A Study of Rice Dwarf Virus in Vector Cell Monolayers by Fluorescent Antibody Focus Counting

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SUMMARY

Infectivity assays of rice dwarf virus (RDV) were done by the fluorescent antibody focus counting technique on vector cell monolayers of the green rice leafhopper, Nephotettix cincticeps. The focus count method was shown to be an accurate and quantitative method for determining RDV infectivities. The optimal pH value for inoculation was about 6.0 in a solution containing 0.1 M histidine HCl and 0.01 M MgCl₂. Below pH 5.5 and above 6.5, the infectivity of RDV dropped rapidly. The optimal adsorption period at 28 °C was dependent upon the RDV concentration. Optimal periods for adsorption with relative RDV concentrations of 10⁻³, 10⁻⁴ and 10⁻⁴.⁵ were about 60, 90 and 120 min, respectively. The period from virion adsorption to penetration into the cell was about 90 to 120 min. Infective progeny virions were first detected 12 h after the initial inoculation. From 12 to 20 h, the growth rate of the virus in the cells was exponential with a doubling time of about 96 min, and then from 20 to 28 h there was little or no further increase in infective virus. When the infectivities of the same inocula were compared by using the focus count and vector insect injection methods, the dilution endpoints were approx. 10⁻⁶ and 10⁻⁴, respectively. The focus count assay method was thus about 100 times more sensitive than vector injection.

INTRODUCTION

Most plant viruses transmitted by leafhopper or planthopper vectors are not mechanically transmissible to plants. The infectivity of most of these viruses has therefore been assayed by injection of virus suspensions into the abdomens of vectors (Black, 1941; Maramorosch, 1955). However, with rice dwarf virus (RDV) this method requires long periods, about 50 to 60 days, before the final results are obtained (Kimura & Fukushi, 1960). Progress on this and similar viruses has therefore advanced slowly compared with that on sap-transmitted plant viruses.

The observation that these insect-borne viruses propagated in their respective insect vectors as well as in host plants led to the conclusion that these viruses possess properties of both plant and insect viruses (Fukushi, 1940). It was therefore expected that the establishment of monolayer cell cultures from the insect vectors would be an effective new experimental system. Chiu & Black (1967) first succeeded in culturing cell monolayers from Agallia constricta (AC), a vector of wound tumour virus (WTV), and demonstrated growth of the virus in AC monolayers.

Cell lines NC and NN, established respectively from embryos of Nephotettix cincticeps and N. nigropictus (both leafhopper vectors of RDV) have already been established, and infection of their monolayers by RDV has been demonstrated (Kimura, 1984, 1985). This paper deals with infection and multiplication of the virus in NC monolayers employing the fluorescent antibody focus counting technique.

METHODS

Preparation of inoculum. Infected leaves were surface-sterilized with 70% ethanol for 3 min, and then rinsed three times with sterile distilled water. The sterilized leaves were ground in 4 times the leaf weight of phosphate buffer (0.1 M, pH 7.3) and the slurry was clarified in a Hitachi 20PR centrifuge at 1470 g for 15 min. The
supernatant was used as a source of inoculum and further dilutions of the inoculum were made in 0.1 M-histidine HCl, 0.01 M-MgCl₂ (His-MgCl₂) solution (pH 6.0).

One ml aliquots of inoculum, at a 5 × 10⁻² dilution, were stored at −80 °C. Later these were thawed in a water-bath at 37 °C, and immediately cooled in an ice-bath. No reduction of the infectivities of the inocula stored frozen was detectable when compared with unfrozen inocula by the cell monolayer technique. Frozen stored samples were therefore used in these experiments.

Preparation of monolayers. Cell monolayers (Liu et al., 1973) for infectivity assays were grown on coverslips of 15 mm diameter, which had been previously washed in ethanol or acetone in an ultrasonicator. About 2 to 3 days after subculturing, cells were harvested from culture flasks, and sedimented by low-speed centrifugation (about 250 g, 3 min). The cell pellets were resuspended in growth medium (Kimura, 1984), a slightly modified Liu and Black's (1976) medium, to give a dilution of about 3.5 × 10⁶ to 5.5 × 10⁶ cells/ml, and thoroughly dispersed. Cell suspensions of 0.12 ml were spread over the entire area of each of two or three coverslips in a plastic dish (60 mm diam.) and enclosed in a sealable glass dish (80 mm diam.) sealed with Parafilm M (American Can Company, Greenwich, Conn., U.S.A.). The freshly seeded coverslips were allowed to remain undisturbed for a minimum period of 2 h to allow cell attachment before being placed in a 28 °C incubator. The cell monolayers on coverslips were used for virus inoculation within 48 h of seeding.

Virus inoculation and incubation of the cell monolayers. For infectivity assays, confluent cell monolayers on coverslips were washed twice with His-MgCl₂ solution and then covered with 0.05 ml of inoculum. Inoculated monolayers were incubated for 1 to 3 h at 28 °C and then washed three times with growth medium. Each coverslip was then covered with about 0.12 to 0.2 ml of medium and incubated at 28 °C for 40 to 48 h.

Detection of virus infection. The direct fluorescent antibody technique (Reddy, 1977) was used for detection of virus infections. The cell monolayers were washed by dipping in a beaker of phosphate-buffered saline (PBS; 0.01 M-phosphate, 0.15 M-NaCl, pH 7.3). After fixation with cold acetone for 5 min they were stained with fluorescent antibody (Chiu & Black, 1969) at 37 °C for 40 to 50 min. Excess stain was removed by dipping in two successive washes of PBS followed by a 30 to 40 min soak in PBS. The stained specimens, mounted in PBS containing 50% glycerol (pH 7–3), were examined under a Nikon fluorescence microscope at ×200 magnification.

Fluorescent antibody focus counts. The method of counting infection units was the 'focus count method' (Spendlove, 1967; Kimura & Black, 1971). An infected cell and any adjoining cells were counted as one infection unit. In order to use the method, the factor for relating diametral zone counts to counts over the whole monolayer on a coverslip was determined. Stained cell foci on 10 coverslip monolayers were counted. The diametral factor for coverslip was determined by dividing the number for the whole coverslip by the average number for horizontal and vertical diametral zones. The data were treated statistically, and the average diametral factor with 95% confidence limits was 10.45 ± 0.45. Numbers of foci per diametral zone count were considered satisfactory if between 4 and 150, but when the number was less than 4, foci over the whole area of the coverslip were counted and used instead (Kimura, 1985).

Vector insect injection method. The insect vector injection method was performed using second instar nymphs, 2 weeks after hatching. Two μl of inoculum was injected into the abdomen of each insect using a glass capillary (50 μm tip diam.). Injected NC insects were confined individually in glass tubes each enclosing three healthy rice seedlings and 5 ml tap water. The exposed rice plants were replaced at intervals of 1 week and the exposed plants were transplanted in a greenhouse. Symptom expression was examined over 50 to 60 days.

RESULTS

Effect of pH on RDV inoculation of vector cell monolayers

The original inocula were diluted with His-MgCl₂ at various pH values. After the start of each inoculation, the pH of a residual sample of inoculum was measured immediately. Each sample was then left in a 28 °C incubator for 90 min before a second pH measurement was made. The changes in pH of the inocula during the 90 min storage at 28 °C were less than 0.05 units. As shown in Fig. 1, several experiments using pH values ranging from 5.0 to 7.5 revealed that the optimum value for RDV inoculum was about pH 6.0. Below pH 5.5 and above pH 6.5, the infectivity of the virus dropped rapidly.

Adsorption and penetration of RDV particles into vector cell monolayers

To test the effects of the concentration of the virus and the volume of inoculum on the optimal interval for virus adsorption, experiments were carried out to determine the most efficient adsorption period at 28 °C with different RDV concentrations. The inoculum was diluted with His-MgCl₂ solution (pH 6.0), and tested at three relative virus concentrations. As shown in Fig. 2, the results indicate that the most efficient adsorption periods for dilutions 10⁻³, 10⁻⁴ and 10⁻⁴.5 were about 60, 90 and 120 min, respectively.
Fig. 1. Efficiency of inoculation with RDV at various pH values in His-MgCl₂ solutions. Conditions: inoculation period, 90 min at 28 °C; incubation period, 42 h, 28 °C; relative concentration of inoculum, 10⁻⁴. The results of two independent experiments are shown.

Fig. 2. Effect of length of inoculation period at different dilutions (○, 10⁻³; ▽, 10⁻⁴; □, 10⁻⁵) of inoculum. Conditions: inoculum at pH 6.0 in His-MgCl₂ solution; incubation period, 42 h, 28 °C; volume of inoculum, 0.05 ml/coverlip.

Fig. 3. Neutralization of infectivity by addition of antiserum to inoculated monolayers at various times after inoculation. Conditions: relative concentration of inoculum, 10⁻²; inoculation period, 30 min at 28 °C; antibody concentration in medium, 1/25 in two experiments (○, ▽); in control (▽), preimmune serum concentration in medium, 1/25; incubation period, 40 h, 28 °C.

It was assumed that RDV particles, adsorbed on the surface of vector cell monolayers, would next enter the cells, perhaps by phagocytosis. To determine the time required for penetration, viral antibody was added at various times after inoculation to neutralize virus remaining outside the cells. Preliminary experiments had been carried out to determine an efficient concentration of antibody for neutralization of virus particles in the cultured cell system, and this titre was 1/6000 when assayed by the micro-precipitin ring test. An efficient neutralizing concentration of the antibody was 1/25 in the medium.

A 10⁻² dilution of inoculum was allowed to adsorb to cell monolayers for 30 min and was then replaced with normal medium. Medium containing antibody was added at 0, 30, 60, 90, 120, 150
and 180 min later and left in contact for 120 min at 28 °C. As shown in Fig. 3, the results indicated that virus particles had almost completely penetrated the cells 90 to 120 min after initial inoculation.

**Growth of RDV in vector cell monolayers**

The growth of RDV in cell monolayers was studied by preparing virus extracts from the inoculated monolayers at various intervals, and assaying these on further monolayers.

Monolayers in flasks were inoculated with $10^{-3}$ inoculum for 60 min at 28 °C. The monolayers were then washed three times with medium, covered with 4 ml medium, and then incubated at 28 °C. At various intervals, the inoculated monolayers were harvested. Usually, the harvest from one flask was taken as a sample for each time point, and the volume of harvested cells was measured by sedimentation in a 5 ml centrifuge tube with a capillary tip graduated to 0.01 ml and accurate to ± 0.002 ml. The cells were frozen and stored at −80 °C with His-MgCl₂ solution of a volume 20 times that of the cell volume. Later, all cell samples in an experiment were thawed at 25 °C in a water-bath at the same time, the cell debris was removed after centrifugation at 1200 r.p.m. for 5 min, and the infectivities of the supernatant fractions were compared by assays on coverslip monolayers.

No infectivity was detected in supernatant fractions prepared during the first 12 h, indicating that residual virus inoculum had been effectively removed by washing three times with medium. Progeny virus was first detected at 12 h but not at 8 h after inoculation (Fig. 4), indicating an eclipse period. From 12 to 20 h, the growth rate of RDV in cells was exponential with a doubling time of about 96 min. From 20 to 28 h, there was little or no further increase of infective virus.

**Comparison of the sensitivity of focus count and vector injection methods for virus assay**

Infectivity assays of the same serially diluted inocula were carried out by focus counting on cell monolayers and by vector injection methods. The number of foci were counted as mentioned above. Under the experimental conditions, the relationship between RDV concentration and
RDV in vector cell monolayers

Fig. 5. Dilution endpoint assay of inoculum by the fluorescent antibody focus count method on vector cell monolayers. Conditions: inoculation period, 2 to 3 h, 28 °C; inoculum at pH 6.03 in His–MgCl₂ solution; incubation period, 44 h, 28 °C. Two independent sets of results are shown.

Fig. 6. Dilution endpoint assay of inoculum by the vector insect injection method. Conditions: age of NC insects, 12 to 14 days after hatching; volume of inoculum, about 0.2 μl/insect; experimental period, 60 days; incubation temperature, 25 (+1) °C. Two independent sets of results are shown.

The number of foci of infected cells was linear in the dilution range from 10⁻⁶ to 10⁻⁴ (Fig. 5). The dilution endpoint by focus counting was between 10⁻⁶.₅ and 10⁻⁶.

Using the insect vector injection method, the relationship between virus concentration and infectivity (no. of transmitters/no. of inoculated insects) was almost linear in the range of virus concentrations 10⁻⁴ to 10⁻² as shown in Fig. 6 and the dilution endpoint of the comparable inoculum by this assay was between 10⁻⁴.₅ and 10⁻⁴.

In this comparison of the two methods of infectivity assay, the focus count assay detected about 100 times less virus than the injection assay.

DISCUSSION

In studies of RDV comparable to those on WTV (Chiu & Black, 1967, 1969) and potato yellow dwarf virus (Chiu et al., 1970; Hsu & Black, 1973), cell lines of NC and NN vector insects were established, and we have shown that infectivity assays are feasible using these vector cell monolayers and a fluorescent antibody focus count technique. Such assays should be useful in research on many leafhopper- and planthopper-borne plant viruses.

The first appearance of infective progeny virus, 12 h after the start of inoculation, was later than that reported for WTV. One reason may be that the growth of NC cells was slower than that of WTV vector AC cells. However, the curve of RDV increase in the cells is similar to that for WTV (Kimura & Black, 1972; Reddy & Black, 1972).

The focus count method employed in this study provides more accurate, more sensitive and more convenient measures of RDV infectivity than does the vector injection method previously used. Moreover, experimental results were available within 40 to 48 h instead of 50 to 60 days as in the insect injection assays.

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REFERENCES


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