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# Antiviral Activity Released from *Aedes albopictus* Cells Persistently Infected with Semliki Forest Virus

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*Aedes albopictus* (mosquito) cells persistently infected with Semliki Forest virus released an agent which inhibited virus production by *A. albopictus* cells infected with homologous virus. Inhibition of virus production was accompanied by a marked reduction in the synthesis of viral RNA and viral proteins. Expression of the antiviral effect was prevented by pretreatment of cells with actinomycin. No analogous antiviral activity was detected in culture fluids of *A. albopictus* cells persistently infected with a flavivirus (Kunjin virus) or a bunyavirus (Bunyamwera virus).

Alphavirus and bunyavirus infection of cultured vertebrate cells leads to gross cytopathic effects, whereas in cultured *Aedes albopictus* cells, infection by these viruses results in a persistent infection characterized by the maintenance of a relatively low level of virus in the culture, the absence of gross cytopathic effects, and a restricted replication of superinfecting related viruses (1–3, 5, 6, 10, 11). Flaviviruses can also establish persistent infections in mosquito cell cultures, although cytopathic effects are commonly observed (7, 10).

The nature of the events which determine the establishment of persistent arbovirus infections in arthropod cells is unknown. Although small-plaque mutants, temperature-sensitive mutants, and defective virus particles are generated in such cultures, they are prominent only after the establishment of persistence, and no data exist to implicate them, or interferon-like agents, as mediators of persistent arbovirus infections of mosquito cells (1, 5, 10).

It is of interest, however, that although alphaviruses and bunyaviruses differ strikingly in genetic constitution and mode of replication, a number of the events which take place during the establishment of persistence by these two groups of viruses show close similarities. An example is the severe restriction on levels of viral protein synthesis in alphavirus- and bunyavirus-infected *A. albopictus* cells by comparison with events in the corresponding infected vertebrate cells (5, 8). Such observations suggest that some common process may underlie the establishment of persistence in mosquito cells infected by arboviruses with diverse replication strategies.

Recently, it has been reported that *A. albopictus* cells persistently infected with Sindbis virus (SIN) release into the medium a low-molecular-weight agent capable of specifically reducing the yield of SIN during the initial, acute phase of infection in mosquito cells (9). The agent is rapidly inactivated on treatment with proteinase K or with heat and is not inactivated by antibody prepared against extracts of SIN-infected BHK cells. The antiviral activity is both virus and cell specific, differentiating it from interferon (9).

In view of the potential significance of this agent, we provide data here on the mode of action of a corresponding antiviral material released from Semliki Forest virus (SFV)-infected *A. albopictus* cells and on attempts to find a similar activity in medium from flavivirus- and bunyavirus-infected *A. albopictus* cells.

Medium containing antiviral activity ("antiviral medium") was prepared by dialysis (at 28°C for 3 days) of persistently infected *A. albopictus* cells against fresh growth medium, as described previously (9). Medium prepared against mock-infected cells was used as a control. We first compared the antiviral activities in medium from *A. albopictus* cells persistently infected with SIN and with SFV. *A. albopictus* cells were preincubated for 48 h in antiviral medium and were infected with homologous virus (multiplicity of infection [MOI] ≃ 20 [9]). Extracellular virus (EV) titers were assayed by plaque formation on Vero cell monolayers. For SFV, EV titers at 24 h postinfection were lowered by 39, 97, and 99%, respectively, after preincubation of cells in medium from 16-, 67-, and 130-day persistently infected cultures; virtually the same result was obtained for SIN (data not shown).

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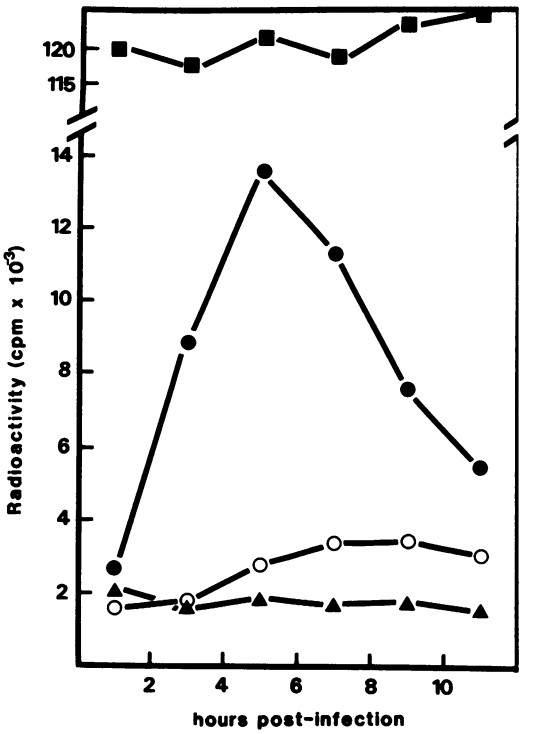


FIG. 1. Effect of antiviral medium on SFV-specific RNA synthesis in *A. albopictus* cells. *A. albopictus* cells were pretreated for 48 h with anti-SFV medium or with medium from mock-infected cells and were infected with SFV (MOI ≃ 50). At zero time, medium was replaced with fresh growth medium. Cells were labeled for 2-h periods with [$^3$H]uridine (5 μCi/ml, 28°C) with or without AMD (3 μg/ml), and incorporation into acid-precipitable material was estimated (5). Mock-infected cells were not preincubated. Symbols: ●, infected cells pretreated with medium from mock-infected cells (plus AMD); ○, infected cells pretreated with anti-SFV medium (plus AMD); ▲, mock-infected cells (plus AMD); ■, mock-infected cells (without AMD).

Other properties of the anti-SFV activity were similar to those previously described for the anti-SIN activity (9). (i) Anti-SFV activity was retained at dilutions of up to $10^{-5}$. (ii) Anti-SFV medium did not interfere with the replication of SIN, Kunjin virus (a flavivirus), or Bunyamwera virus. (iii) Antiviral medium was inactivated on incubation at 56°C for 30 min or on incubation with proteinase K (50 μg/ml) for 30 min at 20°C (data not shown).

To determine whether the anti-SFV material acted late in virus replication by preventing the release of infectious virions from the cell, *A. albopictus* cells were treated with anti-SFV medium or control medium and were infected with SFV (MOI ≃ 20). Cell-associated virus and EV titers were assayed in samples taken at various times after infection. Cells treated with antiviral medium showed a reduction in cell-associated virus titers of approximately 2 log units, which corresponded to the reduction observed in EV titers (data not shown). Thus, the anti-SFV activity was expressed at some stage before the release of infectious virus from the cell.

To determine the effect of the agent on virus-specific RNA synthesis, *A. albopictus* cells treated with anti-SFV medium or with control medium were labeled with [$^3$H]uridine in the presence of actinomycin D (AMD) at various times after SFV infection. Preincubation with antiviral medium markedly depressed AMD-resistant RNA synthesis in infected cells (Fig. 1).

The effect of antiviral material on SFV- and host-specific polypeptide synthesis was examined. *A. albopictus* cells pretreated with anti-

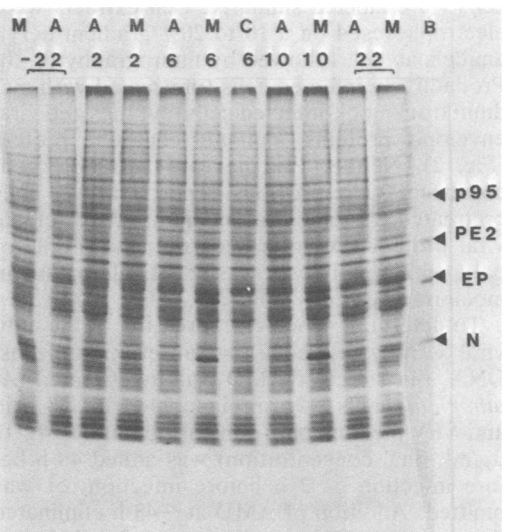


FIG. 2. Effect of antiviral medium on SFV- and host-specific polypeptide synthesis in *A. albopictus* cells. *A. albopictus* cells were pretreated for 48 h with anti-SFV medium or with medium from mock-infected cells. Cells were infected with SFV (MOI ≃ 50) and were labeled for 4 h with $^3$H-amino acids (10 μCi/ml) at various times after infection (5). Electrophoresis was on a 10 to 20% gradient acrylamide slab gel (4, 5). Migration is from top to bottom. Numerals refer to the midpoint of the labeling period (hours postinfection). Abbreviations: M, infected cells pretreated with medium from mock-infected cells; A, infected cells pretreated with anti-SFV medium; C, mock-infected cells with no pretreatment; B, SFV-infected BHK cell extract labeled from 6 to 10 h postinfection. Arrows indicate positions of p95, PE2, envelope proteins, and nucleocapsid protein in BHK cell extract. The mobility of the envelope proteins of SFV grown in mosquito cells is greater than for the envelope proteins of BHK-grown SFV, probably due to the lack of sialylation of the attached carbohydrate chains (see reference 8).

TABLE 1. Effect of actinomycin D on the expression of anti-SFV activity[a]

| Preincubation medium | Time of addition of test medium (h before infection) | Time of addition of AMD[b] (h before infection) | EV titer at 24 h post-infection (PFU/ml) | Inhibition by antiviral medium (%) |
|---|---|---|---|---|
| Anti-SFV | 48 | 48 | $5.5 \times 10^8$ | 8 |
| Mock | 48 | 48 | $6.0 \times 10^8$ | |
| Anti-SFV | 48 | 2 | $5.5 \times 10^7$ | 73 |
| Mock | 48 | 2 | $2.0 \times 10^8$ | |
| Anti-SFV | 48 | NA | $4.6 \times 10^6$ | 99 |
| Mock | 48 | NA | $5.9 \times 10^8$ | |

[a] *A. albopictus* cells were preincubated for 48 h in undiluted anti-SFV medium or in medium from mock-infected cells ("mock") before infection with SFV (MOI ≈ 50). After infection (zero time), normal growth medium was added to cell cultures.

[b] Final concentration, 1 μg/ml. NA, Not added.

SFV medium or control medium were infected with SFV and were labeled with $^3$H-amino acids at various times after infection. Mock-infected cells were labeled similarly. Cell extracts were electrophoresed on a 10 to 20% gradient acrylamide slab gel, followed by fluorography (4, 5). Productively infected cells (pretreated with medium from mock-infected cells) synthesized viral envelope proteins and nucleocapsid protein (Fig. 2). Neither PE2 nor p95 was observed, consistent with previous reports (8). In cells pretreated with antiviral medium, no detectable viral protein synthesis occurred; the profile of polypeptide synthesis was identical to that in mock-infected cells (Fig. 2).

To determine whether expression of the antiviral activity depended on transcription of host DNA, the effect of AMD was investigated. *A. albopictus* cells were preincubated for 48 h in anti-SFV medium or in control medium. AMD (1 μg/ml, final concentration) was added 48 h before infection or 2 h before infection, or was omitted. Addition of AMD at −48 h eliminated the antiviral effect; addition at −2 h led to a slight reduction in the antiviral effect (Table 1). The data suggest a requirement for host RNA transcription for the expression of the antiviral effect, although there was no evidence for enhanced synthesis of specific host proteins after pretreatment with antiviral medium (Fig. 2).

Attempts were made to demonstrate antiviral activity in medium from *A. albopictus* cells at various times up to 4 months after infection with Kunjin virus or Bunyamwera virus. Infected *A. albopictus* cells released no detectable activity into cell culture fluids under conditions in which positive results were obtained for SFV (data not shown).

Our studies show that pretreatment of *A. albopictus* cells with antiviral medium inhibits viral RNA and protein synthesis in cells infected with homologous virus. The action of the antiviral agent is prevented by pretreatment of cells with actinomycin (1 μg/ml), arguing that host RNA transcription is required for the expression of the effect and that induction of a host protein may be required. Thus, the mode of action of the agent has analogies with that of interferon, although the specificity of the antiviral protein distinguishes the two antiviral activities.

Our data do not support a role for the agent in either the establishment or maintenance of persistence in alphavirus-infected *A. albopictus* cells. The time course of appearance of activity in SFV-infected *A. albopictus* cultures is not consistent with evidence that the outcome of alphavirus infection of *A. albopictus* cells is determined early in the virus replication cycle (1, 12). Further, Eaton (2) has pointed to a probable link between the events which determine superinfection exclusion of related alphaviruses and those which lead to persistence. However, the specificity of the antiviral agent described here contrasts with the broad pattern of interference relationships described previously (2).

Finally, the absence of antiviral activity in culture fluids from flavivirus- and bunyavirus-infected *A. albopictus* cell cultures suggests that the activity is specific to alphaviruses and has no significance in relation to infections of arthropod cells by other types of arbovirus.

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## LITERATURE CITED

1. **Davey, M. W., and L. Dalgarno.** 1974. Semliki Forest virus replication in cultured *Aedes albopictus* cells: studies on the establishment of persistence. J. Gen. Virol. 24:453–463.
2. **Eaton, B. T.** 1979. Heterologous interference in *Aedes albopictus* cells infected with alphaviruses. J. Virol. 30:45–55.
3. **Igarashi, A., R. Koo, and V. Stollar.** 1977. Evolution and properties of *Aedes albopictus* cell cultures persistently infected with Sindbis virus. Virology 82:69–83.
4. **Laemmli, U. K.** 1970. Cleavage of structural proteins

during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

5. **Newton, S. E., N. J. Short, and L. Dalgarno.** 1981. Bunyamwera virus replication in cultured *Aedes albopictus* (mosquito) cells: establishment of a persistent viral infection. J. Virol. **38**:1015–1024.
6. **Newton, S. E., N. J. Short, A.-M. Irving, and L. Dalgarno.** 1983. The Mapputta group of arboviruses: ultrastructural and molecular studies which place the group in the *bunyavirus* genus of the family Bunyaviridae. Aust. J. Exp. Biol. Med. Sci. **61**:201–217.
7. **Ng, M. L., and E. G. Westaway.** 1980. Establishment of persistent infections by flaviviruses in *Aedes albopictus* cells, p. 389–402. *In* E. Kurstak, K. Maramorosch, and A. Dubendorfer (ed.), Invertebrate systems *in vitro*. Elsevier/North-Holland Biomedical Press, Amsterdam.
8. **Richardson, M. A., R. W. Boulton, R. S. Raghow, and L. Dalgarno.** 1980. Polypeptide synthesis in alphavirus-infected *Aedes albopictus* cells during the establishment of persistent infection. Arch. Virol. **63**:263–274.
9. **Riedel, B., and D. T. Brown.** 1979. Novel antiviral activity found in the media of Sindbis virus-persistently infected mosquito (*Aedes albopictus*) cell cultures. J. Virol. **29**:51–60.
10. **Stollar, V.** 1980. Togaviruses in cultured arthropod cells, p. 584–620. *In* R. W. Schlesinger (ed.), The togaviruses: biology, structure, replication. Academic Press, Inc., New York.
11. **Stollar, V., and T. E. Shenk.** 1973. Homologous viral interference in *Aedes albopictus* cultures chronically infected with Sindbis virus. J. Virol. **11**:592–595.
12. **Tooker, P., and S. I. T. Kennedy.** 1981. Semliki Forest virus multiplication in clones of *Aedes albopictus* cells. J. Virol. **37**:589–600.