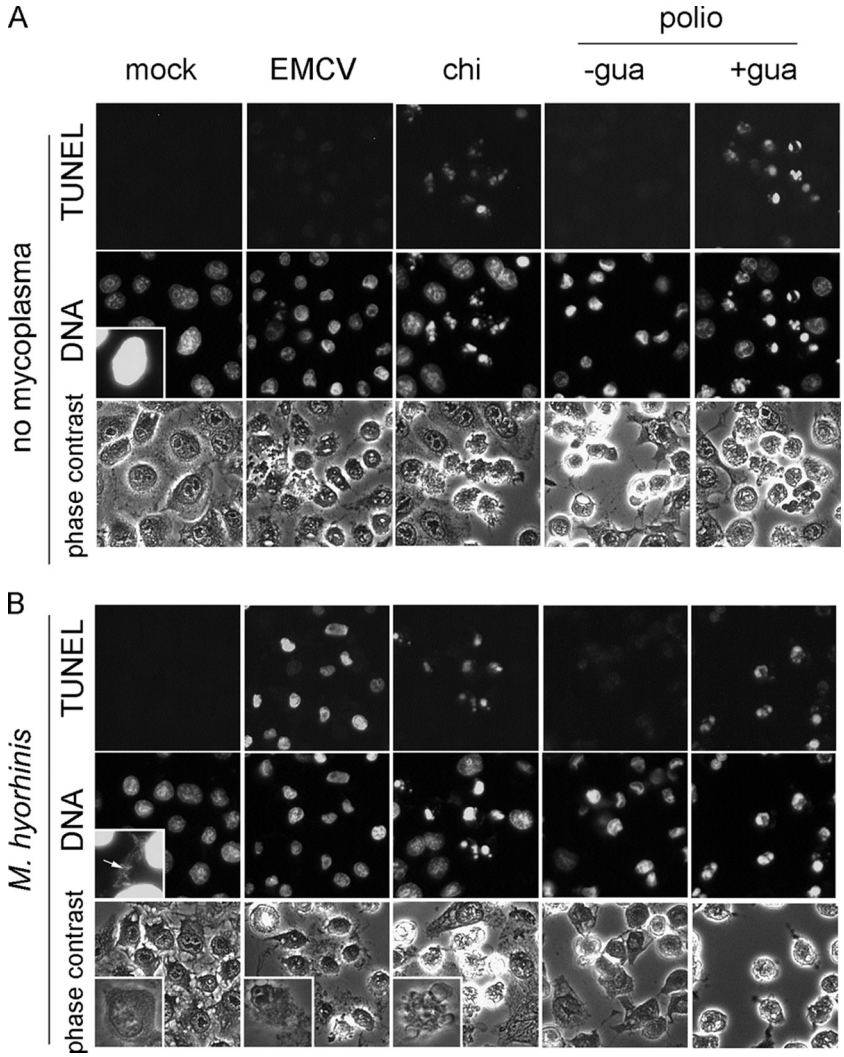

FIG. 1. Effect of EMCV infection on TUNEL staining of mycoplasma-free (A) and mycoplasma-contaminated (B) HeLa cells. HeLa cells were infected at an input MOI of ~100 PFU/cell and were assayed for TUNEL, stained with Hoechst 33342, and inspected under a phase-contrast microscope at 7 h p.i. Mock-infected cells, cells treated with CHI for 5 h, and cells infected with poliovirus at an MOI of ~2,000 PFU/cell for 7 h without (−) or with (+) 100 μg of guanidine-HCl (gua)/ml added at 1.5 h p.i. were used as controls. Insets demonstrate examples of respective individual cells at a higher magnification. Extranuclear DNA in the mycoplasma-contaminated culture is marked by an arrow. The virus-infected cells exhibited nuclear signs of CPE seen in DNA (Hoechst-33342-stained) and phase-contrast panels. Although EMCV infection did not elicit any marked signs of apoptosis in the mycoplasma-free cells, positive TUNEL signals, a hallmark of apoptosis, were clearly seen in the majority of infected mycoplasma-contaminated cells. On the other hand, phase-contrast investigation of the virus-infected cells demonstrated no appreciable blebbing, in contrast to the CHI-treated apoptotic cells (insets). The presence of mycoplasma exerted no appreciable effect on the apoptogenic effect of CHI and on cellular response to productive or restrictive (apoptogenic) infection with poliovirus.

buffer (105 mM β-mercaptoethanol, 3% SDS, 6% glycerol, 90 mM Tris-HCl [pH 6.8]). The probes were resolved in 6 to 15% gradient polyacrylamide gel electrophoresis and analyzed by autoradiography.

Microscopy and image processing. Fluorescence microscopy was performed with Leica DMLS fluorescence microscope equipped with green I3 (for visualization of TUNEL signals) and blue A (for Hoechst-stained nuclei) filter cubes. The pictures were obtained with Leica Digital Camera 100. To stain the nuclei, the cells, prior fixation, were incubated for 30 min with 5 μg of Hoechst 33342 dye/ml added to the culture medium. For staining of dead cells, 0.25% trypan blue in phosphate-buffered saline was used. The color images obtained by microscopy were transformed into the grayscale mode by using Adobe Photoshop 7.0. When appropriate, the brightness and contrast of the whole row of grayscale pictures in a given panel were equally optimized (to make visible details presented in the color images) by using "brightness/contrast" tool of Adobe Photoshop 7.0.

The agarose gels were captured with the DigiDoc-It Imaging System (UVP, LLC). The developed films obtained upon autoradiography were converted into am electronic form by a Snapscan 1212 (Agfa).

RESULTS

The response of HeLa and RD cells to EMCV (but not poliovirus) infection is dramatically altered by the presence of mycoplasma. As we showed recently, infection of mycoplasma-free HeLa cells with wild-type cardioviruses, EMCV or MV, leads to necrotic cytopathic death (70). There is nearly no host DNA degradation, as judged by very faint, if any, TUNEL signal (which reveals DNA breaks) and only quite moderate DNA fragmentation to high-molecular-mass species visible

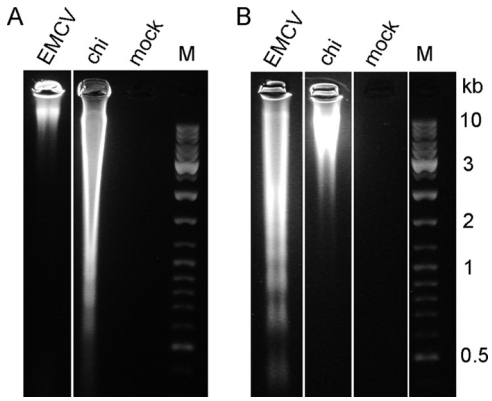

FIG. 2. Effect of EMCV infection on DNA from mycoplasma-free (A) and mycoplasma-contaminated (B) HeLa cells. The cells were infected with EMCV and treated with CHI as indicated in the legend to Fig. 1, and DNA was prepared and investigated by electrophoresis as described in Materials and Methods. The marker slot (M) contained GeneRuler DNA Ladder Mix (Fermentas) showing the DNA length (base pairs). EMCV infection caused extensive DNA degradation in the mycoplasma-contaminated but not mycoplasma-free cells, whereas mycoplasma contamination did not enhance DNA degradation in the CHI-treated apoptotic cells.

upon electrophoretic analysis. Importantly, EMCV-infected cells acquired an antiapoptotic state as revealed by their failure to develop apoptosis in response to the treatment with chemical (e.g., ActD) or viral (restrictive poliovirus infection in the presence of guanidine; see reference 80) apoptosis inducers (70).

A batch of uninfected mycoplasma-contaminated HeLa cells did not exhibit any major signs of pathology: TUNEL-positive cells constituted a minute proportion of the population and, in fact, were absent from the majority of microscopic fields (Fig. 1B), and only minimal degradation of host DNA to high-molecular-mass species could be detected by electrophoresis (Fig. 2B). However, extranuclear punctated staining by permeable DNA dye Hoechst 33342 could be observed, upon overexposure, in the majority of cells (Fig. 1B, arrow), suggesting the presence of a microbial contaminant. The contaminant was identified as mycoplasma by using PCR with appropriate primers and was classified as *Mycoplasma hyorhinis* by sequencing a PCR-amplified variable segment of the 16S rRNA.

The response of these mycoplasma-contaminated HeLa cells to EMCV markedly differed from that of mycoplasma-free cultures, as documented by a very strong TUNEL signal in the overwhelming majority of cells (Fig. 1B) and very intense DNA degradation to heterogeneous fragments, in particular, oligonucleosome-sized ones (Fig. 2B), the both features being typical of apoptosis. The cells, however, did not display such apoptosis-associated signs as plasma membrane blebbing (Fig. 1B, inset) or nuclear fragmentation, although deformation of nuclei and moderate chromatin condensation could readily be observed upon Hoechst-33342 staining (Fig. 1B).These nuclear alterations, typical of CPE associated with EMCV reproduction, did not appear to differ significantly from those observed in mycoplasma-free EMCV-infected cells (Fig. 1A). Remarkably, the presence of mycoplasma did not appreciably change HeLa cell response to poliovirus infection: in line with previous results (3, 80), there was no (or nearly no) signs of apoptosis upon productive infection with this virus, as judged by the TUNEL assay (Fig. 1B) or DNA electrophoresis (see Fig. 4B).

The mycoplasma-contaminated HeLa cells responded to known viral and nonviral inducers of apoptosis, such as abortive poliovirus infection (Fig. 1) and CHI (Fig. 1 and 2) qualitatively in the same way as did mycoplasma-free cells, i.e., by development of TUNEL-positive staining and by DNA fragmentation detectable electrophoretically. The presence of mycoplasma did not markedly alter rapid apoptotic response of HeLa cells to another apoptosis inducer, ActD (not shown).

Extensive DNA degradation, as judged by the TUNEL and DNA electrophoretic assays, could also be observed in mycoplasma-contaminated RD cells (Fig. 3), which are known (69) to be deficient in functions of the apoptotic system, as judged by their inability to develop apoptosis in response to abortive poliovirus infection, as well as to some nonviral apoptosis inducers (Fig. 3).

Thus, the mycoplasma-contaminated cells, in distinction with mycoplasma-free cells, responded to EMCV infection by

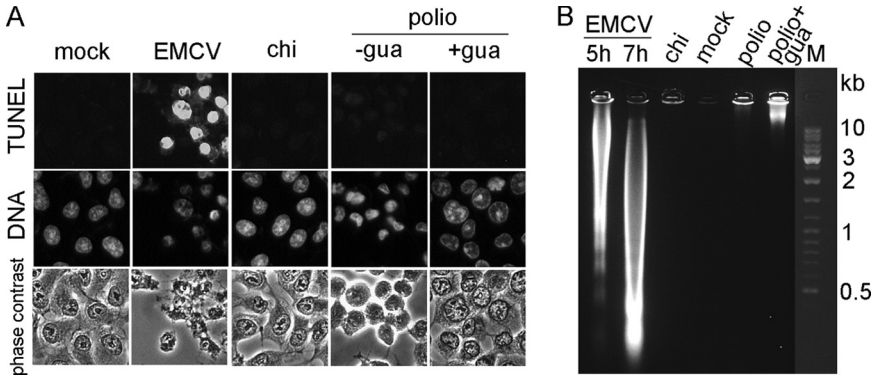

FIG. 3. Effect of EMCV infection on the DNA degradation in mycoplasma-contaminated RD cells. (A) Microscopic examination; (B) DNA electrophoresis. The conditions for EMCV and poliovirus infection and for CHI-treatment were the same as in the legend to Fig. 1. Electrophoretic analysis was performed with DNA samples from EMCV-infected cells taken at 5 and 7 h p.i., from poliovirus-infected cells (with or without guanidine [gua]) at 8 h p.i., and from mock-infected cells at 7 h. EMCV induced a strong TUNEL signals in the cells that failed to develop apoptosis in response to nonviral (CHI) and viral (poliovirus infection in the presence of guanidine) apoptosis inducers. The marker lane was contrasted separately from the rest of the gel to improve bands visibility.

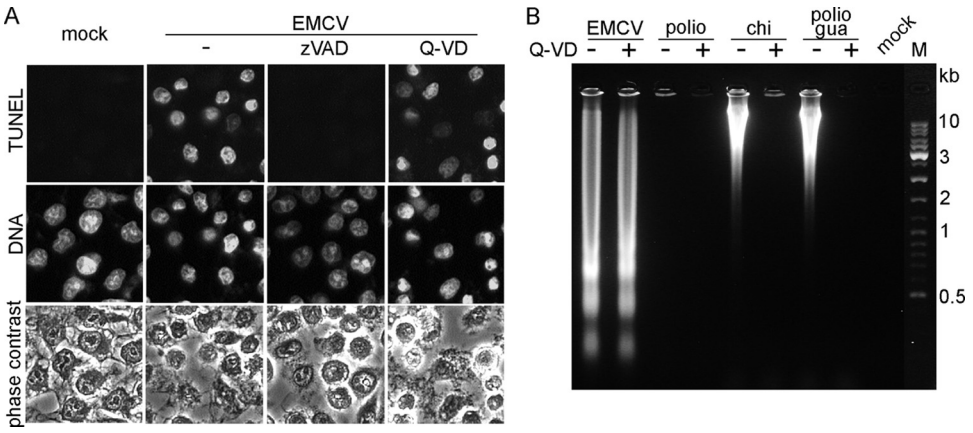

FIG. 4. Effect of caspase inhibitors on DNA degradation in EMCV-infected mycoplasma-contaminated HeLa cells. (A) Disparate effects of zVAD(OMe).fmk and Q-VD. The cells were infected at an input MOI of ~100 PFU/cell and were assayed for TUNEL, stained with Hoechst 33342, and inspected under phase microscope at 8 h p.i. zVAD(OMe).fmk at 100 μM or Q-VD at 20 μM were added to the appropriate samples at the onset of infection. It can be seen that the former inhibitor but not the latter prevented the development of TUNEL signal in EMCV-infected cells. As shown in Fig. 6, different effects of the two inhibitors were not due to differences in their concentrations. (B) Effects of Q-VD on DNA degradation triggered by EMCV infection (8 h p.i.) or by bona fide apoptosis inducers, CHI (5 h p.i.), and restrictive poliovirus infection (8 h p.i. with guanidine added at 1.5 h p.i.) The drug failed to prevent EMCV-triggered degradation of DNA, whereas degradation triggered by the apoptosis inducers was completely suppressed.

extensive DNA degradation the feature usually considered as a hallmark of apoptosis.

EMCV-triggered DNA degradation in mycoplasma-infected HeLa cells could not be prevented by specific caspase inhibitors. The enterovirus-induced apoptosis-related DNA fragmentation is known to be readily preventable by caspase inhibitors, such as zVAD(OMe).fmk (3, 17). When this compound and another broad-spectrum caspase inhibitor Q-VD-OPh (18), hereinafter referred to as Q-VD, were tested for their capacity to prevent the appearance of TUNEL-positive signals in the mycoplasma-contaminated HeLa cells, surprisingly discordant results were obtained. Whereas the former did prevent this apoptosislike feature, the latter failed to do so (Fig. 4A). The failure of Q-VD to affect the EMCV-triggered appearance of TUNEL-positive cells could not be explained by its inability to prevent apoptosis in our system. Indeed, the compound clearly exhibited its antiapoptotic activity in the mycoplasma-contaminated HeLa cells either abortively infected with poliovirus or treated with CHI (Fig. 4B). Moreover, EMCV infection triggered DNA degradation in a significant proportion of mycoplasma-contaminated HeLa-p35 cells constitutively expressing baculovirus antiapoptotic p35 protein (22), as evidenced by both the TUNEL assay (Fig. 5A and B) and DNA electrophoresis (Fig. 5C), although these effects were slightly milder than in the virus-infected control HeLa-MIV cells. On the other hand, the HeLa-p35 cells were fully resistant to the apoptogenic activity of CHI (Fig. 5).

The observation that the effect of zVAD(OMe).fmk sharply deviated from that of another apoptosis inhibitor hinted that the former might have some additional side effect(s) on EMCV reproduction. Indeed, it turned out that this inhibitor prevented the development of virus-induced CPE and fully suppressed the generation of infectious EMCV progeny (Fig. 6A), inhibited replication of the EMCV-based replicon (Fig. 6B), and interfered with processing of the viral polyprotein in a cell-free system. As shown in Fig. 6C, conversion of large precursors into smaller "mature" proteins was inhibited in a drug concentration-dependent mode. This result strongly suggested that the primary target of the inhibitor was the viral protease 3C (or 3CD) responsible for the polyprotein processing. The inhibitory effect of zVAD(OMe).fmk on EMCV reproduction was similarly exhibited in mycoplasma-contaminated and mycoplasma-free HeLa cells and also in L929 cells (not shown). The drug also inhibited reproduction of MV and replication of a MV-based replicon (data not shown). In contrast, Q-VD exerted no significant inhibition of EMCV (or MV) reproduction (see also reference 70) or polyprotein processing (Fig. 6). The distinct effects of zVAD(OMe).fmk and Q-VD could not be explained by the difference in the concentrations used, since the former nearly fully suppressed EMCV reproduction at 20 μM (not shown), whereas the latter did not appreciably affect cardiovirus polyprotein processing at 100 μM (Fig. 6C). Another antiapoptotic drug, DEVD.cmk did not affect MV reproduction either (not shown). These results were consistent with the notion that zVAD(OMe).fmk, in addition to its established anti-caspase activity, also suppressed the activity of the cardioviral 3C (or 3CD) proteases responsible for the viral polyprotein processing. As judged by the results of cell-free translation experiments, zVAD.fmk [i.e., unmethylated form of zVAD(OMe).fmk] also inhibited EMCV polyprotein processing (Fig. 6C) and, by implication, 3C/3CD protease activity, even though the effect of this inhibitor on viral reproduction was markedly less efficient compared to its O-methylated counterpart (not shown), likely due to its poor entry into the cytoplasm. Regardless of the mechanism of the inhibitory effect of zVAD(OMe).fmk on viral reproduction, its ability to prevent EMCV-triggered degradation of DNA of mycoplasma-infected cells suggested that this degradation was coupled to the reproduction of the virus. On the other hand, the failure of Q-VD (which appeared to be essentially devoid of detectable side effects in the systems studied) to prevent this degradation

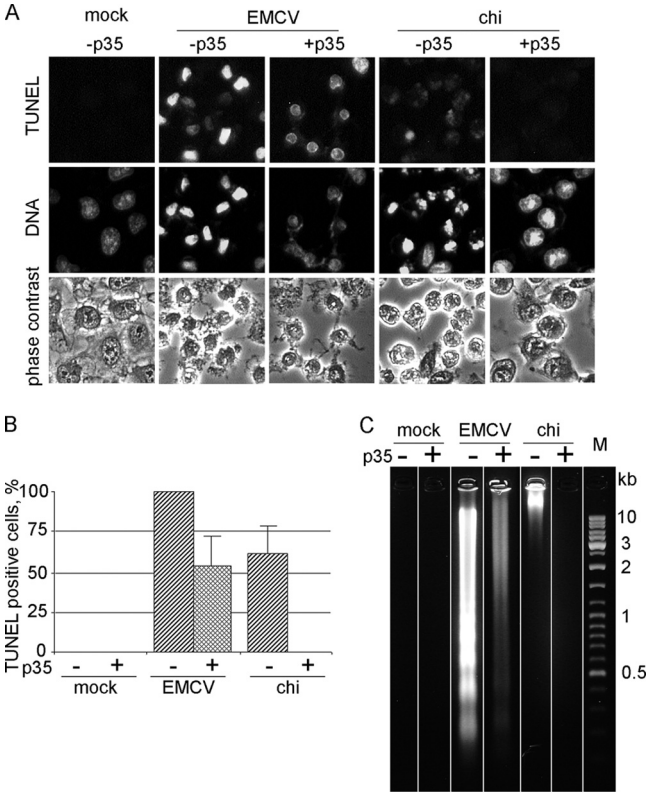

FIG. 5. Effect of baculovirus p35 protein on DNA degradation in EMCV-infected or CHI-treated mycoplasma-contaminated HeLa cells. (A) HeLa-MIV (i.e., transformed with the empty vector) and HeLa-p35 cells were infected with EMCV for 8.5 h or treated with CHI for 4.5 h and processed for the TUNEL assay. (B) Quantification of TUNEL-positive HeLa-MIV (−) and HeLa-p35 (+) cells infected with EMCV or treated with CHI. Average data from two independent experiments. The yield of infectious virus by 8 h p.i. was $\sim 3 \times 10^4$ and $\sim 6 \times 10^4$ PFU/cell in HeLa-MIV and HeLa-p35 cells, respectively. (C) DNA electrophoresis was performed with samples taken from EMCV-infected cells at 8.5 h p.i. and from CHI-treated cells after 4.5 h of incubation with the inhibitor. Expression of p35 completely suppressed the appearance of TUNEL-positive cells and DNA degradation triggered by CHI but only partially diminished these effects in the case of EMCV infection.

suggested that the degradation was due to a caspase-independent mechanism.

Q-VD also did not prevent the appearance of TUNEL-positive signal in EMCV-infected mycoplasma-contaminated RD cells (not shown).

DNA degradation in mycoplasma-contaminated virus-infected cells was accomplished by a microbial enzyme(s). Since the host DNA degradation in mycoplasma-contaminated cardiovirus-infected cells did not appear to be due to the conventional caspase-dependent apoptosis, the nature of the enzyme involved was investigated. To this end, the following approach was used (61). The cell lysates were subjected to electrophoresis in semidenaturing DNA-containing polyacrylamide gels. The gels were washed and incubated at 37°C in a reaction buffer for 14 h, followed by staining with ethidium bromide. The negative bands thus obtained reflected the electrophoretic mobility of the active nucleases. The results demonstrated that EMCV infection was accompanied by a strong activation of several DNase species at 6 to 8 h p.i. (Fig. 7A), with their number increasing during the course of infection. DNase bands with similar mobility but a markedly lower intensity were present in the cultures prior to viral infection as well (Fig. 7A).

Essentially the same pattern of DNase(s) activation was observed during EMCV infection of mycoplasma-contaminated RD cells (Fig. 7B). On the other hand, no, or nearly no, activation, compared to the mock sample, was observed during apoptosis triggered by CHI or during productive or abortive (i.e., apoptosis-inducing) infection with poliovirus at a time corresponding to completion of the reproduction cycle of this virus under the permissive conditions (Fig. 7A). The caspase inhibitor Q-VD did not prevent the EMCV-triggered enhancement of DNase bands (Fig. 7C). Incubation at 37°C of the lysates from mycoplasma-contaminated RD cells, mock-infected or EMCV-infected, lead to an increase in the intensity of DNase bands (Fig. 7D), suggesting that the activation of a preexisting enzyme(s) took place. This effect was observed in both types of samples but was more striking in lysates from the virus-infected cells.

Properties of the enzyme were further studied in extracts of RD cells to minimize possible contributions of host apoptotic proteins (as indicated above, these cells possess a deficient apoptotic machinery). The enzyme(s) was not inhibited by 10 μM 6-DTAF {4-(4,6-dichloro-[1,3,5]-triazin-2-ylamino)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid, also known as DR396} (Fig. 8A), an inhibitor of the DNase gamma (77). It was activated at 200 mM KCl (Fig. 8B), exhibited a marked dependence on the presence of both Ca^{2+} and Mg^{2+} ions (Fig. 8C) and was active under a broad range of pH values with two apparent optimal zones, at pH 6.5 and pH 8.5 to 9.0 (Fig. 8D). These properties were similar to those of mycoplasma DNase(s) (61, 63) and strongly suggested that the enzyme activated upon cardiovirus infection of mycoplasma-contaminated cells was of the mycoplasma origin.

Increased permeability of plasma membrane in EMCV-infected cells as a possible factor involved in mycoplasma DNase activation. The results presented above suggested that infection of mycoplasma-contaminated HeLa cells with EMCV but not with poliovirus strongly activated microbial DNase(s). One of the possible reasons for these discordant effects of the two viruses could be a stronger and earlier increase in plasma membrane permeability in the cardiovirus-infected cells, as evidenced by trypan blue staining. As shown in Fig. 9A, a significant proportion of mycoplasma-free HeLa cells became dye-stained by 7 h p.i. On the other hand, cells undergoing productive poliovirus infection and exhibiting clear cytoplasmic and nuclear signs of CPE remained, at the same time, unstained (appreciable staining could be observed only several hours after completion of viral reproduction; see also reference 69), as were unstained cells with CHI-triggered apoptosis (Fig. 9A). EMCV-elicited permeabilization of the plasma membrane took place also in mycoplasma-contaminated culture, and a proportion of cells became stained already by 5 h p.i., this proportion being markedly increased during the next 2 h (Fig. 9B). The number of TUNEL-positive cells increased in the course of infection in parallel with accumulation of trypan blue-stained cells, and the stained cells were usually found to be TUNEL positive (Fig. 9B). The relevant alteration

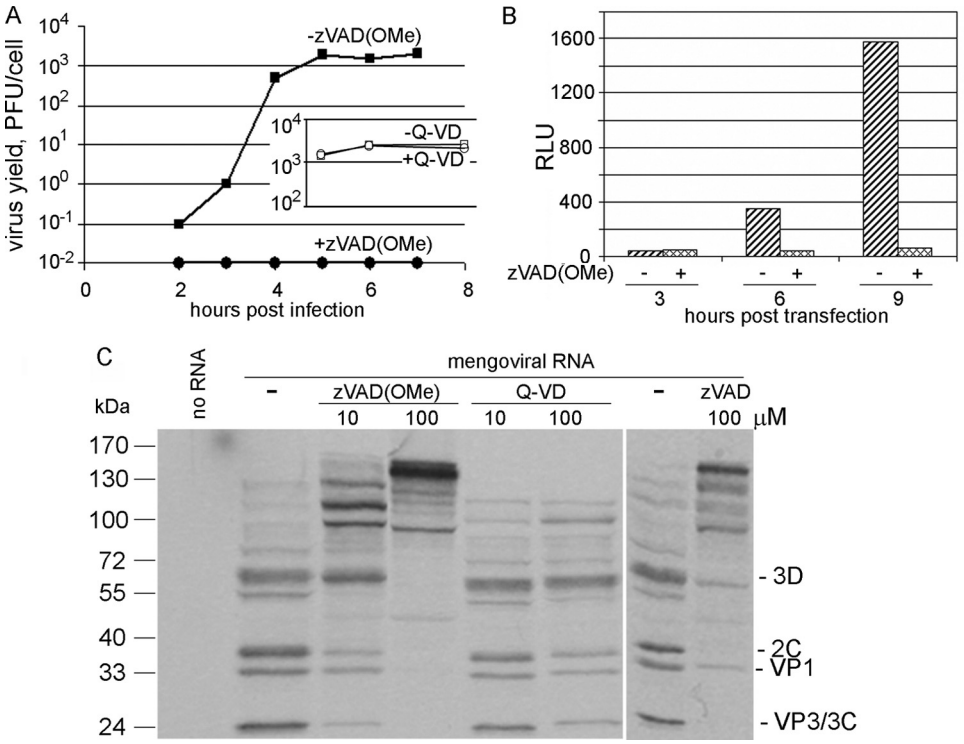

FIG. 6. Effect of caspase inhibitors on cardiovirus reproduction and processing of the viral polyprotein. (A) Effect of zVAD(O-Me).fmk and Q-VD (inset) on the reproduction of EMCV. (B) Effect of zVAD(O-Me).fmk on replication of the EMCV-based replicon. The efficiency of replication is expressed in the relative luciferase units (RLU). (C) Effect of the inhibitors on in vitro processing of the EMCV polyprotein. EMCV RNA was translated in extracts from Krebs-2 cells as described in Materials and Methods in the absence or presence of different concentrations (μM) of zVAD(O-Me).fmk, Q-VD (left panel), or zVAD.fmk (right panel). The generation of infectious progeny, replication of the replicon, and processing of the polyprotein were strongly suppressed by zVAD(O-Me).fmk but were unaffected by Q-VD. The primary target of the drugs, the polyprotein processing, was not inhibited by Q-VD even at 100 μM, the concentration at which zVAD(O-Me).fmk was usually used. The polyprotein processing was also inhibited by zVAD.fmk.

of plasma membrane permeability might facilitate entry of the microorganism (or its protein products) into the animal cells.

One might hypothesize that the DNase involved in DNA degradation in cells with damaged plasma membrane could have originated from the serum, since DNase I is known to be secreted in the bloodstream (74). As shown by the in-gel assay, the serum used in our experiments did not contain detectable amounts of nucleases similar to those described above (data not shown). The serum addition to the mycoplasma-contaminated cells or to EMCV-infected mycoplasma-free cells did not result in DNase activation but rather, in the later case, somewhat inhibited it (not shown), perhaps due to increased cell viability. It became evident in these experiments with EMCV-infected mycoplasma-contaminated HeLa cells that the correlation between the proportion of trypan blue-stained and TUNEL-positive cells was not absolute. At early steps of infection, some microscope fields might contain a significant proportion of the latter but nearly none of the former (not shown). One of the possible explanations for this observation could be a higher sensitivity of the TUNEL assay compared to the trypan blue staining.

DISCUSSION

The present study describes a nontrivial interaction or "cooperation" between an RNA virus and mycoplasma. Cultures of mycoplasma-contaminated cells responded to infection with EMCV with an apoptosis-resembling reaction: the virus-infected cells exhibited a strong TUNEL-positive signal, and their DNAs were degraded to fragments superficially similar to the electrophoretic "ladder" typical of apoptosis. This degradation, however, could not be prevented by a pan-caspase inhibitor and appeared to be caused by DNase(s) of mycoplasma origin. The activation of this DNase and/or its entry into the nuclei was possibly facilitated by an increase in the permeability of the cellular membranes due to the viral infection.

Nature of DNase(s) responsible for DNA degradation in EMCV-infected mycoplasma-contaminated cells. DNA degradation is a hallmark of apoptosis. Several DNases have been shown or proposed to be involved in executing the apoptotic DNA degradation. They could be assigned to two functional classes: cell-autonomous nucleases and waste-management nucleases (60, 74). The former are parts of the apoptotic machinery, whereas the latter are involved in the degradation of DNA of engulfed apoptotic cells by macrophages and other scavenger cells. In the context of the present study, cell-autonomous nucleases appear to be more relevant. Among them, the following enzymes are thought to be most important for apoptotic DNA degradation, CAD (caspase-activated DNase, also known as DFF40), mitochondrial endonuclease G (EndopG),

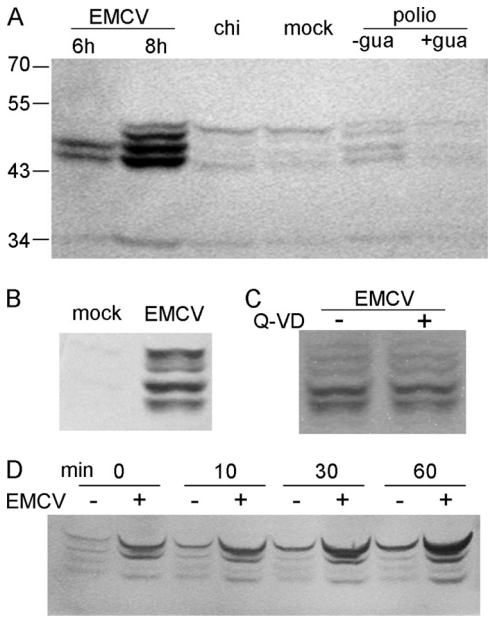

FIG. 7. DNase activation in EMCV-infected mycoplasma-contaminated HeLa cells. (A) DNase bands obtained upon electrophoresis of cell extracts in DNA-containing gels. Extracts were prepared from mycoplasma-contaminated HeLa cells infected with EMCV for 6 and 8 h; mock-infected cells, cells infected with poliovirus (with or without guanidine-HCl [gua], as in the legend to Fig. 1), as well as cells treated with 100 μg of CHI/ml for 4 h, were used as controls. The extracts were analyzed as described in Materials and Methods. The positions of marker proteins with the corresponding molecular masses in kilodaltons are indicated. Marked enhancement of DNase bands in the course of EMCV infection was clearly visible. (B) Extracts were prepared from mycoplasma-contaminated EMCV-infected RD cells at 7 h p.i. DNase activation was obvious. (C) Failure of Q-VD to prevent activation of DNase(s) in EMCV-infected mycoplasma-contaminated HeLa cells. The extract was prepared at 8 h p.i. The inhibitor, 20 μM, was added at the onset of infection. (D) DNase activation upon incubation of extracts from mycoplasma-contaminated RD cells. Lysates prepared from mock-infected (−) and EMCV-infected, (+) cells at 5 h p.i. as indicated in Materials and Methods were incubated at 37°C for the time intervals indicated prior to electrophoresis in the DNA-containing gel. An increase in the band intensity, seen in both types of samples, but especially in those derived from virus-infected cells, indicates activation of the preexisting enzyme(s).

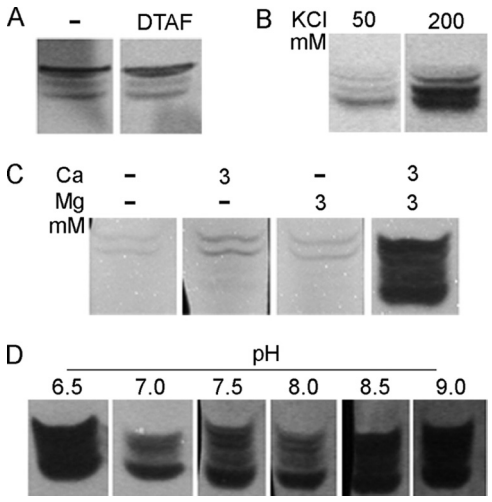

FIG. 8. Properties of the DNase(s) activated by EMCV infection of mycoplasma-contaminated RD cells. Extracts were prepared at 7 h p.i. and analyzed as described in Materials and Methods. The following modifications of the incubation buffer (containing, with the exception of panel C, 3 mM concentrations of $MgCl_2$, $CaCl_2$, and $MnCl_2$) were used: the presence (+) or absence (−) of 10 μM 6-DTAF (A), different concentrations of KCl (B), different concentrations of Mg^{2+} and Ca^{2+} (C), and (D) different pH levels.

and DNase γ. The DNase(s) responsible for the effects described here were clearly distinct from these three nucleases. Although the nuclease activity described in the present study was dependent on the presence of both Mg^{2+} and Ca^{2+}, neither CAD (88) nor EndoG (72, 87) require Ca^{2+}. Furthermore, the latter enzyme is inhibited by KCl concentrations above 60 mM (87). The involvement of DNase gamma was unlikely because of the failure of its inhibitor 6-DTAF, also known as DR396 (77), to suppress the enzyme in our system. The properties of the enzyme studied here were also clearly different from those of a major waste-managing nuclease, lysosomal DNase II, the main difference being an acidic pH optimum of the latter. The involvement of Mg^{2+}/Ca^{2+}-dependent DNase I, another waste-managing nuclease, which could be secreted into the bloodstream, was unlikely because, as mentioned above, our assay failed to detect appreciable amounts of DNases in the serum used and because the addition of serum resulted in a decrease rather than an increase in nucleolytic activity.

On the other hand, the electrophoretic mobility and ionic requirements of the nuclease activated in EMCV-infected mycoplasma-contaminated cells were similar to those known for mycoplasma nucleases (61, 63). Taking into account enzymological similarity and the fact that such nuclease activity could only be detected in mycoplasma-contaminated cells, we conclude that it was of mycoplasma origin.

Influence of mycoplasmas and other "third partners" on the outcome of virus-cell interactions. A fundamental point implicit in the results presented here concerns the potential significance of alterations in virus-cell interactions by the active participation of a third partner, a microorganism or another virus. In our system, the presence of mycoplasma certainly changed the development of cellular pathology of virus-infected cells and perhaps accelerated the virus-induced death.

This issue may be considered in a broader perspective. In natural settings, viral infection rarely takes place under sterile conditions and occurs most often within readily accessible organs, i.e., the gastrointestinal or respiratory tracts. The outcome of virus-cell interaction may be significantly altered by a third participant, a virus or a microorganism. However, how these "third-party" effects influence the mechanisms and outcomes of virus-host interactions is poorly understood. For obvious reasons, most studies have been carried out with human immunodeficiency virus (HIV). In particular, interactions between HIV and different herpesviruses, such as Kaposi sarcoma-associated human herpesvirus 8 and some other herpesviruses, have been a subject of numerous studies. Reproduction of the both partners could be affected (see the recent references (52, 56, and 94). Latent herpesvirus infection may make HIV susceptible to acyclovir, which fails to exhibit anti-HIV effect in cells singly infected with HIV (53). Other viruses, both DNA-

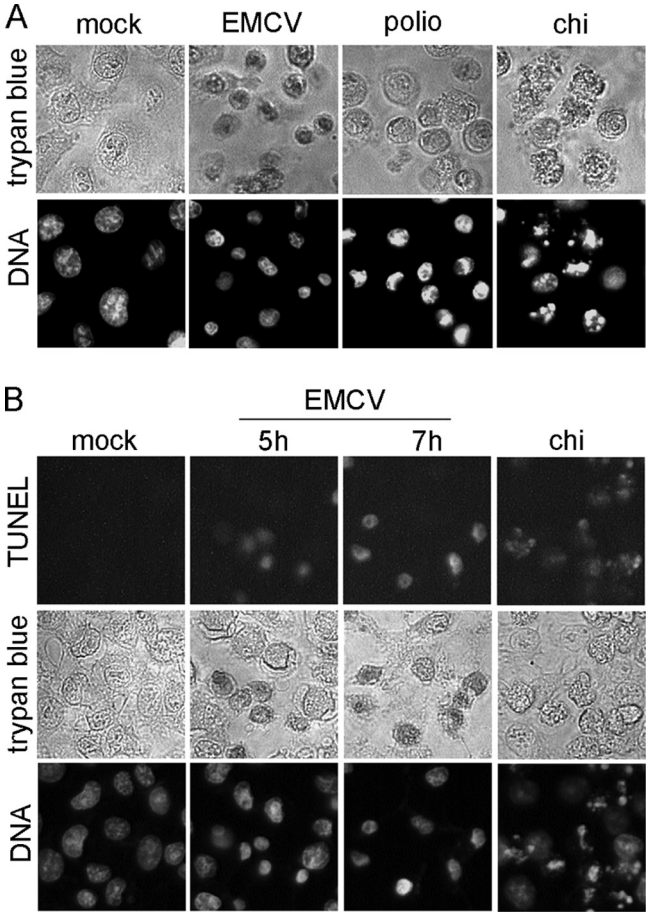

FIG. 9. Membrane alterations in HeLa cells upon EMCV infection as revealed by trypan blue staining. (A) The majority of mycolplasma-free cells undergoing EMCV infection (7 h p.i.) were stained, whereas poliovirus-infected (7 h p.i.) or CHI-treated (5 h) cells were not. Cytopathic changes and apoptotic nuclear fragmentation in the virus-infected and CHI-treated cells, respectively, were clearly seen in the DNA (Hoechst 33342-stained) samples. (B) Correlation between the appearance of trypan blue-stained and TUNEL-positive cells after EMCV infection (5 and 7 h p.i.) of a mycoplasma-contaminated HeLa cell culture. The duration of incubation with 100 μg of CHI/ml was 5 h. The TUNEL-positive virus-infected cells were stained with trypan blue, whereas the CHI-treated apoptotic cells were not.

and RNA-containing viruses, may also interfere with HIV reproduction (33, 84, 90). Nonapparent persistent infection with a virus (e.g., SV5) may modulate the innate immune system of host cells, altering thereby reproduction of another virus in these cells (93).

Prokaryotes, as well as products of their metabolism, are well known to be able to exert a variety of effects on the both innate and adaptive immunity (14, 24, 41, 85, 89). In the context of the present study, modulation of virus-cell interactions by mycoplasmas is of special interest (20). Again, a variety of effects of this microorganism on infection with HIV have been described (58). In particular, it is relevant to mention that the CPE of HIV in certain cell cultures could be observed only if these cultures had been contaminated with mycoplasma. Some data also suggested that mycoplasma coinfection may markedly affect pathogenesis of AIDS, and it was hypothesized that mycoplasma could be an important factor in the HIV epidemics (58).

A recent report demonstrated that mycoplasma infection could activate the NF-κB pathway in cultured cells and protect them from apoptosis induced by genotoxic stress (55). Thus, it is conceivable that under certain conditions mycoplasma contamination may suppress virus-induced apoptosis, an effect that would superficially be the opposite to that described here. This adds one more cautionary note in interpreting the results of virus infection of cultured cells and prompts the necessity of adequate control for mycoplasma contamination.

The results presented here also demonstrate the possibility of modulation of mycoplasma activity by concomitant viral infection. It is conceivable that similar modulatory effects could take place in natural settings as well.

Variability of the mycoplasma effects on cells infected with different picornaviruses. A noteworthy observation reported here is that, in contrast to cardioviruses, poliovirus infection of the contaminated cells did not trigger mycoplasma DNase activation, at least until the full-fledged CPE. The reason for such dissonant behavior of two picornaviruses is unknown, but it is tempting to relate it to different time courses of plasma membrane alterations caused by the representatives of the two picornaviral genera. Indeed, as shown in Fig. 9, a significant proportion of the EMCV-infected cells became accessible to trypan blue dye at the time of exponential accumulation of the viral progeny. In poliovirus infection, such staining could be detected in a significant proportion of the cells only hours after completion of the infectious cycle (69). It may be noted that some activation of mycoplasma DNase could be observed in lysates prepared at such postmortem steps from poliovirus-infected mycoplasma-contaminated cells as well (data not shown).

Although membrane alterations in picornavirus-infected cells have been the subject of numerous studies (16), the exact nature of the plasma membrane permeabilization and damage is not fully understood. A variety of mechanisms operating during distinct steps of viral reproduction have been implicated, but the relevant differences between various picornavirus representatives attracted relatively little attention. Now that we know how diverse are the mechanisms used by various picornaviruses to affect the translation of host mRNA (34, 35, 48, 59, 78, 95), impair nucleocytoplasmic transport (6, 9, 10, 25, 36, 37, 50, 64, 65), suppress innate immunity (7, 26, 32, 38, 62, 68, 83, 91), or affect the apoptotic machinery of the target cells (5, 11, 15, 40, 44, 54, 69, 73, 75, 86), we should not be surprised by the possible variability of the viral effects on plasma membrane integrity. The relevant data are, however, rather scarce.

Ambiguities in the interpretation of the TUNEL assay and of effects of caspase inhibitors. As shown elsewhere (70), productive EMCV infection of HeLa cells results in the acquisition by these cells of an antiapoptotic state. However, if the cells are contaminated with mycoplasma, one may be mislead by the virus' ability to elicit a strong TUNEL-positive signal and extensive DNA degradation, these features which are typical of bona fide apoptosis being erroneously interpreted as evidence for the apoptogenic ability of EMCV. False-positive results of the TUNEL and of some other assays for DNA degradation have been repeatedly described (19, 28, 66), but one should be especially careful in interpreting the results of

such assays in experiments with virus-infected tissue culture cells, known to be not so rarely contaminated with mycoplasmas.

The side effects of widely used and relatively well-characterized caspase inhibitors are another source of potential misinterpretations of virus-cell interactions. The first evidence that the pan-caspase inhibitor zVAD(OMe).fmk is also an inhibitor of proteolytic activity of $2A^{pro}$ of rhinovirus 2 and coxsackievirus B4 was reported by Deszcz et al. (27). In the case of coxsackievirus B3, both 2A and 3C proteases appeared to be suppressed by the drug (57). As far as cardioviruses are concerned, we reported preliminary data on the inhibition of EMCV protease $3C^{pro}$ by zVAD(OMe).fmk in 2006 (50a), and these observations are detailed here. Very recently, similar observations have been made with another cardiovirus, TMEV (76). Thus, some conclusions about the relationships between apoptosis, on the one hand, and virus reproduction, on the other, derived from the experiments in which such inhibitors of apoptosis had been used should perhaps be reconsidered.

ACKNOWLEDGMENTS

We are grateful to E. A. Korotkova and M. L. Yakovenko for help with the control of cell culture mycoplasma contamination.

This study was supported by a joint grant from The Netherlands Organization for Scientific Research-Russian Foundation for Basic Research (to V.I.A.), Russian Foundation for Basic Research grants (to P.V.L. and V.I.A.), a Russian Presidential Grant for Young Scientists (to P.V.L.), and a Scientific School Support Program grant (to V.I.A.). S.V.H. is supported by a mosaic grant from the NWO. F.J.M.V.K. is supported by a VIDI grant from the NWO.

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