Genetic Characteristics of Echovirus Type 9 Strains: Selection and Characterization of Variants

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SUMMARY

Using different methods of selection, three main types of echovirus type 9 variants were obtained from a single isolate derived from a naturally occurring strain. The three variants PL 88, PL 43 and PL 29 differed in a number of genetic characters as follows: virulence for infant mice (v), capacity to grow at 40° as compared to 36.5° (t), cytopathic effect on epithelial cell lines of human origin (c), inhibition by rhesus monkey serum (m) and protection against heat inactivation by Al ions (a). They also differed in the size and time of appearance of plaques on monkey kidney cell monolayers. The PL 88 variant was mouse-virulent, grew at 40° to the same degree as at 36.5°, was not cytopathogenic on epithelial cell lines of human origin, was not inhibited by normal rhesus monkey serum, not protected by Al ions from heat inactivation and produced early large plaques on baboon kidney cell monolayers. Accordingly its genetic markers are \( v^t^c^m^a^- \). The genetic markers of the PL 43 variant are \( v^t^c^-m^a^- \); PL 43 differs from PL 88 only in the v and t markers. Though avirulent for mice, mouse-virulent variants could be selected from it by repeated passage in mice. PL 29 had the characters \( v^-t^-c^+ \). It was distinctly different from both PL 88 and PL 43 by its c marker and produced late and small plaques on baboon kidney cell monolayers. PL 29 was stable in its mouse avirulence and no virulent mutants could be obtained from it by repeated passage in mice. Progeny of PL 29 occurred in two 'subforms', \( m^a^+ \) and \( m^-a^- \). The \( v^-t^-c^+m^a^+ \) form was identical in these characters with both the QUIGLEY and the prototype echovirus type 9, HILL strains.

INTRODUCTION

During outbreaks of aseptic meningitis numerous mouse-pathogenic strains of echovirus type 9 have been isolated (Johnson, 1957; Nihoul, Quersin-Thiry & Weynants, 1957; Godtfredsen & von Magnus, 1957; Tyrrell & Snell, 1956; Wigand & Sabin, 1962). The strains isolated exhibited marked variation in virulence for infant mice, unlike the prototype HILL strain which is avirulent for mice (Ramoiz-Alvarez & Sabin, 1954). In a previous communication (Margalith et al. 1967) we reported a quantitative study of mouse virulence of a number of plaque-purified echovirus type 9 strains. The results indicated that such strains are composed of a mixed virus population of varying degrees of mouse virulence and that there is a positive correlation between virulence for infant mice and the capacity of the virus to multiply at 40° (rct/40 marker).

The purpose of this study was to select mouse-virulent and avirulent variants of
echovirus type 9 and to determine their genetic characteristics. Attempts were made
to select variants of highest mouse virulence by passage in mice and of lowest virulence
by plating at end-point dilution on monkey kidney cell monolayers and to correlate
the virulence character with other genetic markers, especially those that can be
quantified in vitro. In addition, following the observation that the prototype HILL
strain has the capacity to destroy cell lines of human origin, this route of selection was
chosen in order to obtain HILL-like stable mouse-avirulent variants.

METHODS

Primary cell cultures

Monolayers of trypsinized baboon kidney cell cultures grown in medium 199
supplemented with 4% calf serum were used for the preparation of virus stocks, for
virus titrations and for the determination of certain genetic markers. Tube cultures
and monolayers in plates of baboon kidney cells were prepared and used according to
methods previously described (Margalith et al. 1967).

Epithelial cell lines of human origin

KB and human kidney cells were used (Eagle, 1955). The growth medium for KB
cells consisted of Eagle's basal medium containing four times amino acids and
vitamins (Eagle's modified) and supplemented with 15% inactivated calf serum. The
maintenance medium consisted of Eagle's modified medium without calf serum. The
growth medium for human kidney cells consisted of medium 199 supplemented with
10% calf serum and the maintenance medium of 199 + 0.05% bovine serum albumin.

Virus was titrated in cell monolayers in tubes or Petri dishes. Tube cultures were
washed with Hanks's solution and inoculated with 0.2 ml. of the appropriate virus
dilution. After 2 hr adsorption at 36.5°, 2 ml. of maintenance medium was added,
and the tubes incubated at 36.5°. The monolayers in Petri dishes were washed with
Hanks's solution and inoculated with the appropriate virus dilution. After virus
adsorption, the monolayers were overlaid with 4 ml. of 2% Difco special Noble agar
mixed with 4 ml. double strength Eagle's modified medium containing 1% chick
embryo extract. The Petri dishes were incubated in a humidified CO₂ incubator. The
cells were stained 3 to 8 days (according to the virus strain) after inoculation, with a
mixture consisting of 1 ml. Neutral Red (1/3000) and 1 ml. 2% Difco special Noble
agar. Plaques were visible 8 to 24 hr after staining.

Virus

The parent echovirus type 9 strain used in these experiments was derived from an
isolate obtained from a stool sample of a case of aseptic meningitis (Margalith et al.
1967). The strain was passaged a number of times in baboon kidney cell monolayers
and designated sw 2955. For the preparation of stocks of the sw 2955 strain, the
harvested virus was centrifuged at 10,000 rev./min. for 30 min. and the supernatant
fluids were kept frozen at -20°.

The HILL strain prototype echovirus type 9 strain was obtained through the
courtesy of Dr A. B. Sabin. Virus stocks were prepared and titrated in baboon kidney
cells. The QUIGLEY echovirus type 9 strain was kindly supplied by Dr J. L. Melnick.
It was handled similarly to the HILL strain.
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Infectious RNA

Infectious RNA was extracted according to the method described by Darnell et al. (1961). The cell monolayers were pretreated according to the method of Boey (1959). The infectivity titres of the different RNA preparations varied between $10^{3.8} \text{ to } 10^{4.5} \text{ p.f.u./ml.}$, which is about 100,000-fold lower than the titres of the virus stocks from which they were prepared.

Genetic markers

The $v$ marker (virulence for infant mice) was determined according to the method described by Margalith et al. (1967). Strains were designated as virulent ($v^+$) when the p.f.u./LD$_{50}$ ratio ranged from $10^1$ to $10^2$. Virus strains which killed an occasional mouse or none upon inoculation of $10^7$ p.f.u. were regarded as avirulent ($v^-$).

The $t$ marker (rct/40) was determined by a modified rct/40 test (Lwoff, 1959; Margalith et al. 1967).

The $c$ marker (cytopathic effect in human epithelial cells) was determined in monolayer cultures. Strains which caused cytopathic effect in KB cells to a titre similar to that in baboon kidney cells were designated $c^+$; $c^-$ strains did not produce cytopathic effect in human epithelial cell lines even when inoculated with $> 10^7$ p.f.u.

The $m$ marker (inhibition of growth by rhesus monkey serum) was determined by measuring the effect of the serum on the virus titre in baboon kidney cells. All experiments were made with serum inactivated at $56^\circ$ for $\frac{1}{2}$ hr. Strains that showed at least a 100-fold decrease in titre when incubated with undiluted rhesus monkey serum were designated $m^+$. Strains whose titre was the same in the presence and absence of rhesus monkey serum were regarded as $m^-$. 

The $a$ marker (protection against heat inactivation by Al$^{3+}$) was determined by the effect of Al$^{3+}$ on the heat inactivation of the virus (Wallis & Melnick, 1963). Virus of an approximate titre of $10^7$ p.f.u./ml. was heated to $50^\circ$ for $2$ hr in $10$ mAlCl$_3$. A virus suspension in distilled water served as control. Strains protected from heat inactivation by Al$^{3+}$ ions were $a^+$; $a^-$ strains showed the same degree of inactivation in the presence and absence of Al$^{3+}$ ions.

RESULTS

Selection of plaque-purified variants

A stock of the parent sw 2955 isolate was used for the selection of the different variants. Variants of high mouse virulence were obtained by passage of sw 2955 in infant mice. Five consecutive passages were made using 10% (w/v) carcass suspension for each passage. The fifth mouse passage was diluted to the end-point and plated on baboon kidney cell monolayers. From the end-point dilution single plaques were removed, passaged in baboon kidney cells and titrated in parallel in baboon kidney cells and infant mice. For 10 such plaques tested, the p.f.u./LD$_{50}$ ratios varied from $10^{2.6}$ to $10^4$; plaque no. 88 (PL 88) which exhibited the lowest p.f.u./LD$_{50}$ ratios was chosen as a representative $v^+$ variant.

Mouse-avirulent variants were selected by passage of sw 2955 at end-point dilutions on baboon kidney or KB cell monolayers. The mouse-avirulent PL 43 variant was obtained through five consecutive plaque-purifications on baboon kidney cells. At
each passage, plaques were picked from the end-point dilution, propagated in baboon kidney cell tube cultures, and tested for mouse virulence. At each passage, plaque progeny of the least mouse virulence was used for subsequent plating. From the fifth plating, 27 plaques were removed and passaged in baboon kidney cell tube cultures. The harvested virus suspensions were inoculated undiluted in infant mice and titrated in parallel in baboon kidney cell cultures. From the 27 plaques tested, PL 43 was chosen as the representative v⁻ variant. Inoculation of $\geq 10^7$ p.f.u. of PL 43 did not paralyse any of 16 infant mice inoculated. Though apparently mouse-avirulent, the PL 43 variant proved unstable in its avirulence when further passaged in baboon kidney cells and especially in infant mice.

A stable mouse avirulent variant was selected from SW 2955 through passage in KB cell monolayers. This route of selection was chosen when it was found that the mouse-avirulent HILL and QUIGLEY strains were highly cytopathogenic for human epithelial cell lines. SW 2955 was passaged 7 times in human kidney cell cultures using undiluted virus for each subsequent passage. The strain obtained after 7 passages (SW 2955 HuK 7) was found to be as virulent for infant mice as the parent SW 2955. This strain underwent 3 additional blind passages in KB cells when signs of a cytopathogenic effect were observed. Four additional passages in KB cells were made at limit dilution, $10^{-6}$ to $10^{-8}$. At this stage, parallel platings were made in KB and baboon kidney cells. Titres on both cell types were found to be similar. From the baboon kidney cell plates, 28 plaques were picked and propagated in baboon kidney cells. The progeny of all 28 plaques was found to be completely mouse-avirulent. These variants were designated PL 29. Five subsequent parallel passages in baboon kidney cells (by end-point dilution) and in infant mice (undiluted) indicated that these variants were as stable in their avirulence as the HILL and QUIGLEY strains. They were designated v⁻.

The three variants, PL 88, PL 43 and PL 29 were typed for serological identity with echovirus type 9 (HILL) antiserum. All were found to be neutralized to the same extent, a dilution of 1/30 anti-HILL serum neutralizing $\geq 10^6$ TCD 50 of each variant.

Size and day of appearance of plaques on baboon kidney cell monolayers

Plaques of the PL 88 and PL 43 variants appeared 3 to 4 days after plating and were of 5 mm. diameter. Plaques of the PL 29 variant appeared 6 to 8 days after plating and their diameter was 2 mm.

The HILL and QUIGLEY strains formed plaques on baboon kidney cells with great difficulty; often the plates had to be kept 8 to 10 days until barely visible plaques appeared having a diameter of 1 to 2 mm.

Behaviour of the virulent and avirulent echovirus type 9 variants in infant mice

In order to study more thoroughly the pathogenicity or its lack for infant mice of the selected echovirus type 9 variants, growth curve experiments in infant mice were made. Large groups of infant mice were inoculated subcutaneously with doses of $10^6$ to $10^8$ TCD 50 of each of the three variants. Groups of 2 or 3 mice were killed at 6 hr intervals during the first 2 days and then every 24 hr for 6 days. Ten per cent carcass suspensions in medium 199 were titrated in baboon kidney cell tube cultures (Fig. 1). The mouse-virulent PL 88 variant multiplied quickly and reached the level of $10^8$ TCD 50 five days after inoculation, when all infected mice were paralysed. Multiplication of the PL 43 variant was slow and 5 days after infection it reached a titre of
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10^6 TCD50. The infected mice remained healthy. The mouse-avirulent stable PL 29 did not multiply in the mice and no virus could be recovered in samples taken on the fifth and sixth days.

Fig. 1. Growth curves of the virulent and avirulent variants of echovirus 9 in infant mice. All values are means of 4 experiments. ●, PL 88; ○, PL 43; △, PL 29.

Table 1. Capacity of the selected variants to grow at 40°

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log. TCD50/ml. in baboon kidney cells</th>
<th>Mouse virulence (log. LD50/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sw 2955</td>
<td>8·0</td>
<td>8·0</td>
</tr>
<tr>
<td>HILL</td>
<td>8·0</td>
<td>≤ 2·0</td>
</tr>
<tr>
<td>QUIGLEY</td>
<td>7·5</td>
<td>≤ 2·0</td>
</tr>
<tr>
<td>PL 88</td>
<td>7·5</td>
<td>7·5</td>
</tr>
<tr>
<td>PL 43</td>
<td>≥ 7·2</td>
<td>≤ 2·0</td>
</tr>
<tr>
<td>PL 29</td>
<td>8·0</td>
<td>≤ 2·0</td>
</tr>
</tbody>
</table>

Capacity to grow at 40° and mouse virulence

Virus stocks from baboon kidney cells of the selected variants, PL 88, PL 43 and PL 29 were examined for their capacity to grow at 40°, as compared to 36·5° (Table 1). For comparison, results obtained for the parent sw 2955 and the prototype HILL and QUIGLEY strains are included. The LD50 titres of the various strains in infant mice are also presented. There was a positive correlation between mouse virulence and the capacity to grow at 40°.

Behaviour of the variants on epithelial cell lines of human origin

Of the three selected variants, PL 88 and PL 43 did not exhibit cytopathogenicity on KB cell monolayers even when inoculated with virus concentrations of ≥ 10^7 p.f.u./ml. These variants were designated c−. PL 29 produced a distinct cytopathic effect.
when inoculated on KB cell monolayers and formed plaques on KB plates. The QUIGLEY and the prototype echovirus type 9 HILL strain behaved on KB cells similarly to PL 29. All were designated c+.

Fig. 2. Growth curves of PL 88 and QUIGLEY in KB cell monolayers. ○, PL 88; △, QUIGLEY.

Table 2. Inoculation of infected KB cells on baboon kidney cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of KB cells seeded in baboon kidney cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 × 10⁶</td>
</tr>
<tr>
<td>QUIGLEY</td>
<td>--</td>
</tr>
<tr>
<td>PL 88</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* No. of cultures showing cytopathic change/no. of cultures inoculated.

It was of interest to investigate the reasons for the different behaviour of these strains on KB cell monolayers. A c− (PL 88) and a c+ (QUIGLEY) strain were used in these experiments. Three types of experiments were made as follows:

(1) Comparative growth curve experiments on KB cell monolayers were made using a multiplicity of infection of 10 (as determined on baboon kidney cells). The viruses were adsorbed for 2 hr, washed off 5 times with Eagle's modified medium, incubated at 36.5°C, and duplicate samples of cells harvested at different time intervals. The cells were removed with versene, disrupted by freezing and thawing, and titrated on baboon kidney cell monolayers (Fig. 2). Both PL 88 and QUIGLEY multiplied in KB cell monolayers. However, the highest titre reached by PL 88 at 10 hr was 1000 times less than that of the QUIGLEY strain. At 22 hr, the cells infected with PL 88 exhibited no
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visible cytopathic effects whereas cells infected with the QUIGLEY strain were completely destroyed.

(2) In order to study whether the 1000-fold lower titre in KB cell monolayers, infected with PL 88, was due to a lower virus yield per infected cell or to a smaller percentage of cells infected, suspensions of KB cells infected with each of the two viruses were seeded on baboon kidney cell monolayers for assay of infectious centres (Table 2). Each KB cell infected with the QUIGLEY strain produced infective virus; however, following infection with PL 88, only about 1/500 KB cells yielded infectious virus. Thus, it appears that the c- character is due to the low susceptibility of KB cells to the variant.

Table 3. Multiplication of infectious RNA from a c- variant and c- and c+ echoviruses in KB cell monolayers

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Virus titre* in KB monolayers infected with†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA of PL 88 (p.f.u./ml.)</td>
</tr>
<tr>
<td>0</td>
<td>≤ 1.0</td>
</tr>
<tr>
<td>5</td>
<td>≤ 1.0</td>
</tr>
<tr>
<td>10</td>
<td>3.8</td>
</tr>
<tr>
<td>28</td>
<td>4.3</td>
</tr>
<tr>
<td>48</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* In baboon kidney cells.
† M.o.i. 1/150.

Table 4. Effect of rhesus monkey serum and Aβ+ on echovirus type 9 variants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rhesus monkey serum</th>
<th>Heating to 50° 2 hr with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td>PL 88</td>
<td>7.8*</td>
<td>7.8</td>
</tr>
<tr>
<td>PL 43</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>QUIGLEY</td>
<td>7.0</td>
<td>3.0</td>
</tr>
<tr>
<td>PL 29 I</td>
<td>8.2</td>
<td>5.7</td>
</tr>
<tr>
<td>PL 29 II</td>
<td>5.7</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* Log. TCD50/ml.

(3) The results of the assays of infectious centres indicated the possibility that the low infectivity of PL 88 in KB monolayers may have been due to a limited adsorption rather than to a low rate of multiplication. To resolve this problem, washed KB cell monolayers were infected with RNA prepared from the PL 88 variant. KB monolayers infected with the PL 88 variant and with the QUIGLEY strain served as controls. Following adsorption for half an hour for the RNA and 2 hr for each of the viruses, the monolayers were pretreated according to the method of Boeyé (1959), and Eagle’s modified medium was added. The monolayers were incubated at 36-5°C, samples from the supernatant medium were taken at various time intervals and titrated in baboon kidney cells (Table 3). Infectious RNA obtained from the PL 88 variant multiplied extensively.
and reached almost the same titre as the QUIGLEY strain. Thus, it appears that the low infectivity of PL 88 is due to lack of adsorption to the great majority of KB cells, rather than to slow multiplication.

Inhibition of the virus by normal rhesus monkey serum

The three variants were titrated in the presence and absence of normal rhesus monkey serum (Table 4). The titre of PL 88 and PL 43 was the same in the presence and absence of monkey serum (m-). The titre of the QUIGLEY strain showed 1000-fold inhibition in the presence of monkey serum (m+). Progeny of the PL 29 plaques occurred in two forms: m- or m+ (Table 4).

Protection against heat inactivation by Al3+

Strains which were protected against heat inactivation by Al ions, the QUIGLEY and one of the progeny plaques of PL 29 were designated a+; PL 88 and PL 43 were designated a- (Table 4). The plaque progeny of PL 29 gave rise to variation in the a marker; some were a+ and some a-. This correlated in each instance with the m marker. In general, it can be stated that the m and a markers showed positive correlation, each strain occurring in the m-a- or m+a+ forms. All plaque progeny of PL 29, whether in the m+a+ or m-a- form, were stable in their mouse avirulence.

DISCUSSION

The prototype echovirus type 9 strain, HILL (Ramoz-Alvarez & Sabin, 1954) and another isolate of echovirus type 9, QUIGLEY (McLean & Melnick, 1957) are mouse avirulent and r-. They do not revert to v+, thus being stable in their avirulence. These two strains have an additional characteristic, the capacity to cause cytopathic effect in human epithelial cell lines, designated by us c+. Therefore, cell lines of human origin were used for selection of HILL-like variants from SW 2955 and plaques of the c+ type, designated PL 29, were selected. The selection procedure for c+ was cumbersome and involved prolonged passage in KB cells using undiluted material for each passage. Variants of this type are evidently very rare and can be selected only with great difficulty. Variants of the PL 29 type differ from HILL and QUIGLEY by their ability to form plaques on baboon kidney cell monolayers. They may also differ with regard to the two additional markers: m and a. Since PL 88 and PL 43 are m-a-, whereas HILL and QUIGLEY are m+a+, it seemed at first that these two characters may be 'linked' with the c marker. However, progeny plaques of PL 29 were found to occur in the two 'subforms': m+a+ and m-a-, which proved occurrence of 'non-linkage' between c and ma.

Growth curve experiments with the three selected variants in mice and in KB cells provided certain explanations for the differences in the v and c markers. PL 88 multiplied extensively in the mouse, reaching a titre of 10^7 TCD 50 five days after infection and caused paralysis; PL 43 multiplied in the mouse at a lower rate and never exceeded a titre of 10^6 TCD 50. According to Eggers & Sabin (1959), a 'critical level' of 10^8 TCD 50 on the 4th day after inoculation is necessary to cause paralysis in the mouse. The mouse virulent variant, PL 88, is close to meeting this requirement whereas PL 43, though multiplying in the mouse, exhibited 100-fold lower titres on the 4th and 5th day. PL 29, similarly to the QUIGLEY and HILL strains, did not multiply in the
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Although c+ strains adsorbed to every KB cell, the c− variant adsorbed to about 1/500 cells only.

There is undoubtedly a correlation between the various genetic markers of echovirus type 9 variants, as in the case of v and t. The v, t, c interrelationship deserves further study.

REFERENCES


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