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Bunyamwera Virus Replication in Cultured *Aedes albopictus* (Mosquito) Cells: Establishment of a Persistent Viral Infection

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Bunyamwera virus replication was examined in *Aedes albopictus* (mosquito) cell cultures in which a persistent infection is established and in cytopathically infected BHK cells. During primary infection of *A. albopictus* cells, Bunyamwera virus reached relatively high titers ($\simeq 10^7$ PFU/ml), and autointerference was not observed. Three virus-specific RNAs (L, M, and S) and two virion proteins (N and G1) were detected in infected cells. Maximum rates of viral RNA synthesis and viral protein synthesis were extremely low, corresponding to <2% of the synthetic capacities of uninfected control cells. Viral protein synthesis was maximal at 12 h postinfection and was shut down to barely detectable levels at 24 h postinfection. Virus-specific RNA and nucleocapsid syntheses showed similar patterns of change, but later in infection. The proportions of cells able to release a single PFU at 3, 6, and 54 days postinfection were 100, 50, and 1.5%, respectively. Titers fell to 10^3 to 10^5 PFU/ml in carrier cultures. Persistently infected cultures were resistant to superinfection with homologous virus but not with heterologous virus. No changes in host cell protein synthesis or other cytopathic effects were observed at any stage of infection. Small-plaque variants of Bunyamwera virus appeared at approximately 7 days postinfection and increased gradually until they were 75 to 95% of the total infectious virus at 66 days postinfection. Temperature-sensitive mutants appeared between 23 and 49 days postinfection. No antiviral activity similar to that reported in *A. albopictus* cell cultures persistently infected with Sindbis virus (R. Riedel and D. T. Brown, J. Virol. **29:** 51-60, 1979) was detected in culture fluids by 3 months after infection. Bunyamwera virus replicated more rapidly in BHK cells than in mosquito cells but reached lower titers. Autointerference occurred at multiplicities of infection of $\simeq 10$. Virus-specific RNA and protein syntheses were at least 20% of the levels in uninfected control cells. Host cell protein synthesis was completely shut down, and nucleocapsid protein accumulated until it was 4% of the total cell protein. We discuss these results in relation to possible mechanisms involved in determining the outcome of arbovirus infection of vertebrate and mosquito cells.

The Bunyaviridae is a large family of spherical (90 to 100 nm in diameter), enveloped, arthropod-borne viruses. The genome consists of three segments of single-stranded negative-sense RNA designated L (large), M (medium), and S (small) according to size. The only genus as yet accepted within the family is the genus *Bunyavirus*, of which the prototype virus is Bunyamwera virus (BUN) (24). Four proteins have been reported in BUN particles: the nucleocapsid protein (N), a large protein (L), and two glycosylated envelope proteins (G1 and G2). The L protein is a minor component of the nucleocapsids and may be the virion-associated transcriptase (for reviews, see references 2 and 16).

The replication of BUN and certain other members of the *Bunyavirus* genus has been studied extensively in vertebrate cells, in which infection leads to marked cytopathic effects and cell death (2, 16). Only limited information is available about the growth of bunyaviruses in intact mosquitoes (4, 26) and in cultured mosquito cells (1, 11, 12). It is clear, however, that the interaction of bunyaviruses with vertebrate and invertebrate cells is fundamentally different, since in mosquito cells a primary phase of rapid virus growth is followed by a persistent, noncytopathic infection. Similar conclusions have been drawn from studies of alphavirus infections of cultured cells (5, 18-21, 25).

Although a number of hypotheses have been considered, no clear understanding exists of the factor(s) responsible for the establishment by arboviruses of persistent infections, as opposed to cytopathic infections, in mosquito cells (5, 18-20). In this report, the replication of BUN in

Aedes albopictus (mosquito) cells is examined. Emphasis is given to exploring those aspects of virus replication which may be important in the establishment and maintenance of the persistent state.

MATERIALS AND METHODS

Cells. BHK-21 cells were grown in Eagle-BHK medium, and Vero cells were grown in medium 199-LAH medium (5). *A. albopictus* cells were grown in Mitsuhashi and Maramorosch medium supplemented with 20% fetal calf serum (3, 23). *A. albopictus* cells were obtained from S. Buckley (Yale Arbovirus Research Unit); passage no. 50 to 65 were used.

Virus. BUN, obtained from I. Holmes, Department of Microbiology, Melbourne University, was cloned twice by plaque isolation. Infected BHK cell supernatant was used for intracerebral injection into suckling mice. Brains were sonicated in Hanks balanced salt solution supplemented with 0.2% bovine serum albumin (BSA) to give a 10% (wt/vol) suspension which was used as the infecting stock. Small-plaque variants (SPVs) of BUN were cloned by plaque isolation in Vero cells, and infected Vero cell supernatants were used as the infecting stock. Semliki Forest virus, Sindbis virus, and Ross River virus (T48 strain) were obtained from I. D. Marshall (John Curtin School of Medical Research, Australian National University) and were cloned twice by plaque isolation; Barmah Forest virus (a member of the Turlock group of bunyavirus-like viruses [2]) was also obtained from I. D. Marshall. Stocks were infected BHK cell supernatants. Kunjin virus (cloned; MRM 61C) was obtained from E. G. Westaway, Monash University; stocks were prepared from suckling mouse brain.

Plaque assay. Viruses were assayed by plaque formation on Vero cell monolayers (3 to 5 days of incubation under agar overlay containing medium 199, 0.02% DEAE-dextran, and 5% fetal calf serum). Cell-associated virus was estimated by scraping cell monolayers in Hanks balanced salt solution supplemented with 0.2% BSA and sonicating (0°C, 10 s) before plaque assay. Plaques formed by Ross River virus and Barmah Forest virus were larger than those formed by BUN and were easily distinguishable in superinfection experiments.

Infective-center assays. Infective-center assays were performed either on Vero cell monolayers or on *A. albopictus* cell monolayers.

(i) On Vero cell monolayers. Infected BHK or *A. albopictus* cells were detached by scraping, washed three times by centrifugation in growth medium, and dispersed evenly (5). Counted cells were plated in triplicate on Vero cell monolayers and overlaid as in the plaque assay. For assays of infected BHK cells, incubation was at 37°C for 5 days. For assays of infected *A. albopictus* cells, incubation was at 30°C for 2 days, followed by 37°C for 3 days. A previous study (5) has shown that the percentage of infected *A. albopictus* cells determined by this procedure is virtually identical to that obtained by immunofluorescence. Thus, changes in the number of infective centers (see Table 1) are not the result of variations in the efficiency of initiation of infection by plated cells.

(ii) On *A. albopictus* cell monolayers. Monolayers of *A. albopictus* cells were prepared by seeding cells in 1-cm wells on plastic trays (TC grade; Linbro Scientific Inc., Hamden, Conn.). *A. albopictus* cells for assay were detached, washed, dispersed, and seeded in 1 ml of medium at ≃0.75 cell per well. Wells were assayed for virus after incubation either at 28°C for 5 days with 0.3×10^6 cells per well initially or at 28°C for 14 days with 0.1×10^6 cells per well initially. A total of 240 wells were assayed for each percent determination.

Estimation of rates of RNA synthesis. BHK cell monolayers were washed with phosphate-buffered saline and incubated with [5′-^{3}H]uridine (10 μCi/ml; New England Nuclear Corp., Boston, Mass.) in Eagle minimal essential medium. When required, actinomycin D (AMD; a gift from Merck & Co., Inc., Rahway, N.J.) was added to a final concentration of 5 μg/ml 10 min before isotope addition. After labeling, monolayers were washed with phosphate-buffered saline and dissociated in 1 ml of 1% sodium dodecyl sulfate. Samples from ≃10^5 cells were precipitated on Gelman type AE glass fiber disks, washed with 0.25 M perchloric acid and then ethanol, and counted with a toluene-based scintillation fluid in a Beckman LS350 scintillation counter. *A. albopictus* cells were washed before and after labeling in isotonic buffer (0.13 M NaCl, 5 mM KCl, 3.5 mM $CaCl_2$, 4 mM $NaHCO_3$, 0.1% glucose, pH 7.0) and labeled with [5′-^{3}H]uridine (20 μCi/ml) in Grace medium (7). When required, AMD was used at 1 μg/ml. AMD had no significant effect on virus yields in BHK or *A. albopictus* cells whether added before infection or at later times. Precipitated samples from ≃2×10^5 to 10×10^5 cells were prepared and counted as above.

Release of sedimentable labeled material. Supernatants from *A. albopictus* cells labeled with [5′-^{3}H]uridine (as described above) were diluted with 5 volumes of Tris-saline-BSA (TS-BSA; 0.13 M NaCl, 0.02 M Tris [pH 7.4], 0.1% BSA) and centrifuged (30,000 rpm, 2°C, 3 h). (Under these conditions, radiolabeled Semliki Forest virus (≃250S) was completely sedimented.) The pellet was suspended in 250 μl of 1% sodium dodecyl sulfate, and samples (100 μl) were counted as described above.

Electrophoresis of RNA. Infected BHK cells and *A. albopictus* cells were labeled as described above. RNA was extracted (22), and samples from 2×10^5 BHK cells or 4×10^5 to 20×10^5 *A. albopictus* cells were electrophoresed on 0.5% agarose–2.0% acrylamide composite slab gels (17), which were then fluorographed (10).

Analysis of viral nucleocapsids in cytoplasmic extracts. Cell monolayers were extracted with 0.5% Nonidet P-40 (ICI Australia Ltd., Melbourne, Australia) in buffer (0.13 M NaCl, 0.01 M Tris, pH 7.8). Cells were checked for disruption (>95%) by trypan blue staining and centrifuged in an Eppendorf centrifuge (1 min, 0 to 4°C). Cytoplasmic extracts from 1×10^6 to 10×10^6 cells were centrifuged on linear 10 to 30% glycerol gradients in buffer (1 M NaCl, 2 mM EDTA, 0.05 M Tris [pH 7.8]; reference 15) in a Spinco SW27.1 rotor (25,000 rpm, 16 h, 2°C) in a Beckman L5-50 ultracentrifuge. Fractions (100 μl) were assayed for acid-precipitable radioactivity as described above.

Analysis of virus-induced protein synthesis. BHK cell monolayers were washed with phosphate-buffered saline and incubated with ^{3}H-labeled amino

acids (20 μCi/ml; New England Nuclear Corp.) in Eagle minimal essential medium containing 1/10 the normal concentration of amino acids. Monolayers were dissociated in 1% sodium dodecyl sulfate, and samples representing ≃10^5 cells were electrophoresed on discontinuous polyacrylamide gels (9) with a 10 to 20% acrylamide gradient in the resolving gel and a 5% acrylamide stacking gel. Electrophoresis was at 180 V until the tracking dye had travelled 14 cm in the resolving gel. Gels were stained for protein with 0.25% Coomassie brilliant blue in methanol-acetic acid-water (50:10:40, vol/vol), destained in ethanol-acetic acid-water (24:10:66, vol/vol), and fluorographed. Densitometry was on a Transidyne 2955 scanning densitometer, and peak areas were evaluated by triangulation. *A. albopictus* cells were washed before and after labeling with isotonic buffer (described above), and label (10 μCi/ml) was added in this buffer. Electrophoresis samples represented ≃2 × 10^5 cells. The net difference between the densitometric profile of infected and mock-infected cells was used to quantitate viral protein synthesis. Attempts to reduce the background of host cell protein synthesis by pretreatment with AMD were unsuccessful (also see reference 14).

Preparation of labeled BUN. For the preparation of labeled BUN (13, 15, 27), BHK cell monolayers infected at a multiplicity of infection (MOI) of 0.1 were incubated from 6 to 24 h postinfection (p.i.) with a ^{3}H-labeled amino acid mixture (20 μCi/ml) in Eagle minimal essential medium containing 1/10 the normal level of amino acids. The supernatant was clarified by centrifugation, and NaCl and then polyethylene glycol (Koch-Light Labs Ltd., Colnbrook Bucks., England; molecular weight, 6,000) were added to final concentrations of 2.3 and 7% (wt/vol), respectively. The solution was stirred for several hours at 4°C and centrifuged (60 min, 10,000 rpm), and a small volume of TS-BSA was added to the pellet. The pellet was sonicated, and the suspension was centrifuged on a 5 to 25% sucrose gradient containing TS-BSA (60 min, 4°C, 23,000 rpm). Radioactivity and infectivity peaks were coincident and were located near the middle of the gradient; recovery of infectivity was 20 to 50%. For the concentration of virus, peak fractions were diluted with buffer and centrifuged (3 h, 4°C, 30,000 rpm). Uridine-labeled BUN was prepared by labeling with [5′-^{3}H]uridine (10 μCi/ml) in Eagle minimal essential medium containing AMD (1 μg/ml).

Preparation of antiviral medium. For the preparation of antiviral medium (20), *A. albopictus* cells were infected (MOI, ≃5) with BUN, Semliki Forest virus, Sindbis virus, or Kunjin virus and incubated in growth medium at 28°C with subculturing into fresh medium every 5 to 8 days. Uninfected control cultures were mock infected and subcultured similarly. At 112 days after infection or mock infection, cells were resuspended in growth medium by shaking, transferred to sterile pretreated dialysis tubing, and dialyzed against complete growth medium for 72 h at 28°C with gentle shaking. Before use, the medium surrounding the dialysis sac was assayed for the presence of contaminating virus.

RESULTS

Primary BUN infection of *A. albopictus* cells and BHK cells. The growth of BUN in *A. albopictus* cells and BHK cells was compared at temperatures near those optimal for cell growth: 26°C for *A. albopictus* cells and 37°C for BHK cells. For *A. albopictus* cells infected with BUN at an MOI of ≃10, maximum extracellular virus (EV) titers were 2 × 10^7 to 3 × 10^7 PFU/ml at 70 h p.i. (Fig. 1A). At a lower MOI (≃0.01), similar titers were reached, but at later times. Cell-associated virus titers were approximately 1 log unit lower than EV titers (Fig. 1A). No significant difference between cell numbers in infected and control cultures was recorded for the first 5 days of infection, and no cytopathic effects were observed in infected cultures at any stage of infection (data not shown). Maximum EV titers were lower in infected BHK cells (MOI, ≃10) than in *A. albopictus* cells (5 × 10^5 PFU/ml at 20 to 30 h p.i.) but were increased to 5 × 10^6 PFU/ml at a low MOI (0.01) (Fig. 1B). Infected BHK cells showed marked cytopathic effects by 36 h p.i.

Persistent infection of *A. albopictus* cells. *A. albopictus* cells infected with BUN were maintained by subculturing over a 55-day period (Fig. 2). After the primary phase of rapid virus growth (up to 3 days), titers fell gradually, stabilizing at 10^3 to 10^5 PFU/ml. Subculturing led to an immediate fall in EV titer due to virus dilution and then a gradual increase in titer with a peak 4 days later (Fig. 2).

Superinfection of persistently infected *A. albopictus* cells with homologous and heterologous viruses. Persistently infected cultures (Fig. 2) were superinfected at 54 days p.i. with BUN, Ross River virus (an alphavirus), or Barmah Forest virus (a bunyavirus-like virus [2]). There was no increase in titer after superinfection with BUN. Infection with the two heterologous viruses gave rise to normal productive infections (Fig. 3).

Proportion of virus-releasing cells in primary and persistent infections. In preliminary experiments with BUN-infected BHK cells, all cells assayed as infected by 12 h p.i. (data not shown). For *A. albopictus* cells, all cells assayed as infected by 72 h p.i., the time of maximum EV titer (Table 1). Between 72 and 144 h p.i., there was a twofold decrease in the percentage of virus-releasing cells, accompanied by a more pronounced fall in titer from 3 × 10^7 to 1 × 10^6 PFU/ml. By 48 days p.i., titers had fallen to 10^4 PFU/ml (Fig. 2).

Infective-center assays were performed on carrier cultures infected for 48 days by three methods: (i) persistently infected cells were incubated for 5 days on Vero cell monolayers; (ii) cells were incubated for 5 days on confluent *A. albopictus* cell monolayers; and (iii) cells were incubated for 14 days on sparse *A. albopictus* cell monolayers (see above). The proportions of

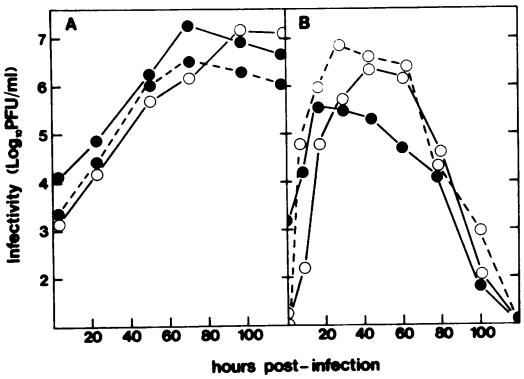

FIG. 1. *Growth of BUN in A. albopictus cells and BHK cells. (A) A. albopictus cells. EV titers: ●——●, MOI ≃ 10; ○——○, MOI ≃ 0.01. Cell-associated virus titer: ●---●, MOI ≃ 10. (B) BHK cells. EV titers: ●——●, MOI ≃ 10; ○——○, MOI ≃ 0.01. Cell-associated virus titer: ○---○, MOI ≃ 0.01.*

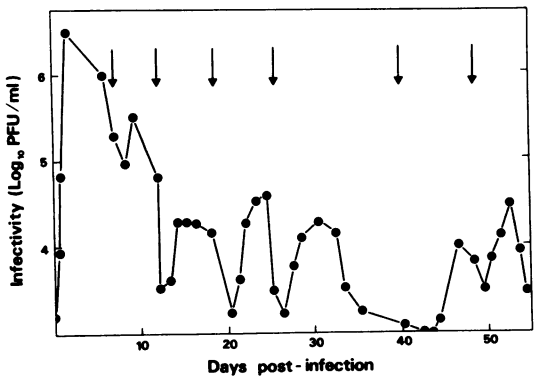

FIG. 2. *Establishment of a persistent infection in A. albopictus cells infected with BUN. A. albopictus cells were infected (MOI, ≃5) under standard conditions and subcultured at intervals (arrows) into fresh growth medium. Symbol: ●, EV titer (wild-type plus SPVs).*

virus-releasing cells obtained by these three procedures were 0.5, 1.0, and 1.5%, respectively. Upon subculturing of persistently infected cultures, the percentage of infective centers rose to 15% by 2 days after subculturing. By 6 days, the proportion of virus-releasing cells had fallen to 0.7% (data not shown).

Generation of SPVs and *ts* mutants and their possible role in persistent infections. SPVs and temperature-sensitive (*ts*) mutants appear in cultures of *A. albopictus* cells persistently infected with alphaviruses (5, 21). It has been proposed that *ts* mutants may be involved in establishing or maintaining persistent vesicular stomatitis virus infections (28). To determine whether SPVs and *ts* mutants appeared during

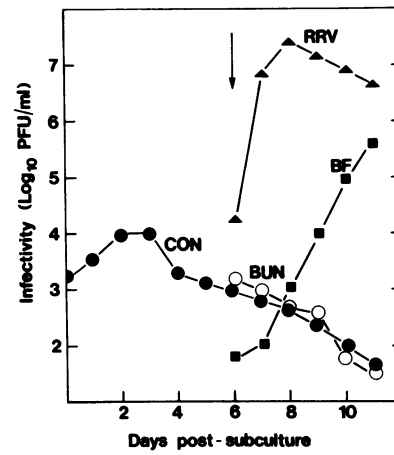

FIG. 3. *Superinfection of persistently infected A. albopictus cells with homologous and heterologous viruses. A. albopictus cells persistently infected with BUN (Fig. 2) were superinfected or mock superinfected at 54 days after primary infection (arrow). Symbols: ●, EV titer of BUN after mock superinfection (CON); ○, EV titer of BUN after superinfection with BUN (MOI, ≃2; BUN); ▲, EV titer of Ross River virus after superinfection with Ross River virus (MOI, ≃2; RRV); ■, EV titer of Barmah Forest virus after superinfection with Barmah Forest virus (MOI, ≃0.01; BF).*

TABLE 1. *Virus-releasing A. albopictus cells during primary BUN infection*[a]

Time after infection (h)	Virus-releasing cells (% of total)	EV titer (PFU/ml)
1	1	3×10^3
24	60	2×10^4
48	89	3×10^5
72	100 (100)[b]	3×10^7
96	66 (65)	2×10^6
144	55 (50)	1×10^6

[a] Infection was under standard conditions (MOI, ≃2).

[b] Numbers within parentheses represent a separate experiment in which triplicate assays were also performed (see text).

BUN infection of *A. albopictus* cells, nine replicate cultures were infected with BUN, and the plaque characteristics (at 37°C) of released virus was determined at various times after infection (Fig. 4). SPVs appeared in each of the nine cultures and showed a similar time course of appearance in each. They represented approximately 5% of EV at 7 days p.i. and 75 to 95% at 66 days p.i. Although the total titer was the same at 14 and 41 days p.i., the absolute number of SPVs increased fivefold over this period. Thus, the proportion of SPVs in the culture did not increase solely because of a greater stability

to inactivation; rather, they must have a growth advantage under the conditions used. Plaque characteristics of virus from persistently infected cultures are shown in Fig. 5.

The temperature sensitivity of virus was determined at various times after infection (Table 2). At 23 days p.i., similar titers were obtained at assay temperatures of 32, 37, and 39°C; hence, most SPVs (25 to 30% of virus) were not *ts* at this time. At 49 days, virus titers at 39°C were one-fifth those at 37°C; a large proportion of SPVs had therefore acquired the *ts* character between 23 and 49 days p.i.

Our data do not support the view that either SPVs or *ts* mutants are involved in the establishment or maintenance of persistence since there was a fall in the percentage of virus-releasing cells (Table 1), in virus titer (Table 1 and Fig. 1 and 2), and in the syntheses of viral RNA, viral nucleocapsid, and viral protein (see below) well before the proportion of these variants was significant. Several cloned stocks were prepared from SPVs isolated from carrier cultures at 23 days p.i. In both BHK cells and *A. albopictus* cells, these clones showed no significant differ-

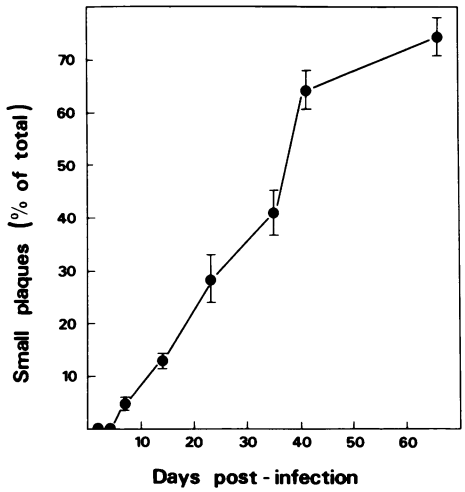

FIG. 4. *Generation of SPVs of BUN in persistently infected A. albopictus cells. Nine replicate cultures of A. albopictus cells were infected with standard BUN (MOI, ≃20) and subcultured at intervals of approximately 7 days. EV samples (taken before subculture) were assayed at 37°C, and the proportion of SPVs in each culture was estimated. Bars indicate the standard deviation of determinations.*

TABLE 2. *Appearance of ts mutants in A. albopictus cells persistently infected with BUN*[a]

Time after infection (days)	Plaque formation at indicated temp relative to 37°C (=1.0)		
	32°C	37°C	39°C
16	0.8	1.0	
23	1.2	1.0	1.2
36	1.4	1.0	
49	1.3	1.0	0.2
55	1.6	1.0	

[a] *A. albopictus* cells were infected with BUN and maintained by subculturing (Fig. 2). EV was assayed under identical conditions at the three temperatures. Incubation was for 7, 5, and 5 days at 32, 37, and 39°C, respectively; 100 to 300 plaques were counted for each determination. The efficiency of plaque formation by wild-type BUN was identical at all temperatures.

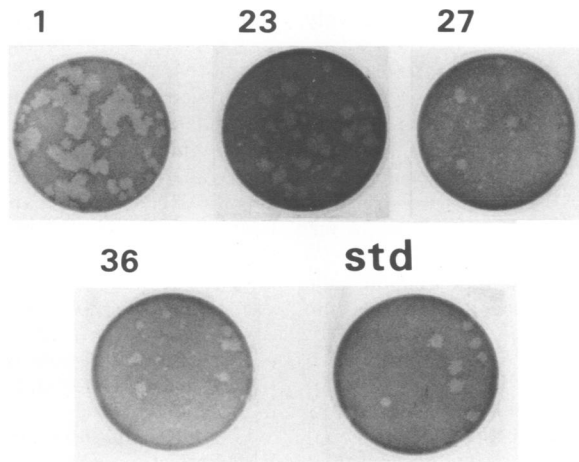

FIG. 5. *Plaque characteristics of virus from A. albopictus cells during acute and persistent infections with BUN. A. albopictus cells were infected with BUN (Fig. 2), and EV from 1, 23, 27, and 36 days p.i. was assayed under standard conditions at 37°C and compared with the BUN used as the infecting stock (std). ×0.6.*

ences from wild-type BUN in yields of virus, kinetics of growth, or effects on the host cell, supporting the conclusions reached above. However, the SPV stocks were significantly less virulent upon intracerebral injection into suckling mice (data not shown).

Lack of antiviral activity in medium from BUN-infected carrier cultures. It has been reported that a dialyzable, cell- and virus-specific material capable of reducing yields of Sindbis virus during the acute phase of infection appears in the culture fluids of *A. albopictus* cells persistently infected with Sindbis virus (20). To determine whether a similar antiviral factor was released from BUN-infected cells, 112-day carrier cultures of BUN-infected or mock-infected *A. albopictus* cells were dialyzed against fresh growth medium (see above), and the resultant fluids were tested for antiviral activity. *A. albopictus* cells were pretreated for 48 h with undiluted medium from infected or control cultures; medium was then removed, cells were infected with BUN (MOI, ≃40), and the total virus yield was determined. Antiviral activity was not detected at significant levels; at 48 h p.i., virus titers of 2×10^7 and 2.7×10^7 PFU/ml were obtained for cultures treated with "anti-BUN" medium and control medium, respectively. Analogous experiments, using growth medium from *A. albopictus* cells persistently infected with Kunjin virus, demonstrated no anti-Kunjin virus activity. The same experiments with medium from Semliki Forest virus and Sindbis virus infections gave an activity which reduced peak EV titers by approximately 150-fold (Semliki Forest virus) and 50-fold (Sindbis virus). As previously reported (20), this activity was virus specific, enhanced by preincubation with cells for 48 h, and retained at a relatively high dilution.

Virus-specific RNA synthesis in *A. albopictus* cells and BHK cells. In *A. albopictus* cells infected with BUN (MOI, ≃5), maximum rates of AMD-resistant RNA synthesis were low, being only 2% of RNA synthetic rates in mock-infected cells without AMD (Fig. 6). Peak viral RNA synthesis occurred at 40 to 50 h p.i., when most cells assayed as infected (Table 1). By 70 h, when EV titers were maximal and all cells were releasing infectious virus (Fig. 1 and Table 1), virus-specific RNA synthesis had fallen to the low level seen in mock-infected cells with AMD (Fig. 6).

To determine whether the apparently low level of viral RNA synthesis in BUN-infected mosquito cells was due to an efficient release of labeled material into the medium, the presence of incorporated radioactivity in culture fluids

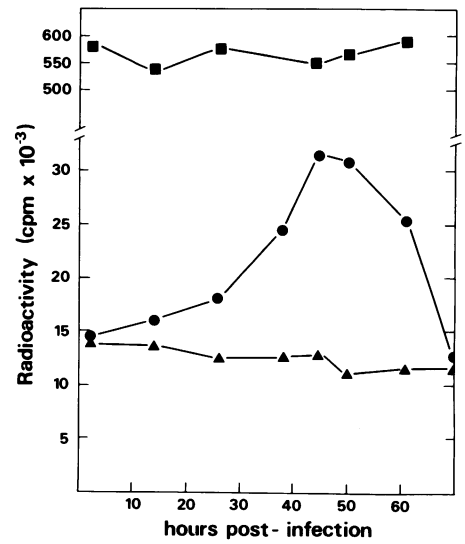

FIG. 6. *AMD-resistant RNA synthesis in A. albopictus cells infected with BUN. Cells were infected with BUN (MOI, ≃5) and labeled in Grace medium with [5'-³H]uridine for 4-h periods, with or without AMD (1 μg/ml). Mock-infected cells were treated similarly. Symbols: ■, incorporation in mock-infected cells without AMD; ●, incorporation in infected cells with AMD; ▲, incorporation in mock-infected cells with AMD.*

was measured after a 4-h pulse of [³H]uridine in the presence of AMD. Incorporation into material which was released during this period represented only 0.08 to 0.33% of the incorporation into cell-associated material at all times up to 3 days p.i. The release of labeled material from mock-infected cells was similar to that from infected cells (data not shown). We conclude that the low level of [³H]uridine incorporation in infected *A. albopictus* cells reflects the low level of viral RNA synthesis.

Labeled RNA from infected mosquito cells showed three virus-specific peaks on agarose-acrylamide gels (Fig. 7). These peaks were identified as the large, medium, and small virus-specific RNAs (L, M, and S) by co-electrophoresis with labeled RNA from BUN-infected BHK cells and with RNA from purified labeled BUN (data not shown). Due to the low levels of viral RNA synthesis in infected cells, residual AMD-resistant incorporation in mock-infected cells represented a relatively large proportion of the total AMD-resistant incorporation in infected cells.

In BHK cells infected with BUN (MOI, ≃5), AMD-resistant RNA synthesis was relatively high, being ≃30% of rates in mock-infected cells without AMD. Virus-specific RNA synthesis

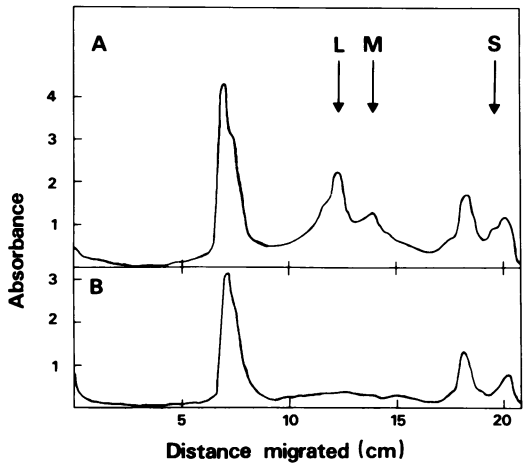

FIG. 7. *Labeled RNA species from A. albopictus cells infected with BUN. Cells infected with BUN (MOI, ≈10) were labeled with [5'-^{3}H]uridine from 48 to 52 h p.i. in the presence of AMD. Mock-infected cells were labeled similarly. RNA was electrophoresed on agarose-acrylamide composite slab gels, and the gels were fluorographed. Densitometry was on a Transidyne 2955 scanning densitometer. Migration is from left to right. (A) RNA from infected cells; positions of marker viral RNAs from infected BHK cells are indicated. (B) RNA from mock-infected cells; the two rapidly migrating species probably represent mitochondrial rRNA's, the synthesis of which is AMD resistant in A. albopictus cells (6). The slowly migrating peak was also seen in BHK cell extracts and banded in the position of host DNA from ethidium bromide staining.*

was maximal at 4 to 8 h p.i. and was maintained over a shorter period than in *A. albopictus* cells. Virus-specific RNA from BHK cells exhibited three major peaks of radioactivity (L, M, and S), which were labeled in the molar ratio 1:1:3 (data not shown).

Nucleocapsid synthesis in infected *A. albopictus* cells. Cytoplasmic extracts from infected *A. albopictus* cells contained three nucleocapsid species (approximately 110S, 90S, and 50S), together with a relatively high proportion of slowly sedimenting labeled material. The three nucleocapsid species cosedimented with the corresponding species from BHK cells. Nucleocapsids were first detected at 6 to 10 h p.i., with rapid labeling at 48 to 52 h being the time of peak viral RNA synthesis (Fig. 6). Nucleocapsid synthesis then fell and was not detectable by 71 to 75 h p.i.; at this time, virus-specific RNA synthesis had also fallen to a low level (data not shown).

Viral and host cell polypeptide syntheses in infected cells. From studies with alphaviruses and other enveloped viruses, it has been argued that there is a close connection between the degree of viral cytopathogenicity and the balance established between the production of viral proteins and their release from the cell (19). In *A. albopictus* cells infected with BUN, two infection-specific proteins were detected (Fig. 8). One of these (N) comigrated with the nucleocapsid protein of purified BUN; the other, which was labeled far less strongly, comigrated with the envelope protein G1 of purified virus. Nucleocapsid protein synthesis was maximal early in infection, at 12 to 14 h p.i., but represented only 1% of the total cellular protein synthesis at this time (Fig. 9). Synthesis then declined, falling to 0.05% of the host synthesis at 48 h p.i. and continuing at around this level until at least 85 h p.i. Synthesis of G1 protein was at too low of a rate to allow quantitation. No accumulation of any viral proteins could be observed upon staining gels of infected cell extracts with Coomassie

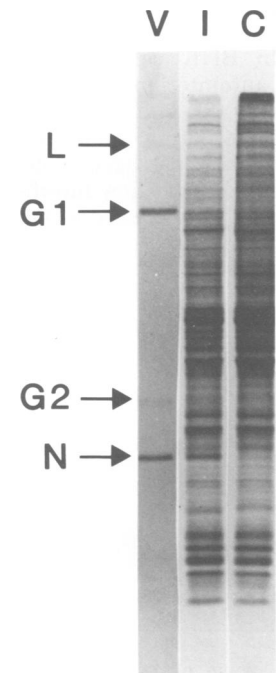

FIG. 8. *Polypeptides in A. albopictus cells infected with BUN. Cells were infected with BUN (MOI, ≈3) under standard conditions and labeled with ^{3}H-amino acids from 12 to 14 h p.i. Mock-infected cells were labeled similarly. Cell extracts, together with purified BUN labeled with ^{3}H-amino acids, were electrophoresed on a discontinuous 10 to 20% gradient slab acrylamide gel and fluorographed. The positions of viral proteins L, G1, G2, and N are indicated. Migration is from top to bottom. V, BUN labeled with ^{3}H-amino acids; I, extract from infected cells; C, extract from control cells.*

brilliant blue. Infection of *A. albopictus* cells had no detectable effect on the synthesis of host cell proteins at any stage of infection.

By contrast, BUN infection of BHK cells led to gross changes in host protein synthesis and high levels of viral protein synthesis. Host protein synthesis was shut down by 14 h p.i. Virus-specific protein synthesis was sustained at relatively high levels from 6 to 24 h p.i. At the time of peak synthesis (8 to 12 h p.i.), the synthesis of infection-specific proteins represented approximately 20% of the level of protein synthesis in mock-infected cells. Nucleocapsid protein accumulated in infected cells; at 18 h p.i., it represented ≃4% of the total cellular protein (N. J. Short, unpublished data).

DISCUSSION

BUN replication in cultured mosquito cells and that in BHK cells differed in a number of important respects. During primary infection of *A. albopictus* cells, relatively high titers were reached and there was no evidence for autointerference. In BHK cells, lower titers were reached and autointerference was observed. In a previous study (8), *A. albopictus* cells showed a low capacity to permit inhibition of standard virus replication by passaged (defective) BUN and were designated "low-interference" cell lines. BHK cells were classed as "high-interference" cells as the replication of standard virus was strongly inhibited by passaged BUN.

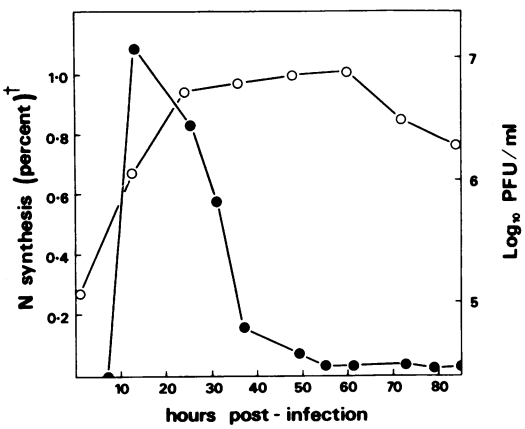

FIG. 9. *Time course of nucleocapsid protein synthesis in A. albopictus cells infected with BUN. Fluorographs of extracts from infected A. albopictus cells (see Fig. 8) labeled at various times after infection were analyzed by densitometry, and the net difference between the profile of infected cells and that of mock-infected cells was used to quantitate viral protein synthesis. Symbols: ●, synthesis of N protein as a percentage of total host cell protein synthesis; ○, EV titer. †, The nucleocapsid protein (N) was the only viral protein synthesized in a sufficient amount to be quantitated against the host cell background.*

Rates of virus-specific RNA synthesis and virus-specific protein synthesis in mosquito cells were only 1 to 2% of the levels in control cells. No accumulation of viral proteins occurred, host protein synthesis was unaltered, and no effects on the cell division rate or cell morphology were observed. In BHK cells, by contrast, viral RNA and protein syntheses occurred at approximately one-quarter of the corresponding synthetic rates in control cells, viral proteins accumulated, and severe cytopathic effects followed.

After the initial phase of rapid virus production in mosquito cells, virus-directed macromolecule synthesis fell, and virus titers dropped to low levels. Carrier cultures were maintained for several months by regular subculturing. Essentially all cells in such cultures excluded superinfecting homologous virus, indicating that all or part of the viral genome is expressed in each cell. Similar observations have been made for mosquito cells persistently infected with alphaviruses (5, 25), but attempts to demonstrate the existence of the complete viral genome in more than a small percentage of cells have been unsuccessful (5, 18). In our study we have attempted to increase the sensitivity of the infective-center assay by incubating mosquito cells with indicator mosquito cells under growth conditions, rather than by plating on Vero monolayers under plaque assay conditions which are potentially suboptimal for mosquito cells. Nevertheless, only 1.5% of the cells from cultures persistently infected for 54 days generated an infective center under these conditions. At least two interpretations are possible: (i) most cells in persistently infected cultures contain defective virus capable of homologous interference, or (ii) the production of infective virus is at such low levels that the proportion which survives normal inactivation contains too few infective particles to initiate an infection. We are not able to distinguish between these possibilities.

The data provided lead to a number of conclusions concerning the establishment of persistence in BUN-infected mosquito cells. Since no evidence was found for heterologous interference, it is unlikely that persistence is mediated by interferon-like agents. Similar conclusions have been reached for persistent alphavirus infections (5, 18). A different type of antiviral activity has been demonstrated in the medium surrounding *A. albopictus* cells persistently infected with Sindbis virus; this factor is both cell specific and virus specific (20). No activity of this type was detected in BUN- or Kunjin virus-infected cultures but was present in Sindbis vi-

rus- and Semliki Forest virus-infected cultures. Therefore, the agent may be specific to alphaviruses.

Wild-type virus was gradually replaced by SPVs in persistent cultures. At later times, virus acquired a *ts* phenotype. Neither SPVs nor *ts* mutants appear to play a role in the establishment of persistence since (i) they appeared well after the repression of virus-directed macromolecule synthesis which accompanied the establishment of low-level infection and (ii) the capacity of cloned SPVs to induce cytopathic effects in BHK cells or persistence in mosquito cells was identical to that of wild-type virus. The appearance of SPVs and *ts* mutants presumably reflects cell growth conditions which favor their selection.

No qualitative differences were observed in the species of RNA, protein, or nucleocapsid generated in mosquito cells as opposed to BHK cells. It is therefore improbable that the viral replication strategy differs in the two cell types. Although viral protein, RNA, and nucleocapsid syntheses were shut down in infected mosquito cells, shutdown per se cannot be responsible for the establishment of persistence since, in BHK cells, viral RNA and nucleocapsid syntheses, but not viral protein synthesis, fell to low levels early in infection (N. J. Short and S. E. Newton, unpublished data).

Perhaps the most important difference between BUN replication in the two cell types was in the high levels of synthesis and accumulation of viral proteins in BHK cells and the relative efficiency of infectious virus production in mosquito cells. Viral protein synthesis in mosquito cells was low and was reduced to barely detectable levels by about 30 h p.i. By contrast, in BHK cells viral protein synthesis was not only rapid but was also sustained at high levels until 30 h p.i. when the first signs of cytopathic effect were visible. At this time, nucleocapsid protein represented approximately 3% of total cell protein (N. J. Short, unpublished data).

Our previous comparative studies on the replication of alphaviruses gave results which are in some respects analogous to those reported here (5, 13, 19). Data from both studies have lent support to the view that, for cultured cells, the outcome of arbovirus infection is determined by the rate of synthesis, clearance, and accumulation of virus-coded proteins.

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