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Markedly reduced severity of Dengue virus infection in mosquito cell cultures persistently infected with *Aedes albopictus* densovirus (*Aal*DNV)

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Abstract

*Aal*DNV-infected C6/36 cells serially passaged for over 10 weeks showed a decline in percentage of anti-AalDNV-positive cells (APC) from an initial 92% to approximately 20%. Cultures of persistent APC were indistinguishable from uninfected cultures by direct microscopy but most stained cells from early APC passages had enlarged nuclei with eosinophilic inclusions, while late APC passages had few and naive cells none. Super challenge of persistent APC cultures did not increase percentage APC and supernatants from persistent APC cultures gave low APC (40%) in naive C6/36 cell cultures. When challenged with dengue virus serotype 2 (DEN-2), naive C6/36 cells showed severe cytopathic effects (CPE) and high mortality within 4 days, as did early passage APC cultures. Remarkably, DEN-2 infections in persistent APC cultures were much less severe, being characterized by reduced DEN-2 infection percentage, retarded DEN-2 virion production, no CPE and no significant mortality. Reasons for rapid reduction in APC and resistance to superinfection upon serial passage remain unproven but may relate to production of *Aal*DNV-defective interfering particles (DIP) by molecular mechanisms still open to speculation. More difficult to explain is cross-protection against DEN-2-induced mortality seen in persistent APC cultures. However, by comparison to work on shrimp viruses, we speculate that this may involve blockage of viral-triggered apoptosis. The phenomena described raise questions regarding the potential for persistent infections by unknown viruses to confound experimental results with insect cell lines.
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Keywords: Densovirus; Dengue virus; C6/36 Cells; Persistent infection; Tolerance

Introduction

Densoviruses form a homogenous group of small isometric DNA containing viruses that may be highly pathogenic for their insect hosts. They belong to the family *Parvoviridae* and comprise very small, un-enveloped viruses that encapsidate complementary single-stranded DNA in the range of 4–6 kb length. Vertebrate parvoviruses are grouped in the subfamily *Parvovirinae*, while those of insects and other arthropods are grouped in the subfamily *Densovirinae* and commonly called densonucleosis viruses (DNVs) or simply densoviruses. There are three densovirus genera *Densovirus*, *Iteravirus*, and *Brevidensovirus* (Bern, 1996; Kerr, 2000; Tijssen and Bergoin, 1995). Several densoviruses have been described from mosquitoes. For example, the first was *Aedes aegypti*

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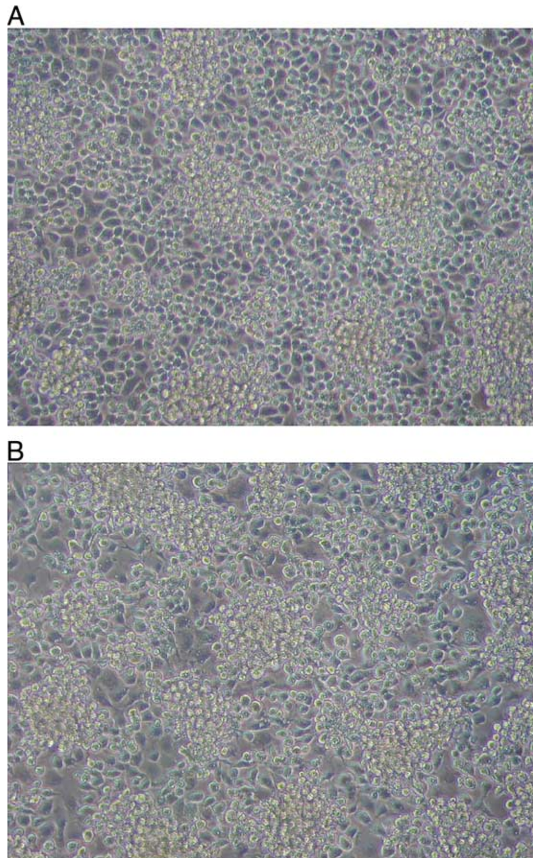

Fig. 1. C6/36 cell cultures mock infected (A) or challenged with *Aal*DNV (B) at 5 days post-challenge, ×200.

densovirus (*Aae*DNV) isolated from fourth instar larvae of a laboratory culture of *Ae*. *aegypti* (Lebedeva et al., 1973). In addition, *Aedes pseudoscutellaris* densovirus (*Ap*DNV) was observed by electron microscopy in an *Ae. pseudoscutellaris* cell line (AP-61) (Gorziglia et al., 1980) and *Aedes albopictus* densovirus (*Aal*DNV) was recovered from a C6/36 subclone of *Ae. albopictus* (Jousset et al., 1993). Another densovirus, *Ath*DNV has been isolated from both *Ae*. *aegypti* and *Ae*. *albopictus* in Thailand (Kittayapong et al., 1999).

The isolation of new viruses from insect colonies and insect cell lines that show no gross signs of infection suggests that cryptic, persistent infections may be fairly common. *Aal*DNV from a C6/36 subclone of *Ae. albopictus* persisted for over 4 years in laboratory culture without showing any cytopathic effects (CPE) (Jousset et al., 1993). The C6/36 subclone is useful for virological study because it is susceptible to several viruses including the arboviruses and especially dengue viruses (Danielova, 1973; White, 1987). It gives good growth and is relatively easy and economical to handle and maintain in the laboratory. However, the propensity C6/36 cells to carry persistent viral infections without CPE raises the possibility that insect cell lines may be infected with unknown cryptic viruses and that these may confound results obtained when they are used for experimental work with another virus such as dengue. To examine the implications of such a persistent infection on dengue experiments, we developed a model using C6/36 cells persistently infected with *Aal*DNV and examined the effect of the persistent infection on a dengue virus serotype 2 super-infection. This required first examining the kinetics of *Aal*DNV infection, including the persistence of the virus through serial cell passages, the infectivity of the virus from passaged culture supernatants and the superinfection of *Aal*DNV persistently infected cells with *Aal*DNV.

Results

Serial passage AalDNV-infected cell cultures

Infection of C6/36 cells with *Aal*DNV did not result in destruction of cells, even though altered morphology was observed (Fig. 1). It was thus possible to subculture the cells and to determine the percentage of putative infected cells during passages by IF staining using flow cytometry. A slight increase in putative infection was observed at the second passage (Fig. 2). Thereafter, the percentage of IF+ cells (i.e., anti-*Aal*DNV-positive cells or APC) progressively decreased from 92% at the second passage to only 29% at the eighth passage. APC seemed to constantly persist at approximately 20% after the ninth passage although cultures showed no destruction cells when compared to serially passaged control cultures. The APC cultures looked healthy and were indistinguishable from uninfected cultures by phase contrast microscopy (Fig. 3).

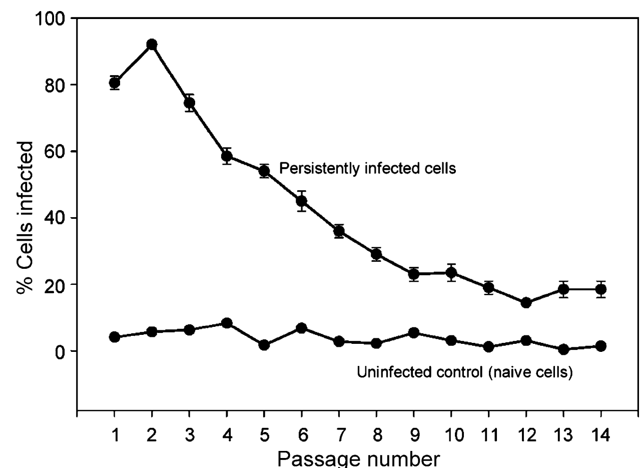

Fig. 2. Percentage of *Aal*DNV IF+ cells in serial passages of C6/36 cells subcultured at 5 day intervals and compared to serial passages of mock-infected cells. Each data point for the *Aal*DNV-infected cells represents the mean ± SD of two replicates.

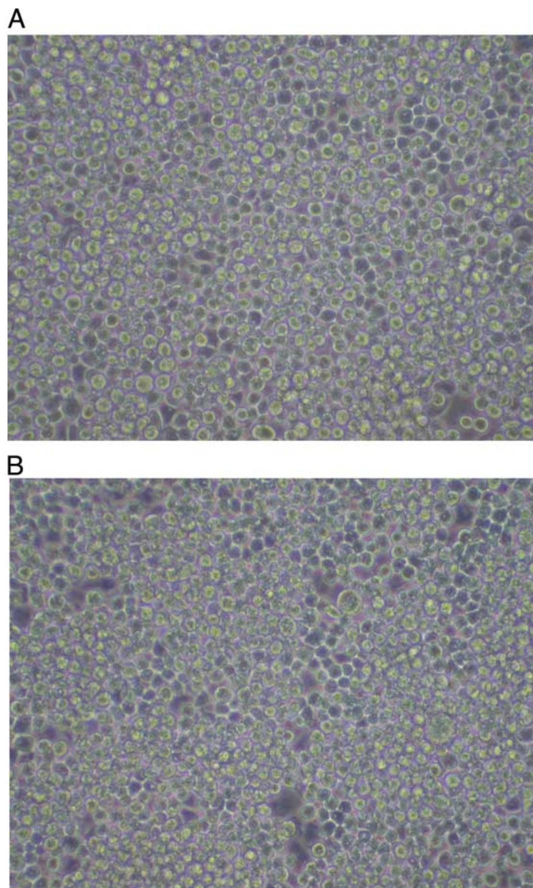

Fig. 3. A mock-infected culture (A) compared with an *Aal*DNV-infected culture (B) at the 14th passage, 200×.

Cytology of AalDNV-infected cells

Light microscopy of H&E stained cell sections from acutely and persistent APC cultures (Fig. 4) revealed cytopathology in the form of enlarged nuclei containing central, eosinophilic to basophilic inclusions. These were not present in naive C6/36 cells but were present in most of the cells in the early passage APC cultures and in few cells in the persistent APC cultures. These results were consistent with those from IF staining using flow cytometry that showed a large percentage of APC in early passage cultures and a low percentage in late passage cultures.

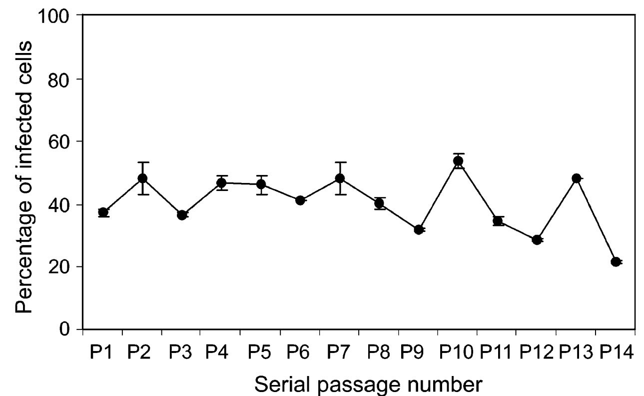

Fig. 5. Percentage of *Aal*DNV-infected cells in naïve C6/36 cell cultures challenged with supernatant from serially passaged cultures persistently infected with *Aal*DNV. Each data point represents the mean ± SD of two replicates measuring the percentage of IF+ cells determined by immunofluorescence staining using flow cytometry.

Infection using serially passaged culture supernatant

When undiluted supernatant from serially passaged infected cultures was used to infect fresh C6/36 cells, APC were detected at 5 days post-challenge. The percentage of APC arising from supernatant solutions decreased somewhat as APC passage number increased but was not very much lower than that for early passages (Fig. 5). The mean percentage of infection was 39.8 (±8 SD). There was no significant difference in cell viability between persistent APC cultures and uninfected cell cultures (data not shown).

Superinfection of APC cell cultures

APC persisted at a low prevalence in serially passaged cells for over 2 months (i.e., 14 passages). Naïve, uninfected cells were maintained in parallel. Challenge of both with *Aal*DNV at the 12th and 14th passages (Fig. 6)

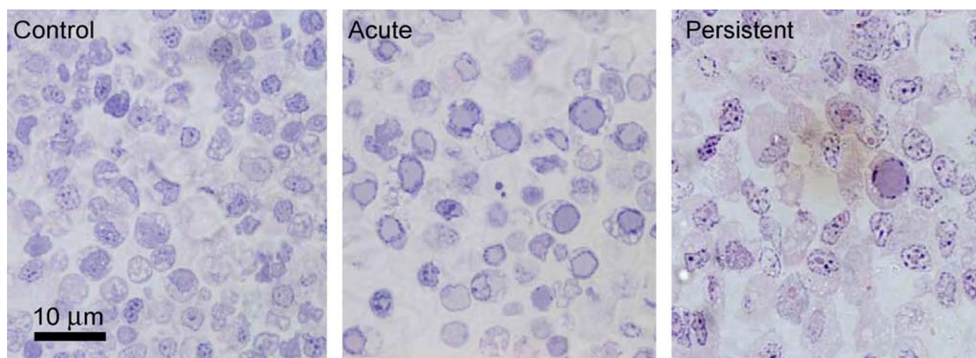

Fig. 4. Histology (H&E staining) of semi-thin sections of C6/36 cells from naïve and *Aal*DNV-infected cultures. The majority of the cells from acutely infected culture (Acute) show enlarged nuclei containing eosinophilic to lightly basophilic central inclusions while only one cell in the micrograph of cells from a persistently infected culture shows an enlarged nucleus with a densely basophilic inclusion.

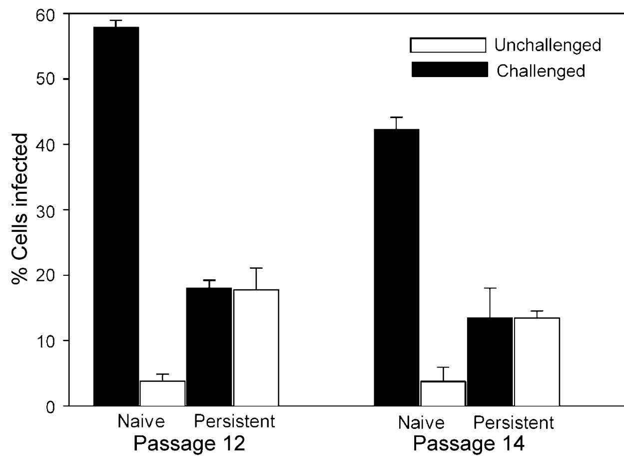

Fig. 6. Results of *Aal*DNV challenges of persistently *Aal*DNV-infected cells (i.e., superinfection tests) at passages 12 and 14 compared to parallel passages of naïve cells. Each data point represents the mean ± SD of two replicates measuring the percentage of IF+ cells determined by immunofluorescence staining using flow cytometry.

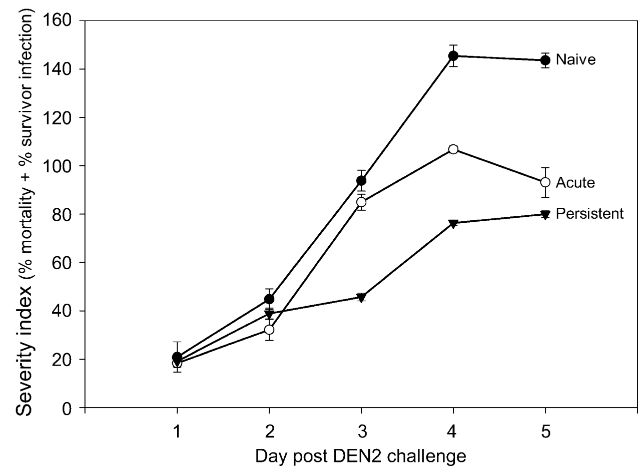

Fig. 7. Severity (% mortality plus % infection in survivors) of DEN-2 infections in C6/36 naïve and *Aal*DNV-infected cell cultures challenged with DEN-2. Each data point for the naïve cultures represents the mean ± SD of four replicates, whereas data points for the *Aal*DNV-infected cultures represent the mean ± SD of two replicates.

revealed no significant increase in APC (i.e., stable at about 20%) for the persistently infected cultures, but a significant difference in percentage APC when compared to parallel-passaged naive cells (58% and 42% infection and P = 0.001 and 0.002, at 12th and 14th passages, respectively). The serially passaged naive cells gave a lower level of infection than did naive cells freshly prepared from storage at −80 °C (see Fig. 2).

Interaction of AalDNV and Dengue virus

Early-passage (about 85% APC) and late-passage (about 25% APC) *Aal*DNV-infected cells were challenged with Dengue serotype 2 (DEN-2) at a multiplicity of infection of 0.08. The cultures were examined for mortality and for percent DEN-2 infection for 5 days post-challenge and severity of infection was assessed using an index calculated by addition of the percent mortality and the percent infection of surviving cells for each culture replicate (i.e., maximum severity 200) (Fig. 7). When compared to naive cells, the severity index was significantly lower ($P < 0.01$) for the late passage APC cultures from day 3 onwards and for the early passage APC cultures from day 4 onwards. The reason for the decline in severity index was the fact that the percentage of APC in surviving cells decreased significantly from day 4 to day 5 in the naive and acutely *Aal*DNV-infected cultures, even as mortality was rising for both over the same interval (Fig. 8).

Cell mortality increased steadily post-DEN-2 challenge for both naive C6/36 cultures and early passage APC cultures. For late passage APC cultures, however, cell mortality was low post-DEN-2 challenge and not significantly different from that for unchallenged cultures of naive cells or late passage APC (Fig. 8).

Phase contrast microscopy revealed severe CPE upon DEN-2 challenge of naive cell cultures and early passage APC cultures but not with late passage APC cultures (Fig. 9). The CPE began at day 3 post-challenge and increased in severity with time. By day 5 post-challenge, when the experiment was terminated, the only indication of infection in late passage APC cultures was a few swollen cells (Fig. 9C).

The dengue titers in supernatants of all challenged cell cultures increased steadily (Fig. 10), although the rate of increase was somewhat retarded in late passage APC cultures when compared to late passage naive cell cultures. The viral titer obtained from persistently *Aal*DNV-infected cells was 125 times lower than that of the naive control at day 4. However, by day 5 post-challenge, there were no significant differences in the DEN-2 titers. In other words,

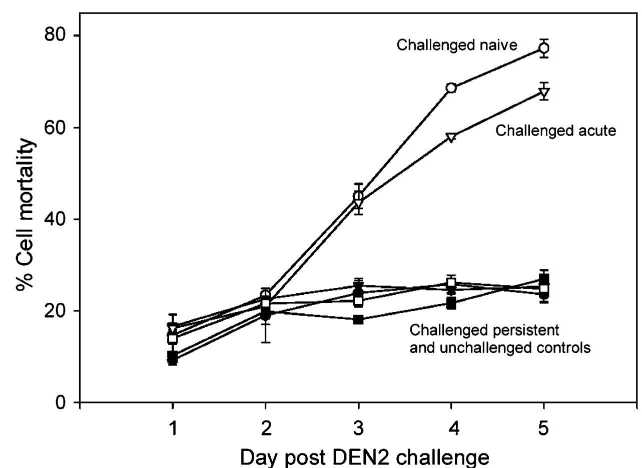

Fig. 8. Percentage mortality at different days for naïve, unchallenged C6/36 cells and naïve and *Aal*DNV-infected C6/36 cells challenged with DEN-2 at 0.08 multiplicity of infection. Each data point for the naïve and naïve/challenged cultures represents the mean ± SD of four replicates, whereas each data point for the other cultures represents the mean ± SD of two replicates.

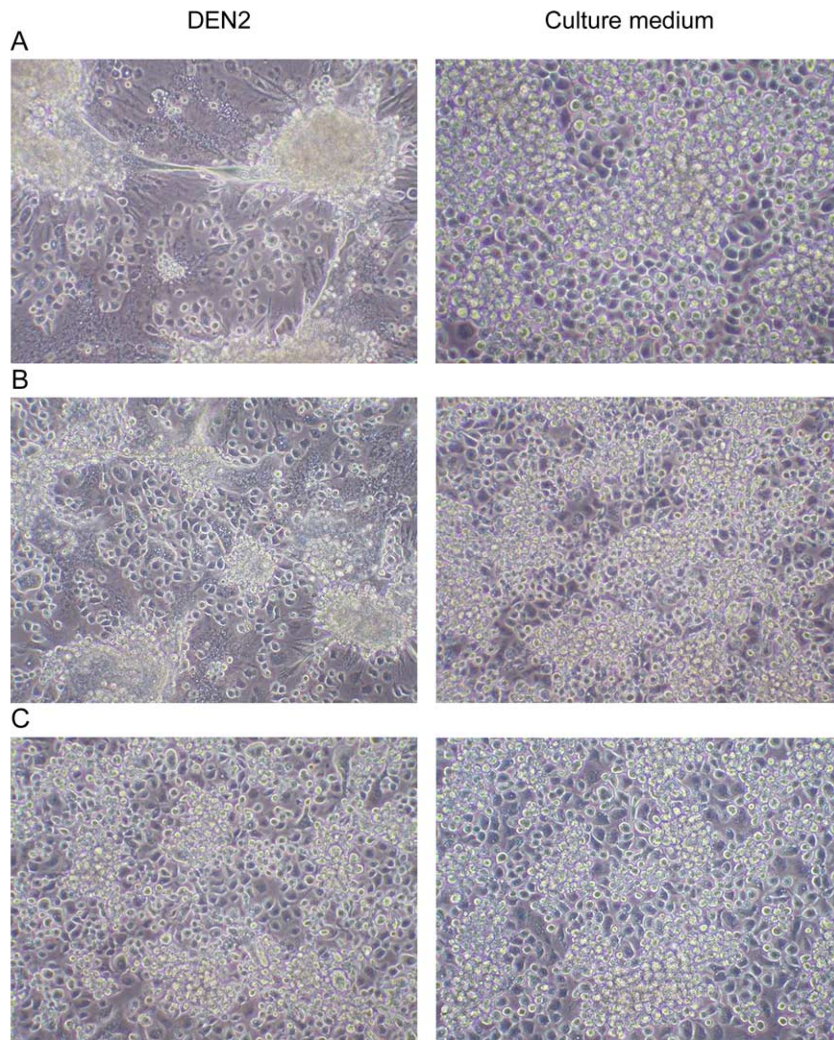

Fig. 9. Comparison of DEN-2 infection in acutely and persistently *Aal*DNV-infected cells. Cells were seeded in 24-well plates and monolayers were inoculated with DEN-2 at 0.08 multiplicity of infection (left panel) and culture medium inoculation (right panel). CPE induced by the infection was analyzed by phase contrast microscopy at day 5 post-inoculation. (A) C6/36, (B) acutely and (C) persistently *Aal*DNV-infected cells, ×200.

production of infectious DEN-2 virions was equal by day 5, whether CPE occurred or not.

Discussion

Our results clearly show that the severity of *Aal*DNV infection was greatly reduced in late passage C6/36 cells cultures when compared to early passage cultures. Specifically, all visible differences with uninfected cultures by phase contrast microscopy disappeared, the proportion of enlarged nuclei containing inclusions and the proportion of APC were greatly reduced. Since an antibody to *Aal*DNV capsid protein was used to test *Aal*DNV-infected cultures, we cannot state that negative cells were uninfected. This does not exclude the possibility that they may contain viral genomic material. On the other hand, there is a wealth of literature on defective interfering viral particles (DIP) of densoviruses and other viruses in interfering with virus infection, freeing the remaining cells uninfected. Regardless of the percentage of cells actually infected in the late passage cultures, the important issue is that the severity of infection was less than in early passage cultures.

The progressive decrease in APC that occurred from the 3rd (75%) to the 11th (20%) passage suggests that cells negative for anti-AalDNV successfully out-competed APC in successive passages until a stable equilibrium was reached. The equilibrium was expected since *Aal*DNV was originally reported as a 4-year persistent contaminant in C6/36 cell lines (Jousset et al., 1993). The supernatants of our serially passaged cultures were capable of infecting naive C6/36 cells at a relatively constant rate that was independent of the percentage of APC at any particular passage. This suggested that either the quantity of functional virions produced was relatively constant or that defective interfering particles (DIP) were also present in varying quantity, like the APC. DIP have long been suggested to interfere with viral production or to compete

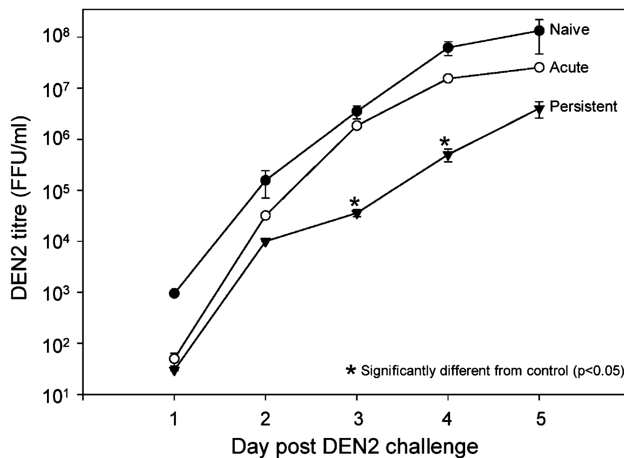

Fig. 10. DEN-2 titer from the supernatant of naïve and *Aal*DNV-infected cell cultures at various days post challenge with DEN-2. Each data point for the DEN-2 challenged naïve cultures represents the mean ± SD of four replicates, whereas those for the DEN-2 challenged *Aal*DNV-infected cultures represent the mean ± S.D. of 2 replicates. Asterisks indicate points significantly different ($P < 0.05$) from the naïve culture challenged with DEN-2. The antibody did not bind with naïve C6/36 cells or with *Aal*DNV persistent or acutely infected cells.

with infective particles for cell surface receptors (Rhode, 1978). It has been shown by electron microscopy that empty particles are mixed with purified *Aal*DNV virions from infected C6/36 cells (Jousset et al., 1993). Similar phenomenon was also seen in porcine parvovirus (PPV) (Choi et al., 1987), parvovirus H–I (Rhode, 1978), and in parvovirus minute virus of mice (MVM) (Clement et al., 2001). Varying quantities of DIP could explain the fluctuations in Fig. 5 and the fact that maximum percentage infection obtained using culture supernatants from persistently infected cells (approximately 40%) was considerably less than that (over 90%) obtained with purified viral preparations. However, other explanations are possible and more work would be needed to prove the DIP contention.

The resistance of late passage APC cultures to *Aal*DNV re-infection corresponds to a process called superinfection exclusion with a homologous virus. This has been reported in several viruses and is defined as the phenomenon where cells infected with one virus cannot be productively infected with the same or a closely related virus at some later time. Cultured C6/36 cells persistently infected with Dengue virus type 1 were found to be resistant to superinfection with Dengue type 3 after 20 h of primary infection (Dittmar et al., 1982). *Ae. albopictus* cell lines persistently infected with Sindbis virus excluded the replication of both homologous (variant strains) and heterologous alphaviruses but exclusion was restricted to alphaviruses (Karpf et al., 1997). Bunyamwera virus persistently infected C6/36 cells were also resistant to superinfection with Bunyamwera virus and other bunyaviruses (Elliott and Wilkie, 1986). Most simply, superinfection exclusion may result from the production of DIP that compete for host cell receptors, but the phenomenon may also involve intracellular host factors such as interferon, interferon-like substances or other antiviral substances.

Putting aside possible mechanisms, we return to the facts that persistent infections tend to be less severe than early infections and that they reduce the severity of superinfections by homologous viruses. If the surprising ability of persistently *Aal*DNV-infected cells to better withstand DEN-2 infection proves to be a general phenomenon, it may indicate a second advantage arising from persistent viral infections in arthropods. It might be called superinfection suppression by a heterologous virus, since the persistently *Aal*DNV-infected cell cultures became infected with DEN-2 at a significantly lower percentage than did naive cells. However, it might be more precise to call it superinfection disease suppression since the most striking outcome was not the reduced percentage of viral infection but the reduced severity of disease. In substance, our results do not differ from an earlier report in which persistent St. Louis encephalitis virus infections provided resistance to superinfection with a homologous virus but not with heterologous Japanese encephalitis virus and yellow fever virus (Randolph and Hardy, 1988). In both cases, the heterologous viral infection was successful.

Again, the critical issue is not reduced infection rate but reduced disease, and an important question that follows is whether similar reduction in disease might occur in whole organisms. A recent publication on shrimp viruses indicates that it may. It has been shown (Tang et al., 2003) that a prior infection with a densovirus (infectious hypodermal and hematopoietic necrosis virus or IHHNV) gave protection against lethal challenge with heterologous white spot syndrome virus (WSSV), a dsDNA virus in the new family *Nimaviridae* (Mayo, 2002). It must be stressed that the prior IHHNV infection did not prevent WSSV infection, but simply prevented mortality resulting from that infection. It is difficult to explain this phenomenon by invoking defective interfering particles from the heterologous virus.

The mechanism underlying cross-protection from disease by a heterologous virus would be of much interest. Here, it prevented CPE and mortality but did not prevent DEN-2 infection or the production of infective DEN-2 virions. In addition, the fact that percent DEN-2 infection for the surviving cells declined from day 4 to day 5 in both the naive cells and acutely *Aal*DNV-infected cells suggests that adaptation to DEN-2 may have already begun in these cultures. It would be interesting to subculture the surviving cells for many passages to determine whether persistently DEN-2 cultures and persistently, dually infected *Aal*DNV/DEN-2 cultures could be obtained. Natural, persistent, dual infections do occur in mosquitoes that carry both *Aal*DNV and DEN-2 (and perhaps even other viruses in various combinations).

The phenomenon of natural, dual to multiple infections also occurs commonly in shrimp (Flegel, 2001; Flegel et al., 2002) and has been proposed to result from a process called "viral accommodation" (Flegel, 2001; Flegel and Pasharawipas, 1998). In addition, recent publications have shown that prior exposure of older, juvenile shrimp to inactivated viral particles or envelope proteins may protect them from lethal viral challenge for a short period of time (Namikoshi et al., 2004). By contrast, persistently infected shrimp appear to maintain protection as long as they remain infected (Venegas et al., 2000). Since treatment with inactivated viruses or coat proteins runs out relatively quickly, it should not be called vaccination and compared to long-term protective, antibody-based, immunological memory such as that gained by vaccination in vertebrates. There is no evidence that shrimp or other arthropods have a comparable defense system (Johansson and Soderhall, 1996; Sritunyalucksana and Söderhäll, 2000). Rather, the evidence in hand, including that presented here, suggests that homologous protection may result from interference by DIP and that "memory", when it exists, is provided by continuous presence of the pathogen (i.e., "the virus is the memory"). This being the case, one would expect protection to be limited by the range of interfering particles produced. In other words, it would be expected to have specificity, as has earlier been proposed (Flegel, 2001). However, cross-protection between *Aal*DNV and DEN-2 is both unexpected and intriguing.

It has been proposed (Flegel, 2001; Flegel and Pasharawipas, 1998) that mortality from viral disease outbreaks in shrimp is a result of viral-triggered apoptosis and that the process of viral accommodation prevents triggering by some unknown process. In support of this proposal, evidence for increasing numbers of apoptotic cells prior to shrimp death has been demonstrated for two lethal shrimp viruses (Khanobdee et al., 2002; Sahtout et al., 2001; Wongprasert et al., 2003). Further work is needed to determine whether cell death in the DEN-2 infected C6/36 cultures is the result of triggered apoptosis and whether presence of a persistent *Aal*DNV infection prevents this triggering.

In conclusion, we have shown that mosquito cell cultures persistently infected with a densovirus at a low level are protected from heavy infections by the same virus and also protected from severe disease, but not infection, by a heterologous virus. These phenomena may be indicative of general arthropod processes that employ persistent viral infections to decrease the severity of viral disease. If such processes exist, cryptic infections by unknown viruses in continuous insect cell lines may be common and might confound experimental results with known viruses. In addition, it implies that biological control of insects with viral pathogens would be unlikely to succeed in the long run, since target populations would rapidly adapt to the pathogen by developing protective, persistent infections (Flegel, 2001).

Materials and methods

Cells and viruses

Aedes albopictus cell line (C6/36) (cloned Singh's *Aedes albopictus* cell line) was maintained in Leibovitz's medium (L-15), containing 10% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 1.2% antibiotic (Penicillin G and Streptomycin). Cells were prescreened for freedom from *Aal*DNV by PCR assay before use.

PS clone D (porcine kidney fibroblast cell line) was used for dengue titration and was maintained in L-15 medium, containing 10%FBS, 10%TPB and 1.2% antibiotic.

Aedes albopictus densovirus (*Aal*DNV) used throughout this study was isolated by cultivation of inoculum from *Aedes aegypti* mosquitoes in *Aedes albopictus* cell lines (C6/36) at the section of Biology, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT, USA. This virus was kindly provided by Dr. Pattamaporn Kittayapong, Faculty of Science, Mahidol University. Sequencing of a nearly full-length DNA clone revealed 98% sequence identity to a previously described *Aal*DNV (Boublik et al., 1994) and only about 77% identity to previously described *Aae*DNV (Boublik et al., 1994). It was deemed appropriate to label the virus as *Aal*DNV. *Aal*DNV was maintained in the laboratory of the Division of Medical Molecular Biology, Faculty of Medicine Siriraj Hospital, Mahidol University. Cultivation of densovirus was performed by inoculation of 2 ml virus stock in the C6/36 cells in T-162 culture flask at room temperature for 2 h with constant rocking. Then, 18 ml of L-15 medium containing 10% FBS were added, cultivation was continued for 6 days at room temperature. The virus supernatant was collected by centrifugation at 10,000 rpm at 4 °C for 10 min. This virus stock assigned as 21st passage was kept aliquots in L-15 containing 20% FBS at −70 °C.

Dengue virus serotype 2 (DEN-2) strain 16681 used throughout this study was maintained in the laboratory of the Division of Medical Molecular Biology, Faculty of Medicine Siriraj Hospital, Mahidol University. The DEN-2 stock was prepared by amplification in C6/36 monolayer cultures. Cells were grown to confluence in 75 cm^2 tissue culture flasks and 1 ml of the viral suspension was added to the monolayer. After incubation on a rocking shaker at room temperature for 2 h, growth medium was added, followed by further incubation at room temperature until 80% CPE was observed. The supernatant was collected in aliquots and stored at −70 °C.

*Aal*DNV infection of C6/36 cells was carried out in 25 cm^2 culture flasks (Costar, Corning). Cells were seeded at a normal subculture split ratio of 1:5 (approximately 2×10^6 cells/flask). After 36–48 h of culture, the supernatant was decanted and 1.0 ml of the viral suspension was inoculated onto flasks of confluent cells. The cells were incubated on a

rocking shaker at room temperature for 2 h, and then fresh medium was added to each flask to make up 5 ml of total volume. The cells were incubated further at room temperature for 5 days. Growth medium, processed in the same way was used in place of viral suspension for mock infections.

Serial passage of *Aal*DNV-infected cell cultures was carried out in 25-cm^2 culture flasks, according to the infection protocol. At 5 days post-infection, supernatant was removed, 5 ml of fresh growth medium was added and cells were detached by knocking the flask. The cell suspension was mixed thoroughly before transfer to a new flask, where growth medium was topped up to 5 ml. Cells were subcultured at 1:10 dilution. The same procedure was repeated at each passage, at 5-day intervals. Mock-infected cells were run in parallel to the *Aal*DNV-infected cells and served as the negative control.

Superinfection of *Aal*DNV persistently infected cells with *Aal*DNV was accomplished in 25-cm^2 culture flasks. After several passages, *Aal*DNV-infected cell monolayers were superinfected with 1 ml of a 10-fold diluted *Aal*DNV, according to the normal infection protocol. Five days post-infection, cells were detached and kept for determination of the percentage of infection by antibody assay.

Interaction of *Aal*DNV and dengue virus was examined in 25 cm^2 culture flasks following the infection protocol. At 5 days post-infection, the supernatant was removed and 5 ml of fresh medium was added to the flask. Cells were detached and mixed thoroughly before being transferred to a new flask. The growth medium was then topped up to 5 ml. After a monolayer had formed, both *Aal*DNV and mock-infected cells were separately seeded in 24-well plates at 5×10^5 cells/well for 24 h prior to infection. After removing medium, 200 μl of the dengue 2 supernatant was added and incubated at room temperature for 2 h, followed by fresh medium addition to make up 1 ml of total volume. Every day, cells were mixed thoroughly, counted for the percentage of viability and then transferred to a sterile tube and clarified by centrifugation. The supernatant was collected in aliquots and stored at −70 °C for determination of dengue viral titer. Cells were kept for determination of the percentage of infection.

Titration of dengue virus was carried out using 96-well microtiter plates (Costar, Corning). PS clone D was seeded with 2×10^4 cells/well 24 h prior to infection, and 100 μl of the 10-fold serially diluted viral suspension in 1% FBS L-15 medium was added, with normal medium as the negative control. Plates were incubated at room temperature for 2 h followed by addition of 1% gum tragacanth in 3% FBS 2X L-15 and further incubation in a humidified chamber for 3 days before immunostaining. After removing some overlayer medium from each well, cells were washed three times with PBS, fixed with 3.7% formaldehyde for 10 min at room temperature and then treated with 1% Triton X-100 in PBS for another 10 min. After five washes with PBS, cells were incubated with monoclonal antibody against the dengue virus envelope protein (4G2) for 30 min at 37 °C in a humidified chamber. Cells were washed three times with PBS before incubation with anti-mouse IgG rabbit immune serum coupled to HRP (P0260, DAKO, Denmark) under the same conditions. After triple washing, the substrate solution was added, the stained foci were visible within 5 min and the reaction was terminated by washing cells with PBS. Cells were examined under a light microscope. Foci were counted to calculate the viral concentration in the supernatant.

Immunostaining for flow cytometry

After infection, cells were washed once with fresh medium and fixed with 2% formaldehyde in PBS for 1 h at room temperature. Two washing steps were performed using 0.1% triton X-100 in PBS. Cells were incubated in a 1000-fold mouse anti-*Aal*DNV ascites fluid for *Aal*DNV (kindly provided by Dr. Francoise-Xaviere Jousset, Station de Recherches de Pathologie Comparee, Saint Christol-lez-Ales, France, (Jousset et al., 1993) and 4G2 for dengue infection at room temperature for 1 h. Then, cells were washed twice and incubated with anti-mouse IgG rabbit immune serum conjugated with FITC (F0261, DAKO, Denmark) at a final concentration of 4 μg/ml, for 30 min at room temperature in the dark. After incubation, cells were washed once, suspended and then analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA). The mock-infected C6/36 cells were run in parallel and served as negative controls. At least 5000 cells were gated by light scatter and collected in a list mode manner. Data analysis was performed using Cell Quest software (Becton Dickinson). The percentage of positive cells was determined on FITC fluorescence histograms using a region defined according to mock-infected cells.

Statistical comparisons

All results are expressed as means ± standard deviations. Statistical comparisons were carried out using ANOVA with SPSS computer software version 8.0 (SPSS Inc., Chicago, IL, USA) and differences were considered significant when $P \leq 0.05$.

Cytology

Samples of cultured cells were fixed with 2% formalin in 0.1 M PBS (phosphate buffered saline, NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.2 g and H_20 to 1L) and stored at 4 °C for 10 months before being embedded in LR-White (London Resin Co. Ltd.) according to the LR white manual. Semi-thin sections were cut and stained with hematoxylin and eosin (H&E).

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