Study of the One-Step Growth Curve of Equine Infectious Anemia Virus by Immunofluorescence

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Primary horse leukocyte cultures were inoculated with 2 or 10 50% tissue culture infective doses (TCID_{50}) of equine infectious anemia (EIA) virus per cell, and the titer of cell-associated and fluid-phase virus was determined from 1 to 72 hr post-inoculation (PI). Cover slips were collected from 4 to 72 hr PI and stained for EIA viral antigen by the indirect immunofluorescent (FA) technique. Viral replication was detected after a latent period of approximately 18 to 24 hr and reached peak titers of approximately 10^{4.5} to 10^5 TCID_{50} /0.5 ml from 48 to 72 hr PI. The fluid phase contained 10^1 to 10^5 TCID_{50} /0.5 ml more virus than the cells. Viral antigen was first detected by FA from 18 to 24 hr PI. Approximately 75% of the cells contained antigen in their cytoplasm 72 hr PI. The FA technique is a sensitive method for detecting EIA virus in horse leukocyte cultures.

Equine infectious anemia (EIA) is a persistent viral infection of horses characterized by anemia, hypergammaglobulinemia, hepatitis, glomerulitis, and lymphoproliferative changes. The pathogenesis of the disease (5, 6, 10, 13) and some of the characteristics of the agent (9, 12, 19, 20) have been investigated only recently. The development of an in vitro cell culture system for virus propagation using primary peripheral horse leukocyte cultures (HLC) by Kobayashi (8) in 1961 made possible laboratory study of the agent. Since the agent persists in infected horses, it was assumed that an immune response did not develop. Within the last few years, however, anti-EIA virus-specific antibodies were detected by complement-fixation (6, 11), complement-fixation inhibition (14), neutralization (10, K. Kobayashi, J. B. Henson, and J. R. Gorham, Fed. Proc. 28:429, 1969), precipitation (1, 18, 19), and immunofluorescent (2, 13, 22) techniques.

Infection of HLC by EIA virus has been detected by the development of a cytopathic effect (CPE) (6, 8, 9), formation of CF antigen (6, 10, 11), and immunofluorescence (2, 22). Kono et al. (12) recently described the one-step growth curve of EIA virus using CPE as the indicator of infection. The present study was carried out to elucidate further the one-step growth curve of EIA virus in HLC and to determine the sensitivity of indirect immunofluorescence (FA) as a means of detecting infection.

**MATERIALS AND METHODS**

**Viral propagation.** An isolate of EIA virus from Wyoming passed 50 times in HLC and cloned by the limiting dilution technique was used. The agent was propagated in HLC as previously described by Kobayashi and Kono (9) with slight modifications. Leukocytes for culture were obtained from a single donor that was shown to be free of herpes viruses by three serial passages of blood and of leukocytes in rabbit kidney cultures. The leukocytes from this donor remained viable in culture for over 14 days.

Two milliliters of medium 199 with 50% calf serum containing 3 x 10^5 to 4 x 10^5 leukocytes per ml were placed in a glass petri dish (30-mm diameter). Cultures to be examined by immunofluorescence contained a cover slip (20 mm²) placed flat on the bottom of the dish. Incubation was at 37°C in an atmosphere of 5% CO₂. The medium and nonattached cells were removed after 24 hr and replaced with 2 ml of whole bovine serum. After 5 days, the cultures were infected with EIA virus at an input multiplicity of 2 or 10 50% tissue culture infective doses (TCID_{50}) per attached cell. The inoculum was allowed to remain in contact with the cells for 90 min at 37°C. The cultures were then washed twice and maintained in medium 199 containing 25% bovine serum.

**Virus titration.** Duplicate cultures were harvested at various times from 1 to 72 hr postinfection (PI). The medium was collected and centrifuged for 5 min at 5,000 rev/min, and the virus titer of the supernatant fluid was determined. The cells remaining adherent to the petri dishes were scraped from the surface with a rubber policeman. The cells and the sediment from the centrifuged medium were then disrupted by sonic treatment at 20 kc/sec for 3 min. Virus titration was carried out by serial 10-fold dilutions using HLC as described by Kobayashi and Kono (9).

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Immunofluorescence. Cover slips were collected from petri dishes containing infected or normal cultures from 4 to 72 hr PI to demonstrate viral antigen by immunofluorescence. The cover slips were washed twice with phosphate-buffered saline (PBS pH 7.4), fixed in acetone for 5 min at room temperature, and stained by the indirect fluorescent antibody (FA) technique as described by Ushimi et al. (22). The anti-EIA viral antibody source was serum with a neutralizing titer of 1:50 collected from a horse infected with a Wyoming isolate of EIA virus. Goat anti-equine globulin with a titer of 1:128 determined by gel diffusion was labeled with fluorescein isothiocyanate at an F/P ratio of 1:8. The final protein concentration of the conjugate was adjusted to 10 mg/ml. The conjugate was absorbed once with 100 mg of calf liver powder per ml and used at a dilution of 1:8. The cover slips were incubated with anti-EIA serum (immune horse serum) for 2 hr at 37 C, washed with PBS for 15 min, and stained with fluorescein-tagged anti-equine gamma globulin at 37 C for 40 min. They were then washed in PBS for 10 min and mounted in a mixture of glycerine (90%) in 0.05 M carbonate-bicarbonate buffer (pH 9.5). The cells were examined with a Zeiss Ultraphot microscope with an ultraviolet (UV) light source.

RESULTS

Titers of cell-associated and fluid-phase virus. The titer of cell-associated and fluid-phase virus at an input multiplicity of 10 TCID50 per cell is shown in Fig. 1. Approximately 10⁵ to 10⁴ TCID50/0.5 ml of virus were found in both the fluid-phase and cell-associated materials at 1 hr PI. During the following several hours, this residual virus gradually decreased. Between 18 and 24 hr PI, both fluid-phase and cell-associated virus rapidly increased and continued to rise almost exponentially until 36 hr PI. The amount of virus then increased more slowly and reached a maximum titer of about 3 × 10⁶ TCID50/0.5 ml from 48 to 72 hr PI for the fluid-phase virus and

10⁵ TCID50/0.5 ml for the cell-associated virus 72 hr PI. The propagation curves for both fluid-phase and cell-associated virus were similar, but the fluid-phase virus titer was always higher (Fig. 1). A cytopathic effect (CPE) was first detectable at 60 hr PI and was more pronounced, but still not marked, at 72 hr PI.

The results of inoculation of HLC with 2 TCID50 per cell are shown in Fig. 2. The growth curve was similar to that obtained with an input multiplicity of 10 TCID50 per cell. Both cell-associated and fluid-phase virus gradually decreased until 8 hr PI. Rapid increase in virus titer then occurred from 18 until 36 hr PI for the cell-associated virus and until 48 hr PI for the fluid-phase virus. The rapidity of virus propagation then increased gradually until 72 hr PI. The maximum titers were 3 × 10⁶ TCID50 per 0.5 ml in the medium and 3 × 10⁷ TCID50 per 0.5 ml in the attached cells 72 hr PI.

Immunofluorescence. Viral antigen in HLC inoculated with an input multiplicity of 10 TCID50 per cell was first detected 18 hr PI by FA. A small proportion of the cells (0.7%) (Table 1) had weak cytoplasmic fluorescence, and a rare cell showed strong cytoplasmic fluorescence (Fig. 3). The number of fluorescent cells then gradually increased, and fluorescence was detected at the cell periphery of some cells 24 hr PI when about 6% of the cells contained antigen. Thirty hours PI the number of infected cells rapidly increased until approximately 19% of the attached cells were fluorescent. The fluorescence was cytoplasmic and was present at the cell periphery (Fig. 4). Approximately 30% of the cells had cytoplasmic fluorescence 36 hr PI. The cytoplasmic fluorescence increased in brightness and was present in approximately 50% of the cells 48 hr PI. From 70 to 85% of the cells had cytoplasmic fluorescence 60 and 72 hr PI. Fluorescence detected at the cell periphery had
TABLE 1. Relationship between occurrence of viral antigen by immunofluorescence and cell-associated virus titer

<table>
<thead>
<tr>
<th>Inoculum (MOI)</th>
<th>Time after inoculation (hr)</th>
<th>Cell-associated virus titer</th>
<th>Per cent fluorescent cells</th>
<th>Intensity of fluorescence</th>
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<tbody>
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<td>$10^2$</td>
<td>ND*</td>
<td>ND</td>
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<tr>
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<td>4</td>
<td>$10^2$</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>8</td>
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<td>12</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>18</td>
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<td>0.7</td>
<td>+</td>
</tr>
<tr>
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<td>24</td>
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<td>5.7</td>
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<td>72</td>
<td>$10^1.6$</td>
<td>76.2</td>
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</table>

* Multiplicity of infection [number of 50% tissue culture infective doses (TCID$_{50}$) per cell].

Log TCID$_{50}$/0.5 ml.

* Number of cells counted were the total observed in 10 high-power fields.

** = none; + = slight cytoplasmic fluorescence, ++ = moderate cytoplasmic fluorescence, +++ = intense cytoplasmic fluorescence.

* Not done.

almost disappeared and there had been a loss of approximately 30% of the attached cells due to CPE by 72 hr PI. (Fig. 5).

Viral antigen was first detected in the cell cytoplasm 24 hr PI with an input multiplicity of 2 TCID$_{50}$ per cell. Approximately 10% of the cells had cytoplasmic fluorescence 30 hr PI and some had weak peripheral fluorescence. The number of infected cells increased until approximately 20 to 30% of the attached cells were fluorescent 36 to 48 hr PI. The cytoplasmic fluorescence then increased in intensity with about 50% of the cells showing bright fluorescence 60 hr PI. Approximately 75% of the cells were fluorescent 72 hr PI.

Viral antigen was initially detected by immunofluorescence at both input levels 18 to 24 hr PI when the virus titer initially increased and was approximately $10^2$ TCID$_{50}$/0.5 ml. The relationship between the occurrence of immunofluorescence and virus titer is given in Table 1.

DISCUSSION

In one-step growth curve experiments, Kono et al. (12) reported a latent period of approximately 21 hr post-inoculation of HLC with 4 TCID$_{50}$ of EIA virus per cell. Rapid viral replication began 20 to 24 hr PI. Our studies were conducted to investigate further the one-step growth curve of EIA virus in HLC with two levels of infection and to determine when viral antigen appeared in the infected HLC by using the indirect FA technique. The results of the growth curve studies were similar to those reported by Kono et al. (13). There was an eclipse period of approximately 24 hr. Following this latent period, both cell-associated and fluid-phase virus showed a rapid increase in titer, although there was approximately $10^{1.2}$ to $10^2$ TCID$_{50}$/0.5 ml more fluid-phase than cell-associated virus.

Our results were similar to those reported by Thormar (21) for the growth cycle of visna virus in which a latent period of approximately 20 hr followed by the period of rapid viral growth was demonstrated. Thormar detected highest virus titer 50 hr after inoculation, and the fluid phase had $10^4$ TCID$_{50}$/0.5 ml more virus infectivity than did the cell phase. Harter et al. (4) also studied the kinetics of visna viral replication and indicated that viral propagation was demonstrable after a latent period of about 24 hr.

When the indirect immunofluorescent technique was used to detect viral antigen in infected HLC in this study, initial cytoplasmic fluorescence was shown at 18 to 24 hr PI. The virus titers in these cells were $10^{2.25}$ TCID$_{50}$ to $10^{2.5}$ TCID$_{50}$. Harter et al. (3) were able to detect visna viral antigen by FA approximately 24 hr PI.

Viral antigen was detected either on the cell surface or at the periphery of the cytoplasm from 24 hr PI to 48 hr PI at the higher virus input, but fluorescence did not seem to be concentrated at the cell periphery after 60 hr PI. Staining of the cell at this location is consistent with budding of EIA virus from the cell surface. Several workers (7, 12, 20) have examined infected HLC with the electron microscope and have described budding of EIA virus from the cell surface.

The appearance of the fluorescence in the infected cells was limited to the cytoplasm in the present study. Similar results have been reported by Ushimi et al. (22) using the indirect method and Crawford et al. (2) when the direct procedure was employed. Crawford et al. (2) have suggested that the cells supporting EIA viral propa-
Fig. 3. Immunofluorescent stain of horse peripheral leukocytes 18 hr after inoculation with EIA virus at an input multiplicity of $16 \text{TCID}_{50}$ per cell. $\times 750$.

Fig. 4. Immunofluorescent stain of horse peripheral leukocytes 30 hr after inoculation with EIA virus at an input multiplicity of $10 \text{TCID}_{50}$ per cell. $\times 750$.

gation in HLC are macrophages. EIA viral antigen was also demonstrated in the cytoplasm of macrophages in the tissue of infected horses by McGuire et al. (13) by using the direct immunofluorescent technique.

According to Moore et al. (16), intranuclear inclusions have been demonstrated in continuous cell lines derived from horse leukocytes inoculated with EIA-infected plasma or HLC-passaged material. Intranuclear inclusions or intranuclear fluorescence were not demonstrated in the present study or in other published work (2, 13, 22). Furthermore, Moore et al. (15) suggested that EIA virus was a parvovirus based upon size (30 nm), nucleic acid type (deoxyribonucleic acid), and lack of ether sensitivity. These findings are in contrast to the reports by a number of other workers (7, 12, 19, 20). The latter have
indicated that the virus is approximately 70 to 140 nm in diameter and composed of ribonucleic acid. It appears, however, that deoxyribonucleic acid is indirectly involved in EIA viral replication (12).

Our data suggest that immunofluorescence is a sensitive method for detecting low levels of EIA virus in infected HLC. It was possible to detect antigen approximately 24 hr PI by FA while CPE was not discernable until 60 hr PI. The amount of CPE was slight at the latter time and was still not marked 72 hr PI. These findings and other experiences with CPE in HLC (6) indicate that FA is a more sensitive and specific indicator of EIA viral infection. Furthermore, the results reported here substantiate the growth curve data for EIA virus described by Kono et al. (12).

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LITERATURE CITED


