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Résumé

VIRUS DE LA FIEVRE APHTEUSE : PROPRIETES D'UN CLONE VIRAL ISOLE DE LA POPULATION ORIGINALE PAR LA TECHNIQUE DE DILUTION FINALE. — Nous avons préparé par dilution finale à 29 °C un clone de virus à partir d'une souche de virus de la fièvre aphteuse de type O et nous avons étudié les propriétés qui distinguent ce virus de la population originale. Ce virus se caractérise par une multiplication plus intense à 29 °C qu'à 37 °C. Il présente une capside fragile qui entraîne son inactivation précoce par la température, par les solutions acides et par la trypsine. Chez le souriceau, ce virus manifeste un pouvoir pathogène atténué et retardé. Ce virus a conservé son activité immunogène et l'on peut donc envisager utiliser la technique présentée pour préparer rapidement des virus destinés à servir de vaccins vivants.

Many investigators have described the methods of attenuation of foot and mouth disease virus and its different properties before and after attenuation (Sellers *et al.*, 1959 ; Bengtsson *et al.*, 1963 ; Asso *et al.*, 1964 ; Goldsmit and Barzili, 1964 ; Prunet *et al.*, 1964). One of these methods is the isolation or cloning of the mutants at the temperature lower than that for the growth of the virus (Wittmann and Ahl, 1964 ; Asso *et al.*, 1966 ; Asso, 1967 ; Asso *et al.*, 1969) either by the previous chemical mutagenesis or by the natural selection. These modified viral clones can be selected naturally, from the original virus population, consisting of the virus with heterogenic properties (Asso *et al.*, 1966 ; Asso, 1967). Moreover, these modified viral clones, if selected critically, are non-pathogenic to the natural hosts like cattle, pig, but simultaneously retaining their immunogenicity and multiplication ability in the same hosts (Wittmann and Ahl, 1964 ; Asso *et al.*, 1966), and this is easily understandable by the pathogenic behaviour of FMD virus which is essentially related to its stability outside in the infected cell and its replicative efficiency at the body temperature of the host (Asso *et al.*, 1969).

This paper describes, the method of the selection of a clone and the comparison of its properties with the original virus, such as growth character at the different temperatures, inactivation by heat, low or acidic pH, exposure to 37 °C and 4 °C ; plaque characters and mice pathogenicity.

Material and Methods

Cell culture

Monolayers of foetus lamb muscle cells (LM cells) between 15th and 30th passage of their

primary culturing were utilised throughout the work. The cell growth medium used was Earle's lactalbumin hydrolysate yeast extract medium with 10 % calf serum (Ely 10 %) at pH 7.2 ; whereas for the virus growth Earle's lactalbumin hydrolysate medium without serum (El) was used. The pig kidney cells (PK cells) from a cell line were grown in the monolayers using Ely 10 % medium.

Viruses

« Original » or uncloned virus was FMD Type O virus previously adapted to the cell cultures. The cloned virus was selected from the original virus, in LM cells at 29 °C. The original virus was always studied at 37 °C, whereas the cloned virus at 29 °C, except their growth characters at the different temperatures.

Infectivity assays

24-48 h old LM cells grown in Nunc or Costar microplates [1] (20×10^3 cells in each well) and incubated at 37 °C in a CO_2 incubator (5 % CO_2 atmosphere) were used in the assays or titrations of the virus. $TCID_{50}$ were calculated on the basis of cytopathic effect (CPE) observations in the minimum of six wells, each well with 0.1 ml of the tenfold dilution of the virus, between 48-72 h of the incubation.

For the virus titrations at 29 °C, the microplates were sealed with the scotch tape after one hour's pre-incubation at 37 °C in a CO_2 incubator to provide CO_2 and humid atmosphere to the cells.

The assaying of PK cell-produced virus was carried out by the monolayer plaque assay technique, using 24-48 h old PK cells in Bellco tubes (8×10^5 cells/tube). After 30 minutes'adsorption of the virus, the cells were overlaid with 0.5 % concentrated agarose mixed with El medium. After 24-48 h of the incubation, the plaques were observed by staining with Lugol's iodine (0.5 % solution) after removing agarose from the tubes. Plaque forming units (PFU) per ml of the virus were determined.

Selection of the virus clone

Terminal dilution technique.

The original virus was titrated for infectivity, at its tenfold terminal dilutions (based on $TCID_{50}$) by inoculating many wells with 0.1 ml of virus-dilution in each of the well of the microplate. The microplates were incubated at 29 °C and virus clones were selected from the wells showing nearly 100 % CPE, after 48 h of the incubation. The selected viral clones were cultivated up to 10 ml quantities as shown in figure 1. The final selection of the cloned virus, from these cultivated viral clones, was done on the basis of their ability to grow more quickly at 29 than at 37 °C and for which the comparative titrations were carried out at the two temperatures.

Growth characters at different temperatures

These were studied by cultivating the virus at 29, 33 and 37 °C, in precisely regulated suspension culture waterbaths. After 30 minutes' adsorption of the virus on PK cell monolayers with a virus cell ratio of 1/3, the cells were trypsinized and suspended in El medium. The single cycle growth of the virus was studied by assaying it at the different time intervals. The comparison among the growth at the three temperatures was made on the basis of the maximum growth of the virus, during the single growth cycle.

Plaque characters

100 $TCID_{50}$ in 0.1 ml of the suspension of the virus, were inoculated on 24-48 h old PK cells. The rest of the method used was the

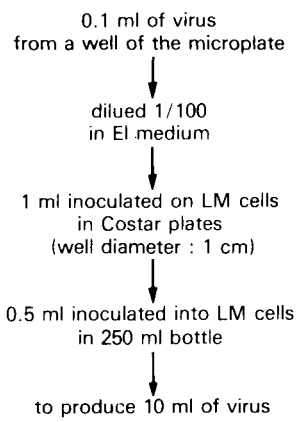

Fig. 1. — Selection of the virus clone : terminal dilution technique.

1 Nunc : made in Denmark ; Costar : made in U.S.A..

Table 1. — Selection of the viral clone.

No of clone	Titre/ml at 29 °C	Titre/ml at 37 °C
1	$10^{6.4}$	$10^{6.8}$
14	$10^{6.0}$	$10^{6.8}$
16	$10^{6.8}$	$10^{6.8}$
21	$10^{6.0}$	$10^{4.0}$
23	$10^{7.0}$	$10^{7.0}$

same, as for described for plaque assay and the two viruses under study were incubated at both 29 and 37 °C.

Studies on the virus inactivation

The inactivation of the virus was studied by exposing 1 000 $TCID_{50}$ of the virus in 1 ml aliquots in distilled water to the different inactivating agents. After the inactivation treatments, virus was assayed in tissue cultures in comparison to the control non treated virus.

1. *Inactivation at 37 and 4 °C* was studied by keeping the virus for overnight (16 h) in the incubator and the refrigerator respectively for titrating it next morning. Control virus remained, non-exposed to these temperatures or otherwise under similar conditions.

2. *Inactivation by heat* was studied by heating the virus at 50 °C in a precisely regulated waterbath. Virus was sampled and transferred to icebath at 10 and 20 min intervals and titrated for infectivity. Control virus remained in icebath until titrated or otherwise under similar conditions.

3. *Inactivation by acidic pH* was studied by exposing the virus [1] to pH 6.4 and 6.0 respectively for 10 min at the room temperature, in the solution of El medium plus potassium-phosphate buffer (KH_2PO_4, 0.65 M). Immediately after 10 min, virus-acid solutions were adjusted to pH 7.3 by sodium-phosphate buffer (Na_2HPO_4. 12 H_2O, 0.65 M) and the virus suspension titrated. Control virus suspension remained at pH 7.3 or otherwise under similar conditions.

1 : The final dilution of the virus was done in the respective acid solutions instead of distilled water.

Mice pathogenicity

Five 4-day-old suckling mice were inoculated intraperitoneally with 0.1 ml containing 100 $TCID_{50}$ doses of the virus. These mice were observed up to ten days of the inoculation along with the control non-inoculated mice.

Results

Selection of the viral clones

Using the terminal dilution technique, many viral clones were obtained at 29 °C, from the original virus which was not adapted at 29 °C for its growth. However, as seen in Table 1 only five clones were able to grow with high titres at 29 °C and only one (i.e., No 21) yielded a higher titre at 29 °C than at 37 °C. Therefore, No 21 was selected as a clone virus for further studies.

Growth of the virus at the different temperatures

Table 2 shows the results of the growth in PFU/ml of both original and cloned viruses at the three temperatures. From the results of the virus growth at the different time intervals, it was found that both original and cloned viruses have maximum growth at the end of 5 h at all three temperatures studied.

The cloned virus showed more than two hundred times more growth at 29 than at 37 °C, whereas the original virus had two hundred times more growth at 37 than at 29°C.

Table 2. — Growth characters at the different temperatures

Virus	PFU/ml after 5 h of growth at 29 °C	33 °C	37 °C
Original	1.8×10^4	3.6×10^6	3.7×10^6
Cloned	2.0×10^6	1.7×10^4	0.8×10^4

Table 3. — Plaque characters

Virus	Plaque size (mm)	
	37 °C	29 °C
Original	> 2	< 1
Cloned	> 2	> 2

Plaque characters

As seen from table 3, the original virus gave large plaques only at 37°C but small plaques at 29 °C, whereas the cloned virus selected from the original virus gave large plaques at both the temperatures, irrespective of the growth character difference observed in Table 2.

Virus inactivation by the different agents

As shown in table 4, it was observed that the cloned virus was more sensitive to inactivation by overnight exposure at 37 and 4 °C, heating to 50 °C for 10 min, and exposure to pH 6.4 for 10 min than that of the original virus. However, the original virus was inactivated by heating to 50 °C for 20 min or exposure to pH 6.0 for 10 min.

Mice pathogenicity

The original virus was found totally pathogenic causing 100 % mortality with clinical symptoms within 24-48 h of the inoculation, whereas the cloned virus inoculated mice showed clinical symptoms like paralysis after 120 h of the inoculation and only 20 % mortality after 168 h of the inoculation.

Action of trypsin (Panina, 1965)

This technique was attempted to increase the tissue culture infectivity titre of the cloned virus at 29 °C. But as shown in table 5, the titre of the virus has decreased at 29 °C and increased at 37 °C, as determined by the comparative titrations of the viruses, produced in three consecutive cell culture passages at 29 °C. We used crude trypsin (Difco) diluted 1.25 g/l in PBSA saline.

Discussion

A purpose of the present study was to select and to investigate the characters of the fragile temperature sensitive mutants already present in the heterogenous population of the original virus. For such a kind of the selection, the terminal dilution technique, which has been used, is more advantageous than the other methods like chemical mutagenesis prior to the selection (Mowat *et al.*, 1969 ; Pfefferkern, 1969 ; La Bonnardière, 1971 ; Maes, 1972 ; Mackenzie *et al.*, 1975 ; Richmond, 1975) because of the preferential chemical inactivation by the mutagen employed of the most of the fragile temperature sensitive population. The terminal dilution technique is very simple and easily reproductive method for the type of selection carried out in this investigation.

The determination of the growth characters of the virus at the different temperatures is an important marker for selecting and studying the viral clone at the temperature(s) lower than that of its growth. From our unpublished studies the determination of « rt » marker (Asso, 1967 ; Asso *et al.*, 1969) of the cloned virus showed it be 38.5 °C.

The growth character at different temperatures is a useful marker, because inside the body of an animal it determines the number of virus particles produced by each of the infec-

Table 4. — Virus inactivation

Virus	before inactivation	after inactivation by					
				50 °C		10 min	
		37 °C	4 °C	10 min	20 min	pH 6.4	pH 6.0
Original	3	1.48[a]	2.83	0.96	0	1.76	0
Cloned	3	0	0.54	0.79	0	1.05	0

a : number of virus in log.

Table 5. — Action of trypsin on cloned virus

Cell culture passage n°	Titre/ml	
	29 °C	37 °C
Before trypsin	$10^{6.0}$	$10^{4.0}$
After trypsin		
1	$10^{5.0}$	$10^{6.0}$
2	$10^{5.0}$	$10^{6.0}$
3	$10^{5.0}$	$10^{6.0}$

ted cells. Thus, in these experiments it was emphasised and showed that this marker was reliable for selection and differentiation of the cloned virus from the original virus.

It was also observed that the plaque character of the virus did not change after its cloning and hence is not related to the change in the growth character of the virus at 29 and 37 °C.

The nature of the capsid of FMD virus determines its stability outside the infected cell and also its pathogenicity by its spread inside and outside the animal body (Asso *et al.*, 1969). In the studies, the unstable nature of the capsid of the cloned virus is well reflected by its high degree of sensitivity to all the inactiviting agents studied, unlike the stable capsid of the original virus which has a less degree of sensitivity to the same inactivating agents. The change from stable to unstable nature for the capsid can be explained biochemically by finding that the unstable viral capsid when analysed showed five polypeptides, whereas the stable viral capsid showed only four. This is explainable by the split of VP_2 protein of the stable capsid (Laporte, 1972).

We interpret the usefulness of the trypsin action, for increase in the infectivity titre of the virus through elimination of its trypsin sensitive population which interferes in the total growth of the virus population. And this is quite evident from our experience wherein the cloned virus with its fragile trypsin-sensitive nature of the capsid was suppressed in its growth and therefore, the titre of the remaining trypsin resistant population had increased.

A difference was found out in the degree of mice pathogenicity of cloned and original virus because of the changed nature of the capsid of cloned virus and its reduced ability to grow at the body temperature of the host. However the cloned virus retained its antigenicity as observed in the serum neutralisation tests performed (unpublished data).

Therefore, in our opinion the virus with the characters like :
1. less production of the infectious virus particles at the body temperature of the host,
2. unstability outside the infected cell,
3. no change in antigenicity,
can be a good virus for its use as a live (modified) vaccine.

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Summary

Using the terminal dilution technique, a cloned virus was selected in a tissue culture systems from a heterogenous population of the original virus. The different markers studied differentiated the cloned virus from the original virus on the basis of its following characters :
1. ability to grow more efficiently at 29 than at 37 °C.
2. unstability of its capsid as seen by :
a. high degree of sensitivity to heating at 50 °C for 10 min of exposure to pH 6.4 for 10 min.
b. more inactivation by overnight exposure to 37 and 4 °C.
3. late pathogenicity in the suckling mice.
The practical usefulness of a simple technique of the selection of viral clones and the possibilities of such clones to be used in the live (modified) vaccine are discussed.

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