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Cytopathogenicity of Classical Swine Fever Viruses that do not Show the Exaltation of Newcastle Disease Virus is Associated with Accumulation of NS3 in Serum-Free Cultured Cell Lines

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ABSTRACT. Pestiviruses can be distinguished as two biotypes, cytopathogenic (cp) and noncytopathogenic (noncp), by the morphological changes that they induce during growth in cultured cells. In this study, the cp phenotype of several classical swine fever viru ses (CSFV) was evaluated by the detections of the nonstructural proteins NS2–3 and NS3 using immunoprecipitation and Western blotting in di fferent porcine cell lines. Most CSFVs that showed the exaltation of Newcastle disease virus (END) phenomenon (END^+ viruses) did not induce cytopathic effect (CPE) in any cell line, and detections of NS2–3 and NS3 showed a strong signal for NS2–3 in the END^+ virus-infected cells. However, clear CPE was observed in serum-free cultured cells (FS-L3 and CPK-NS) infected with viruses that induce intrinsic interference but did not show the END phenomenon (END^- viruses), and signal of NS3 was strongly detected than that of NS2–3 in these cells at 72 hr after infection. As the results of the analysis of FS-L3 cells infected with ALD (END^+ virus) and ALD-END^- virus (END^- virus) at several incubations, the signal of NS3 detected was strengthened with CPE that become evident progressively. These results suggest that CPE is associated with the accumulation of NS3, which is promoted in serum-free cell lines infected with END^- viruses. Thus, indicating there is a close relationship between CPE and the quantity of NS3 produced in END^- CSFV infection.
KEY WORDS: CSFV, cytopathogenicity, NS2–3, NS3.

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Classical swine fever is a highly contagious and often fatal disease of pigs that is characterized by fever and hemorrhages and can run either an acute or chronic course. The causative agent of this disease is classical swine fever virus (CSFV), which is classified as a member of the family *Flaviviridae*, belonging to the genus *Pestivirus* [31]. This genus also includes bovine viral diarrhea virus (BVDV) and border disease virus of sheep, both of which share antigenicity with CSFV. Pestiviruses are small positive-sense, single-stranded RNA viruses, which have a length of approximately 12.3–12.5 kb [3, 17]. The open reading frame, which is flanked by 5'- and 3'-noncoding regions, codes for a polyprotein of about 4,000 amino acids that is processed by viral and cellular enzymes.

One of the biological properties of pestiviruses is the existence of two biotypes, cytopathogenic (cp) and noncytopathogenic (noncp), which are distinguished by the morphological changes that they induce during growth in cultured cells. The noncp pestiviruses replicate without inducing a cytopathic effect (CPE), while cp viruses cause lysis of appropriate cultured cells. Cytopathogenic viruses are frequently reported for BVDV and some cp BVDVs have insertions of cellular sequences, duplication of the viral genome, or contain defective interfering (DI) particles [16, 20, 21]. Most CSFV field isolates that show the exaltation of Newcastle disease virus (END) phenomenon [8] are noncp in porcine cell cultures. On the other hands, cp strains of CSFV are rare. Recently, some reports have indicated that cp CSFV are composed of DI particles, containing subgenomic RNA, and noncp helper viruses, containing full-length viral genomes [1, 7, 15, 18, 19]. Moreover, it has been shown that some strains of CSFV that do not show the END phenomenon but show intrinsic interference [5], induce clear CPE in serum-free cultured cell lines, such as FS-L3 and CPK-NS [24, 26, 27]. END phenomenon-negative (END^-) viruses can be prepared from a minor population within a field strain by more than one method. For example, the Japanese live vaccine GPE^- strain was prepared by long-term serial passage, whereas the ALD-END^- strain was prepared by the reverse plaque formation method [5, 11]. Although END^- viruses were initially assayed by the interference method with western equine encephalomyelitis virus (WEEV) or vesicular stomatitis virus [4, 29], direct and/or indirect methods using serum-free cultured cell lines have made the assay of CSFV by observation of CPE possible [26, 27]. However, there are no reports of END^- viruses being isolated independently from animals suffering from CSF in field outbreaks to date.

Molecular studies in the BVDV system have identified differences in the proteins present between cells infected with cp and noncp viruses. The nonstructural protein NS3 can be found together with NS2–3 in the cells infected with cp BVDV, but only NS2–3 is detected after infection with noncp BVDV [10, 16]. Therefore, the detection of NS3 was recognized as a specific marker for cp BVDV. Similarly, it has been reported that cells infected with CSFV DIs contain much more NS3 than those inoculated with noncp viruses

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[15]. However, the cytopathogenicity of END$^-$ viruses has not been analyzed molecularly in detail. Here we report that there is a correlation not only between END$^-$ viruses and CPE, but also between CPE and accumulation of NS3 in cells infected with cp CSFVs.

MATERIALS AND METHODS

Viruses: CSFV ALD and Ames strains were used as wild-type strains. ALD-END$^-$ and Ames-END$^-$ strains were isolated by Dr. N. Ogawa (National Veterinary Assay Laboratory) from ALD and Ames strains respectively by the reverse plaque formation method [5]. The CSFV GPE$^-$ strain is an attenuated virus that was isolated during serial passage of the ALD strain and has been used in Japan as a live vaccine. After each strain (ALD, Ames, ALD-END$^-$, Ames-END$^-$ and GPE$^-$) was recloned by biological method described previously [1], the cloned viruses were used for further experiments.

The CSFV WB82 strain was isolated from a wild boar suffering from CSF in 1982, and contains cp DI particles that cause cytopathogenicity [1]. The WB82/E$^+$ strain was cloned from the WB82 strain as a helper virus for replication of the DI particles [1]. CSFV Miyazaki/80, Ogatamura/68, Shimane/80, Yamagata/80, Fukushima/80 and Chiba/80 isolates were isolated during CSFV outbreaks in Japan [25].

After cloned viruses and 6 field isolates were propagated in FS-L3 cells in serum-free culture at 37°C for 3 or 4 days, they were reevaluated by the END method and the interference method [4, 8, 13, 29] (Table 1). Then, they were titrated by the direct and/or indirect CPE method in CPK-NS cells [27] and stored at –80°C until use.

Cells: The stable porcine kidney cell lines FS-L3 and CPK-NS were grown as described by Sakoda *et al.* [24, 26, 27]. The PK-15 cell line was grown as monolayer in MEM containing 0.295% TPB, 5% inactivated fetal bovine serum (FBS) and 1.125 mg of sodium bicarbonate per m*l*. Primary swine kidney (SKp) and testicle (STp) cells were prepared from pestivirus-free and anti pestivirus antibody-free pigs and grown in MEM containing 10% inactivated FBS and 1.125 mg of sodium bicarbonate per m*l*.

Immunoprecipitation: Each cell line was grown as monolayer in a 35 mm diameter dish, infected with viruses at an m.o.i. of 0.1 and subsequently incubated for 72 hr in a 5% CO_2 incubator. After infection the cells were lysed with 0.5 m*l* of lysis buffer {0.1 M Tris-HCl [pH 7.5], 0.1 M NaCl, 1% [vol/vol] Nonidet-P40, 1% [vol/vol] Triton-X 100 and a protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Germany) per 50 m*l* of lysis buffer} at 4°C for 1 hr. The cell lysates were centrifuged at 20,000 × g for 30 min at 4°C, and then 2 μl of anti-NS3 monoclonal antibody (mAb) JCU/BVD/CF10 (JCU TropBio, Australia) were added and reacted to the supernatants for 1 hr at 4°C. Then, proteins were precipitated from the lysates by the addition of 50 μl of 50% (vol/vol) protein G sepharose (Pharmacia Biotech) followed by incubation for 1 hr at 4°C. The precipitates were collected by centrifugation at 2,500 × g for 30 sec and washed 4 times with lysis buffer. Precipitates were resuspended in 20 μl of sodium dodecyl sulfate (SDS)-sample buffer (4% [vol/vol] SDS, 2% [vol/vol] glycerol, 1% [vol/vol] 2-mercaptoethanol, 0.1 M Tris-HCl [pH 6.8], 0.01% [wt/vol] bromophenol blue) and boiled for 5 min prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and Western blotting: Ten μl of SDS-sample buffer containing the precipitated proteins was electrophoresed in 7.5% (wt/vol) polyacrylamide gels. After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes, and the membranes were blocked overnight at 4°C with 2% (wt/vol) skimmed milk in PBS containing 0.1% (vol/vol) Tween-20 (PBST). Rabbit anti-NS3 polyclonal antibody which was harvested from a rabbit immunized with recombinant NS3 proteins was used to detect both NS2–3 and NS3 proteins by Western blotting. Rabbit anti-NS3 polyclonal antibody, at a dilution of 1:500 in 5% (wt/vol) skimmed milk in PBST, was allowed to react with the membranes for 1 hr at room temperature. After the reaction, the membranes were washed thrice with PBST. Peroxidase-conjugated anti-rabbit IgG was allowed to react with the membranes for 1 hr at room temperature, the membranes were washed and developed with ECL Plus Western Blotting Detection Reagent substrate solution (Amersham Bioscience K.K., NJ, U.S.A.). After signals of NS2–3 and NS3 were quantified with an ATTO Densitgraph Ver4.0 software, ratios between NS2–3 and NS3 were estimated.

RESULTS

Observations of CPE in cells infected with END$^+$ and END$^-$ viruses: Each virus was inoculated into FS-L3, CPK-NS, PK-15, SKp and STp cells at an m.o.i. of 0.1 and the induction of CPE was examined over 7 days (Table 1). CPE was not observed in any cells infected with END$^+$ viruses during 7 days. However, the END$^-$ viruses induced CPE in serum-free cultured cells such as FS-L3 and CPK-NS cells from 2 days after inoculation, although CPE was not observed in SKp and STp infected with END$^-$viruses during 7 days. In PK-15 cells infected with END$^-$ viruses, CPE was not clearly observed but the number of detached cells was increased. WB82 strain containing the DI particles induced strong CPE in all cell cultures 2–3 days after inoculation, but no CPE was observed in any cells infected with WB82/E$^+$ virus during 7 days.

Detection of NS2–3 and NS3 proteins: Three days after the viruses were inoculated into each cell-type at an m.o.i. of 0.1, nonstructural proteins NS2–3 and NS3 were detected by immunoprecipitation using anti-NS3 mAb and Western blotting, and the signals were quantified (Figs. 1 and 2). From each cell infected with cloned END$^+$ viruses, the NS2–3 signal with a molecular mass of 120 kDa was detected in addition to a weak NS3 signal with a molecular mass of 80 kDa (NS2–3, 55–100%; NS3, 0–45%). For END$^-$ virus infections, the signal of NS2–3 was stronger than that of NS3 in PK-15, SKp and STp cells (NS2–3, 53–

Table 1. END phenomenon, interference phenomenon and CPE observations on cells infected with cloned CSFVs and 6 field isolates

		END[a]	Interference[b]	Observations of CPE				
				FS-L3	CPK-NS	PK-15	SKp	STp
Cloned viruses	ALD	+	–	–	–	–	–	–
	Ames	+	–	–	–	–	–	–
	WB82/E$^+$	+	–	–	–	–	–	–
	GPE$^-$	–	+	+	+	(+)[c]	–	–
	ALD-E$^-$	–	+	+	+	(+)	–	–
	Ames–E$^-$	–	+	+	+	(+)	–	–
DI[d]	WB82	ND[e]	ND	+	+	+	+	+
Field isolates	Ogatamura/68	+	–	–	–	–	–	–
	Miyazaki/80	+	–	–	–	–	–	–
	Shimane/80	+	–	–	–	–	–	–
	Yamagata/80	+	–	–	–	–	–	–
	Fukushima/80	+	–	–	–	–	–	–
	Chiba/80	+	–	–	–	–	–	–

a) exaltation of Newcastle disease virus phenomenon. b) interference phenomenon with WEEV. c) clear CPE was not observed but detached cells were increased. d) CSFV containing DI particles. e)Not determined.

100%; NS3, 0–47%). However, the NS3 signal was stronger than that of NS2–3 in FS-L3 and CPK-NS cells infected with the END$^-$ viruses (NS2–3, 0–39%; NS3, 61–100%). WB82 infection caused a strong NS3 signal in all cells (NS2–3, 0–22%; NS3, 78–100%). Each cell infected with 6 field isolates gave similar results as the cloned END$^+$ virus infections. Namely, the signals of NS2–3 was stronger than that of NS3 in field isolate infections (NS2–3, 52–91%; NS3, 9–48%) (Fig. 2).

After ALD and ALD-END$^-$ viruses were inoculated into FS-L3 cells at an m.o.i. of 0.1, observations of CPE and detections of NS2–3/NS3 were examined out at several incubations (Fig. 3). Although ALD virus did not induce CPE during 72 hr incubations, CPE was observed in cells infected with ALD-END$^-$ virus at 48 hr after inoculation, and CPE become evident at 72 hr after inoculation (Fig. 3A). Although NS2–3 was clearly detected in FS-L3 cells infected with ALD-END$^-$ virus in addition to weak signal of NS3 after 36 hr of incubation (NS2–3, 82%; NS3, 18%), NS3 was strongly detectable in addition to NS2–3 at 48 hr postinoculation (NS2–3, 55%; NS3, 45%) and the strength of NS3 signal was increased at 72 hr after inoculation (NS2–3, 36%; NS3, 64%) (Fig. 3B). In the case of ALD virus infection, ration between NS2–3 and NS3 was similar to that of ALD-END$^-$ virus at 36 hr postinoculation (NS2–3, 84%; NS3, 16%) but the rations of NS2–3/NS3 were almost maintained up to 72 hr of incubations (NS2–3, 73–78%; NS3, 21–27%) (Fig. 3B).

DISCUSSION

Before immunodetections of nonstructural proteins NS2–3 and NS3, biological phenotype of CSFVs prepared in this study was reevaluated in accordance with some reports about the cp phenotype [1, 7, 15, 18, 19, 26, 27]. CPE was not observed in any cells infected with END$^+$ viruses (ALD and Ames) and 6 field isolates. In contrast, END$^-$ viruses (ALD-E$^-$, Ames-E$^-$, and GPE$^-$) induced clear CPE only in serum-free cell cultures FS-L3 and CPK-NS (Table 1). The WB82 strain, which contains cp DI particles, induced strong CPE in all cell lines tested, but the WB82/E$^+$ virus did not induce CPE in any cell lines as well as END$^+$ viruses. These results suggest that the END$^-$ viruses as well as the CSFV containing DI particles should be classified as a cp virus, and that the mechanism of CPE induced by END$^-$ virus differ from that of DI particles, because induction of CPE by END$^-$ viruses is dependent on the type of cell culture. Thus, there is a factor in these cells that allows the induction of CPE by END$^-$ viruses. Moreover, these results help to analysis of correlations between the cytopathogenicity and detections of NS2–3/NS3 in cp CSFV infections.

NS2–3 and NS3 within virus-infected cells were analyzed by immunodetection at 72 hr after inoculation. Each protein was detected and the signal of NS2–3 was stronger than that of NS3 in infection with END$^+$ viruses and 6 field isolate (NS2–3, 52–100%; NS3, 0~48%) (Figs. 1 and 2). However, it was found that a larger amount of NS3 was present within serum-free cultured cells infected with an END$^-$ virus and within any cells infected with WB82 strain (NS2–3, 0–39%; NS3, 61–100%) (Fig. 1), and also that CPE was observed in these infections at 72 hr after inoculation. It is recognized that NS3 is over-expressed in cp CSFV related with DI particles and also that NS3 is a specific marker for cp viruses in BVDV systems [10, 15]. In our study, results showed that NS3 was strongly detected not only in any cells infected with virus containing DI particles but also in serum-free cultured cells infected with an END$^-$ virus. Therefore, our results suggest that the presence or production of a large amount of NS3 within cells would be associated with CPE induced by cp CSFV including not only DI particles but also END$^-$ viruses. In particular, it is likely that both the CPE and the strong signal of NS3 shown by the END$^-$ virus

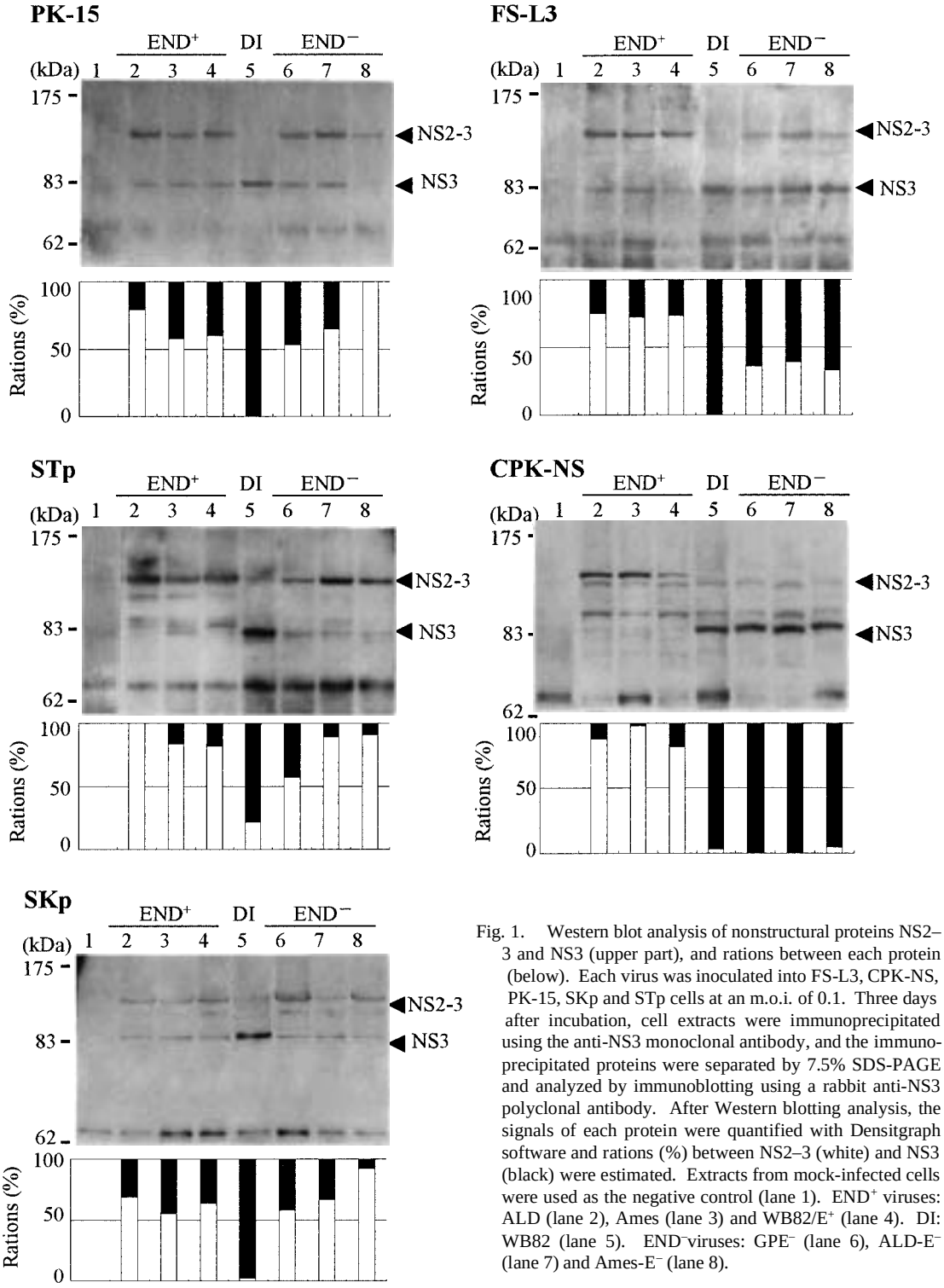

Fig. 1. Western blot analysis of nonstructural proteins NS2–3 and NS3 (upper part), and rations between each protein (below). Each virus was inoculated into FS-L3, CPK-NS, PK-15, SKp and STp cells at an m.o.i. of 0.1. Three days after incubation, cell extracts were immunoprecipitated using the anti-NS3 monoclonal antibody, and the immunoprecipitated proteins were separated by 7.5% SDS-PAGE and analyzed by immunoblotting using a rabbit anti-NS3 polyclonal antibody. After Western blotting analysis, the signals of each protein were quantified with Densitgraph software and rations (%) between NS2–3 (white) and NS3 (black) were estimated. Extracts from mock-infected cells were used as the negative control (lane 1). END$^+$ viruses: ALD (lane 2), Ames (lane 3) and WB82/E$^+$ (lane 4). DI: WB82 (lane 5). END$^-$viruses: GPE$^-$ (lane 6), ALD-E$^-$ (lane 7) and Ames-E$^-$ (lane 8).

infection are dependent on a cell-originated factor because they were related characteristics were only exhibited in certain cell lines.

Although there were some other signals except for the signals of NS2–3 and NS3, most of those signals could be excluded by the comparison with the mock-infected cells. However, FS-L3 cells infected with END$^-$ virus were used for analyzing kinetics of NS2–3 and NS3 (Fig. 3), because there were few other signals detected in FS-L3 compared with CPK-NS cells (Fig. 1). NS2–3 was strongly detected in

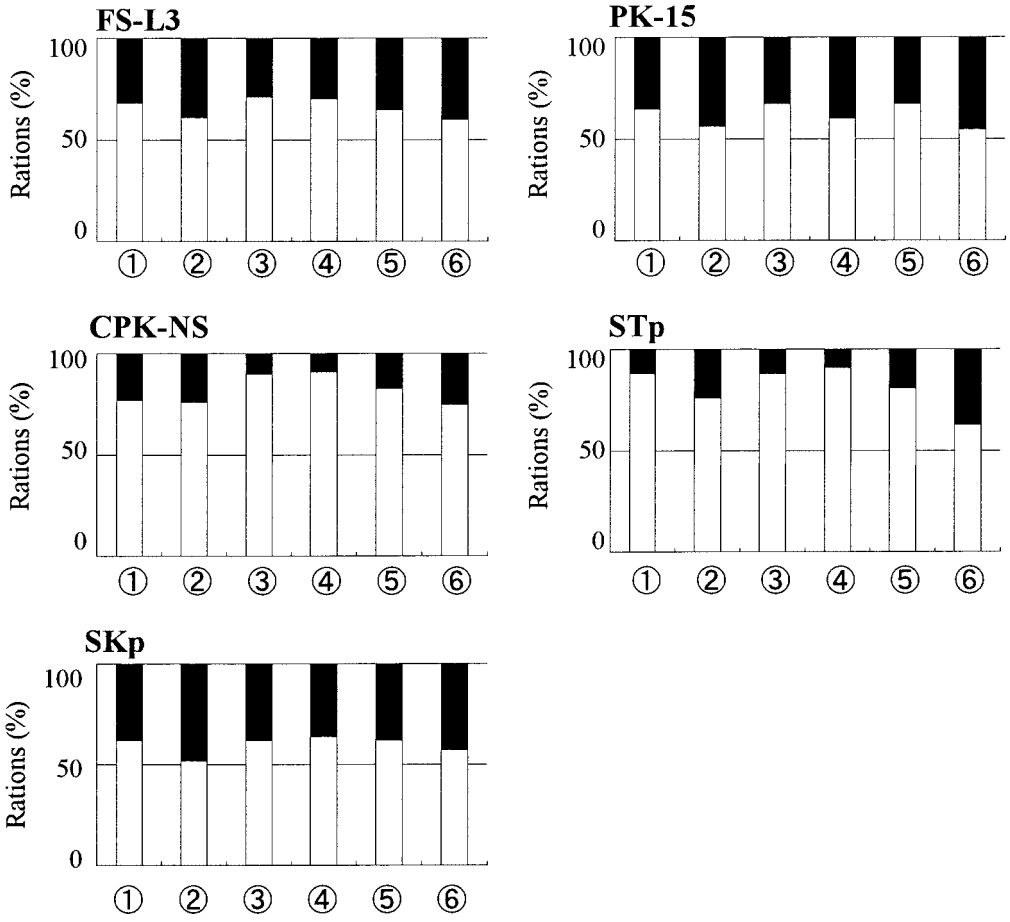

Fig. 2. Rations between NS2–3 and NS3 quantified in cells infected with field isolates. After NS2–3 and NS3 in the cells were detected by immunoprecipitation and Western blotting, the signals of each protein were quantified and rations (%) between NS2–3 (white) and NS3 (black) were estimated. Ogatamura/68 (①), Miyazaki/80 (②), Shimane/80 (③), Yamagata/80 (④), Fukushima/80 (⑤), Chiba/80 (⑥).

ALD and ALD-END$^-$ virus-infected FS-L3 cells that did not show CPE at 36 hr after inoculation (NS2–3, 84 and 82%; NS3, 16 and 18%). However, it was found not only that NS3 was strongly detected in ALD-END$^-$ virus-infected cells at 48 hr after inoculation but also that strength of NS3 signals in the cells was increased at 72 hr after inoculation as CPE became evident (NS2–3, 36–55%; NS3, 45–64%) (Fig. 3). Molecular analysis of cp BVDV strains has also revealed that NS3 is produced by NS2–3 cleavage in many cases [10] and that either insertions of cellular ubiquitin-coding sequences or cIns encoding Jiv in the viral genome are also associated with the cp phenotype [14, 20–23, 30, 32]. In the case of cp CSFV, the detection of NS3 could be often understandable in any cells infected with DI particles because a subgenomic RNA contained in DI particles of cp CSFV lacks the genome region from N^{pro} to NS2 [1, 15, 19]. However, the GPE$^-$ strain has a standard pesitivirus genome without neither subgenomic RNA in DI particles nor the insertions shown for cp BVDV [6, 26]. In our studies, a subgenomic RNA was not detected in cells which showed CPE induced by END$^-$ viruses (data not shown). Therefore, our results suggest that NS2–3 expressed from END$^-$ CSFV is efficiently cleaved in serum-free cell cultures. Furthermore, the mechanism of NS2–3 cleavage by END$^-$ CSFV may be different from cp BVDV systems. Accordingly, it is possible that the NS2–3 protein derived from END$^-$ viruses is more susceptible to cleavage than that from END$^+$ viruses.

It had previously been recognized that CSFV isolates express NS3 whether they are a cp or noncp biotype, whereas the expression of NS3 is strictly correlated with the cp biotype for the BVDV strains [10, 16]. In our study, indeed, a slight accumulation of NS3 almost occurred in infection of END$^+$ viruses and 6 field isolates. Some CSFV isolates are composed of both END$^+$ virus as the major virus and END$^-$ virus as the minor virus (Ogawa, unpublished data). However, our results on NS3 detection could omit speculation that a subpopulation with a different NS2–3 cleavage behavior was required, because not only field viruses but also molecular clones were examined in this study. Kümmerer and Thiel have suggested that for BVDV

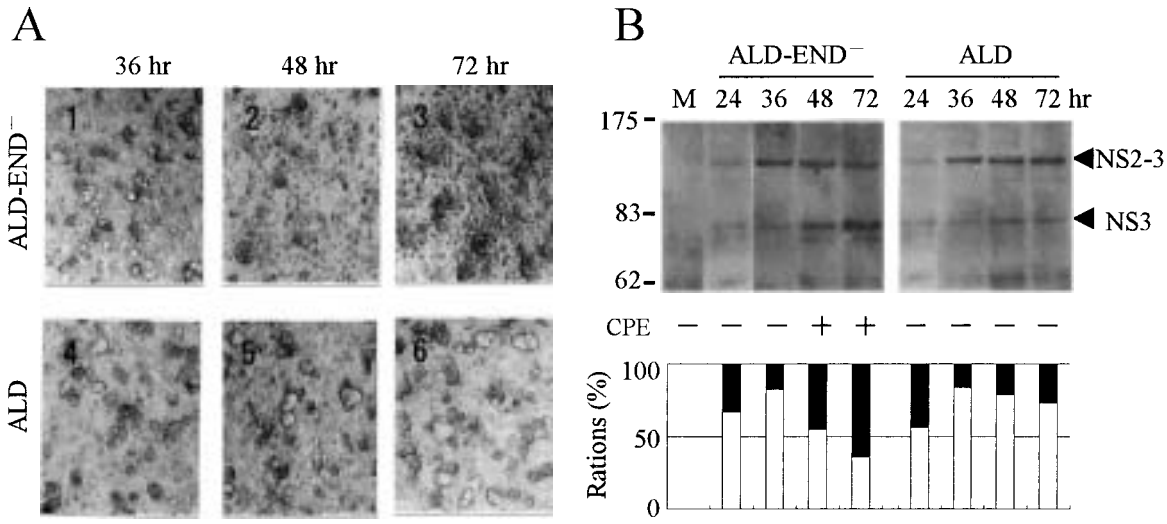

Fig. 3. Kinetics of NS2–3 and NS3 in FS-L3 cells infected with CSFVs. A: The morphological changes of FS-L3 infected with ALD-E⁻ and ALD viruses at 36 (1 and 4), 48 (2 and 5) and 72 hr (3 and 6) after infection. B: The upper part shows Western blot analysis and the results of CPE observation. Below, the rations between NS2–3 and NS3. After ALD-E⁻ and ALD viruses were inoculated at an m.o.i. of 0.1, cell extracts were examined at 24, 36, 48 and 72 hr after infection by immunoprecipitation and Western blotting. Signals of each protein were quantified with Densitgraph software and rations between NS2–3 (white) and NS3 (black) were estimated. Extracts from mock-infected cells at 72 hr after infection were used as the negative control (M).

Oregon induction of cell lysis is dependent on the presence of a certain amount of NS3 within the cell, and also suggested that there seems to be a threshold value somewhere between 48 and 61% of NS2–3 cleavage efficiency that separates noncp and cp BVDV [9]. In our studies, CPE was not detected in cells infected with END⁺ viruses and field isolates at 72 hr after inoculation, and the ration of NS3 for these viruses was between 0 and 48%. On the other hand, END⁻ viruses and WB82 showed a NS3 ration of 61% or higher in cells that showed clear CPE at 72 hr incubation. Therefore, our results suggest that a threshold value is somewhere between 48 and 61% of NS2–3 cleavage efficiency that separates noncp and cp viruses, as well as BVDV. Inefficient cleavage may indeed explain the reason why CSFV is noncp although the virus expresses NS3. However, the ration of NS3 in PK-15 cells infected with END⁻ viruses was similar to that in cells that did not show CPE though detached PK-15 cells were slightly increased. The same results were shown in another swine kidney cell lines such as CPK and SK-L (data not shown). Namely, an amount of NS3 or a threshold value of cleavage efficiency that separates noncp and cp CSFV could differ from that of BVDVs. Further studies are needed to determine in detail whether the cytopathogenicity of CSFV is due to the expression levels of NS3 or NS2–3 cleavage efficiency, and whether the detachment of PK-15 cells infected with END⁻ virus relates to an accumulation of NS3 or to another factor.

A report shows that CSFV infected cells appear as hazy plaques when viewed against an indirect light source though the molecular analysis had not been shown in that report [12]. Moreover, some reports recently point out the significance of the amount of double-stranded (ds) viral RNA being synthesized in infected cell for determining the cytopathogenicity of the BVDV [2, 28]. Although it has not been clarified whether the cytopathogenicity of cp pesitiviruses is due to the expression of NS3 or also due to an increased rate of ds viral RNA synthesis, our results would be helpful for analysis of mechanism of CPE in cp pestivirus infections. Further studies to elucidate the significance of NS3 expression, the mechanism of cleavage of NS2–3 and the END phenomenon are required to enhance our understanding of cytopathogenicity of END⁻ CSFV and pestiviruses in general.

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