Multiplication of Canine Parvovirus in CRFK Cells

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ABSTRACT. Canine parvovirus was inoculated into CRFK cells synchronized with thymidine treatment and into the asynchronous cells which were dividing actively. The number of cells bearing intranuclear inclusion bodies was much larger in the former. In the synchronized cells, both infectivity and hemagglutination titers began to rise in the cellular phase 12 hr postinoculation (p.i.) and in the extracellular phase 24 hr p.i., then reached the maximum 48 and 72 hr p.i., respectively. Intranuclear inclusion bodies appeared 10 hr p.i., and typical ones with chromatin margination and halo formation were seen 14 to 16 hr p.i. In the immunofluorescence studies, specific fluorescence was detected first in the cytoplasm 4 hr p.i. It appeared in the nuclei 6 hr p.i. and spread throughout the nuclei 10 hr p.i. Its intensity reached the maximum 14 to 16 hr p.i. At 20 hr p.i., viral antigen was observed again in the cytoplasm. In the immunoperoxidase studies, development of the stained substance was similar to that in the immunofluorescence method. Immunoperoxidase technique was useful to detect the viral antigen as well as immunofluorescence technique except a weak staining at the early stage of infection. When the virus was inoculated into the synchronized cells 1, 3, 5 or 7 hr after the release from thymidine block, the number of cells bearing intranuclear inclusion bodies increased most rapidly in the cells inoculated 3 hr after the release, and the fluorescence spread throughout the nuclei 8 hr p.i. in the cells inoculated 3 or 5 hr after the release.—**KEY WORDS:** canine parvovirus, CRFK cells.

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In the early studies on the *in vitro* multiplication of autonomous parvoviruses (genus *Parvovirus*), it was shown that the virus multiplication depended on the physiological conditions of the host cells. This dependence was reported in Kilham rat virus (RV) [29, 30], bovine parvovirus [21], H-1 virus [11, 22], Lu III virus [24], minute virus of mice (MVM) [28] and porcine parvovirus (PPV, KBSH-virus) [26]. From these studies, it was revealed that the virus multiplication was associated with the function(s) of the host cells in the S to G₂ phases, probably in the late S phase [11, 21, 22, 24]. Recently, this relationship was also confirmed in feline panleukopenia virus (FPLV) [6], but the precise replication cycle of FPLV and its relationship to the physiological conditions of the host

cells remain unknown.

As for canine parvovirus (CPV), which is antigenically related closely to FPLV and mink enteritis virus (MEV) [2, 31], little is known about its multiplication characteristics *in vitro*. The present report describes the multiplication of CPV in CRFK cells and its relationship to the cell cycle. Besides usual manners of virus titration and cell staining, immunoperoxidase technique was carried out together with immunofluorescence technique to detect the viral antigen.

MATERIALS AND METHODS

Virus, cell and assay: Cp 49 strain of CPV and CRFK (Crandell feline kidney) cells were used. Stock virus fluid was prepared, and virus titration and hemagglutination (HA) test were performed, as described before [1]. CRFK cells were synchronized at early S phase by treating with thymidine (TdR) [6,

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24]. Two ml of CRFK cells in growth medium ($0.8-1.0 \times 10^5$ cells/ml) were seeded into Leighton tubes with coverslip and incubated at 37°C for 24 hr. Then growth medium was replaced with the medium containing 3 mM TdR, and the cells were further incubated for 20 hr. Asynchronous, actively dividing cells were prepared as follows [18]. Two ml of CRFK cells in growth medium ($1.0-1.2 \times 10^5$ cells/ml) were dispensed into Leighton tubes with coverslip and incubated at 37°C for 24 hr. The media were removed, and the cells were washed with serum-free medium before the virus inoculation.

Examination of inclusion bodies: Stock virus fluid was inoculated into both synchronized and asynchronous cells. Multiplicity of infection (m.o.i.) was 1 TCID₅₀/cell. Coverslips were fixed in methanol at room temperature for 5 min and stained with May-Grünwald Giemsa (MGG) solution [1]. The ratio of the cells bearing intranuclear inclusion bodies was expressed as a percent of cells examined (at least 2,000 cells were observed).

Growth curve: Virus was inoculated into TdR-treated cells in tissue culture tubes (m.o.i. was 1 TCID₅₀/cell). At each harvest, the culture fluid was centrifuged at 1,000 rpm for 10 min. The supernatant was termed as extracellular phase and stored at -70°C . The pellet was resuspended in the original volume of serum-free medium, and the suspension was poured into the tubes again. It was frozen and thawed 3 times, sonicated and centrifuged at 3,000 rpm for 10 min, then the supernatant was stored at -70°C as cellular phase.

Immunofluorescence technique: Immunofluorescence studies were performed by the indirect method. Anti-CPV serum was prepared in rabbits [1] and diluted 1:64 in phosphate-buffered saline (PBS). FITC-conjugated anti-rabbit IgG goat serum (Miles-Yada Ltd., Israel) was also diluted 1:64 in PBS. Both sera were absorbed with the ace-

tone-treated uninoculated CRFK cell powder (10 mg/ml) at room temperature for 1 hr before use. Coverslips were washed with PBS, fixed in acetone at room temperature for 10 min, washed again with PBS, then incubated with anti-CPV serum at 37°C for 45 min in a moist chamber. They were washed with 3 changes of PBS for 5 min each, and stained with the FITC-conjugated serum as described above. The washing was repeated, and coverslips were mounted in buffered glycerin. The observation was carried out by a Nikon type 104 fluorescence microscope.

Immunoperoxidase technique: The virus was inoculated into the TdR-treated cells (m.o.i. was 1 TCID₅₀/cell). Immunoperoxidase staining was carried out by the unlabeled antibody (PAP) method [27]. Coverslips were fixed in acetone, treated with normal goat serum for 20 min, then incubated with anti-CPV serum (1:128) for 15 hr. Anti-rabbit IgG goat serum (1:32, Litton Bionetics, Inc., USA) was placed on the coverslips for 45 min. PAP complex (1:128, Miles-Yada Ltd., Israel) was applied for 45 min. Sera except normal goat serum were absorbed with the acetone-treated CRFK cell powder. The staining solution consisted of 0.01% H₂O₂ and 0.025% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M tris-HCl buffer (pH 7.6). Coverslips were immersed in the solution at room temperature for 15 min. After washing they were counterstained with hematoxylin solution.

Inoculation of the virus at different stages of CRFK cells: After the TdR treatment, cells were washed 3 times with serum-free medium, and growth medium was added to each Leighton tube. TdR block was released at this time. The virus was inoculated 1, 3, 5 or 7 hr after the release (m.o.i. was 0.5 TCID₅₀/cell). Coverslips were stained with MGG solution or applied to the immunofluorescence studies, and were observed as described above.

Fig. 1. Production of intranuclear inclusion bodies in CRFK cell cultures inoculated with Cp 49 strain (○ : synchronized cells, ● : asynchronous cells).

RESULTS

Production of inclusion bodies: Percentage of cells bearing intranuclear inclusion bodies is shown in Fig. 1. Inclusion bodies were first observed 10 hr postinoculation (p.i.) in both synchronized and asynchronous cells. Only a few cells (<1%) had inclusion bodies 10 hr p.i., and until 12 hr p.i., the percentage was almost equal in both cells. From 14 to 72 hr p.i., the number of the cells bearing inclusion bodies was larger in synchronized cells than in asynchronous cells. At 72 hr p.i., many cells were detached from coverslips, and the remaining cells were rounded and darkly stained.

Growth curve and cytopathology: Fig. 2 illustrates a growth curve of Cp 49 strain in synchronized CRFK cells. In the cellular phase, both infectivity and HA titers began to rise 12 hr p.i., and reached the maximum

48 hr p.i. In the extracellular phase, they began to rise 24 hr p.i. and reached the maximum 72 hr p.i. The titers were always higher in the cellular phase than in the extracellular phase.

Rounding and detachment of the cells were the most significant cytopathic changes. Rounding cells were evident 24 hr p.i. Some cells were detached from the glass surface 48 hr p.i. At 96 hr p.i., most cells were detached, and the remaining cells were rounded and tended to make clumps.

Morphogenesis of inclusion bodies: Fig. 3-a shows an uninoculated CRFK cell. Nuclei were somewhat larger in synchronized cells than in asynchronous cells. They were stained uniformly except relatively chromatin-free zone around nucleoli. The earliest sign of the intranuclear inclusion body was observed 10 hr p.i. (Fig. 3-b). Nuclei were stained unevenly, and amorphous eosinophil-

Fig. 2. Growth curve of Cp 49 strain in synchronized CRFK cell culture (○: infectivity, ●: HA titer, —: cellular phase, ---: extracellular phase).

ic substance appeared between the nucleolus and the nuclear membrane. At 12 hr p.i., such substance surrounded the nucleolus and occupied the most part of the nucleus (Fig. 3-c). There was a clear pale region around nucleolus. From 14 to 16 hr p.i., the inclusion bodies were stained homogeneously, and chromatin margination and halo formation were evident (Fig. 3-d). At 20 hr p.i., inclusion bodies began to darken (Fig. 3-e), and densely stained ones were dominant 48 and 72 hr p.i.

Other changes detected 20 hr p.i. are shown in Fig. 3-f. Among the cells bearing intranuclear inclusion bodies, nuclear atrophy was observed. In these cells, inclusion bodies were stained less densely with unclear halo. Amorphous substance similar to that which made up the intranuclear inclusion body was seen in the cytoplasm. Its amount increased subsequently (Fig. 3-g), but the cells containing such cytoplasmic substance were observed sporadically throughout the examination.

There was no obvious difference in the

morphogenesis of inclusion bodies between synchronized and asynchronous cells.

Immunofluorescence studies: In uninoculated cells, no fluorescence was observed throughout the observation. Specific fluorescence appeared and spread in the same way in both cell systems. It was first detected at the perinuclear region in the cytoplasm 4 hr p.i. (Fig. 4-a), where it persisted in many cells (approximately 70% of the cells) in following 6 hr. At 6 hr p.i., viral antigen was detected within the nuclei (Fig. 4-b). The fluorescence was localized near the nuclear membrane or occasionally seen as granules within the nuclei. At the same time, intracytoplasmic fluorescence disappeared from these cells. Intranuclear fluorescence spread in the nuclei and became more intense 8 hr p.i. (Fig. 4-c), then it filled the nuclei 10 hr p.i. (Fig. 4-d), and its intensity reached the maximum 14 to 16 hr p.i. (Fig. 4-e). The nuclei were swollen in these cells. At 20 hr p.i., the fluorescence reappeared in the cytoplasm (Fig. 4-f), increased thereafter, and intranuclear fluorescence disappeared concurrently.

Fig. 3. Intranuclear inclusion body formation in CRFK cells inoculated with Cp 49 strain. MGG staining. $\times 1,600$. a: noninoculated, b: 10 hr, c: 12 hr, d: 16 hr, e and f: 20 hr, g: 30 hr postinoculation.

Fig. 4. Immunofluorescence in synchronized CRFK cells inoculated with Cp 49 strain. $\times 1,600$. a: 4 hr, b: 6 hr, c: 8 hr, d: 10 hr, e: 14 hr, f: 20 hr, g and h: 24 hr postinoculation.

Fig. 5. Immunoperoxidase staining in synchronized CRFK cells inoculated with Cp 49 strain. $\times 1,600$. a: 10 hr, b: 16 hr, c: 20 hr, d: 28 hr postinoculation.

At 30 hr p.i., the most of the fluorescence was seen in the cytoplasm (Fig. 4-g), and in some cells, nuclear membrane also fluoresced intensely (Fig. 4-h).

Immunoperoxidase studies: Viral antigen was visualized as brown colored products. There was no staining in uninoculated cells. Development of the viral antigen was similar to that of the immunofluorescence. Positive staining was first seen at the perinuclear region in the cytoplasm 6 hr p.i. At 8 hr p.i., it was detected as brown granules in the nuclei. These early stainings were less intense compared with the fluorescence at the same time. Intranuclear staining was obvious 10 hr p.i. (Fig. 5-a) and filled the nuclei 12 hr p.i. It was most intense 14 to 16 hr p.i. (Fig. 5-b). The cytoplasmic staining reappeared 20 hr p.i. (Fig. 5-c), then it spread through-

out the cytoplasm (Fig. 5-d).

Effect of the host cell cycle on the inclusion body production and the development of the fluorescence: Production of the intranuclear inclusion bodies is shown in Fig. 6. The number of cells bearing inclusion bodies increased most rapidly in the cells inoculated 3 hr after the release from TdR block. On the other hand, the increase was markedly slow in the cells inoculated 7 hr after the release. Morphogenesis of inclusion bodies was the same as described above in each cell system. Intranuclear fluorescence appeared earliest in the cells inoculated 3 hr after the release (4 hr p.i.), and filled the nuclei 8 hr p.i. in the cells inoculated 3 or 5 hr after the release. In the other cell systems, it appeared and spread in the same way as previously described.

Fig. 6. Production of intranuclear inclusion bodies in synchronized CRFK cell cultures inoculated with Cp 49 strain at different stages of cell cycle. Inoculated 1 (●), 3 (■), 5 (▲) and 7 (○) hr after the release from thymidine block.

DISCUSSION

The multiplication of CPV *in vitro* has not been intensively studied. Only a few reports have mentioned the viral antigen by immunofluorescence [7, 16, 19]. In FPLV, morphogenesis of inclusion bodies [9, 13, 17, 18] and immunofluorescence studies [9, 13] were reported. A similar study was performed on MEV [12], but it lacked detailed description.

Among above reports, intranuclear inclusion bodies were first detected 8 hr p.i. [18] or 12 hr p.i. [9, 13] in FPLV, and 24 hr p.i. [12] in MEV. In the studies of O'Shea and Studdert [18], high m.o.i. of the virus was inoculated into actively dividing cells. On the other hand, low m.o.i. of the virus was inoculated into non-conditioned cells in other reports [9, 12, 13]. In the present study, inclusion bodies were first detected 10 hr p.i. in both TdR-treated cells and asynchronous

cells (m.o.i. was 1 TCID₅₀/cell), and, when lower m.o.i. of the virus was inoculated, they appeared later. Inclusion body formation seemed to be affected by m.o.i. and the physiological conditions of the host cells.

The morphogenesis of intranuclear inclusion bodies by CPV was similar to that of FPLV [9, 13, 17, 18] except the intracytoplasmic substance shown in Figs. 3-f and 3-g, which seemed to be of nuclear origin and was not pointed out in any previous reports. Though the number of the cells with such intracytoplasmic substance was small even in the late stage of infection, many of these cells might be detached easily from coverslips during MGG staining because of the degeneration, and only a few of them might be observed after the staining.

Previous immunofluorescence studies *in vitro* on CPV [7, 16, 19], FPLV [9, 13] and MEV [12] did not mentioned details of the

development of fluorescence, and their results were inconsistent each other. In CPV, specific fluorescence was first detected in the cytoplasm [7] or nuclei [16, 19]. Then it spread throughout the nuclei and reappeared in the cytoplasm in the late stage of infection [7, 16], while Paradiso *et al.* [19] did not refer to this late cytoplasmic fluorescence. In the present study, the fluorescence appeared in early (4 hr p.i.) and late (20 hr p.i.) stages of the infection in agreement with Eugster's report [7]. In FPLV and MEV, however, virus-specific fluorescence first appeared in the nuclei, and the late intracytoplasmic fluorescence was not observed [9] or not mentioned [12, 13].

The development of intranuclear fluorescence corresponded with the morphogenesis of intranuclear inclusion bodies. The fluorescence spread throughout the nuclei 10 hr p.i., when inclusion bodies by MGG staining were first observed. At 14 and 16 hr p.i., the fluorescence was most intense, and inclusion bodies were typical with clear halo. After 20 hr p.i., the fluorescence spread into the cytoplasm, and nuclear atrophy and intracytoplasmic substance shown in Fig. 3-f were observed. Intranuclear inclusion body formation seemed to be associated with the synthesis of viral antigen. Considering this correspondence and the growth curve shown in Fig. 1, it may be concluded that CPV matures in nuclei with inclusion body formation, then newly synthesized virus particles are released into the cytoplasm.

Immunofluorescence technique was applied to other parvoviruses, i.e., RV [3, 8, 14], H-1 virus [10], Lu III virus [24], MVM [20] and PPV [15, 26]. Intranuclear and late intracytoplasmic fluorescence was observed in all reports except one [14]. However, the results varied on the early intracytoplasmic fluorescence, which was usually detected when relatively high m.o.i. of virus was inoculated [3, 8, 15, 24, 26]. In spite of some experiments and discussions, its nature

has not been clearly shown. It is regarded as inoculum origin [15], and is discussed by Siegl [23] that "the development of intracytoplasmic fluorescence reflects nothing but the uptake of an excess of viral antigen by the infected cells". Nevertheless, there is some possibility that the early intracytoplasmic fluorescence is the viral antigen already synthesized in the infected cells [24-26].

CPV antigen *in vitro* was detectable by immunoperoxidase method in the present study, as well as *in vivo* studies reported previously [4, 5]. The development of the viral antigen detected by this method was consistent with that in immunofluorescence method. However, early intracytoplasmic and intranuclear antigens were shown more clearly by immunofluorescence method, which was suitable to detect the early viral antigen *in vitro*. But, in immunoperoxidase method, samples are preserved well for a long time and can be observed by a light microscope. This method will be useful in further studies on CPV.

As shown in Fig. 1, much more inclusion bodies were produced in the TdR-treated cells than in actively dividing asynchronous cells. Nevertheless, there was no obvious difference between both cells about the morphogenesis of inclusion bodies and the development of the fluorescence. Because cells were actively dividing in the latter, some cells might be suitable for the virus multiplication. The number of cells bearing inclusion bodies increased most rapidly in the cells inoculated 3 hr after the release from TdR block (Fig. 6). Moreover, the fluorescence appeared in the nuclei 4 hr p.i. and filled them 8 hr p.i., 2 hr earlier respectively, in the cells inoculated 3 or 5 hr after the release than in the cells inoculated 1 or 7 hr. So, intranuclear fluorescence developed nearly at the same time in the cells inoculated 1 hr and in the cells 3 hr after the release. Similar results were reported in Lu III virus [24]. Though the cell cycle of CRFK cells is not clear, the

results described above suggest that multiplication of CPV is associated with the function(s) of the host cells in middle to late S phase like other parvoviruses [11, 21, 22, 24].

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要 約

イヌ・パルボウイルスの CRFK 細胞における増殖: 平沢 勉・辻村直美・小西信一郎 (東京大学農学部家畜微生物学教室)——イヌ・パルボウイルス (CPV) の CRFK 細胞における増殖について検討した。チミジン処理により同調した細胞および分裂増殖のさかんな非同調の細胞にウイルスを接種したところ、核内封入体の形成率は同調細胞においていちじるしく高かった。同調細胞における感染価およびHA価を経時的に測定したところ、両者とも細胞層では12時間後から、液層では24時間後から上昇し、細胞層では48時間後に、液層では72時間後に最大となった。核内封入体は接種10時間後に出現し、14～16時間後にはクロマチンの辺在化およびハローを有する典型的核内封入体がみられ、封入体は以後濃染した。蛍光抗体法における特異蛍光は接種4時間後に細胞質において認められ、6時間後核内に出現し、10時間後核内全域に広がった。14～16時間後には、最も強い核内蛍光が観察された。20時間後には再び細胞質に特異蛍光が出現、拡大するとともに、核内蛍光は減弱した。酵素抗体法における発色部位の推移は蛍光抗体法のそれとほぼ同様であったが、初期の細胞質および核内における発色は微弱であった。同調解除1～7時間後の細胞にウイルスを接種したところ、解除3時間後に接種した細胞において核内封入体の増加は最も速かで、3または5時間後接種の細胞においては、接種8時間後に特異蛍光は核内全域に広がった。