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# CYTOPATHIC AND PATHOGENIC PROPERTIES OF DENGUE TYPE 2 VIRUS REPRODUCED IN QUAIL EMBRYO FIBROBLAST CULTURES

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*Summary.* — Quail embryo fibroblast cultures are able to reproduce dengue type 2 virus up to high titres measured by $TCID_{50}$ values. The reproduced virus at low passage levels was antigenically closely related to the original virus reproduced in newborn mouse brains in vivo, but was loosing its virulence for these animals.

*Key words*: *dengue virus*; *quail embryo fibroblasts*

## *Introduction*

Primary quail embryo fibroblast (QEF) cultures proved to be a suitable cell system for the reproduction of several arboviruses (Takayama and Simizu, 1973; Kańtoch *et al.*, 1978) as well for the production of measles vaccine (Andzhaparidze *et al.*, 1969; Vasileva and Boichuk, 1970). On the other hand, there has been an increasing interest in the development of an in vitro cell system for dengue virus reproduction also in respect of specific immunoprophylaxis (Halstead *et al.*, 1973; Halstead and Palumbo, 1973; Eckels *et al.*, 1976; Hotta, 1978).

All observations mentioned above encouraged us to study the possibility of using QEF cultures for dengue virus reproduction and testing its basic biological properties. Dengue virus type 2 was used as a model.

## *Materials and Methods*

*Virus.* Dengue type 2 strain originally received as lyophilized infected mouse brain (30th passage) in 1963 from the School of Hygiene and Tropical Medicine, London, was used. After resuspending, it was reproduced in 1—2 days old Swiss newborn mice. In further experiments, virus reproduced in intracerebrally (i.c.) inoculated newborn mice and in different cell cultures was used. Infected mouse brain was used as "seed virus" in cell culture experiments.

*Animals.* Most experiments were carried out on 1—2 days old Swiss newborn mice, and both Swiss and inbred CFw mice weighing 6—8 g and 18—22 g. Swiss and CFw mice showed a similar susceptibility to the virus, the observed differences in susceptibility did not exceed 0.5 log mic $LD_{50}$.

*Cell cultures.* For virus reproduction, the following cell cultures were employed: *Cercopithecus aethiops* primary monkey kidney cells (PMKC), Vero and QEF. PMKC — trypsinized cells

**Table 1. Pathogenicity of dengue type 2 virus for mice**

| Mice | Route of inoculation | Infecting dose (smic $LD_{50}$) | Mean incubation period (days)* | Mean survival time (up to 14 days) | Harvested virus titre (log smic $LD_{50}$) |
|---|---|---|---|---|---|
| Newborn | i.c. | $10^{3.5}$ | 4 | 4.0 | 4.5 |
| | i.p. | $10^{4.5}$ | 10 | 10 | 1.5 |
| 6–8 g | i.c. | $3 \times 10^{3.5}$ | 8– 9 | 8.45 | 4.25 |
| | i.p. | $5 \times 10^{4.5}$ | – | 14 | 0 |
| 18–20 | i.c. | $3 \times 10^{3.5}$ | 6–11 | 10.4 | 3.5 |
| | i.p. | $5 \times 10^{4.5}$ | – | 14 | 0 |

* – = no paralytic symptoms.

($2.5 \times 10^5$ cells/ml) were grown in Hanks' salt solution with 0.5% lactalbumin hydrolysate, 4% inactivated calf serum, 0.05% $NaHCO_3$ and antibiotics. EDTA-dispersed Vero cells ($5 \times 10^4$ cells/ml) were grown in medium 199 (95%) with inactivated calf serum (5%) and antibiotics. QEF cultures were prepared from 9 days old embryos according to methods described by Lityńska (1979): $4 \times 10^5$ cells/ml in medium 199 (75%) + Hanks' salt solution (15%) + inactivated calf serum (10%) + antibiotics.

*The virus titres* were determined by the following tests: *a*) by i.c. inoculation of newborn mice with 0.015 ml volumes (smic $LD_{50}$); *b*) by i.c. inoculation of 6–8 g and 18–22 g mice with 0.03 ml volumes (mic $LD_{50}$); *c*) by intraperitoneal (i.p.) inoculation of newborn mice with 0.15 ml volumes (smip $LD_{50}$); *d*) by i.p. inoculation of older mice with 0.5 ml volumes (mip $LD_{50}$); and *e*) by inoculation of cell cultures with 0.1 ml volumes per tube ($TCID_{50}$). The in vivo titres were calculated on the 14th day after infection (p.i.) and in vitro on the 7th–10th day, using the Reed and Muench formula.

*Mean survival time* of infected animals was calculated according to Grunert *et al.* (1965):

$$\text{MSD} = \frac{\Sigma[f(d-1)]}{N},$$

where f = number of paralyzed or dead animals on day "d" and N = number of animals tested.

*Virus identification.* Haemagglutination inhibition (HI) and indirect immunofluorescence tests were used for serological identification of the viruses reproduced in vivo and in vitro. Anti-dengue type 2 hyperimmune mouse sera were obtained after 2–3 inoculations of infectious mouse brain ($1.5 \times 10^5$ smic $LD_{50}$ per immunizing dose) or of virus reproduced in QEF cultures ($5 \times 10^6$ $TCID_{50}$ per immunizing dose).

*Haemagglutinating antigen* was obtained according to Clarke and Casals (1958); 8 units of antigen and an 8% suspension of goose red cells were used.

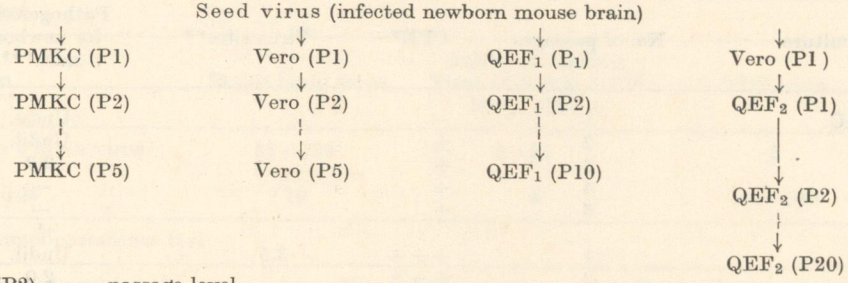
*Indirect immunofluorescence test.* Infected cell cultures and mouse brain smears fixed in cold acetone, the immune sera mentioned above and conjugated anti-globulin serum (Sevac, Prague), were used.

## *Results*

### *Dengue type 2 virus virulence in vivo*

Establishment of mouse susceptibility to the dengue type 2 strain was necessary because of two reasons: passaging of seed virus in mouse brains and controlling the virulence of cell culture-adapted virus. Two criteria were adopted – incubation period (days passing up to the first visible paralytic

**Table 2. Scheme of dengue type 2 virus adaptation in vitro**




(P1), (P2), ... = passage level.

symptoms) and survival time. In newborn mice, the survival time was equal to the incubation period both after i.c. and i.p. infection. In i.c. infected older mice the incubation period was 2—3 times longer, showing a greater variation in 18—22 g mice. The survival time increased with age. According to the criteria mentioned above both 6—8 g and 18—22 g mice were more resistant to i.p. virus inoculation than newborn mice (Table 1).

*Virus adaptation and reproduction in vitro*

The virus adaptation procedure which included passages in PMKC (5 passages), Vero cells (5 passages) and QEF cultures in two variants — $QEF_1$ (direct passages in these cells) and $QEF_2$ (one passage in Vero followed by 20 passages in QEF cells) is schematically illustrated in Table 2.

The inoculated cell cultures were observed for a cytopathic effect (CPE) for up to 14 days. Virus harvests from each passage level were collected and stored at −70° C for testing the virulence for suckling mice and assay of the harvested virus titre (Table 3).

Virus reproduction was very low in PMKC and was decreasing with passages in Vero cells. Different results were obtained in QEF cultures, which showed a high susceptibility to the virus: the virus titres increased with passage level (up to $10^{7.0}$ log $TCID_{50}$), especially in $QEF_1$, when the virus was directly transferred to QEF (see Table 2).

The harvested virus was not virulent for newborn mice (i.c. inoculation) already since the first passages in QEF cultures.

*Identification of the passaged virus*

The virus identification was carried out by HI and indirect immunofluorescence tests, using the seed virus and its further passages in mouse brains as control (Table 4).

The HI test gave positive reactions between seed virus (brain virus) and homologous immune serum (control) and between seed virus (brain virus) and immune serum against dengue virus of the 10th and 16th passages.

Tick-borne encephalitis (TBE) virus, strain Klodobok, used as an additional control, gave cross reactions according to the known antigenic relationships of flaviviruses (Berge, 1975; Schlesinger, 1977; Hotta, 1978).

**Table 3. Pathogenicity of dengue type 2 virus passaged in cell cultures**

| Cell culture | No. of passages | CPE* | Virus titre** | Pathogenicity for newborn mice*** |
|---|---|---|---|---|
| PMKC | 1 | − | | Undil. |
| | 2 | ± | | Undil. |
| | 3 | + | | 2.0 |
| | 4 | + | | − |
| | 5 | + | | − |
| Vero | 1 | +++ | 3.5 | Undil. |
| | 2 | +++ | | 2.0 |
| | 3 | ++ | 1.5 | 2.5 |
| | 4 | + | | 1.0 |
| | 5 | + | 1.75 | Undil. |
| $QEF_1$ | 1 | ± | 1.0 | − |
| | 2 | ++ | 1.75 | − |
| | 3 | +++ | 5.5 | − |
| | 4 | +++ | 7.5 | − |
| | 5 | +++ | 7.0 | − |
| | 6−10 | +++ | 7.0−7.5 | − |
| $QEF_2$ | Vero P1 | +++ | | Undil. |
| | 1− 2 | +++ | 1.0 | − |
| | 3− 7 | +++ | 1.5−3.5 | − |
| | 8−10 | +++ | 5.0−6.0 | − |
| | 11−16 | +++ | 5.5−6.5 | − |
| | 17−20 | +++ | 6.0−7.0 | − |

* ±, +, ++, +++ = intensity of CPE; − = no CPE.
** Titre (log $TCID_{50}$) of reproduced virus assayed in QEF cultures.
*** − = no pathogenicity in vivo; undil. = undiluted virus suspension tested.

Immunofluorescence tests showed two phenomena: *a*) at first passage levels and up to the 10th passage, intense or moderate fluorescence was found with anti-seed virus antibodies which confirmed the close antigenic relationship of QEF-passaged virus to seed brain virus; and *b*) at higher passage levels (passages 13−18), the intensity of fluorescence decreased and was observed in a much lower number of cells, in spite of a high titre of virus and an intense CPE.

## *Discussion*

The replication of dengue viruses, including type 2, in mice is known (Berge, 1975; Schlesinger, 1977); it was especially proved for newborn mice (Meiklejohn *et al.*, 1952). The viruses can also be reproduced in several established and primary cell cultures (Sinarachatanant and Olson, 1973; Shiraki and Hotta, 1977; Hotta, 1978). The present data proved the primary QEF cultures to be also reproducing the dengue virus. Both mouse brain virus and virus previously passaged through Vero cells reproduced in them,

**Table 4. Serological identification of dengue type 2 virus reproduced in QEF cultures**

HI test

| Antigen | Mouse brain virus | Antiserum against Virus at P10 and P16 levels ($QEF_2$) | TBE virus |
|---|---|---|---|
| Dengue 2 brain virus | 32—128 | 8—32 | 2 |
| TBE virus | 10 | 4 | 160—320 |

Immunofluorescence test

| Virus reproduced in | Virus passage level | Fluorescence intensity |
|---|---|---|
| QEF | 1 | +++ |
| | 2 | ++ |
| | 5 | ++ |
| | 10 | ++ |
| | 13 | ± |
| | 16 | ± |
| | 18 | ± |
| Vero | 1 | +++ |
| | 4 | ++ |
| Newborn mice | 33 | +++ |

±, ++, +++ = low, moderate and intense fluorescence.

although the results of the adaptation procedure suggested a decisive role of the cells used. A regular increase in the reproduced virus titre in QEF cultures (up to $10^7$ $TCID_{50}$) was directly connected with the passage level. This observation is in agreement with those on the high susceptibility of QEF to other arboviruses (Nawrocka *et al.*, 1980).

The observed virus adaptation tt QEF was accompanied by a complete loss of virulence for mice. HI and immunofluorescence tests proved the virus at low passage levels to be still antigenically similar to virus reproduced in mice; virus at high passage levels was more distant. These two facts may suggest a selection of viral particles of a high activity in vitro and of low pathogenicity in vivo or some antigenic shift of the virus at high passage levels or both phenomena. The nature of these changes remains obscure.

The immunogenicity of QEF-adapted virus will be the subject of a subsequent report.

## References

Andzhaparidze, O. G., Dorofeev, V. M., Kolganova, I. S., Mertslina, T. G., Sokolova, T. M., Biryukova, E. G., Garkushenko, L. G., Nikulina, Z. V., Kropotova, N. S., Yablokova, M. L., Dosser, E. M., and Ermakova, M. N. (1969): Scientific basis and experience of large-scale production of live measles vaccine L-16 (in Russian). *Vop. Virus.* **14**, 13—18.

Berge, T. O. (Ed.) (1975): *International Catalogue of Arboviruses*, 2nd ed., Public Health Service, CDC, Atlanta.

Clarke, D. H., and Casals, J. (1958): Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Amer. J. trop. Med. Hyg.* **7**, 561—573.

Eckels, K. H., Brandt, W. E., Harrison, V. R., McCown, J. M., and Russel, P. K. (1976): Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. *Infect. Immun.* **14**, 1221—1227.

Grunert, A. R., Mcgahen, J. W., and Davies, W. L. (1965): The in vivo antiviral activity of l-adamantanamine (Amantadine). I. Prophylactic and therapeutic activity against influenza viruses. *Virology* **26**, 262—269.

Halstead, S. B., Casals, J., Shotwell, H., and Palumbo, N. E. (1973): Studies on the immunization of monkeys against dengue. I. Protection derived from single and sequential virus infections. *Amer. J. trop. Med. Hyg.* **22**, 365—374.

Halstead, S. B., and Palumbo, N. E. (1973): Studies on the immunization of monkeys against dengue. II. Protection following inoculation of combinations of viruses. *Amer. J. trop. Med. Hyg.* **22**, 375—381.

Hotta, S. (1978): *Dengue and related tropical viruses.* Kobe.

Kańtoch, M., Nawrocka, E., and Lityńska, J. (1978): Cytopathic and immunogenic activity of arbo and measles viruses reproduced in Japanese quail embryo fibroblasts. Internat. Symp. "Cell substrates and their use in the production of vaccines and other biologicals", 23—26 Oct. 1978, Lake Placid, N.Y., U.S.A.

Lityńska, J. (1979): Wplyw pasazy in vitro na biologiczne wlasciwosci atenuowanych szczepów wirusa odry. I. Replikacja oraz wlasciwosci antygenowe. *Med. dośw. Mikrobiol.* **31**, 111—120.

Meiklejohn, G., England, B., and Lennette, E. H. (1952): Propagation of Dengue virus strains in unweaned mice. *Amer. J. trop. Med. Hyg.* **1**, 51—58.

Nawrocka, E., Bednarz, K., and Kańtoch, M. (1980): Replication of arboviruses in Japanese quail embryo fibroblasts. In: *Proc. Internat. Symp. "New aspects in ecology of arboviruses"*, Smolenice, June 11—15, 1979, in press.

Schlesinger, R. W. (1977): *Dengue viruses.* Virology Monographs 16, Springer-Verlag, Wien, New York.

Shiraki, K., and Hotta, S. (1977): Dengue-1 virus permissiveness of established cell line cultures. *Microbiol. Immunol.* **21**, 411—415.

Sinarachatanant, P., and Olson, L. C. (1973): Replication of dengue virus type 2 in *Aedes albopictus* cell culture. *J. Virol.* **12**, 275—283.

Takayama, N., and Simizu, B. (1973): Japanese quail embryo cells as a host of group A arboviruses. *Jap. J. Microbiol.* **17**, 199—203.

Vasileva, G. A., and Boichuk, L. M. (1970): Elaboration of production technology of live measles vaccine L-16 in Japanese quail embryo tissue culture (in Russian). In *Spetsificheskaya Profilaktika Kori*, Mat. nauchno-prakt. Konf., 6—8 Oct. 1969, Leningrad.