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Isolation of a Singh's *Aedes albopictus* Cell Clone Sensitive to Dengue and Chikungunya Viruses

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SUMMARY

Twenty clones were isolated from cultured *Aedes albopictus* (Singh) cells in the presence of anti-Chikungunya (CHIK) virus serum. Each clone was tested for its yields of Dengue (DEN) viruses, types 1, 2, 3 and 4, and also CHIK virus. Clone C6 showed the highest yield of each virus tested. Forty-three clones obtained by recloning C6 in the presence of anti-DEN sera showed almost the same virus yields as C6. One of the clones, C6/36, showed mild to extensive cytopathic effects several days after virus infection, in contrast to the original uncloned (SAAR) cells. Fluorescent antibody staining revealed that the amount of virus antigen accumulated in the cytoplasm was almost the same in every cell in the case of clone C6/36, while it was highly heterogeneous for uncloned SAAR cells. Growth curves of the viruses indicated that clone C6/36 gave a significantly higher yield for each virus than uncloned SAAR cells up to 7 days after infection.

Virus sensitivity of the C6/36 clone did not change by growing the cells with the medium used for uncloned SAAR cells, nor did the virus sensitivity of uncloned cells increase in medium used for clone C6/36. However, the C6/36 clone became resistant to CHIK virus, but not to DEN or Sindbis viruses, after incubation with the medium used for another *A. albopictus* cell line (SAAK). The transfer of the specific resistance to CHIK may be mediated by some latent virus related to CHIK.

INTRODUCTION

Dengue (DEN) and Chikungunya (CHIK) virus infections have been among the most important virus diseases in Southeast Asia causing haemorrhagic manifestations and, in the case of DEN, sometimes shock and death of infected children (Hammon *et al.* 1960; Halstead, 1966). The viruses are transmitted by *Stegomyia* mosquitoes, such as *Aedes aegypti* or *A. albopictus* (Clarke & Casals, 1965).

The establishment of cell lines from these mosquitoes (Peleg, 1966; Singh, 1967) allows investigations of the growth of arboviruses, including DEN and CHIK, in simplified cell cultures and may clarify the basic biology of the growth of these viruses in vector mosquitoes. Since these cell lines were prepared from a mixed population of embryos or larvae, they were possibly genetically heterogeneous. This paper describes an attempt to isolate a genetically homogeneous cell clone from Singh's *A. albopictus* cells, in order to obtain a uniform host cell system which is sensitive to DEN and CHIK viruses.

METHODS

Viruses. Dengue viruses, type 1 (DEN-1) Hawaiian strain at the 15th passage in suckling mouse brain (SMB), type 3 (DEN-3) H-87 strain at the 26th passage in SMB, type 4 (DEN-4) H-241 strain at the 43rd passage in SMB, Chikungunya (CHIK) virus African strain at the 174th passage in SMB, and Sindbis (SIND) virus were obtained from Dr Sompop Ahandrik, Virus Research Institute, Bangkok, Thailand. Dengue virus type 2 (DEN-2) New Guinea B strain which had been passaged in KB and BHK21 cells was obtained from Dr S. Hotta, Kobe University School of Medicine. These viruses were passed one to three more times in SMB in this laboratory. Stocks of viruses were prepared as 10% homogenates of infected SMB in PBS (Dulbecco & Vogt, 1954) and the supernatant fluids obtained after low-speed centrifugation were stored at −70 °C. Dengue viruses were plaque purified on LLC-MK_2 cells under agar overlay (Russell *et al.* 1967) and were then inoculated to uncloned Singh's *A. albopictus* (SAAR) cells. After 7 days incubation at 28 °C with virus maintenance medium (see below), infected culture fluids were harvested and stored in aliquots at −70 °C. Seeds of DEN viruses were prepared by inoculation of the aliquots once more into SAAR cells and the infected culture fluids were harvested after 4 days incubation at 28 °C and kept at 4 °C to be used in the experiments within 1 week.

Seeds of CHIK and SIND viruses were prepared in BHK21 cells after plaque purification as described by Igarashi & Fukai (1969).

Infectivity titration of viruses. Virus infectivities were assayed by counting fluorescent foci formed on BHK21 cells which had been grown in Lab-Tek 8-chamber slides (Miles, Ill., U.S.A.) with slight modifications of the method described for DEN-4 (Igarashi & Mantani, 1974). With CHIK and SIND viruses, alcohol-washed tragacanth gum (Wako Pure Chemical Industries Ltd, Osaka, Japan) was introduced into the virus maintenance medium at 1% final concentration in order to localize the infected cells by preventing spread of progeny viruses. After 2 h virus adsorption at 37 °C, BHK21 cells in 8-chamber slides were incubated with virus maintenance medium in 5% CO_2 atmosphere at 28 °C for 16 h for SIND, at 37 °C for 16 h for CHIK, at 37 °C for 2 days for DEN-2 and DEN-4 and at 37 °C for 3 days for DEN-1 and DEN-3 viruses, respectively. Infected cells were then stained by the indirect fluorescent antibody technique to count the foci of cells having virus antigen. Infectivities of viruses were recorded as focus forming units (f.f.u.) per ml.

Cells. Two lines of *A. albopictus* (Singh) cells of different origins were used. One was obtained from Dr V. Stollar, CMDNJ, Rutgers Medical School, N.J., U.S.A. The cell line had been adapted (Igarashi *et al.* 1977) to a medium composed of 10% foetal calf serum (FCS) in a mixture of 1 part MM medium (Mitsuhashi & Maramorosch, 1964) and 9 parts Eagle's medium (Eagle, 1959) supplemented with 0·2 mM each of non-essential amino acids (E medium). The cell line was further adapted to grow with a cell growth medium consisting of 10% FCS in E medium only. This cell line is designated SAAR. Another cell line was obtained from Dr S. Hotta, Kobe University. The cell line was originally grown with 20% FCS in MM medium. By gradual replacement of MM medium with E medium over several months, the cell line was also adapted to grow with the same cell growth medium as SAAR (10% FCS in E medium). This cell line is designated SAAK. Both SAAR and SAAK cell lines were grown at 28 °C and were transferred once a week at 1:20 split unless otherwise specified.

BHK21 cells and LLC-MK_2 cells were obtained from Dr S. Hotta, Kobe University, and Dr S. Inoue, National Institute of Health, Tokyo, Japan, respectively. These cell lines were grown at 37 °C with 10% FCS in Eagle's minimal essential medium (Eagle, 1959).

Cloning of A. albopictus *cells.* The modified method of Puck & Marcus (1955) was used without feeder layer. The procedure (Igarashi *et al.* 1977) involved (1) seeding single cell suspensions into plastic Petri dishes and (2) picking up visible colonies one by one with pieces of filter paper and transferring them to separate containers with cell growth medium. After 1 to 2 weeks incubation at 28 °C, cells in each clone grew to sufficient numbers. Plating efficiency was 10 to 30%.

Assay of virus yields from cloned A. albopictus *cells.* Replicate cultures of each clone were prepared in Lab-Tek 8-chamber slides by seeding 0·4 ml of 1×10^5 cells/ml suspension in cell growth medium in each chamber. After 3 days incubation at 28 °C in 5% CO_2 atmosphere, each chamber contained 2 to 4×10^5 cells forming a monolayer. The culture medium was removed and 0·1 ml of seed virus was inoculated into each chamber at input multiplicities of 0·1 f.f.u./cell for DEN-1, 3 and 4 viruses and 1 f.f.u./cell for DEN-2 and CHIK viruses. After 2 h for virus adsorption, residual virus was removed and cell sheets were washed twice with 0·5 ml of PBS and incubated with 0·4 ml/chamber of virus maintenance medium (2% FCS in E medium) at 28 °C in 5% CO_2 atmosphere. Incubation was for 2 days for CHIK, 3 days for DEN-2 and DEN-4, and 4 days for DEN-1 and DEN-3. Virus infectivities in the media were then determined as described above.

Fluorescent antibody staining and antisera. Cell preparations either on 8-chamber slides or on coverslips were fixed with cold acetone at −20 °C for 20 min and then stained with diluted anti-virus sera at room temperature for 40 min in a humidified chamber. Antisera against CHIK and SIND viruses were prepared in rabbits by two intramuscular inoculations, with a 4-week interval, of a 1:1 mixture of purified virus and Freund's complete adjuvant. Purified virus preparations were obtained from infected BHK21 cell culture fluids as described by Igarashi *et al.* (1970). Rabbits were bled 1 to 2 weeks after the second immunization. Neutralizing titres of the antisera were about 1:10000 by the 50% plaque reduction method. These sera were used at 1:300 dilution. Rabbit antisera against DEN viruses were kindly supplied by Y. Okuno of this Department and were used at 1:150 dilution. All the undiluted anti-virus rabbit sera were heat inactivated at 56 °C for 30 min.

Specimens which reacted with anti-virus sera were washed three times in PBS and stained with fluorescent isothiocyanate-conjugated goat antibody globulin to rabbit 7S gamma-globulin (Hyland Division Traverol Laboratories, Inc., Calif., U.S.A.) at 1:70 dilution at room temperature for 40 min. The specimens were washed three times with PBS, mounted with a 3:2 mixture of glycerol and PBS, and were observed under an interference type fluorescent microscope.

Determination of chromosome numbers. Chromosomes were flattened without manual squashing (Parker, 1961). Cells were seeded on 11×40 mm coverslips in Leighton tubes using 2 ml of 1×10^5 cells/ml suspension per tube. After 3 days, incubation at 28 °C, cells were treated with colchicin (E. Merk AG, West Germany) at 0·005% final concentration for 6 h. The coverslips were treated with hypotonic solution (1:5 dilution of PBS with distilled water) at room temperature for 30 min, followed by fixation with acetic alcohol (1:3 mixture) for 10 min. Coverslips were dried at room temperature and were stained with a few drops of 2% orcein in acetic acid.

RESULTS

Virus yields from cloned cells

Twenty clones were isolated from *A. albopictus* (SAAR) cells in the presence of 1% anti-CHIK serum. The purpose of adding anti-CHIK serum to the cloning medium was to obtain clones free from CHIK virus latently infecting certain *A. albopictus* cell lines (Cunning-

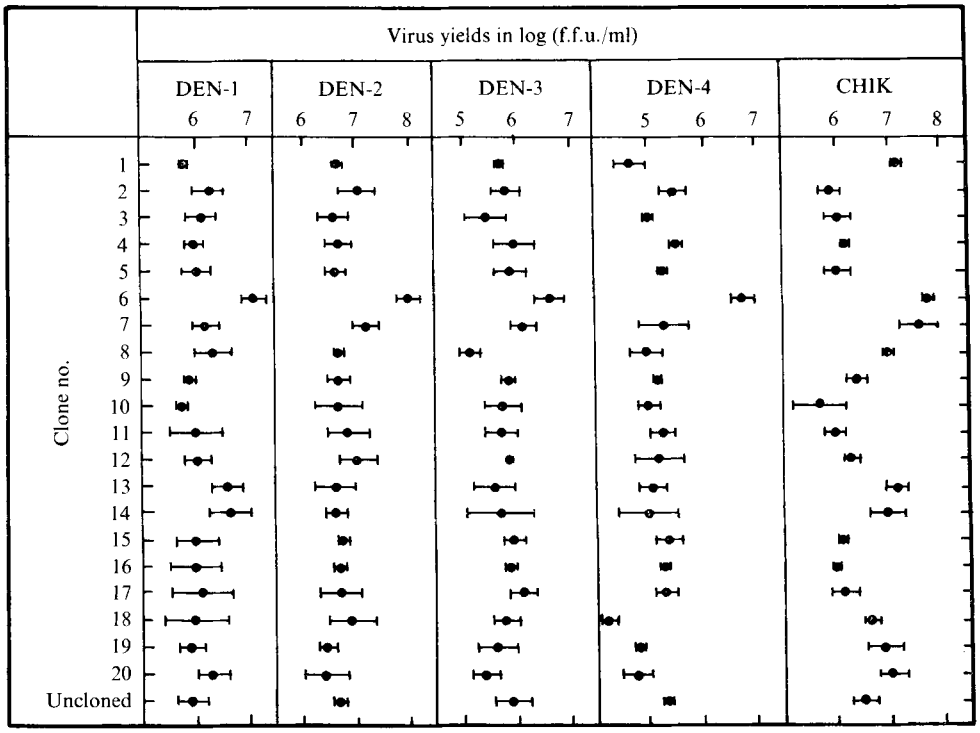

Fig. 1. Virus yields for Singh's *A. albopictus* (SAAR) cell clones. Twenty clones isolated from SAAR cells in the presence of 1 % anti-CHIK serum and original uncloned cells were tested for their yields of DEN-1, DEN-2, DEN-3, DEN-4 and CHIK viruses as described in Methods. Averages of three experiments are shown with their standard errors represented by horizontal bars.

ham *et al.* 1975; Hirumi *et al.* 1976). In the case of *A. albopictus* cells persistently infected with SIND virus, several virus-free clones could be isolated when cloning was performed in the presence of anti-SIND serum (Igarashi *et al.* 1977). Each clone was tested for its virus yields after inoculation with a fixed amount of each virus. Virus yields varied from clone to clone and clone C6 gave the highest virus yield for every virus tested. Most of the other clones gave more or less similar yields as the original uncloned SAAR cells. Some clones gave higher yields than uncloned cells for only one or two kinds of the viruses tested (Fig. 1). The differences between the yields from the C6 clone and those from uncloned SAAR cells were statistically significant. Clone C6 was subcultured for 10 weeks in the presence of 2·5 % of each antiserum against each type of DEN viruses. Then recloning was performed in the presence of 1 % of each anti-DEN sera. Anti-DEN sera were used in order to eliminate some latent viruses related to DEN if such viruses were present in clone C6. Forty-three clones were isolated and virus yields were tested for each clone as above. All the clones re-isolated from clone C6 showed almost the same level of virus yields as clone C6 (data not shown). One of the re-isolated clones, C6/36, was studied further. Replicate cultures of C6/36 clone and original uncloned SAAR cells prepared in 2 oz rubber-stoppered prescription bottles were inoculated with DEN-1, DEN-2, DEN-3, DEN-4 or CHIK virus. Assay of the virus infectivity every day in the medium provided the growth curves of these viruses (Fig. 2). Initial rates of virus growths were almost the same for clone C6/36 and for uncloned SAAR cells. However, virus yields in uncloned SAAR cells reached a plateau 2 to 3 days after infection and then declined as virus production stopped. For the C6/36 clone,

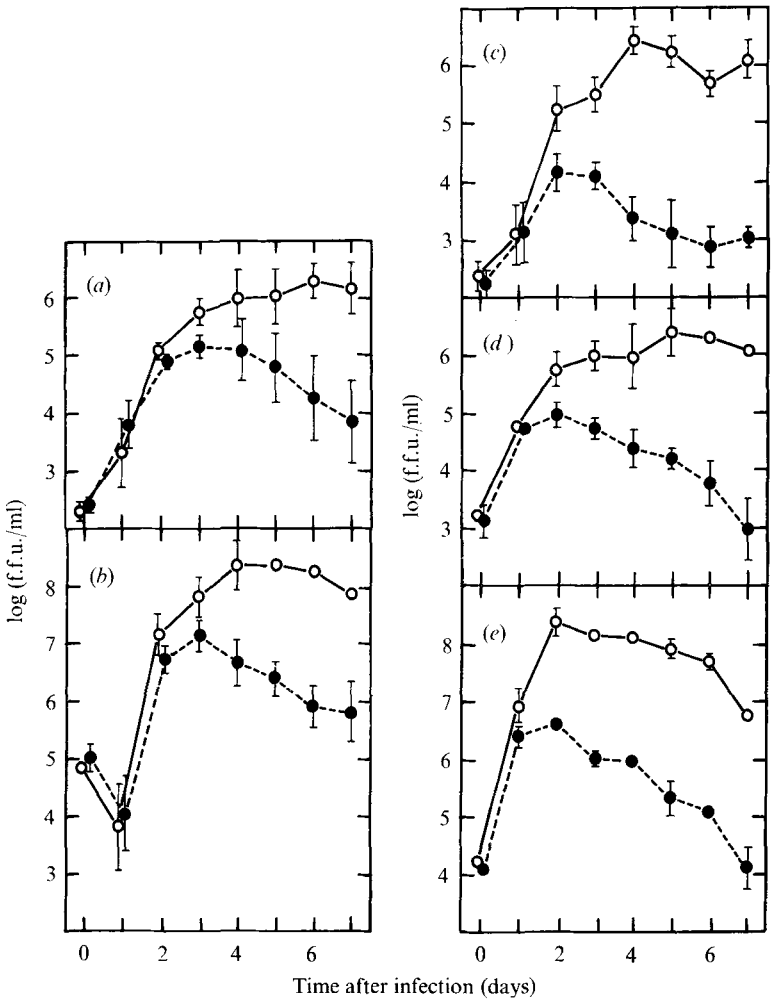

Fig. 2. Virus growth curves for clone C6/36 and uncloned SAAR cells. Replicate cultures of clone C6/36 and original uncloned SAAR cells were prepared in 2 oz rubber-stoppered prescription bottles by seeding 5×10^5 cells in 5 ml of cell growth medium per bottle. After 3 days incubation at 28 °C, each bottle contained 3 to 5×10^6 cells. Medium was removed from each bottle and cells were infected with either, (*a*) DEN-1, (*b*) DEN-2, (*c*) DEN-3, (*d*) DEN-4, or (*e*) CHIK virus. Input multiplicities were 0·1 f.f.u./cell for DEN-1, DEN-3 and DEN-4, whereas 1 f.f.u./cell was used for DEN-2 and CHIK. After 2 h virus adsorption at 28 °C, residual virus was removed and cell sheets were washed twice with 4 ml of PBS and incubated with 5 ml of virus maintenance medium per bottle. Portions of infected culture fluids were taken every day after incubation at 28 °C and were assayed for infective virus as described in Methods. Virus growth curves in clone C6/36 (○——○) and in uncloned SAAR (●– – –●) cells are shown as averages of three experiments with standard errors represented by vertical lines where they are larger than the circles of the points.

virus infectivity remained at a high level up to 7 days after infection except for CHIK. As a result, virus yields in the C6/36 clone were significantly higher than those in uncloned SAAR cells in the late stage of infection.

In order to test the possibility that high virus yields from clone C6/36 might have resulted from higher efficiency of virus adsorption to this clone than to uncloned SAAR cells, replicate cultures of the C6/36 clone and uncloned SAAR cells were infected with the viruses as

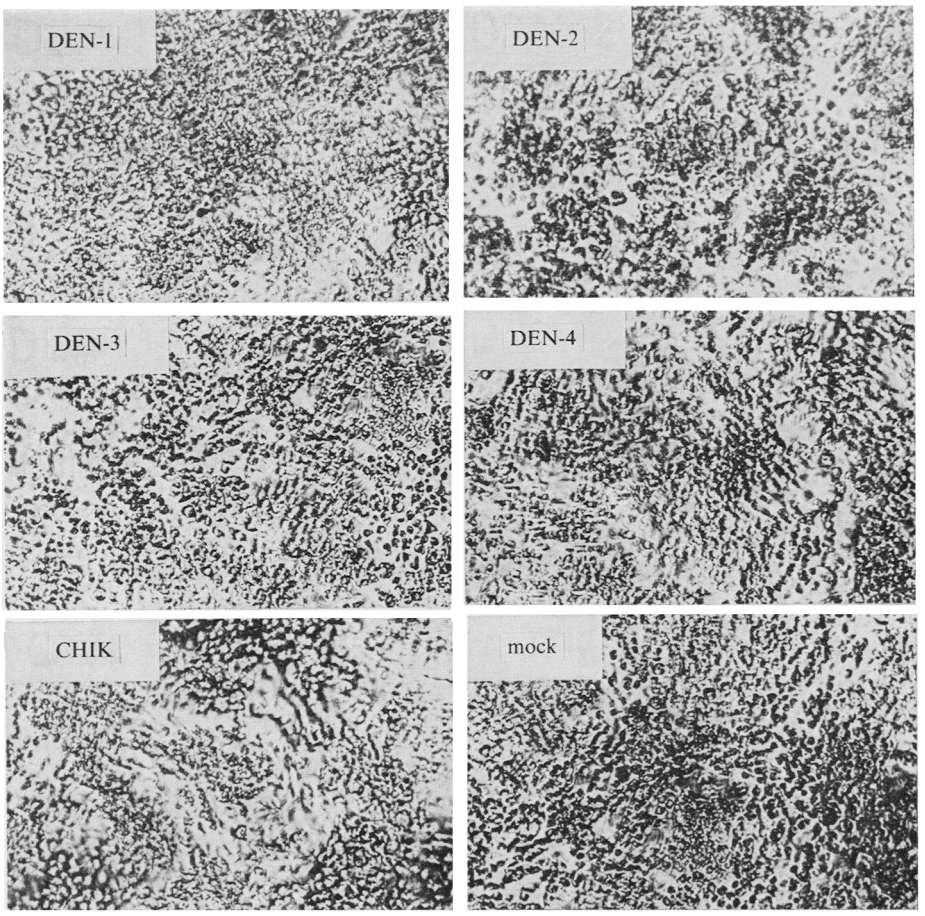

Fig. 3. Uncloned *A. albopictus* (SAAR) cells infected with DEN or CHIK viruses. Replicate cultures of uncloned SAAR cells were infected with DEN-1, DEN-2, DEN-3, DEN-4 or CHIK virus as described in the legend of Fig. 2. Photomicrographs were taken by an inverted microscope 6 days after incubation at 28 °C. Mock-infected cells are shown as a control.

described in the legend of Fig. 2. After 2 h adsorption, the residual virus was taken out and infectivities were assayed. No significant difference was observed between the infective virus remaining after adsorption to the C6/36 clone and to uncloned SAAR cells. After the residual virus was removed, cell sheets were washed twice with PBS and infected cells were suspended in virus maintenance medium at 4 °C (5 ml/bottle). Portions of cell suspensions were used to determine cell concentrations and the rest were serially diluted in the cold for infective centre titration. Diluted cell suspensions were inoculated on to BHK21 cells prepared in 8-chamber slides (0·1 ml/chamber). After 2 h adsorption at 28 °C, cells were incubated under virus maintenance medium at 28 °C in 5% CO_2 atmosphere for 24 h for CHIK, 4 days for DEN-2 and DEN-4, or 5 days for DEN-1 and DEN-3. The BHK21 cells were then fixed and processed for focus counting as described in Methods. Almost the same number of infective centres were recorded from infected C6/36 cells and uncloned SAAR cells for every virus tested. Even when the stock of DEN-2 or CHIK viruses with 10 times the concentration used in the above experiments were inoculated on to clone

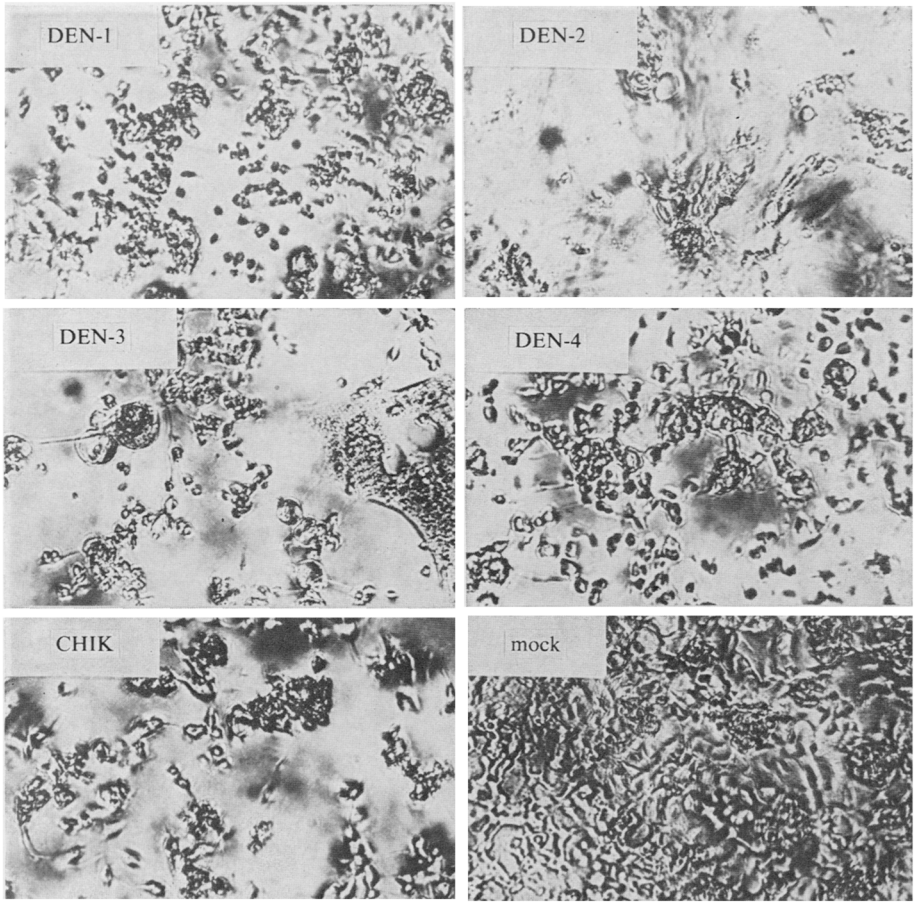

Fig. 4. *A. albopictus* clone C6/36 cells infected with DEN or CHIK virus. Replicate cultures of the clone C6/36 cells were infected with DEN-1, DEN-2, DEN-3, DEN-4 or CHIK virus as described in the legend of Fig. 2. Photomicrographs were taken by an inverted microscope 6 days after incubation at 28 °C. Mock-infected cells are shown as a control.

C6/36 or uncloned SAAR cells, the maximum virus titres obtained from uncloned SAAR cells were similar to those shown in Fig. 2 and they were always lower than the maximum virus titres from the C6/36 cells, although the time required to reach the maximum titres was shortened by about 1 day. The basic pattern of virus growth curves was essentially the same as shown in Fig. 2, when stocks of viruses prepared from infected C6/36 cells were inoculated. Thus, the higher virus yields from the infected C6/36 cells than from uncloned SAAR cells probably did not result from the more efficient adsorption of the seed viruses to the C6/36 clone than to uncloned SAAR cells.

No apparent cytopathic effect (c.p.e.) was observed in uncloned SAAR cells (Fig. 3). Most of the cells survived and the number of cells continued to increase until the 7th day after infection. Thus the levelling off and decline of the virus yields from uncloned SAAR cells 2 to 3 days after infection cannot possibly be due to the decrease in the viable cells supporting virus production. In contrast, clone C6/36 showed marked to moderate c.p.e. for every virus tested (Fig. 4). Patterns of c.p.e. were different from virus to virus. Cells infected with DEN-2 showed marked giant cell and syncytium formation beginning 2 to 3

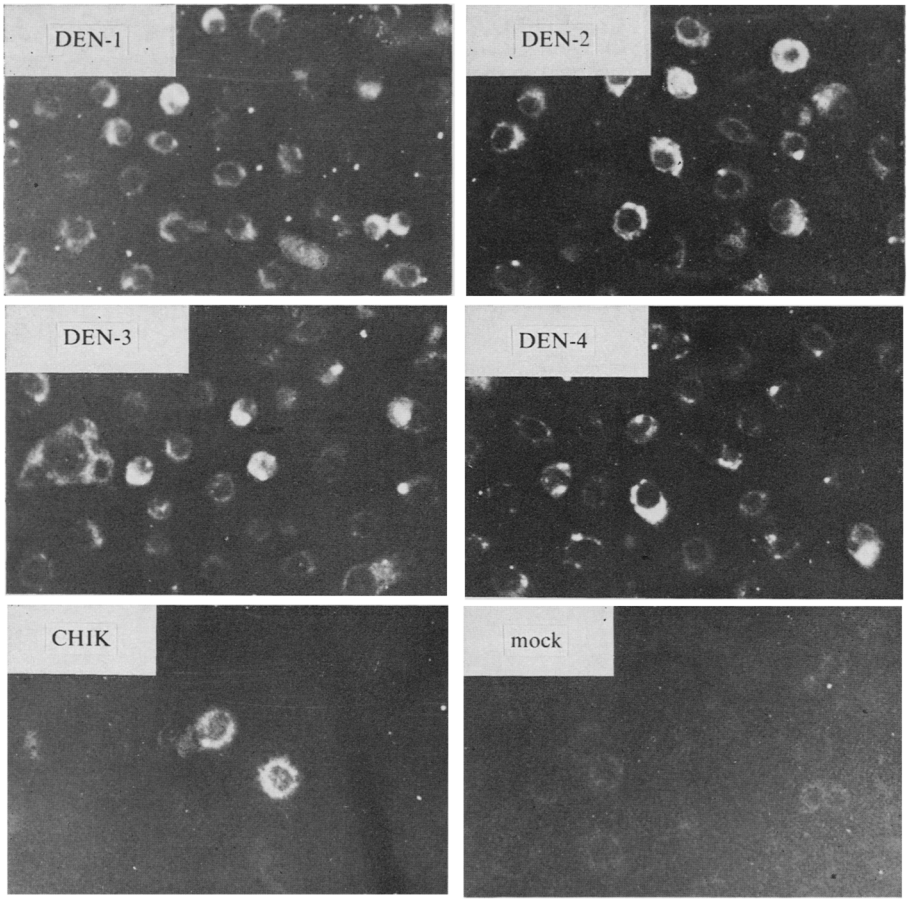

Fig. 5. Immunofluorescence of uncloned *A. albopictus* (SAAR) cells infected with DEN or CHIK virus. Replicate cultures of uncloned SAAR cells were prepared on coverslips in Leighton tubes by seeding 2 ml of 1×10^5 cells/ml suspension per bottle. After 3 days incubation at 28 °C, cells were infected as described in the legend of Fig. 2. Infected cells on coverslips were harvested after 2 days incubation for CHIK, 3 days for DEN-2 and DEN-4, or 4 days for DEN-1 and DEN-3, and were stained by the indirect fluorescent antibody technique as described in Methods. Mock-infected cells are shown as a control, which were stained with a mixture of antisera against five different viruses used in the test.

days after infection. In the case of DEN-1, DEN-4 and CHIK viruses, on the other hand, cell rounding was prominent. DEN-3 infected cells showed both cell rounding and giant cell formation.

Fluorescent antibody staining of infected uncloned SAAR cells revealed that only a portion of the cell population accumulated virus antigen in the cytoplasm and the intensity of the fluorescence varied from cell to cell (Fig. 5). In contrast, all the infected clone C6/36 cells accumulated virus antigen in their cytoplasm and the intensity of the fluorescence was similar in every cell. Remarkable cell surface projections in CHIK-infected C6/36 cells may reflect active virus budding from plasma membranes (Fig. 6). When specimens were harvested earlier for fluorescent antibody staining, 1 day after infection of CHIK and 2 days after infection of DEN viruses, there was no significant difference between the infected C6/36 lonec and uncloned SAAR cells in terms of the proportion of fluorescent positive

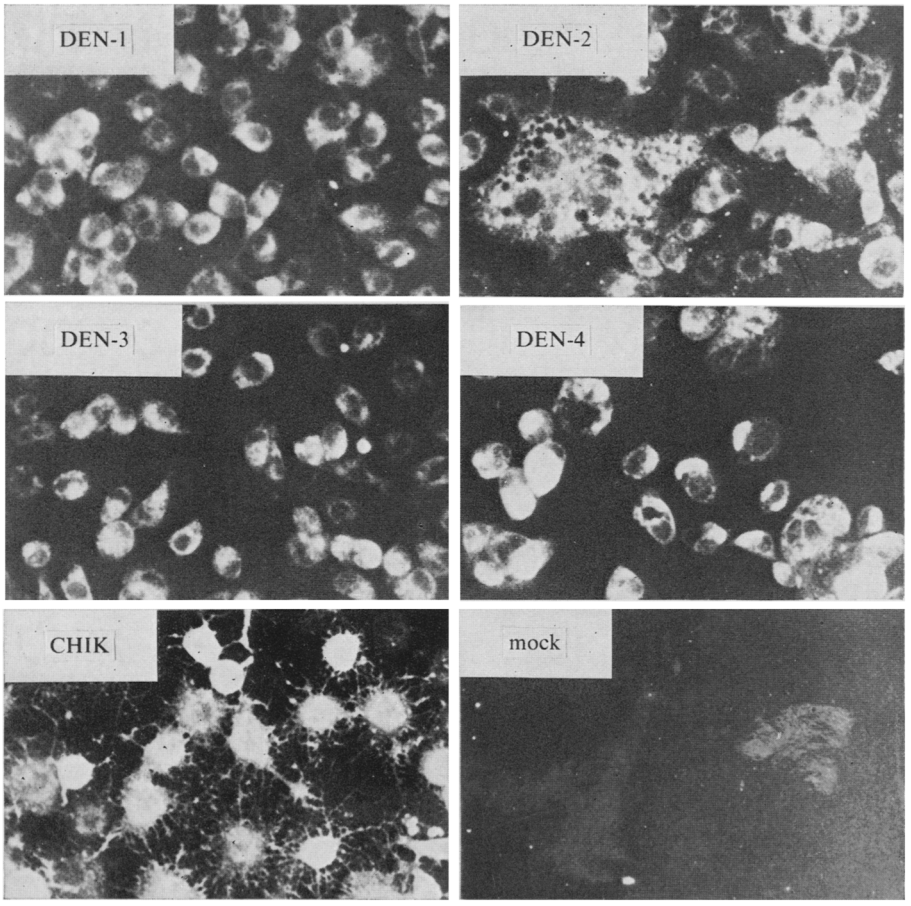

Fig. 6. Immunofluorescence of *A. albopictus* clone C6/36 cells infected with DEN or CHIK virus. Replicate cultures of clone C6/36 cells were prepared on coverslips and infected with DEN-1, DEN-2, DEN-3, DEN-4 or CHIK virus and stained by the indirect fluorescent antibody technique as described in the legends of Fig. 2 and 5.

cells and the intensity of the fluorescence in individual cells, as expected from similar initial growth rates of viruses in these two kinds of cells.

Chromosome numbers were determined for clone C6/36 and uncloned SAAR cells (Fig. 7). In both cases, the majority of the cells had XY type $2n = 6$ chromosomes with some $4n$ types (Fig. 8), and sometimes $8n$ types in SAAR cells. However, the distribution of chromosome numbers was significantly more uniform in the C6/36 clone than in uncloned SAAR cells ($P < 0{\cdot}001$).

Transfer of CHIK virus resistance from an A. albopictus *cell line to virus-sensitive clone C6/36*

Several reports have described the virus contamination of Singh's *A. albopictus* cell line (Cunningham *et al.* 1975; Hirumi *et al.* 1976). Such contaminating viruses could alter the sensitivity of the cells to other viruses by homologous or other interference mechanisms (Stollar & Shenk, 1973). An experiment was performed to test the possible transfer of virus resistance or susceptibility from the sensitive clone C6/36 to the relatively resistant uncloned

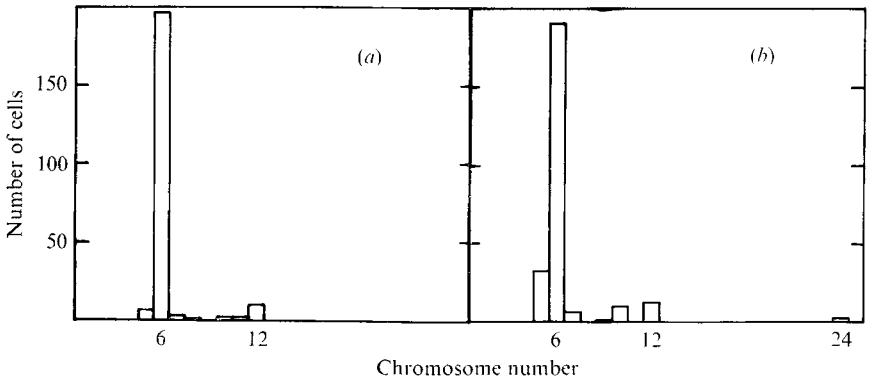

Fig. 7. Chromosome numbers of *A. albopictus* clone C6/36 and uncloned cells. Cultures of (*a*) clone C6/36 and (*b*) uncloned SAAR cells were prepared on coverslips and processed to give metaphase preparations as described in Methods.

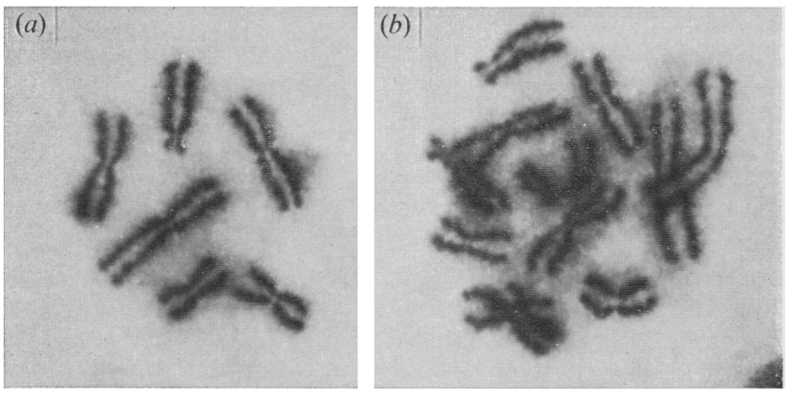

Fig. 8. Metaphase chromosomes of *A. albopictus* cells. Typical diploid (*a*) and tetraploid (*b*) metaphase chromosomes observed in the experiment of Fig. 7 are shown.

cells, or *vice versa*, through cell-free medium which had been used for growing each cell (Table 1). Uncloned SAAR or SAAK cells were grown for 6 weeks with the medium used for clone C6/36. Neither of the treated cell lines was found to increase its virus yield after this treatment. Also, the virus yields from C6/36 clone did not change after 6 weeks incubation in the medium used for the original uncloned SAAR cells. However, the yield of CHIK virus from clone C6/36 decreased markedly, to the level from SAAK cells, after incubation of the clone with the medium used for SAAK cells (SAAK medium). On the other hand, yields of SIND and DEN viruses did not change after the same treatment. Assay of the residual infectivity remaining after virus adsorption did not reveal any significant difference between the adsorption efficiency of CHIK virus to the sensitive clone and to the resistant cells. This transfer of specific resistance to CHIK virus by SAAK medium was established by overnight incubation. However, the transfer of the resistance was not established when SAAK medium was pretreated by heat (56 °C, 30 min), acid (pH 2, 4 °C, 2 h) or anti-CHIK serum (1:100 dilution, 28 °C, 2 h). Thus the resistance to CHIK virus was probably not mediated by interferons or interferon-like substances but rather by some virus related to CHIK, present in SAAK medium. However, the infectivity of this putative virus was not detectable by conventional plaque or fluorescent focus assay on BHK21 or LLC-MK_2 cells.

Table 1. *Alteration of virus sensitivity of* A. albopictus *clone C6/36 cells by incubation with the medium used for growing an uncloned* A. albopictus *cell line (SAAK)*

Host cells	Pretreating medium*	Virus yields in log (f.f.u./ml)† CHIK	SIND	DEN-1	DEN-2	DEN-3	DEN-4
C6/36	—	7·4	7·2	6·0	7·7	6·0	5·3
C6/36	SAAR	7·7	7·1	6·0	7·5	5·6	5·7
C6/36	SAAK	4·4	6·9	5·5	7·1	5·4	5·2
SAAR	—	6·1	5·6	4·8	5·4	3·0	4·8
SAAR	C6/36	6·2	5·9	5·0	5·9	3·6	4·9
SAAK	—	4·2	6·0	5·4	6·5	5·4	5·4
SAAK	C6/36	5·0	5·9	5·2	6·1	4·3	4·9

* Media used for growing C6/36 clone, uncloned SAAR or SAAK cells were passed through a Millipore type HA filter and were used to treat each clone or cell line for 6 weeks.

† Replicate cultures of each clone or cell line were prepared in Lab-Tek 8-chamber slides (1×10^5 cells/ml, 0·4 ml/well). After 3 days incubation at 28 °C, each well contained 2 to 4×10^5 cells. Medium was removed and seed viruses were inoculated (0·1 ml/well) at input multiplicities of 0·1 f.f.u./cell for DEN-1, DEN-3 and DEN-4 viruses, and 1 f.f.u./cell for DEN-2, CHIK and SIND viruses. After 2 h virus adsorption, residual virus was removed and cells were incubated under virus maintenance medium at 28 °C. Infective virus titre in the media was assayed after 2 days incubation for CHIK and SIND, 3 days for DEN-2 and DEN-4, and 4 days for DEN-1 and DEN-3 viruses, respectively.

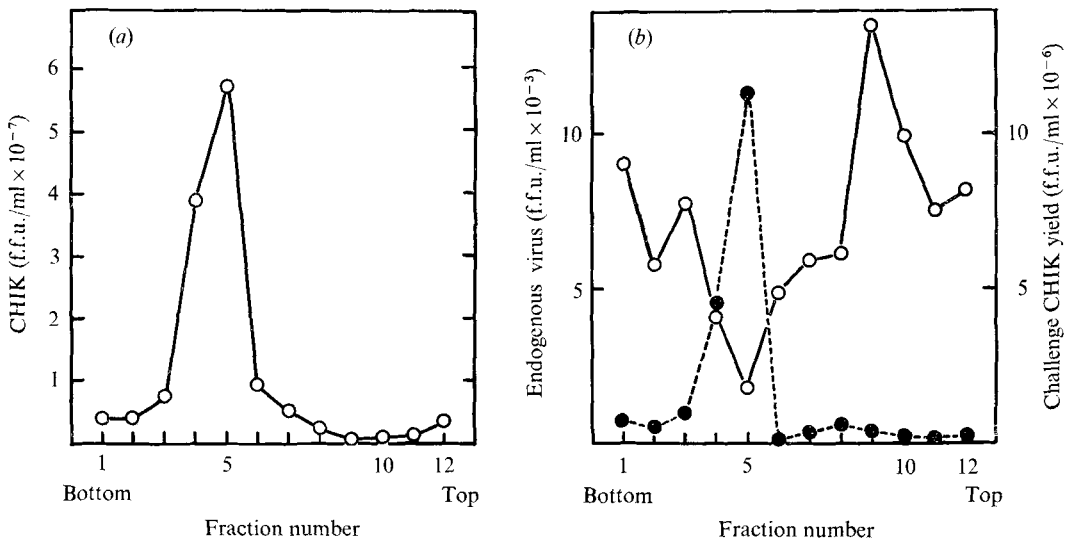

Fig. 9. Sucrose gradient sedimentation of standard CHIK virus and latent virus from *A. albopictus* SAAK cells. (*a*) The seed virus (0·5 ml) of standard CHIK was centrifuged through 4·5 ml of 15 to 30% sucrose gradient on 0·1 M-NaCl, 0·01 M-tris-HCl, 0·001 M-EDTA, pH 7·4, containing 0·2% bovine plasma albumin fraction V (Armour), at 35000 rev/min for 60 min at 4 °C in a Beckman SW 50·1 rotor. Fractions were assayed for CHIK virus infectivity on BHK21 cells (○——○). (*b*) *A. albopictus* SAAK cells were seeded at 1×10^5 cells/ml using 5 ml of suspension per 2 oz rubber-stoppered prescription bottle and were incubated at 28 °C for 3 days. Culture medium was harvested and centrifuged as in (*a*). Each fraction was assayed for its infectivity of endogenous virus by focus counting on C6/36 cells (●---●). A portion of each fraction was mixed with an equal volume of diluted standard CHIK virus (1×10^6 f.f.u./ml) and 0·1 ml of each mixture was seeded on to the C6/36 cells prepared in each well on Lab-Tek 8-chamber slides. The yield of standard CHIK virus (○——○) was assayed as described in Methods.

It was found that groups of cells containing cytoplasmic antigen reacting with anti-CHIK serum were observed by immunofluorescence in C6/36 cells when SAAK medium was inoculated and the cells were incubated at 28 °C for 20 h. This enabled us to assay the infectivity of this latent virus in SAAK medium. The SAAK medium was centrifuged through sucrose gradients and each fraction was assayed for its endogenous virus infectivity on C6/36 cells by focus counting. Fig. 9(*b*) shows a single peak of endogenous virus infectivity sedimenting at the same rate as the standard CHIK virus run in parallel (Fig. 9*a*). Each fraction of Fig. 9(*b*) was mixed with diluted standard CHIK virus and then inoculated on to C6/36 cells. The yield of standard CHIK virus was reduced most when the fraction having the highest infectivity of endogenous virus was present (Fig. 9*b*).

These results support the idea that the SAAK cell line is latently infected with some virus closely related to CHIK virus, and that the resistance to CHIK was transferred to C6/36 clone by infection with this latent virus present in SAAK medium. Later it was found that SAAK medium produced marked c.p.e. by syncytium formation in C6/36 cells and produced plaques under agar overlay after 3 days' incubation at 28 °C. The same kind of latent virus was found in an SAAK cell line newly obtained without adaptation to E medium.

DISCUSSION

Established lines of mosquito cells were derived from a large number of embryos or larvae (Peleg, 1966; Singh, 1967) and were apparently mixed populations. Cytopathic effects were described in Singh's *A. albopictus* cells infected with DEN-2 virus by Paul *et al.* (1969), whereas in the same virus–cell system, Sinarachatanant & Olson (1973) did not observe any c.p.e. Suitor & Paul (1969) observed c.p.e. in DEN-2 infected *A. albopictus* cells when the cells were cultivated in plastic containers but not in glass containers. We observed marked c.p.e. following DEN or CHIK virus infection in glass containers with cloned cells but not with uncloned cells. This indicates that the host cell population may be a determinant for c.p.e. Paul *et al.* (1969) and Buckley (1969) reported that CHIK virus caused no apparent c.p.e. in an *A. albopictus* cell line in spite of continuous virus production. On the other hand, Yunker & Cory (1975) reported plaque formation with the same virus–cell system.

Cell clones of *A. albopictus* which responded differently in terms of c.p.e. to SIND virus were described recently (Sarvar & Stollar, 1977). One of the clones showed marked c.p.e. and the other did not. Still, both clones produced almost the same level of virus yield. In this study, marked c.p.e. seemed to be associated with higher virus yield in the clone. Virus yields from uncloned SAAR cells might be low because the cell line is a mixed population of sensitive and less sensitive cells. However, most of the clones yielded almost the same virus titre as uncloned SAAR cells and completely virus-resistant clones were not obtained in this study.

In clone C6/36, virus remained at a high level for up to 7 days after infection, whereas in uncloned cells infectivities tended to level off 2 to 3 days after infection and then declined, although the initial rates of virus growth were almost the same in these two kinds of cells. No significant difference was observed between the virus adsorption to the cloned and uncloned cells as far as we tested. This suggests that some nutritional limitation or regulatory mechanism is effective in uncloned SAAR cells. Such regulation of virus production was observed in *A. albopictus* cells recently infected with Semliki Forest virus (Davey & Dalgarno, 1974) or persistently infected with SIND virus (Igarashi *et al.* 1977), and appeared to be related to shut off of virus RNA synthesis. Although we have not looked at the virus

RNA synthesis in cloned and uncloned cells, the virus-sensitive C6/36 clone may lack efficient regulatory mechanism for virus RNA synthesis and virus production or may be less demanding metabolically and nutritionally.

Another *A. albopictus* cell line (SAAK) is contaminated with some latent virus. This virus seems to be closely related to CHIK, since SAAK medium transferred specific resistance to CHIK to the C6/36 clone and this transfer was blocked by previous incubation with anti-CHIK serum. The infectivity of this latent virus was not demonstrated by the conventional assay methods for standard CHIK virus using vertebrate cells. However, it was easily detectable by virus-sensitive mosquito cell clone C6/36. The presence of latent CHIK virus (Cunningham *et al.* 1975) or CHIK-related virus (Hirumi *et al.* 1976) in Singh's *A. albopictus* cell line has been described and the virus recovered from such contaminated cells differed from standard CHIK in terms of virulence or antigenicity (Buckley *et al.* 1976). As described in this report, such latent viruses in mosquito cell lines may be more easily detectable by a sensitive mosquito cell clone than by vertebrate cells. A latent virus in Peleg's *A. aegypti* cell line (Stollar & Thomas, 1975) was detected by Singh's *A. albopictus* cell line but not by vertebrate cells.

Geographical variation was reported in the vector competence of *A. albopictus* mosquitoes to CHIK or DEN viruses (Gubler & Rosen, 1976; Tesh *et al.* 1976) and seemed to be at least in part genetically controlled. In this report, heterogeneity of the sensitivity to virus, as tested by the virus yields, was observed in individual cell clones even when the original cell line was derived from a population of mosquito larvae from a single location. The observations described in this report were obtained at the cell culture level and may be quite unrelated to the phenomenon in the natural environment. We do not know when and how the SAAK cells were contaminated with the virus related to CHIK. However, if the observations at cell culture level apply to the level of whole mosquitoes or mosquitoes in natural environments, then vector mosquito populations may become resistant to or at least less sensitive to certain arboviruses, not only by genetically controlled factors but also by the prevalence in mosquitoes of latent viruses closely related to the arboviruses. Such latent viruses in mosquitoes may be more easily detected by virus-sensitive mosquito cells than by vertebrate hosts.

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