ESTABLISHMENT OF A CLONE OF BHK-21 CELLS FAVORABLE TO RUBELLA HEMAGGLUTININ PRODUCTION

It has been shown that BHK-21 cells infected with rubella virus are able to synthesize hemagglutinating virions (Stewart et al, 1967, Vaheri et al, 1967). The rubella hemagglutinin (HA) is now being widely employed in assay of rubella antibody by hemagglutination-inhibition test in place of the time-requiring procedures for infectivity neutralization (Parkman et al, 1964).

Trials to demonstrate HA in rubella infected primary green monkey kidney cells, Vero cells (an established cell line from green monkey kidney, Yasumura and Kawakita, 1963, Liebhaber, Riordan and Horstmann, 1967) and an rabbit cornea cell line SIRC cells (Leerhøy, 1966) revealed that only Vero cells produce HA of a low level when the infected cultures were carefully maintained for more than 20 days.

In experiments concerning HA production by rubella virus, we used maintenance medium without supplement of calf serum, because it usually contains inhibitory substance(s) to rubella HA (Stewart et al, 1967). The removal of calf serum from the culture medium immediately after infection, resulted in a spontaneous degradation of cells within a week, and consequently in a low yield of HA.

In order to improve the viability of BHK-21 cells in the absence of calf serum and to obtain better yields of HA: clonal selections of the cells were performed after the method described by Puck, Marcus and Cieciura (1956). The results are presented in this communication.

M33 strain of rubella virus had been passed 32 times through green monkey kidney cells, twice through cynomolgus monkey kidney cells and 16 generations through BHK-21/WI-2 cells (Vaheri et al, 1965), before supplied from Dr. Ohtawara of this laboratory. The virus was passed four times or more in BHK-21/WI-2 cells before use in this experiment. The virus seed was a maintenance media of BHK-21/WI-2 cells pooled on the 5-6 day of infection with rubella virus. It was divided into small vials after a clarification by low speed centrifugation, and stored at -80°C.

BHK-21/WI-2 cells, passage history of which was unknown, had been maintained in this laboratory for more than one year by Dr. Ohtawara.

For growth and maintenance of BHK-21 cells, Earle's balanced salt solution containing 0.5% lactalbumin hydrolyzate, 0.1% yeast extract, 100 μg/ml of streptomycin, 100 units/ml of penicillin and 5% calf serum was used.

The cells were grown in a medium containing calf serum until a confluent monolayer was formed. Then, they were inoculated with virus at the highest possible multiplicity of infection (moi), placed for 2 hr at room temperature, washed well with phosphate buffered saline (PBS) and fed with culture medium without calf serum.

HA titration was performed in test tubes. Serial twofold dilutions of HA were made in 0.2 ml of PBS of pH 7.4 containing 0.1% bovine serum albumin (Armour’s fraction V). Two-tenths ml of goose red blood cells (RBC) suspension in PBS (OD at 490 mμ, 1 cm light path =0.45) was added to each tube and incubated at 7°C for 1 hr. HA units were determined as a reciprocal of the highest dilution that gave complete aggluti-
nation pattern of RBC on the bottom of the tube.

Infectivity of the inoculum virus was determined by the plaque method. SIRC cells were obtained by the courtesy of Dr. Leerhøj, the Statens Seruminstitut, Copenhagen, Denmark. The cells were grown in monolayers in 50 ml plaque bottles and were inoculated with 0.5 ml of a rubella virus suspension diluted tenfold in Hanks' balanced salt solution. After one hour's adsorption at room temperature, the monolayers were washed once with PBS and overlayed with 3 ml per bottle of medium 199 containing 0.9 % Noble agar, 0.001 % DEAE-dextran and 3 % calf serum. After 4 or 5 days of incubation at 37 C, 3 ml per bottle of above agar medium containing 0.008 % neutral red was added. Plaques were counted usually 20 hr later.

Typical time course of HA production by the original BHK-21/WI-2 cells infected with rubella virus is shown in Fig. 1.

![Fig. 1. Synthesis of HA by original BHK-21/WI-2 cells infected with rubella virus.](image)

Monolayers of 2 day old BHK-21/WI-2 cells in 200 ml bottles were placed in contact with 1.0 ml of rubella virus suspension for 2 hr at room temperature, washed once with PBS and fed with 10 ml per bottle of serum free medium (see text). Multiplicities of infection (moi) were 0.3 and 0.003 PFU/cell. Two bottles were employed for each moi. Five-tenths ml aliquots of media were withdrawn daily, pooled and assayed for HA after clarification by low speed centrifugation. On the 5th day of infection, media of all bottles were changed.

As shown in the figure, the higher the moi at which cultures were infected, the faster was the appearance of HA in the medium and the higher was the peak reached. In this experiment, observations were terminated on the 6th and 7th days of infection because of the nonspecific extensive deterioration of the cells.

Clonization of BHK-21/WI-2 cells were carried out in 60-mm Petri-dishes. About 100 viable cells per dish were incubated in Eagle's basal medium supplemented with 20 % of newborn calf serum at 37 C, under the humidified atmosphere containing 5 % of CO2. Average plating efficiency was around 60 %.

Twenty fresh isolates thus obtained were grown in 200 ml bottles and tested for their stability in serum-free medium. Five clones designated as 1M, 4M, 7M, 8M and
11M exhibited a satisfactory stability. Their growth characteristics in the presence of calf serum were not altered from the parent BHK-21/WI-2 cells.

Fig. 2 summarizes the time course of HA production in these clonal cells.

HA was best induced by clone 7M reaching a maximum titer of 1024 units/0.2 ml. The rest of the clones were also sensitive to rubella virus, but their responses in HA production varied considerably. Partial nonspecific deterioration of monolayers of clones 4M, 8M and 11M were noticed on the 11th day of infection. This will explain the rapid disappearance of HA in the culture media of these clones. Focal cell-fusing areas were often seen in clone 7M and less frequently in clone 1M on the 11th and 13th days of infection.

![Graph of rubella HA synthesis](image)

Fig. 2. Synthesis of rubella HA by freshly isolated clones of BHK-21/WI-2 cells.

Experimental conditions were the same as those described in the footnote for Fig. 1. Media of all bottles were changed on the 5th day of infection. The virus inoculum contained $1.3 \times 10^6$ PFU per ml per bottle (estimated moi=about 0.1 PFU/cell).

Based on these findings, clone 7M was chosen as a host cell suitable for the preparation of rubella HA. The homogeneity of cell population of this clone in the production of rubella HA was then examined by re-cloning. Results are shown in Fig. 3.

Arbitrarily chosen four sub-clones from BHK-21/WI-2/7M cells were inoculated with rubella virus and their HA productivities were compared. From the figure, it is clear that the deviation in HA accumulation curves of these sub-clones was less prominent as compared with those clones shown in Fig. 2. The main characteristics of clone 7M, i.e., the tolerance to the removal of calf serum, relatively high HA productivity and its morphological appearance consisting mostly of elongated fibroblasts, were all retained in these sub-clones.

This seems to imply that the clone 7M may be regarded practically as homogeneous cells which are favorable for mass preparation of rubella HA for diagnostic and sero-epidemiological purposes.

The clone 7M tolerated freezing with dimethylsulphoxide and keeping below $-80^\circ$C. This made possible the preservation of large pools of 7M without endangering the useful characteristics by variation through multiple passages.
Fig. 3. Synthesis of rubella HA by four sub-clones of BHK-21/ WI-2/7M.

Experimental conditions were the same as those described in the footnote for Fig. 1. Media of all bottles were changed on the 4th day of infection. The virus inoculum contained \(1.5 \times 10^6\) PFU per ml per bottle (estimated moi=about 0.1 PFU/cell).

HA produced by rubella infected BHK-21/WI-2/7M gave a single peak at the density of 1.185 g/ml in tris-buffered sucrose gradient. This value was in good agreement with those obtained by McCombs and Rawls (1968) on rubella infectivity. Therefore, in this system, the high potency of HA may be released into the medium in close association with the virion.

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