An Agent in the *Aedes aegypti* Cell Line (Peleg) which Causes Fusion of *Aedes albopictus* Cells

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Cell cultures of the *Aedes aegypti* (Peleg) cell line contain an agent that causes marked syncytium formation of *Aedes albopictus* cells. A plaque assay was used to measure the growth of the cell fusing agent (CFA) in *A. albopictus* cultures, and it was found to have a latent period of about 12 hr. It failed to replicate in or cause fusion of BHK, KB or Vero cells. The CFA was sensitive to ether and to deoxycholate and sedimented in a sucrose velocity gradient more slowly than did Sindbis virus. The preliminary evidence suggests that the CFA may be similar to the group B togaviruses.

INTRODUCTION

It has generally been observed that infection of cultured mosquito cells with any one of several group A togaviruses results in virus replication to high titer but without any cytopathic effect (CPE). Such observations have been made for Sindbis, Semliki Forest, eastern equine encephalitis, and Ross River valley viruses in either *Aedes albopictus* or *Aedes aegypti* cells (Peleg, 1968; Stevens, 1970; Stollar and Shenk, 1973; Davey et al., 1973).

In contrast, several reports have documented striking CPE, usually involving syncytium formation, after infection of *A. albopictus* cells with one of several group B togaviruses (flaviviruses). For example, Paul et al. (1969) recorded such observations 3–4 days following infection with Japanese encephalitis (JE), West Nile or dengue viruses. Suitor and Paul (1969) also noted syncytium formation 4–5 days after infection of *A. albopictus* cells with dengue virus. Two other reports described plaque formation on monolayers of *A. albopictus* cells infected with group B togaviruses (Suitor, 1969; Cory and Yunker, 1972).

More recently, Germiston virus (GV), a member of the Bunyamwera group, and Wesselsbron virus (WV), a member of the group B togaviruses, were shown to cause extensive cell fusion not only of *A. albopictus* cells but also of several vertebrate cell lines (Djinawi and Olson, 1973). However, with both of these viruses the phenomenon occurred within 3 hr after infection and therefore appeared to represent fusion from without.

In this report, we present evidence pointing to a hitherto undetected agent in the *A. aegypti* cell line of Peleg. The agent was recognized only by its ability to cause fusion of *A. albopictus* cells. The evidence presented in this report suggests that the agent may be a group B togavirus. Pending precise identification, the agent is referred to simply as the cell fusing agent (CFA).

MATERIALS AND METHODS

**Cells, media, and viruses.** The *A. albopictus* cells were from the established cell line derived by Singh (1967) from mosquito larvae. They were grown as monolayer cultures at 28° in the medium of...
Mitsuhashi and Maramorosch (1964) (MM medium). Fetal calf serum was added to a final concentration of 10%.

The A. aegypti cells were from the established cell line derived by Peleg (1968) from mosquito embryos. They were grown in a medium consisting of eight parts Kitamura's solution B (Kitamura, 1965) and two parts of MM medium. Fetal calf serum was added (10% final concentration) along with NaHCO₃ to adjust the pH to 6.8. These cells were also grown as monolayers at 28°C.

The BHK-21, KB and Vero cells were all obtained from the American Type Culture Collection. The KB and Vero cells were grown in Eagle's minimal medium (Eagle, 1959) and the BHK cells in the HT medium of Rouse et al. (1966), all three with 10% calf serum supplements. Wild type Sindbis virus (SV-W) was prepared from the medium of infected chick cells (Stollar and Shenk, 1973).

PBS-1 contains 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.2 mM Na₂HPO₄. PBS-2 contains 132 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, and 8.2 mM Na₂HPO₄.

Preparation of stock of CFA. Medium from cultures of A. aegypti cells was centrifuged at low speed and the supernatant fluid was added, undiluted, to A. albopictus cell monolayers. After 60 min at room temperature the residual inoculum was removed and the cultures fed with MM medium and incubated at 28°C. When cell fusion had become extensive (about 72 hr later), medium was harvested and centrifuged at low speed. The supernatant fluid constituted the working stock of CFA and was used to prepare subsequent stocks in A. albopictus cells.

Electron microscopy. Cells were washed, scraped from petri dishes, centrifuged at 4°C, fixed in 1.5% glutaraldehyde for 1 hr and then stored in sodium phosphate buffer (0.1 M, pH 7.3) overnight at 4°C. Pellets were rinsed, fixed in 2% osmic acid for 1 hr, dehydrated and embedded in Epon 812. Sections were cut on a Porter-Blum MT-2 microtome and stained with uranyl acetate and lead citrate. The sections were then examined and photographed in a Philips EM300 microscope at 60 kV.

RESULTS

Microscopic Examination of A. albopictus Cells Infected with CFA

Normal uninfected A. albopictus cells are shown in Fig. 1A. The amount of cytoplasm is relatively small. Occasional binucleate cells are sometimes seen (not shown) but syncytium formation is not evident.

At 60 hr after infection with CFA, suggestions of syncytium formation were evident. By 76 hr there were many large syncytia usually involving over 90% of the cells on the plates. Figures 1B and 1C illustrate cell cultures at this stage. In all experiments, scattered small islands of unfused cells remained. After the stage of massive syncytium formation and resultant death of most of the cells, the unfused cells continued to divide, eventually giving rise to an A. albopictus cell culture of almost normal appearance. Such cultures could be distinguished, however, by increased numbers of binucleate cells and by scattered, small but obvious syncytia containing approximately 6–12 nuclei. The cultures never returned to a stage of large syncytium formation as seen 72 hr after the initial infection.

In order to examine further what the nature of the cell fusing agent might be, A. albopictus cells infected with CFA were examined by electron microscopy. Figure 2A shows a section taken from cells infected 76 hr previously. Several nuclei are seen within the same cytoplasm. At scattered sites in the cytoplasm there are several virus-like particles (Fig. 2B). They are located within dilated regions of rough endoplasmic reticulum which are filled with amorphous material. The particles are approximately 50 nm in diameter, appear to have an outer membrane and are embedded in the amorphous material. These viral particles were not seen in sections taken earlier (24 or 53 hr), and they were not seen in normal A. albopictus cells. It should be noted, however, that even in the 76-hr sample the viral particles...
**Fig. 1.** Aedes *albopictus* cells as seen by light microscopy. Cells were stained with Giemsa. A, Normal cells, \( \times 576 \); B, cells infected with CFA 76 hours previously, \( \times 228 \); C, cells infected with CFA 76 hours previously \( \times 576 \).
were not very numerous and had to be searched for. Whether these particles have any causal relationship to the cell fusion process remains to be clearly demonstrated.

**Plaque Assay of CFA**

*A. albopictus* monolayer cultures were exposed to various dilutions of the CFA for 60 min at room temperature. The inoculum was then removed and the cultures were overlaid with 5 ml of medium that consisted of one part 1.8% Noble agar in PBS-1 and one part MM medium. After 4 days, 2 ml of 0.025% neutral red was added to each culture. The neutral red was removed the next day, at which time plaques were generally visible, although it was usually easier to wait one more day to count them.

Figure 3 shows plaques on cultures infected with CFA. Often the plaques themselves, which were clear, were surrounded by a more intensively stained red ring. Microscopic examination showed that in the plaque itself there was very little neutral red visible, but evidence of syncytium
formation. Surrounding the plaque one could often see cells more intensely stained with neutral red than cells in normal areas. At very early times it sometimes appeared that "red" plaques were formed which then changed into clear plaques.

**Growth Curve of CFA**

With the ability to quantitate the CFA readily it was possible to examine the growth curve. Figure 4 shows the levels of extracellular and cell-associated virus at various times after infection. There was a latent period of approximately 12-hr duration followed by a 60-hr rise period. By 72 hr there was a leveling off in the amount of virus produced.

Two points can be made with respect to the growth curve. Cell fusion was not evident at all until 60 hr, and it did not become extensive until about 72 hr. Thus syncytium formation is a late event not occurring until the "growth curve" has been almost completed. Second, the nature of the growth curve, with a latent period of 12 hr, is compatible with that of a group B
Fig. 3. Plaque assay of CFA on monolayer cultures of Aedes albopictus cells. The procedure for plaque assay was described under Materials and Methods. The plates shown are 60 mm in diameter.

Fig. 4. Growth of CFA in Aedes albopictus cells. Monolayers of A. albopictus cells in 60-mm petri dishes were infected with CFA at an input m.o.i. of 0.1 PFU/cell. After a 90-min adsorption period at room temperature the CFA was removed and cultures fed with 5 ml of MM medium per plate. Incubation was at 28°C. At various times after infection, samples were then taken to measure both extracellular and cell-associated CFA. Assay was by plaque formation on A. albopictus monolayer cultures. To obtain cell-associated virus, the cells from one plate were washed, suspended in 2 ml of PBS-1 (containing 0.2% BSA), frozen and thawed twice, and then centrifuged at low speed. The supernatant was collected and taken to represent cell-associated virus.

togavirus, at least as seen in mammalian cells (Schulze, 1964; Stollar et al., 1967). The virus-like particles seen in Fig. 2 are also consistent with the size and structure of known group B togaviruses (Matsumura et al., 1971).

Infection of Different Mosquito and Mammalian Cell Lines with CFA

Three mammalian cell lines were exposed to CFA (approximate m.o.i. = 1 plates were infected with CFA at an input m.o.i. of 0.1 PFU/cell. After a 90-min adsorption period at room temperature the CFA was removed and cultures fed with 5 ml of MM medium per plate. Incubation was at 28°C. At various times after infection, samples were then taken to measure both extracellular and cell-associated CFA. Assay was by plaque formation on A. albopictus monolayer cultures. To obtain cell-associated virus, the cells from one plate were washed, suspended in 2 ml of PBS-1 (containing 0.2% BSA), frozen and thawed twice, and then centrifuged at low speed. The supernatant was collected and taken to represent cell-associated virus.
FUSION OF Aedes albopictus CELLS

PFU/cell) and incubated at 34°. In no case was there any cell fusion, cytopathic effect or evidence of replication of CFA (Table 1, Experiment I).

When A. aegypti cells were exposed to CFA, no cell fusion or CPE was observed but there was an increase in the titer of CFA at 72 hr as compared to 2 hr (Experiment I). A. aegypti cell cultures chronically infected with SV responded in a similar fashion. That this rise in titer probably represented, at least partially, endogenous production of CFA by A. aegypti cells, which were the original source of CFA, is supported by the rise in titer observed in A. aegypti cultures that were mock infected (Experiment II).

The effects of CFA on the normal A.

### TABLE 1

**RESPONSE OF DIFFERENT MAMMALIAN AND MOSQUITO CELL LINES TO CFA**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line or subline</th>
<th>Infected with CFA</th>
<th>Titer of CFA (PFU/ml)</th>
<th>CPE at 55 hr</th>
<th>CPE at 72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 hr</td>
<td>55 hr</td>
<td>72 hr</td>
</tr>
<tr>
<td>I</td>
<td>BHK</td>
<td>+</td>
<td>4.7 x 10^3</td>
<td>&lt; 10^3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Vero</td>
<td>+</td>
<td>1.7 x 10^3</td>
<td>&lt; 10^3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>KB</td>
<td>+</td>
<td>1.6 x 10^3</td>
<td>&lt; 10^3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A. albopictus (normal)</td>
<td>+</td>
<td>5.5 x 10^2</td>
<td>2.6 x 10^2</td>
<td>4+</td>
</tr>
<tr>
<td></td>
<td>A. albopictus (SV)^a</td>
<td>+</td>
<td>1.5 x 10^3</td>
<td>7.2 x 10^5</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>A. albopictus (CFA)^c</td>
<td>+</td>
<td>6.0 x 10^3</td>
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<tr>
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<td>A. aegypti (normal)</td>
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<td>8.7 x 10^4</td>
<td>5.5 x 10^4</td>
<td>0</td>
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<tr>
<td></td>
<td>A. aegypti (SV)</td>
<td>+</td>
<td>2.7 x 10^4</td>
<td>7.0 x 10^5</td>
<td>0</td>
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<tr>
<td>II</td>
<td>A. albopictus (normal)</td>
<td>+</td>
<td>1.2 x 10^5</td>
<td>4.3 x 10^5</td>
<td>3+</td>
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<tr>
<td></td>
<td>A. albopictus (normal)</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A. albopictus (SV)^v</td>
<td>+</td>
<td>2.2 x 10^3</td>
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<td>0-1+</td>
</tr>
<tr>
<td></td>
<td>A. albopictus (SV)^v</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0</td>
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<tr>
<td></td>
<td>A. albopictus (CFA)^g</td>
<td>+</td>
<td>4.3 x 10^3</td>
<td>4.6 x 10^6</td>
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<td></td>
<td>A. albopictus (CFA)^g</td>
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<td>7.5 x 10^3</td>
<td>1.4 x 10^6</td>
<td>0</td>
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<tr>
<td></td>
<td>A. aegypti (normal)</td>
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<td>8.5 x 10^2</td>
<td>5.5 x 10^4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A. aegypti (normal)</td>
<td>-</td>
<td>1.2 x 10^2</td>
<td>3.5 x 10^5</td>
<td>0</td>
</tr>
</tbody>
</table>

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^a CFA was added to cells (approximate m.o.i. = 1 PFU/cell) for 60 min at room temperature and then removed; cells were fed with the appropriate medium and incubated at 34°C in the case of vertebrate cells and at 28°C in the case of mosquito cells. Cytopathic effect or syncytial formation was graded on a 0–4+ scale. Samples of medium were taken at the times indicated and assayed by plaque formation on A. albopictus cells.

^b These cells were initially infected 11 months previously with SV-W. Sindbis virus was usually present in the medium at levels near 10^5 PFU/ml and gave rise only to small plaques (Stollar and Shenk, 1973).

^c These cells were initially infected 4 months previously with CFA.

^d Cultures not infected with CFA were mock infected with PBS-2 containing 0.2% BSA.

^e None detectable.

^f Cultures were initially infected 17 months previously with SVW.

^g Cultures were initially infected 10 months previously with CFA.
albopictus cells were as described above. By 72 hr in Experiment I and by 55 hr in Experiment II, there was dramatic cell fusion and a marked rise in the amount of CFA. A. albopictus cultures chronically infected with Sindbis virus showed a similar response although both the CPE and the CFA yield were slightly less. In Experiment II, the cell fusion observed in the chronically infected cells at 55 hr after superinfection was distinctly less than in the normal A. albopictus cells after primary infection with CFA. By 72 hr, this difference was minimal.

It was pointed out above that after exposure of A. albopictus to CFA a small number of cells survived and eventually grew into a confluent culture. Such cultures, designated A. albopictus (CFA), have now been carried for several months and are distinguished from normal A. albopictus cell cultures only by occasional small areas of syncytium formation. They continue to produce CFA and in this way resemble cultures of A. albopictus persistently infected with SV (Stollar and Shenk, 1973). Although, in Experiment I, A. albopictus (CFA) cultures "superinfected" with CFA seemed to yield significantly less CFA than either A. albopictus or A. albopictus (SV) cultures, in Experiment II both "mock infected" and superinfected A. albopictus (CFA) cultures produced yields of CFA equivalent to that from normal A. albopictus cells. The latter result suggests that the CFA being produced in each instance represents mainly the endogenous production by these cells. In no case did superinfection of A. albopictus (CFA) cells with CFA lead to cell fusion. Thus, in this respect, these cells are resistant to superinfection.

Properties of the CFA

Two further experiments were performed to help characterize the CFA. First, sucrose velocity gradient centrifugation was used to estimate its size. CFA was mixed with Sindbis virus and sedimented through a 15–30% sucrose gradient. Fractions of the gradient were then assayed for (1) SV-W on chick cells and (2) CFA on A. albopictus cells. As seen in Fig. 5, the CFA sedi-

mented much more slowly than SV and at a rate consistent with that of group B togaviruses (Boulton and Westaway, 1972).

Second, evidence for the presence or absence of a viral envelope was sought by testing for sensitivity to deoxycholate or to ether. Table 2 shows that the infectivity of CFA as measured by plaque formation on A. albopictus cells was reduced both by deoxycholate and by ether by nearly 10,000-fold to a nondetectable level. We conclude that the CFA is probably an enveloped particle.

DISCUSSION

Although we have not specifically identified the agent that causes fusion of A. albopictus cells, some circumstantial evidence suggests to us that it may be similar to group B togaviruses. First, the growth curve of the CFA, like that of group B togaviruses, had a latent period of approximately 12 hr. Second, the infectivity of the CFA was abolished by deoxycholate and by ether, suggesting that the CFA is enveloped or has an essential lipid

![Fig. 5. Sucrose gradient centrifugation of CFA. CFA and SV were mixed and centrifuged through a 16-ml 15–30% (w/w) sucrose gradient. The sucrose was prepared in TNE buffer (0.1 M NaCl, 0.001 M EDTA, and 0.05 M Tris pH 7.6). Centrifugation was at 25,000 rpm for 3 hr in an SW27 rotor. Fractions were collected with an ISCO density gradient fractionator and assayed for SV on chick cells and for CFA on A. albopictus cells.](image-url)
Third, in thin sections of *Aedes albopictus* cells infected with CFA, spheroidal particles about 50 nm in diameter were found. This corresponds to the size of group B togaviruses. Finally, the CFA sedimented in a sucrose velocity gradient more slowly than Sindbis virus, and at a rate consistent with that expected for a group B togavirus. In addition, a number of examples have been cited above in which the fusion of *A. albopictus* cells followed infection with a group B togavirus.

If indeed CFA is a group B togavirus, this would be yet another example of fusion of mosquito cells by a virus of this group. Several reports already referred to involved fusion of *A. albopictus* cells by dengue, West Nile or JE viruses and depended on replication of the virus. In previous experiments, our strain of type 2 dengue virus replicated only to low titers in mosquito cells, and there was no suggestion of cytopathic effect or cell fusion (Stevens, 1970). Experiments by Igarashi with JEV and *A. albopictus* cells have shown the importance of the strains used and their passage history in achieving high virus titers (Igarashi et al., 1973). Cell fusion was noted occasionally in the experiments of Igarashi but appeared to be dependent to a large extent on the strain of JEV used (Igarashi, personal communication). Similar observations were made with respect to dengue virus in *A. albopictus* cells (Sweet and Unthank, 1971).

Another report that described cell fusion by Germiston and Wesselsbron viruses differed from our observations in two respects. First, fusion by CFA appeared late, was related to replication and was a manifestation of fusion from within rather than from without. Fusion by GV and WV appeared early, did not require viral replication and exemplified fusion from without. Secondly, fusion by CFA was specific to *A. albopictus* cells whereas both GV and WV fused vertebrate as well as mosquito cells.

If the CFA is a togavirus, the question might be posed as to why it did not replicate in any of the vertebrate cell lines tested, lines usually susceptible to togaviruses. One explanation might be that after a prolonged period of being carried in mosquito cells the CFA had become temperature sensitive (Shenk et al., 1974). With this in mind, in the experiment recorded in Table 1, the vertebrate cells were maintained at 34° after infection with CFA. This temperature, however, might still have been nonpermissive. Lower temperatures have not been tested.

One of the most interesting problems posed by the togaviruses relates to their ability to replicate in both insect and vertebrate cells, but often without CPE in the former as opposed to their destructive effects in the latter. It is evident, however, that even in mosquito cell cultures the cellular responses are variable due to factors not clearly understood at present but which bear further examination.

This report underlines the importance of carefully monitoring insect cell cultures for unsuspected viral agents. Togaviruses in mosquito cells, like oncornaviruses in vertebrate cells, may not be readily apparent by any obvious visible effects on the infected cells. This may be due to the fact that no CPE occurs at the time of the initial infections or that CPE does occur initially but that the cultures recover and thereafter appear quite normal. We observed such recovery of *A. albopictus* cultures after infection with CFA, and Paul et al. (1969) described a similar phenomenon after infection with either JE, WN or dengue viruses. A somewhat similar situation was observed with human cells (KB) chronically infected with dengue virus (Schulze and Schlesinger, 1963). Togaviruses, therefore, would not be suspected under such circumstances unless specifically looked for either with a suitably

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**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Titer of CFA (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$1.2 \times 10^8$</td>
</tr>
<tr>
<td>Ether</td>
<td>$&lt;2.5 \times 10^5$</td>
</tr>
<tr>
<td>0.05% Na DOC</td>
<td>$&lt;2.5 \times 10^5$</td>
</tr>
</tbody>
</table>

* A stock of CFA was treated with ether or sodium deoxycholate as described by Hsuing (1973) and then assayed by plaque formation on *A. albopictus* cells.

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...
sensitive indicator system or by specific immunological means. In the case of the CFA, only a chance observation led to its detection.

It is not yet known when the A. aegypti cells might first have been exposed to the CFA and which of the sublines might also contain the agent or, indeed, if the CFA may have evolved endogenously in A. aegypti (Schlesinger, 1971).

Tests to identify specifically the CFA are now underway as well as further investigations into the biochemistry of the agent and its replication.

ACKNOWLEDGMENTS

Since these experiments have been completed, Dr. J. Casals (Yale Arbovirus Research Unit, New Haven, CT) has been kind enough to help in the identification of the CFA. Preliminary experiments using polyvalent anti-group B togavirus sera in complement fixation tests failed to give a positive result. This result does not, however, definitively eliminate the possibility that the CFA is a group B togavirus (flaviviruses).

We thank Dr. R. W. Schlesinger for his critical reading of the manuscript and for his suggestions.

We are also extremely grateful to Mrs. Lily Tao for her expert help with these experiments. Preliminary experiments with CFA were begun by Ted Bass while he was a student at Rutgers Medical School.

REFERENCES


