Demonstration of Interference between Dengue Virus Types in Cultured Mosquito Cells using Monoclonal Antibody Probes

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(Accepted 11 November 1981)

SUMMARY

Cultured Aedes albopictus cells (clone C6/36), persistently infected (PI) with dengue virus type 1 (dengue-1) were found resistant to superinfection with dengue virus type 3 (dengue-3). This was determined by indirect immunofluorescent (IF) staining of cultures using monoclonal antibody against a dengue-3 type-specific antigen. Dengue-1 PI cultures stained with this antibody 3 days after superinfection with dengue-3 virus (m.o.i. of 2) had dengue-3 antigen in 0.1 to 1.0% of the cells. Control cultures infected with dengue-3 at the same multiplicity contained dengue-3 antigen in greater than 90% of the cells. The resistance to superinfection was not interferon-mediated, and occurred within 20 h after primary infection. In cultures simultaneously infected with two dengue virus types, one virus type was excluded from replication in most cells. A small population of cells was also found (about 1%) that contained type-specific antigen of both dengue virus types.

INTRODUCTION

The dengue viruses are group B togaviruses (flaviviruses) that form a complex of four closely related antigenic types (types 1 to 4). Each of the four virus types can be transmitted by infected mosquito vectors (Aedes aegypti or Aedes albopictus) to man, where they often cause a self-limiting, febrile disease. Locations endemic for dengue virus infection often harbour two or more dengue types (Nimmannitya et al., 1969; Bancroft et al., 1979) resulting in individual hosts (man or mosquito) being exposed to, and possibly becoming infected with, multiple dengue virus types. The biology of coinfection with two dengue virus types in either of these hosts is, however, poorly understood. In live mosquitoes, coinfection with two taxonomically distinct (heterologous) togaviruses can result in interference between the viruses (Sabin, 1952; Altman, 1963), or independent replication (Chamberlain & Sudia, 1957; Lam & Marshall, 1968). Coinfection of mosquitoes with two strains of the same togavirus (homologous viruses), as reported by Davey et al. (1979) for Sindbis virus, can result in interference if there is more than a 24 h interval between infection with each virus. In contrast, the two viruses replicate independently if the mosquito is infected with a mixture of both viruses. In cultured mosquito cells, interference has been reported between homologous Sindbis viruses (Stollar & Shenk, 1973; Riedel & Brown, 1977, 1979), and between heterologous togaviruses (Eaton, 1979). With dengue virus, cultured mosquito cells persistently infected for 50 weeks (22 weeks for dengue-3) release a temperature-sensitive virus strain which results in the cultures becoming resistant to superinfection with wild-type strains of the four dengue virus types (Igarashi, 1979).

In the present investigation, we have found that cultured mosquito cells infected with a single dengue virus type for 20 h are resistant to superinfection with other dengue virus types (heterotypic dengue viruses). This was determined by using monoclonal antibodies with
indirect immunofluorescent (IF) staining to detect dengue type-specific antigen in individual cells. This technique was also used to examine interference between two dengue virus types in cultures simultaneously infected with both types.

**METHODS**

**Virus.** Dengue virus types 1 (Hawaii), 2 (New Guinea C) and 4 (H241) were obtained from Robert Shope (Yale University, New Haven, Conn., U.S.A.). Dengue virus type 3 (PR-6) was obtained from Philip Russell (Walter Reed Army Medical Center, Washington, D.C., U.S.A.). Virus stocks were passed in suckling mouse brain (SMB) from Swiss white mice (Clarke & Casals, 1958) and stored as 20% SMB suspensions in borate-buffered saline (0.12 M-NaCl, 0.05 M-borate buffer pH 9) at -70 °C.

**Cell cultures.** Baby hamster kidney cells (BHK-21) were grown in BHK medium: Eagles minimal essential medium (MEM; Flow Laboratories) containing 10% tryptose phosphate broth, 10% foetal bovine serum (FBS), 50 units/ml penicillin, and 30 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified, 5% CO₂ atmosphere. Cultured A. albopictus cells (clone C6/36) and mouse lymphocyte hybridoma cells were cultured as described previously (Dittmar et al., 1980).

**Antibodies.** Antibody against dengue-3 antigen for use in the infectious virus titration was obtained from mouse hyperimmune ascites fluid as described by Cardiff et al. (1971). Dengue-3-infected SMB was used as the immunogen. Anti-dengue virus monoclonal antibodies were obtained from mouse lymphocyte hybridomas. The preparation and characterization of these antibodies were reported previously (Dittmar et al., 1980). One of these hybridomas (no. 31) secreted antibody which was shown to be specific for dengue-3 antigen by haemagglutination inhibition (HI) and indirect IF antibody assays. Conditioned medium from this hybridoma was used as a source of anti-dengue-3 monoclonal antibody (D-3 MCA). Another hybridoma (no. 20) secreted antibody that had equivalent HI and IF antibody titres against all four dengue virus types (D-C MCA). It was not determined if this antibody had activity against other togaviruses. Conditioned medium from these cells was also used as a source of antibody. Monoclonal antibody against dengue-1 type-specific antigen (D-1 MCA), which has not previously been reported, was obtained by the same methods used for D-3 MCA and D-C MCA, except that dengue-1-infected SMB was used as the immunogen.

**Indirect immunofluorescent (IF) staining.** C6/36 cells grown in tissue culture slide chambers (Lab-Tek, Napierville, Ill., U.S.A.) were rinsed with phosphate-buffered saline (PBS) pH 7.4 and then fixed for 15 min in -20 °C methanol. After fixation, the slides were rinsed once in PBS and flooded with undiluted, conditioned hybridoma medium for 30 min. All staining procedures were done at room temperature unless otherwise indicated. After this incubation, the slides were rinsed three times with PBS with a 5 min incubation between each rinse. The slides were then flooded with fluorescein-conjugated goat anti-mouse immunoglobulin (Cappell Laboratories, Cochranville, Pa., U.S.A.) for 30 min, then rinsed three times with PBS. The slides were mounted with Flo-Tex (Lerner Laboratories, Stamford, Conn., U.S.A.), and examined using a Leitz ortholux fluorescent microscope with an HBO 200 W mercury lamp and the following filters: BG-38 heat-absorbing, BG-12 exciter and K-510 suppression. The percentage of cells containing IF antigen reported for a given culture was the average percentage found for three groups of 100 examined cells. If this value was less than 1%, 700 more cells were examined and the percentage of IF cells was determined based upon 1000 examined cells.

**Infection or superinfection of C6/36 cells.** Control or dengue virus-infected C6/36 cells were grown in four-well tissue culture slides to a density of 1 x 10^5 cells/well. Cultures were then rinsed once with PBS and inoculated with 0.2 ml of diluted, dengue virus-infected SMB.
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(m.o.i. of 2). The inoculum was adsorbed for 120 min at 28 °C, then removed by rinsing three times with PBS. C6/36 medium containing 1% FBS (C6/36 maintenance medium) was then added to each well (0.4 ml) and the slides incubated for 3 days at 28 °C. The inoculum used was sufficient to infect about 90% of the cells in each culture. This was determined by indirect IF staining of infected cultures with D-C MCA at 2 days post-infection, which was the earliest time that antigen could be detected. Cultures in experiments were routinely stained at 3 days post-infection.

Metabolic inhibitors. Actinomycin D or puromycin (Sigma) in C6/36 maintenance medium was added to C6/36 cultures in four-well tissue culture slides 30 min before infection with dengue-1 virus (m.o.i. of 2). After infection, the inhibitors were again added to the cultures, incubated for 24 h, and then removed by rinsing three times with PBS. After rinsing, the cultures were superinfected with dengue-3 virus (m.o.i. of 2), incubated for 3 days, and then stained by indirect IF staining. Control C6/36 cultures, not exposed to dengue virus and in 30 ml tissue culture flasks, were included with each concentration of actinomycin D used. These cultures were exposed to [3H]uridine (5 μCi/ml) during the 24 h incubation period. The cultures were then rinsed three times with PBS, and the amount of incorporated [3H]uridine determined as described by Davey & Delgarno (1974).

Infectious virus titration. Infectious virus was quantified in BHK-21 cells by the fluorescent focus assay described by Igarashi & Mantani (1974). Briefly, 0.1 ml of virus sample was adsorbed to a monolayer of BHK-21 cells in a well of an eight-well tissue culture slide for 120 min at 37 °C. The wells were then rinsed three times with PBS and 0.3 ml of BHK medium containing 5% FBS added. After incubation for 72 h, the slides were stained by indirect IF staining with a 1/40 dilution of anti-dengue-3 hyperimmune mouse ascites fluid, and the number of focus-forming units (f.f.u.) counted.

Rate of virus adsorption. Control or dengue-1 PI C6/36 cells were grown in 24-well tissue culture plates until confluent (about 5 × 10^5 cells/well). The wells were then rinsed once with PBS, 0.2 ml of C6/36 maintenance medium containing 5 × 10^5 f.f.u. of dengue-3 added, and the plates incubated at 28 °C. Samples of 0.1 ml were removed from duplicate wells at the indicated times and assayed for the number of f.f.u. A control in which dengue-3 was not added to dengue-1 PI cultures was used to determine the amount of dengue-1 released during the experiment (120 min). This amount was less than 0.2% of the number of f.f.u. in the dengue-3 inoculum.

Dual staining. Identification of individual cells containing dengue-1 or dengue-3 type-specific antigen, or both, was done using D-1 MCA and D-3 MCA with a modification of a sequential, indirect immunoperoxidase (IP)—indirect IF technique described by Lechago et al. (1979). Methanol-fixed cultures were flooded with 1% H₂O₂ in 75% methanol for 45 min to remove endogenous peroxidase activity. The slides were then rinsed once in PBS, flooded with D-3 MCA for 30 min, then rinsed three times with PBS. Peroxidase-conjugated goat anti-mouse immunoglobulin (Cappell Laboratories) was added to the slides, incubated for 30 min, and then rinsed three times with PBS. Peroxidase substrate (5 mg/ml diaminobenzidine, 0.3% H₂O₂ in PBS) was added to the slides, incubated for 3 min, and then rinsed three times with PBS. The slides were then stained using the indirect IF technique with D-1 MCA. After staining, the slides were mounted with Flo-Tex and examined by bright field and fluorescence microscopy.

RESULTS

Infection of C6/36 cells with dengue-3

Anti-dengue virus monoclonal antibodies were used with indirect IF staining to identify individual cells containing dengue-3 type-specific or dengue type cross-reactive antigen. Table
Fig. 1. Induction of resistance to dengue-3 superinfection in C6/36 cultures after infection with dengue-1. Cultures were infected with dengue-1 at an m.o.i. of 2, then superinfected with dengue-3 (m.o.i. of 2) at the times indicated. Cultures were stained 3 days after dengue-3 superinfection using an indirect IF technique with D-3 MCA (■), or D-C MCA (□), and the percentage of antigen-containing cells determined.

Table 1. Comparison of the percentage of control or dengue-1 PI* C6/36 cells containing dengue virus antigen after infection with dengue-3

<table>
<thead>
<tr>
<th>C6/36 cells</th>
<th>Antibody used for immunofluorescent staining</th>
<th>% Antigen-containing cells with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No virus</td>
</tr>
<tr>
<td>Control</td>
<td>D-3 MCA</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>D-C MCA</td>
<td>0</td>
</tr>
<tr>
<td>Dengue-1 PI</td>
<td>D-3 MCA</td>
<td>0</td>
</tr>
<tr>
<td>Dengue-1 PI</td>
<td>D-C MCA</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

* Dengue-1 persistently infected C6/36 cells (four passages, 4 weeks). † Cultures were infected at an m.o.i. of 2 and stained 3 days later.

1 shows that greater than 90% of control C6/36 cells contained dengue-3 antigen 3 days after infection with dengue-3. The same percentage of cells also stained with D-C MCA. In contrast, dengue-1 PI cultures contained only a small percentage (0.1 to 1.0) of cells with dengue-3 antigen after infection with dengue-3 virus. When D-C MCA was used for staining, dengue-1 PI cultures contained greater than 90% fluorescent cells both before and after dengue-3 superinfection. From these results, it was concluded that the antigen identified with D-C MCA in the dengue-1 PI cultures was dengue-1-directed, indicating that dengue-1-infected cells were predominantly resistant to superinfection with dengue-3.

**Induction of resistance to dengue-1 superinfection**

The phenomenon of resistance of dengue-1 PI cultures to superinfection with dengue-3 was further examined by determining the interval required for C6/36 cells to develop resistance after infection with dengue-1. Fig. 1 shows that resistance to dengue-3 superinfection was first detected at 8 h post-infection with dengue-1, where the percentage of cells containing dengue-3 antigen was reduced from about 50 to 35. The proportion of dengue-3-infected cells steadily declined after 8 h to less than 1% at 20 h post-infection. Parallel cultures stained with D-C MCA consistently contained greater than 90% fluorescent cells, demonstrating that cells which did not contain dengue-3 antigen were infected with dengue-1 virus.
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Fig. 2. Effect of puromycin on the percentage of cells containing dengue-3 antigen in dengue-1-infected cultures superinfected with dengue-3. C6/36 cultures were exposed to puromycin for 30 min before, and 24 h after, infection with dengue-1 (m.o.i. of 2). Puromycin was then removed and the cultures superinfected with dengue-3 (m.o.i. of 2). After 3 days, the cultures were stained using an indirect IF technique with D-3 MCA and the percentage of cells containing dengue-3 antigen determined.

Fig. 3. Adsorption of dengue-3. Virus was added to control (●) or dengue-1 PI (○) cultures and incubated at 28 °C. Samples were removed at the times indicated and assayed for the remaining focus-forming units (f.f.u.).

Table 2. Effect of metabolic inhibitors on the percentage of cells developing dengue-3 antigen in C6/36 cultures infected with dengue-1, and then superinfected after 24 h with dengue-3*

<table>
<thead>
<tr>
<th>Metabolic inhibitor</th>
<th>Antibody used for IF staining</th>
<th>% Antigen-containing cells treated with inhibitor at concn. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>D-3 MCA</td>
<td>0 (0) 0.001 (70) 0.01 (85) 0.1 (&gt;95) 1</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>D-C MCA</td>
<td>0.1-1-0 1.0-0.1-0 0.1-1-0 0.01-1-0 0-1-0 1-0 1-0 1-0 1-0 1-0</td>
</tr>
<tr>
<td>Puromycin</td>
<td>D-3 MCA</td>
<td>&gt;90 &gt;90 &gt;90 20-30 20-30</td>
</tr>
<tr>
<td>Puromycin</td>
<td>D-C MCA</td>
<td>&gt;90 &gt;90 &gt;90 50-60 50-60</td>
</tr>
</tbody>
</table>

* Cells stained 3 days after superinfection with dengue-3 virus.
† Values in parentheses indicate percentage reduction of [3H]uridine incorporation in control C6/36 cells exposed to actinomycin D.
‡ Cell destruction.

In cultures infected with both types less than 8 h apart, about 50% of the cells developed dengue-3 antigen. In contrast, cultures singly infected with dengue-3 virus at the same input multiplicity (m.o.i. of 2) (Table 1) contained dengue-3 antigen in greater than 90% of the cells. Therefore, it can be concluded that in the cultures infected with both virus types less than 8 h apart (before resistance to superinfection was induced) about 40% of the cells exposed to dengue-3 virus did not develop dengue-3 antigen.

Effect of metabolic inhibitors upon the induction of interference

Cultures of C6/36 cells were treated with actinomycin D before and after infection with dengue-1 to determine if cellular transcription was required for inducing resistance. Table 2 shows that actinomycin D had no effect upon resistance to dengue-3 superinfection, since a maximum level of resistance (0-1 to 1-0% of the cells containing dengue-3 antigen) was found at all concentrations of actinomycin D tested (0-001 to 1 µg/ml). The incorporation of
Table 3. Comparison of the percentage of cells containing dengue type-specific antigen in dengue virus-infected C6/36 cultures* 3 days after superinfection with heterotypic dengue virus

<table>
<thead>
<tr>
<th>Virus used for superinfection</th>
<th>Antibody used for IF staining</th>
<th>% Antigen-containing cells infected with</th>
<th>Dengue-1</th>
<th>Dengue-2</th>
<th>Dengue-3</th>
<th>Dengue-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dengue-1</td>
<td>Dengue-2</td>
<td>Dengue-3</td>
<td>Dengue-4</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>D-1 MCA</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D-3 MCA</td>
<td>0</td>
<td>0</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td></td>
</tr>
<tr>
<td>D-C MCA</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>0.1-1.0</td>
<td>0.1-1.0</td>
<td>0.1-1.0</td>
<td></td>
</tr>
<tr>
<td>D-I MCA</td>
<td>0</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
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<tr>
<td>D-3 MCA</td>
<td>&gt;90</td>
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<td>D-C MCA</td>
<td>&gt;90</td>
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<td>D-I MCA</td>
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<td>D-C MCA</td>
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</tbody>
</table>

* All dengue virus-infected cultures were used at 2 weeks post-infection.

| [3H]uridine into control C6/36 cultures was also determined at each of the concentrations of actinomycin D tested. At an actinomycin D concentration of 0.1 μg/ml, uridine incorporation was reduced by more than 95%, demonstrating that cellular RNA synthesis was inhibited by the actinomycin D exposure.

Cultures were also tested for the effect of puromycin on the induction of resistance to dengue-3 superinfection. At puromycin concentrations of 0.001 and 0.01 μg/ml, the induction of resistance was not affected, since only 0.1 to 1.0% of the cells were permissive to superinfection with dengue-3 (Table 2). In contrast, 20 to 30% of the cells were permissive at puromycin concentrations of 0.1 and 1 μg/ml. The effect of puromycin on the induction of resistance was further examined to determine if the number of dengue-3 permissive cells present after dengue-1 infection and exposure to puromycin was related to the concentration of puromycin (Fig. 2). Over a range of puromycin concentrations from 0.03 to 0.07 μg/ml, the percentage of dengue-3-infected cells increased from less than 1% to about 25%.

Rate of virus adsorption

The rate of adsorption of dengue-3 to control or dengue-1 PI cultures was compared to determine if dengue-1-directed cell surface proteins interfered with the adsorption of dengue-3. The rate of dengue-3 adsorption was the same for both cultures (Fig. 3).

Superinfection of dengue-3-infected cultures with dengue-1

The phenomenon of resistance in dengue-1-infected cultures to superinfection with dengue-3 was further studied to determine if dengue-3-infected cultures were resistant to dengue-1 superinfection. These experiments, however, required dengue-1-specific monoclonal antibody for identification of dengue-1-infected cells. To obtain this antibody, mouse lymphocyte hybridomas were prepared from mice immunized with dengue-1-infected SMB. One of these hybridomas secreted antibody (D-1 MCA) that stained dengue-1 PI cells, but not dengue-2, -3, or -4 PI cells. When indirect IF staining was used.

Using this antibody, dengue-1-infected cells were identified in dengue-3 PI cultures superinfected with dengue-1 (Table 3). Less than 1% of the superinfected cells developed dengue-1 antigen, demonstrating resistance of the dengue-3-infected cultures to superinfection with dengue-1. Cultures infected with either dengue-2 or dengue-4 were also tested for resistance to superinfection with dengue-1 or dengue-3. Results in Table 3 show that, in all superinfected cultures, 0.1 to 1.0% of the cells contained antigen of the superinfecting virus, indicating resistance of the cultures to superinfection.
Simultaneous infection of C6/36 cells with dengue-1 and dengue-3

Simultaneous infection of C6/36 cultures with two dengue virus types was investigated using a dual staining technique with D-1 MCA and D-3 MCA (Fig. 4). A control of dengue-3 PI cells stained with this technique showed dark-brown intracellular staining with bright field microscopy, indicating areas of peroxidase activity. No fluorescence was detected in these cells. In contrast, dengue-1 PI cells showed bright fluorescence, but no peroxidase activity. No cells were found in either culture that stained with both antibodies.

Cultures of C6/36 cells were then infected with both dengue-1 and dengue-3 at an m.o.i. of 2. At 2 days post-infection, type-specific antigen of each virus was found in 20 to 30% of the cells (Table 4). At 4 days post-infection, the percentage of antigen-containing cells increased to 40 to 50% for each type. In contrast, singly infected control cultures contained antigen in
about 90% of the cells at 2 days post-infection, demonstrating that virus antigen synthesis was delayed in the simultaneously infected cultures. The data in Table 4 also show that most cells in these cultures contained antigen of only one virus type, even though the majority of these cells were initially exposed to both types. These data are consistent with the data in Fig. 1, where about 40% of dengue-3-infected cells in simultaneously infected cultures did not contain dengue-3 antigen.

At days 6, 13 and 20, cells were found that contained antigen of both virus types (Table 4). These cells appeared relatively late in infection (day 6), and then decreased in number on days 13 and 20.

**DISCUSSION**

We have found that cultured C6/36 cells infected with each of the four dengue virus types are resistant to heterotypic dengue virus superinfection with dengue virus types 1 or 3. This resistance was inducible within 20 h after primary dengue virus infection as demonstrated in cultures infected with dengue-1, and then superinfected with dengue-3. This relatively short period is in marked contrast to the 50-week interval reported by Igarashi (1979) for resistance to occur in dengue-1 PI cultures, indicating that we have not observed the same phenomenon. The possibility that the resistance is interferon-mediated is also unlikely, since the induction of resistance was not sensitive to actinomycin D. In addition, other investigators have shown that mosquito cells do not express interferon activity (Peleg, 1969; Murray & Morahan, 1973; Stollar & Shenk, 1973; Davey & Delgarno, 1974; Kascak & Lyons, 1974).

Interference between togaviruses in cultured mosquito cells has consistently been shown between strains of the alphavirus, Sindbis virus (homologous interference) (Stollar & Shenk, 1973; Riedel & Brown, 1977, 1979), and recently between Sindbis virus and other alphaviruses (heterologous interference) (Eaton, 1979). The interference between dengue virus types (heterotypic interference) is a unique example of togavirus interference in cultured mosquito cells. Since the dengue viruses are classified as distinct antigenic types, and not homologous virus strains, the interference between them is not of the homologous type. In contrast, the interference between dengue virus types is not a clear example of heterologous togavirus interference, since the dengue viruses are all members of a unique togavirus taxonomic subgroup (dengue subgroup) that consists of antigenically distinguishable viruses which have the same biological and clinical properties (heterotypes). Furthermore, resistance was demonstrated without using mutant dengue virus strains obtained by prolonged passage in cultured mosquito cells. Whether or not dengue virus heterotypic interference is related to the homologous or heterologous interference reported for other togaviruses, or is a separate phenomenon, will depend on a complete understanding of the mechanisms involved. We have determined that the induction of resistance in dengue virus-infected cells is sensitive to puromycin, indicating that protein synthesis is required. In addition, the mechanism of resistance does not involve interference with adsorption of heterotypic dengue virus to dengue virus-infected cells. These findings indicate that dengue heterotypic interference has a virus-directed intracellular mechanism.

The interference found between dengue virus types was not limited to resistance of infected cultures to superinfection. In cultures simultaneously infected with two dengue virus types, one type was excluded from replication in cells infected with both, except for a small population of cells (about 1%) that contained antigen of both virus types. The virus type excluded was random, since the input multiplicity (m.o.i. of 2) and the percentage of cells that contained type-specific antigen were the same for both virus types. Interference in these cultures was also suggested by a delay in the appearance of dengue type-specific antigen when compared to singly infected cultures.
These results are consistent with an interference phenomenon (heterotypic exclusion) reported by Legault et al. (1977) for interference between serotypes of vesicular stomatitis virus (VSV), a rhabdovirus, in a cultured vertebrate cell system (mouse cells). Simultaneous infection of these cultures with two VSV serotypes resulted in individual cells infected with one serotype or the other. The percentage of cells infected with each serotype was proportional to the input multiplicity of each. When cultures were infected with a single VSV serotype for 1.5 h, they became resistant to superinfection with heterotypic VSV. In dengue virus-infected C6/36 cells, resistance to superinfection required a longer interval for induction (about 20 h), which might reflect differences between the viruses and the cultured cells used. One proposed mechanism of heterotypic exclusion with VSV was thought to be similar to the non-interferon-mediated interference (intrinsic interference) described by Marcus & Carver (1967). The effect of intrinsic interference in Sindbis virus-infected vertebrate cells is to inhibit the replication of superinfecting VSV prior to the synthesis of new VSV RNA (Hunt & Marcus, 1974). It is not known if intrinsic interference occurs in mosquito cells, but it can be induced by West Nile virus, a group B togavirus, in cultured vertebrate cells (Marcus & Carver, 1967), and has been proposed by Murphy (1975) as a mechanism for modulation of togavirus infection in mosquitoes.

An interesting aspect of C6/36 cultures simultaneously infected with two dengue viruses is that a small population of infected cells (about 1%) contained antigen of both dengue types. This finding indicates that heterotypic interference with dengue virus was not fully efficient, and suggests that genetic or non-genetic interactions could occur between dengue virus types. Hammon (1973) proposed that dual dengue virus infection in man or mosquitoes could result in genetic recombinants with the ability to cause dengue haemorrhagic fever. Studies with Sindbis virus, however, have shown a unique replication process for that alphavirus in mosquito cells (Gliedman et al., 1975), which was interpreted by Riedel & Brown (1977) as a cause for very limited complementation between Sindbis virus strains in dually infected cultures. If dengue virus follows a similar replication process in cultured mosquito cells, interaction between virus types in dually infected cells would be unlikely.

Our findings of mixed dengue virus infection in cultured mosquito cells might reflect the biology of such infection in live mosquitoes. If so, we suggest that infection of mosquitoes with two dengue virus types could have two possible outcomes. Infection with two virus types within a short interval (i.e. before resistance to superinfection is induced) could result in mixed infection. Independent replication of both types in a single mosquito suggests that the two viruses could be transmitted simultaneously. The effect of this upon the pathogenesis of dengue infection in man is unknown. In contrast, if a mosquito were infected with one type for a longer interval, it could become resistant to infection with a second dengue virus type. This would make the individual mosquito an ineffective vector for all other dengue types. Whether such resistance affects the ecology of dengue virus and its epidemiology remains to be studied.

This work is in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology, University of Miami, Florida for D. Dittmar.

REFERENCES


(Received 20 July 1981)