Phenotypic Changes in the Flavivirus Kunjin after a Single Cycle of Growth in an Aedes albopictus Cell Line

By MAH LEE NG† AND E. G. WESTAWAY*

Department of Microbiology, Monash University, Clayton, Melbourne, Australia 3181

(Accepted 31 March 1983)

SUMMARY

The properties of Kunjin virus produced during acute infections of Aedes albopictus (Aal) mosquito cells were compared with those of the virus progeny from the C6/36 clone of mosquito cells and from Vero cells. Titres of $10^8$ p.f.u./ml or greater were obtained from all cells, but significant haemagglutinin activity was associated only with progeny from Vero and C6/36 cells. Kunjin virus from Aal cells adsorbed to goose erythrocytes and blocked haemagglutination by virus from Vero or C6/36 cells. High titres of virus from Aal cells were obtained only when the cells were grown in the presence of 10% or higher concentrations of foetal calf serum, and large losses were encountered when this virus was concentrated by pelleting or by precipitation with polyethylene glycol, and during rate-zonal sedimentation through sucrose gradients. Because of these losses, and because of poor incorporation of $[^35S]$methionine in Aal cells, only analytical amounts of labelled structural proteins were recovered. Electropherograms showed that the presumptive envelope protein E of Kunjin virus from Aal cells migrated more rapidly than E of virions grown in C6/36 and Vero cells, and that the core (C) and membrane-like (M) proteins were not detectably labelled. All the observed changes in phenotype were reversed during one cycle of growth in Vero cells.

INTRODUCTION

Flaviviruses share with many other arthropod-borne viruses the ability to replicate in mammalian, avian and insect hosts, and also in cell cultures derived from these hosts (Westaway, 1980). Relatively high yields of flaviviruses are obtained during acute infection in mosquito cell lines, and persistent infections are readily established; some flaviviruses, including Kunjin virus, induce syncytium formation in these cells (Igarashi, 1978, 1979; Ng & Westaway, 1980; Stollar, 1980).

When examining the progeny of Kunjin virus grown in Aedes albopictus (Singh) cells, we were surprised to find that despite high yields of infectious virus during acute infections, there was virtually a complete lack of associated haemagglutinin activity. This observation prompted a comparison of the growth and properties of Kunjin virus replicating in Vero (mammalian) cells, the A. albopictus cells and the C6/36 clone derived from these mosquito cells (Igarashi, 1978).

METHODS

Viruses and cells. The strains of Kunjin virus, dengue-2 virus and Japanese encephalitis virus have been described previously (Westaway, 1966). Virus pools were prepared from a 10% suspension of infected suckling mouse brain. Vero cells and the A. albopictus (Singh) cell line (Aal cells) were grown and maintained as described previously (Ng & Westaway, 1979). C6/36 cells supplied by Dr A. Igarashi were grown at 28 °C in Eagle’s basal medium supplemented with 10% foetal calf serum (FCS) (heated at 56 °C for 0.5 h) and non-essential amino acids (0.2 ml of each), adjusted to pH 7.2 with NaHCO₃.

Labelling of virions during growth, concentration and purification. Vero cells and C6/36 cells were infected with 10 p.f.u./cell and incubated at 37 °C in Eagle’s minimal essential medium containing 0.1% bovine serum albumin.

† Present address: Department of Microbiology, National University of Singapore, Kent Ridge 0511, Singapore.
(MEM + BSA), supplemented with non-essential amino acids (0.2 mM of each) for C6/36 cells only. Aal cells were infected similarly and maintained at 28 °C in modified Mitsuhashi and Maramorosch medium (Buckley, 1969) with the supplemented amino acid components of MEM replacing lactalbumin hydrolysate, plus 10% FCS. All cells were labelled from 8 h to either 30 or 48 h with 200 μCi [35S]methionine in maintenance medium with the methionine content reduced to 15 μg/ml for Vero cells; for the two mosquito cell lines 75 μg/ml of unlabelled methionine was required for the support of efficient virus replication (see Results).

The virus yields from cell culture fluids were clarified, concentrated by precipitation with polyethylene glycol (PEG), and purified by rate-zonal sedimentation through 5 to 25% (w/v) sucrose as previously described (Westaway & Reedman, 1969).

Haemagglutination (HA) assay. This was based on the method of Clarke & Casals (1958), using microtitre trays. Lipid inhibitors of HA in infected culture fluids were removed by acetone extraction (Clarke & Casals, 1958). The optimum pH for HA at 37 °C was 6.4 for Kunjin virus grown in Vero cells and 6.0 for virus grown in C6/36 cells.

Reagents and isotopes. Actinomycin D (Act D) was a gift from Merck, Sharp & Dohme, Australia. [35S]Methionine (sp. act. 1300 Ci/mmol) and the 3H-labelled amino acid mixture (code TRK440) were obtained from Amersham International.

RESULTS

Growth curves and yields of Kunjin virus in mosquito cells

The latent periods and virus yields were similar in both cell lines maintained with FCS, but the yield at 28 °C in C6/36 cells was about 50-fold less when BSA replaced FCS (Fig. 1). The large decline in virus yields after 3 days at 37 °C was apparently due to cell death. As reported previously (Ng & Westaway, 1980) syncytium formation was induced in Aal cells at 28 °C, whereas C6/36 monolayers remained unchanged until 5 days post-infection when cells rounded and detached. Although the HA titres in culture fluids of C6/36 cells reached 4 units (at 28 °C) or 16 units (at 37 °C), no HA was obtained from infected Aal cells.

Labelling, concentration and purification of virions

In order to reduce contamination by serum proteins during PEG precipitation of virions prior to purification, infected Aal cells were incubated in medium containing 10% dialysed FCS from which some of the protein was eliminated before use by precipitation with 6% PEG; under these conditions, the yield still reached 10⁸ p.f.u./ml at 28 °C. For labelling virions from Aal cells with [35S]methionine, the methionine concentration was increased to 75 μg/ml because the yield of infectious virus was reduced by 100-fold when the concentration was only 15 μg/ml, as used for labelling in Vero cells. A similar inhibition of Sindbis virus replication in A. albopictus cells caused by methionine deficiency has been reported by Stollar (1978).

Virus yields in culture fluids from infected cells were compared after being concentrated 20- to 30-fold by precipitation with 6% PEG; less than 10% of the p.f.u. remained in each of the supernatant fluids. The recovery of p.f.u. from the precipitates was 30% and 61% from the C6/36 and Vero cells respectively, but only 2 to 4% from Aal cells. We obtained better recoveries of virus from Aal cells (16 to 29% of p.f.u.) by pelleting from culture fluids through a 5% sucrose cushion in Tris–saline buffer (0-12 mM-NaCl, 0-012 M-Tris–HCl pH 7) for 3 h at 25000 rev/min in an SW28 rotor at 4 °C, but only a trace amount of HA (4 units or less at an optimum pH of 6-0) was detectable. After purification of the resuspended virus pellets by rate-zonal centrifugation, the recovery of infectivity (Fig. 2a) and HA (32 to 64 units; results not shown) in the peak from C6/36 cells (fractions 8 and 9) was greater than or equal to the recovery from Vero cells. However, the peak of slow sedimenting haemagglutinin (SHA) in fractions 25 to 28 (HA titre 32) of the harvest from Vero cells (not shown) was absent from harvests of C6/36 cells; a similar lack of SHA production during dengue-2 virus infection of A. albopictus cells has been reported by Sinarachatanant & Olson (1973). The peak of infectious virus and the coincident peak of cpm for virions from Aal cells were 1000-fold and about 100-fold less respectively, than from C6/36 cells, and no HA activity was detected in any of the gradient fractions. These experiments were repeated three times with similar poor recoveries. Furthermore, no labelled infectious peak of Kunjin virus was obtained after Aal cells were labelled post-infection with 100 μCi 3H-labelled mixed amino acids in medium with reduced concentrations of amino acid supplements. Thus,
Mosquito cells induce changes in Kunjin virus

Fig. 1. Growth curves and yields of Kunjin virus Aal and C6/36 cells at (a) 28 °C and (b) 37 °C. Total harvests were collected at the specified times for p.f.u. and HA assays. □, HA activity released from C6/36 cells; ●—●, virus released from Aal cells in growth medium supplemented with 20% FCS; ●—○, yield of infectious virus from C6/36 cells in medium supplemented with 10% FCS; ○—○, yield of infectious virus from C6/36 cells in medium supplemented with 0.1% BSA.

virus from Aal cells appeared to be strikingly more fragile during concentration or purification, compared to virions of similar titre grown in C6/36 and Vero cells. In contrast, dengue-2 and Japanese encephalitis viruses grown in Aal cells at 28 °C (titres 10^7 p.f.u./ml) were pelleted from culture fluids with recoveries of 60% and 50% respectively, of infectious virus, and 32 to 64 HA units.

Particle counts of Kunjin virus were compared after pelleting virions of similar infectious titre from C6/36 and Aal cells through a sucrose cushion (see previous paragraph); resuspended
Fig. 2. Purification by rate-zonal sedimentation of Kunjin virus labelled with $^{35}$Smethionine during growth in Aal, C6/36 and Vero cells. The virus preparations in (a) and (b) were concentrated for centrifugation as described in Methods. (a) Recovery of infectious virus from Aal cells (●---●), C6/36 cells (■---■) and Vero cells (○---○); (b) radioactive counts for the fractions from the sucrose gradients in (a), with symbols as in (a); (c, d) ct/min of sedimentation profiles of unconcentrated preparations from mock-infected (c) and Kunjin virus-infected (d) Aal cell culture fluids. All preparations were centrifuged through 5 to 25% (w/v) sucrose gradients for 3 h at 25,000 rev/min in an SW28 rotor, and 1 ml fractions were collected from the base of the tube. Sample sizes for ct/min were 10 μl for all fractions from Vero and C6/36 cell preparations, and 100 μl for Aal cells.
pellets were stained with ammonium molybdate on carbon-coated Formvar on grids. Particles from either cell type appeared identical by negative staining and their counts were not significantly different.

Because of the fragility of Kunjin virus from Aal cells, we attempted to purify it for further characterization with minimum processing of culture fluids. Infected Aal cells were labelled with $[^{35}\text{S}]$methionine in a vol. of only 1 ml, which was later applied directly to a purification gradient (Fig. 2d); fluid from a mock-infected culture was processed similarly (Fig. 2c). An
improved radioactive peak (compared to Fig. 2b) in the expected position of the virion (fraction 24, Fig. 2d) had an infectious titre of $10^6$ p.f.u./ml and an HA activity of 2 to 4 units at optimum pH 6-0, which was inhibited with acetone-extracted antiserum (Clarke & Casals, 1958) to Kunjin virus. From the uninfected culture fluid only, a peak of radioactivity was observed in the 70S to 80S region (fraction 24, Fig. 2c). These results were reproducible. Virus selection did not occur in Aal cells because Kunjin virus harvested from Vero cells at 24 h after infection with virus from Aal cells had properties similar to virus progeny obtained using standard infectious pools. Thus, the fragile nature of Kunjin virus from Aal cells is probably due to a phenotypic change in the virus acquired during replication in these cells.

**Adsorption of virions to goose erythrocytes**

Kunjin virus grown in each of the three host cell types was pelleted and then diluted so that each preparation represented $10^6$ p.f.u. in 1 ml of borate-buffered saline (pH 9) + BSA. A 1 ml amount of the virus adjusting diluent at the appropriate pH (see Methods) containing a 1/300 suspension of goose erythrocytes was added to 1 ml of each virus suspension, and the mixtures were shaken slowly at room temperature for 30 min; macroscopic clumping of the erythrocytes was observed only in the tubes containing virus grown in Vero or C6/36 cells. The erythrocyte suspensions were then filtered on membrane filters (average pore diam. 0.22 μm); plaque assays of the filtrates showed that the infectivity was reduced by 90% for Aal virus and by 100% for virus grown in Vero and C6/36 cells. Hence, despite the lack of efficient haemagglutination, nearly all the Kunjin virus from Aal cells was apparently adsorbed to goose erythrocytes.

**HA blocking activity of Kunjin Aal virus**

Pelleted Kunjin Aal virus was resuspended in borate-buffered saline + BSA: the trace amounts of HA were diluted to less than 2 HA units before the suspension was mixed with an equal volume of goose erythrocytes, the final pH being adjusted to 6-0. The mixture was shaken at room temperature for 30 min before aliquots were added to equal volumes of 8, 4, 2 and 0 HA units in Kunjin virus-infected culture fluids from C6/36 cells and from Vero cells (final pH adjusted to 6-4 for Vero cell-grown virus), and then incubated at 37 °C for 45 min to allow haemagglutination to occur. The ability of the erythrocytes to be agglutinated by 8 HA units or less of Kunjin virus grown in Vero and C6/36 cells was completely blocked after being pre-adsorbed with virus from Aal cells.

**Structural proteins of Kunjin virus from Aal cells**

[$^{35}$S]Methionine-labelled proteins in virions purified after growth in C6/36 cells, or in Aal cells followed by a single cycle of growth in Vero cells, were electrophoresed; they comprised the expected structural proteins E, C and M (Fig. 3). However, the presumptive E protein from purified Aal virions migrated slightly faster than expected, and C and M were not detected (Fig. 3a). This result was reproducible with different preparations of virions (Fig. 3b). The particles sedimenting in the 70S to 80S region from culture fluids of uninfected Aal cells (Fig. 2c) were labelled in a protein equivalent in migration to E from the Aal cell virions (Fig. 3a).

**Kunjin virus-specified proteins labelled in mosquito cells**

Infected cells were pretreated for 1 h with 1 μg/ml Act D before being labelled with [$^{35}$S]methionine from 24 to 28 h post-infection in the presence of 1·5 μg/ml methionine. Incorporation of acid-precipitable ct/min into C6/36 cells was about 50% greater than into Vero cells, whereas incorporation into Aal cells (both mock-infected and Kunjin virus-infected) was 15- to 30-fold less than in C6/36 cells at either 28 or 37 °C. The rate of protein synthesis in Aal cells is thus much slower than in C6/36 cells. When labelled virus-infected cytoplasma was electrophoresed, the migration in slab gels of P51(E), the intracellular non-glycosylated form of envelope protein E, and P14(C), the intracellular equivalent of core protein C, was the same irrespective of the infected cell source, i.e. Vero, Aal or C6/36 cells (results not shown). These results shed no light on the apparent difference in migration of E from virions labelled in Aal cells (Fig. 3).
Fig. 3. Electrophoresis in 8% SDS-phosphate gels of Kunjin virus structural proteins labelled with $^{35}$S)methionine during growth in Aal, C6/36 (C6) and Vero cells. All samples except A/V Vn are from the purification gradients in Fig. 2. The structural proteins are designated according to the recently amended nomenclature system (Westaway et al., 1980) in which E, C and M represent the envelope protein (formerly V3), the core protein (V2) and the membrane-like protein (V1) respectively; GP19 (formerly NV2) is the glycoprotein associated with SHA, the slow sedimenting HA released from infected Vero cells (Westaway & Shew, 1977). Un, Uninfected cells; Vn, virion. In (a) Aal Vn and Vero Vn are the respective virus peaks in Fig. 2(b); Un Aal 70S, Aal 70S and Vero SHA represent the 70S to 80S region of gradients in Fig. 2(c) (fraction 24), 2(d) (fraction 25) and 2(b) (fractions 25 to 28) respectively. In (b) Aal Vn is the virus peak (fraction 9) in Fig. 2(b); A/V Vn is the Kunjin virus progeny after growth in Aal cells followed by transfer to Vero cells for a single cycle of growth. Particles were pelleted from the relevant fractions after dilution to less than 5% sucrose, in an SW56 rotor for 3 h at 55,000 rev/min; the recoveries of cts/min after pelleting were only about 20% for the Aal virus peak, and 10% for the 70S peak from mock-infected Aal cells. Gel (b) was run short relative to gel (a). The open arrowheads (•) indicate the E protein associated with virions and with the 70S to 80S region in preparations from infected Aal cells. Samples were boiled for 2 min in SDS before electrophoresis as described previously (Westaway & Shew, 1977).

DISCUSSION

The phenotypic changes induced in Kunjin virus during acute infections of Aal cells appear to be associated with altered properties of E, the envelope protein. The changes comprise: (i) negligible amounts of HA activity despite high p.f.u. titles; (ii) the ability of such virions to bind to erythrocytes and block HA by active haemagglutinin grown in other cells; (iii) consistent instability in virion structure and infectivity when subjected to precipitation by PEG, pelleting or rate zonal sedimentation; (iv) increased migration of the (presumptive) E protein during electrophoresis, and lack of detectable labelling of C and M proteins with $^{35}$S)methionine.

The lability of Kunjin Aal virus during centrifugation and the loss of HA activity were not observed with two other flaviviruses, or with Kunjin virus grown in C6/36 mosquito cells. The
apparently smaller size of E was unexpected, because the intracellular precursor of E labelled in each of the three cell lines migrated identically. Recently it was shown in this laboratory that Kunjin E lacks carbohydrate (Wright, 1982); hence, there is no analogy with the incomplete glycosylation and faster electrophoretic migration of envelope proteins of alphaviruses grown in A. albopictus cells (Luukkonen et al., 1977). A reduction in size of Kunjin E could result from post-translational trimming in Aal cells, as yet undetected.

Despite the relatively high yields of Kunjin virus from Aal cells, we were unable to recover sufficient amounts of purified virus to characterize E by tryptic peptide mapping, or to compare E with the host protein of similar size associated with a 70S to 80S particle (Fig. 3a). These consistently poor recoveries were compounded by the inefficient incorporation of $^{35}$S-methionine in Aal cells compared to incorporation in the other two cell lines. A larger protein (mol. wt. 54000) was reported to be released from C6/36 cells (Reigel & Koblet, 1981), and enhanced synthesis starting 2 days post-infection of a smaller protein (mol. wt. 43000, associated with a nuclear ribonucleoprotein particle) was reported in alphavirus infections of the A1 Cl 19 clone of A. albopictus cells (Eaton, 1982). We have not observed enhanced synthesis of any host protein during Kunjin virus infection, and have not detected any evidence of an adventitious agent in uninfected Aal cells by electron microscopy of thin sections or by intracerebral inoculation of culture fluids into suckling mice (Ng & Westaway, 1979; unpublished results).

Although virions containing the faster migrating E protein lacked significant HA activity, they retained the capacity to attach to erythrocytes. The HA blocking tests indicated that the sites of adsorption were equivalent to those of the parental virus. Furthermore, when trace amounts of HA were detected after virus concentration, this HA was blocked by antiserum to parental Kunjin virus. The phenotypically changed progeny also retained high infectivity for both Vero cells and mosquito cells, and continued to cause syncytium formation efficiently in acute and persistently infected Aal cells (Ng & Westaway, 1980). These results argue against incorporation or substitution into virions, in lieu of E, of the host protein released into culture fluids, but the possibility cannot be completely excluded at present. The anomalous nature of the virion structure, namely smaller E and lack of detectable C and M proteins, suggests that this is the basic reason for instability of structure and lack of HA activity. The morphogenesis of flaviviruses remains undefined but may occur by a condensation process rather than by budding of a nucleocapsid, which has never been unequivocally demonstrated (Murphy, 1980). In thin sections of Kunjin virus-infected Aal cells, the morphology of accumulating virions appeared normal, and no budding was observed (M. L. Ng & E. G. Westaway, unpublished results).

Further characterization of the phenotypic changes in Kunjin Aal virus awaits development of improved methods of preserving the integrity and infectivity of virus particles during purification. Such information may help overcome the present dearth of knowledge in flavivirus research regarding the detailed architecture and mode of assembly of virions, the mechanism of haemagglutination and syncytium formation in infected mosquito cells.

This work was supported by grants from the National Health and Medical Research Council of Australia and the Special Research Fund of Monash University. Ms M. Goodman and Ms Khim Hoe provided valuable assistance with electron microscopy. The advice and criticism of Dr Peter Wright are much appreciated.

REFERENCES


Mosquito cells induce changes in Kunjin virus


(Received 10 August 1982)