A Comparative Study of Related Alphaviruses – a Naturally Occurring Model of Antigenic Variation in the Getah Sub-group

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SUMMARY

The relative susceptibilities of six cell culture lines were compared using six closely related alphaviruses. Plaque morphology was used as a parameter for biological differences of these viruses in parallel with plaque reduction neutralization tests. One virus showing mixed plaque sizes was plaque purified and the two variants thus obtained were compared by in vivo and in vitro methods. The implications of plaque variants within a mixed virus population are considered along with possible natural selective mechanisms in the evolution of these related viruses of synpatric distribution.

INTRODUCTION

The twenty or so Togaviruses belonging to the alphavirus genus have been shown by Karabatsos (1975) and Chanas, Johnson & Simpson (1976) to be subdivisible into three antigenic complexes. The largest of these, headed by Venezuelan equine encephalitis virus can be further divided into smaller sub-sets. Four such viruses, Getah (GET), Bebaru (BEB), Sagiyama (SAG), and perhaps Ross River (RR) are closely related. Two additional strains, Itakura (ITAK) and Haruna (HAR) isolated in Japan, were once considered as separate viruses but do not appear in the current Arbovirus Catalogue (Berge 1975), having been found identical to SAG and GET respectively. We therefore undertook to study these viruses closely in order to determine any biological and antigenic differences that may exist between them, and the influence of recent passage history in the evolution of the sub-group.

Plaque variants occur with many viruses (Takemoto, 1966; Pedersen, Slocum & Robinson, 1972; Jahrling, 1976) and we have already indicated that plaque variation existed in this sub-group (Chanas et al. 1976). At least one of these, the HAR strain of GET consisted of variants producing distinctly mixed populations of plaques. We report here studies on small plaque (SP) and large plaque (LP) purified variants of HAR, undertaken to determine any corresponding differences in biological properties.

METHODS

Viruses and antisera. Immune antisera, except for those against the two HAR variants, were prepared in New Zealand White rabbits by two 1·0 ml intramuscular inoculations. Hyperimmune sera were produced as described previously by Chanas et al. (1976). Equal volumes of virus seed and Freund’s complete adjuvant were thoroughly shaken for 30 min. The inoculations were spaced 10 days apart and the rabbits were bled 10 days after the last
inoculation. The antisera against the HAR variants were prepared by intraperitoneal inoculations of adult white mice with 0.25 ml of seed; the intervals were as for the rabbits. Details of the viruses are shown in Table 1. All seeds consisted of 20% SMB suspension in phosphate buffered saline. A new seed of HAR virus was obtained from Dr A. Oya, National Institute of Health, Tokyo, and fresh stock was prepared to eliminate the possibility of our seed having been contaminated with another virus during previous experiments.

**Tissue culture.** Six cell lines were used: hamster BHK21, monkey Vero and LLC-MK2, pig PS, Xenopus toad XTC₂ (Pudney, Varma & Leake, 1973) and mosquito *Aedes pseudo-scutellaris* M61 (Varma, Pudney & Leake, 1974). The cell lines were all cultured and maintained in L15 (Leibowitz, 1963) medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and antibiotics. Incubation was at 37 °C except for XTC₂ and M61 cells which were incubated at 28 °C.

**Virus assays.** Titrations were performed in disposable tissue culture plates (Linbro, model FB16-24 TC multi-dish). Tenfold dilutions were made with 2% FBS, 10% TPB and antibiotics in the plates containing 0.45 ml of L15 medium. Cell suspension (0.5 ml) containing 500000 cells/ml was then added, and incubated at the appropriate temperature for 4 h. One ml of overlay consisting of equal volumes of carboxymethyl cellulose salt (3%) and double strength L15 supplemented with 2% FBS, 10% TPB and antibiotics was also added. The plates were then incubated inside sealed plastic bags and stained (0.1% naphthalene black in 10% acetic acid) after 4 and 8 days.

**Cross-neutralization tests.** A plaque reduction neutralization test was used to check the results previously reported by us (Chanas *et al.* 1976). Tenfold virus dilutions (0.5 ml) were mixed with a constant dose of antisera (0.5 ml). After 1 h at 37 °C, 0.5 ml of L15 containing 500000 Vero cells/ml was added. After a further 4 h incubation, 1 ml of overlay was added. All dilutions were prepared in L15 with 2% FBS. Serum dilutions are indicated in Table 2.

**Plaque purification.** The 50% end-point dilution of the HAR seed was delivered dropwise into a micro-tissue culture plate (M29 ART Gibco). Vero cells and overlay were added as previously described. After 2 days incubation all wells with single plaques were recorded and the plate was incubated a further 4 days. The supernatants of all wells recorded were then transferred to a corresponding well in a Linbro plate. Vero cells and overlay were again added and after 4 days incubation about 2 ml of supernatant from each well was separately harvested and the plate was stained. The supernatants from all wells showing homogeneous LP or SP variants were passaged into 200 ml glass medical flat bottles containing confluent Vero monolayers. The viruses harvested from these were intracerebrally inoculated into suckling mice from which an SMB seed was made. The plaque morphology of all variants obtained was checked in Vero cells and classified as LP, SP or mixed. All subsequent experiments were performed with the same pools of homogeneous or heterogeneous viruses.

**Interference testing.** A chequerboard challenge titration was devised to determine any direct virus interference by the LP and SP HAR variants. Tenfold dilutions of one variant were delivered in lines down a micro-tissue culture plate containing Vero cells. Three hours later the cells were challenged with tenfold dilutions of the other variants in rows across the plate. Overlay was added after overnight incubation. The variant(s) from each well were checked after 4 days by titration in Linbro plates.

**Mouse inoculations.** Suckling mice were inoculated intradermally (i.d.), intraperitoneally (i.p.) or intracerebrally (i.c.) with 0.01 ml of suspension containing 10⁴⁻⁰ infective doses/ml to produce virus stocks or to study virulence.

**Mosquito feeding and maintenance.** *Aedes aegypti*, from a colony maintained at the
Table 1. Tissue culture characteristics of six arboviruses

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>BHK</th>
<th>Vero</th>
<th>LLC-MK²</th>
<th>PS</th>
<th>XTC₁</th>
<th>M6t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 days</td>
<td>8 days</td>
<td>4 days</td>
<td>8 days</td>
<td>4 days</td>
<td>8 days</td>
</tr>
<tr>
<td>Beharu MM 2354* P.11</td>
<td>5-7 mm clear irregular</td>
<td>14 mm clear regular</td>
<td>5-8 mm clear irregular</td>
<td>Overgrown</td>
<td>3 mm turbid irregular</td>
<td>4-6 mm irregular turbid</td>
</tr>
<tr>
<td>Haruna JaG AN 185 P.10</td>
<td>2-3 mm round</td>
<td>5-7 mm irregular mixed population clear</td>
<td>1 mm round</td>
<td>5-7 mm irregular mixed population clear</td>
<td>2 mm</td>
<td>5-7 mm mixed population clear</td>
</tr>
<tr>
<td>Getah MM 2021 P.10</td>
<td>4-7 mm clear slightly irregular</td>
<td>10-12 mm clear regular</td>
<td>1-4 mm clear irregular wide range</td>
<td>3-7 mm clear irregular wide range</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Itakura Original P.9</td>
<td>6-8 mm clear round</td>
<td>10-12 mm clear round</td>
<td>5-7 mm round turbid</td>
<td>9-12 mm round clear</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Sagiyama Original P.8</td>
<td>2 mm clear irregular</td>
<td>2-3 mm clear irregular</td>
<td>1-2 mm clear irregular</td>
<td>1-2 mm clear irregular</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Ross River T.48 P.6</td>
<td>2-3 mm clear round</td>
<td>3-4 mm clear irregular</td>
<td>4-5 mm clear irregular</td>
<td>5-6 mm clear irregular</td>
<td>-ve</td>
<td>3-4 mm clear irregular</td>
</tr>
</tbody>
</table>

* Strain.
† Suckling mouse pass number.
Entomology Department of the London School of Hygiene and Tropical Medicine, were allowed to feed on mixtures of 20% suspension of human erythrocytes in phosphate buffered saline (PBS) supplemented with 5% FBS and containing $10^4$ p.f.u./ml of the HAR prototype or variants. The method is as described by Johnson & Varma (1975).

*Testing mosquitoes for infectivity.* Ten days after feeding, pools of six mosquitoes were ground up in 1 ml of L15 medium with 2% FBS and antibiotics and spun at 2000 rev/min for 10 min. Samples of supernatant (0.05 ml) were removed and diluted tenfold in Linbro trays as previously described.

**RESULTS**

*Plaque morphology in various cell lines*

Details are shown in Table 1. Apart from the average overall size we recorded whether the plaques were clear or turbid and whether the shape of the plaques was round or irregular. We also noted any mixed populations of plaques as in the case of HAR prototype or wide range of plaque sizes as in GET (Fig. 1). Three strains, HAR, GET, and ITAK, proved to be inconsistent in XTC cells. On several occasions plaques apparent at 4 days had disappeared by day 8. To test this we titrated ITAK six times in XTC cells and stained one row every 3 days. The plaques were clearest between 3 and 6 days, becoming difficult to read by day 9 and disappearing completely by day 18, after which the cells looked normal. All other tissue culture lines yielded reproducible results. On a few occasions we were unable to take readings at 8 days. This was because the plaques had overgrown the space available in the wells (16 mm). None of the viruses formed plaques in M61 cultures although virus replication occurred. Virus ($10^2$ to $10^5$ p.f.u./ml) could be recovered from tube cultures of M61 cells 8 to 40 days after inoculation with any of the six viruses. However, in the cases of HAR-LP, GET, ITAK and BEB, the plaque morphology of the recovered viruses had altered. When plated in Vero cells they showed a distinctly mixed population of plaques similar to that of HAR prototype. The mixed plaque composition was found to remain stable through two further Vero passages. HAR, GET, ITAK and SAG did not appear to grow in LLC-MK. 

![Fig. 1. Plaque morphology in Vero cells after four days. (a) RR, (b) SAG, (c) ITAK, (d) GET, (e) BEB, (f) HAR prototype, (g) HAR-SP, (h) HAR-LP. Compiled from wells in a single culture plate. Well diameter 16 mm.](image-url)
Table 2. Cross-neutralization tests with six arboviruses*

<table>
<thead>
<tr>
<th>Virus</th>
<th>HAR-prototype</th>
<th>HAR-LP</th>
<th>HAR-SP</th>
<th>GET</th>
<th>SAG</th>
<th>ITAK</th>
<th>BEB</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAR-prototype</td>
<td>4:33†</td>
<td>4:0</td>
<td>4:0</td>
<td>4:1</td>
<td>2:4</td>
<td>3:2</td>
<td>0:9</td>
<td>1:1</td>
</tr>
<tr>
<td>HAR-LP</td>
<td>4:0</td>
<td>4:0</td>
<td>3:9</td>
<td>4:0</td>
<td>2:5</td>
<td>2:6</td>
<td>&lt;0:5</td>
<td>0:9</td>
</tr>
<tr>
<td>HAR-SP</td>
<td>4:0</td>
<td>4:2</td>
<td>4:2</td>
<td>4:1</td>
<td>0:8</td>
<td>3:0</td>
<td>&lt;0:5</td>
<td>0:5</td>
</tr>
<tr>
<td>GET</td>
<td>3:0</td>
<td>1:3</td>
<td>2:1</td>
<td>3:9</td>
<td>2:0</td>
<td>2:7</td>
<td>1:0</td>
<td>1:0</td>
</tr>
<tr>
<td>SAG</td>
<td>2:9</td>
<td>2:0</td>
<td>2:5</td>
<td>3:1</td>
<td>2:5</td>
<td>2:2</td>
<td>1:0</td>
<td>0:8</td>
</tr>
<tr>
<td>ITAK</td>
<td>2:0</td>
<td>2:4</td>
<td>1:2</td>
<td>2:5</td>
<td>0:5</td>
<td>2:0</td>
<td>1:4</td>
<td>1:0</td>
</tr>
<tr>
<td>BEB</td>
<td>0:9</td>
<td>&lt;0:5</td>
<td>&lt;0:5</td>
<td>1:0</td>
<td>&lt;0:5</td>
<td>&lt;0:5</td>
<td>3:0</td>
<td>&lt;0:5</td>
</tr>
<tr>
<td>RR</td>
<td>1:0</td>
<td>&lt;0:5</td>
<td>&lt;0:5</td>
<td>1:1</td>
<td>0:6</td>
<td>1:3</td>
<td>1:0</td>
<td>2:1</td>
</tr>
</tbody>
</table>

* Using immune (2-dose) sera.
† Antibody dilution.
‡ Log plaque reduction neutralization index.

although Stim (1969) did show plaques with GET. SAG did not grow in XTC. In these cases recovery of virus from the cultures was unsuccessful.

Cross-neutralization

Small antigenic differences within the group were detectable in the plaque reduction neutralization tests using immune sera. The neutralization index of HAR prototype antiserum was highest against the prototype followed by HAR-LP and HAR-SP (Table 2). Otherwise HAR prototype and GET were antigenically very close; HAR-SP being closer to GET than HAR-LP. SAG and ITAK could be distinguished only with SAG antiserum, whilst HAR-LP could be distinguished from GET only when using HAR-LP antiserum. This was not true using hyperimmune antisera which were equally non-discriminatory.

In each case, we also examined the plaque morphology at the breakthrough dilution. Where differences were detected between this and our original estimations, virus from the ‘breakthrough’ well was passaged on to fresh cells to find out if it bred true. We were thus able to isolate HAR-SP variant from HAR prototype in the presence of GET and HAR-LP antisera, and HAR-LP variant from HAR prototype in the presence of HAR-SP antiserum. A small plaque variant of ITAK was also obtained in the presence of antiserum against the HAR prototype.

Mouse inoculations

All the viruses killed 1-day-old mice within 2 days when inoculated i.c. HAR prototype and two variants killed within 36 h. By the i.d. route death times were extended over 4 days in day-old mice, with 100% mortality by HAR-LP within 2 days, HAR prototype 3 days, and HAR-SP 4 days. Twelve-day-old mice similarly inoculated with HAR-LP, had 100% mortality within 5 days, with HAR prototype 6 days and with HAR-SP, 9 days (Table 3).

Five-day-old mice inoculated i.p. showed no significant differences in their mean death times. All mice were dead within 3 days. Re-isolation of the virus from the brains of the i.d. and i.p. inoculated mice was attempted and similar virus yields of $10^{5.0}$ to $10^{6.0}$ were obtained regardless of virus or route of inoculation. Mouse passaged HAR-LP and HAR prototype viruses appeared unchanged though the latter had a substantially reduced SP sub-population. Mouse passaged HAR-SP consisted of mixed plaques of approximately equal numbers.
Table 3. Mouse survival time after intradermal inoculation of HAR

<table>
<thead>
<tr>
<th>HAR-prototype</th>
<th>HAR-LP</th>
<th>HAR-SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-old* 12-day-old</td>
<td>Day-old 12-day-old</td>
<td>Day-old 12-day-old</td>
</tr>
<tr>
<td>1 0/20†</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>2 6/20</td>
<td>1/20</td>
<td>19/20</td>
</tr>
<tr>
<td>3 20/20</td>
<td>4/20</td>
<td>20/20</td>
</tr>
<tr>
<td>4 13/20</td>
<td>11/20</td>
<td>20/20</td>
</tr>
<tr>
<td>5 9/20</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>6 20/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Age of mice.
† Cumulative number of deaths out of a total of 20.

Passage through Aedes aegypti mosquitoes

We obtained virus yields of 2.9 logs from HAR-LP, 2.1 logs from HAR prototype and 0.6 logs from HAR-SP when mosquitoes were tested ten days after feeding. The yield from HAR prototype and HAR-LP was of LP and from HAR-SP of SP, only.

Interference

In spite of virus-cell multiplicities of up to 20:1 in our primary infection of cells and challenge multiplicities of much lower order, mixed populations of HAR were always recoverable irrespective of sequence of infection and challenge. No direct virus interference could be detected in Vero cells.

Discussion

The differences in plaque size of the viruses described in this report are real and reproducible. With the exception of the M61 passage, the viruses remained unchanged following passages in SMB and in cell cultures. Close antigenic relationships between these viruses may reflect a common ancestry based on natural selection of antigenically heterogeneous sub-populations. Henderson, Shah & Wallis (1965) suggested that the antigenic properties of arbovirus strains can be defined by the qualitative array of constituent virus sub-populations and the quantitative distribution of these types through the total strain population. In the case of HAR prototype the cross-neutralization data must reflect the cumulative effect of the prototype antiserum against its two sub-population variants.

There are various mechanisms through which such a system could operate in the evolution of new virus strains. (a) Host susceptibility differences may play an important role in determining the composition of a virus strain (Henderson, 1968; Issel et al. 1975). (b) Heterologous antibody may have differentially selective properties. (c) Chance selection due to low dose infection may occur.

While this work was done under artificial conditions, parallels may still be drawn with natural situations.

Host susceptibility differences do seem to exist and these may extend to sub-population variants to a greater or lesser degree. Examples of this may be the lack of growth in XTC cells by SAG and the reappearance of LP variant in the virus isolated from the brains of the mice inoculated i.p. or i.c. with HAR-SP virus. The emergence of normally undetectable SP
variants in the viruses HAR-LP, GET, ITAK and BEB after a single passage in M61 cells may be another indication of this, as is the reported attenuation of arboviruses that occurs during passage in cultured mosquito cells (Peleg, 1971; Singh, 1971). Once the SP sub-populations had been enhanced in this way, their presence remained stable through two passages in Vero cells which we attempted.

That antibodies can be differentially selective was shown by the extraction of different variants from some of these viruses in the presence of heterologous antisera. One way crossing in neutralization tests exhibited by many arboviruses (De Madrid & Porterfield, 1974; Chanas et al. 1976) and shown here by the GET sub-group may also be explained in terms of sub-population variants. An antisera made against a mixed virus population of two or more variants will neutralize both the mixed or any separate constituent variant equally. Antiserum made against the latter however will be less effective against the mixed virus, as any neutralization will be masked by the remaining variant(s) breaking through the neutralization end point.

Chance selection due to low dose infection resulting from low viraemia or high infectivity threshold could work in much the same way as plaque purification techniques, a new host becoming infected only with the virus sub-population present at the highest concentration (Taylor & Marshall, 1975).

The synpatric distribution and the heterogeneous populations of some of these viruses suggest that any mutations do not necessarily confer to their progeny any survival advantage or disadvantage, as indicated by the lack of interference by the two HAR variants. In a stable ecosystem with a constant and high input of new susceptible hosts, such variants will persist along with the existing ones until a new selective force favours one variant.

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REFERENCES


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