Release of Arboviruses from Cells Cultivated with Low Ionic Strength Media (36831)

TAKEO MATSUMURA, KAZUKO SHIRAKI, SUSUMU HOTTA,
AND TERUMASA SASHIKATA

Department of Microbiology, Kobe University School of Medicine and Division of Pathology, Hyogo Cancer Center, Kobe, Japan 650

Although it has been reported that low salt concentration of culture media inhibited production of viruses in tissue culture (1–3), mechanism(s) underlying the phenomena have not yet been fully clarified. A previous study, on the other hand, indicated that high concentrations of magnesium chloride in culture media caused increase of extracellular yields of dengue virus in Vero cells, that, however, was not observed in BHK-21 cells (4). The present paper describes experiments in which production of arboviruses (Chikungunya and dengue) in BHK cells cultured in media of low or high NaCl concentrations was examined. An emphasis was also put on electron microscopic observations of the culture cells for the purpose of providing a morphological basis of the phenomena.

Materials and Methods. Cell cultures. The BHK-21 cells were dispersed by trypsin and the monolayer cultures were prepared in 60 mm petri dishes throughout the experiments.

Media. Culture medium was Eagle's MEM supplemented with 5% bovine serum (heat-inactivated) and 60 μg/ml kanamycin. After infection of virus, the bovine serum was replaced by 0.4% bovine serum albumin (BSA, Fraction V, Armour, USA). Concentrations of sodium chloride in the medium were regulated as follows: 20 parts of Earle's solution (5 times concd and deprived of NaCl), 20 parts of 2% BSA, 20 parts of amino acid–vitamin mixture (5 times concd as those for Eagle's MEM) plus 0.146% L-glutamine were mixed, to which desired parts of 6.8% NaCl and sterile distilled water were added to make 100 parts.

In the present study, "normal medium" (abbreviated to N-medium) contained 0.68% NaCl, and low or high NaCl concentration medium (abbreviated to L-medium or H-medium) was prepared by changing the amounts of 6.8% NaCl solution added. In most instances, a medium containing 0.27% NaCl (40% of the physiologic concentration) was used as the L-medium (see Results below).

Viruses. Chikungunya (abbreviated to CHIK), African strain, passed in BHK-21 cell, and dengue type 1 (abbreviated to DEN-1), Mochizuki strain, passed in 2- to 3-wk-old mice intracerebrally, were used.

Infectivity assay. A modification of the method by Aoki of this laboratory (personal communication) was adopted. Confluent monolayers of BHK-21 cells grown in 60 mm petri dishes were washed once with Hanks' balanced salt solution (BSS) and inoculated with 0.5 ml of virus suspension appropriately diluted in Hanks' BSS containing 0.2% BSA. After leaving the cultures at 37° for 90 min for virus adsorption, the remaining inoculum was sucked and 9 ml of overlay medium (Eagle's MEM containing 5% bovine serum and 1.0% methyl cellulose) (5) was added. After incubation at 37° for 3 days (for CHIK) or 5 days (for DEN-1), the cultures were stained with the solution of 0.04% crystal violet in alcohol including 0.8% ammonium oxalate to count plaques (6).

Electron microscopy. The cells, either virus-infected or control noninfected, were examined by an electron microscope (Akashi S-500, Japan). Technical specifics of fixation, embedding and sectioning were the same as described previously (7). Direct magnifications were ×15,000 to 30,000 throughout the studies.

Results. Effect of salt concentration in medium on production of infective virus. Ta-

TABLE I. Extracellular Yields of CHIK and DEN-1 Viruses in BHK-21 Cell Cultures[a] Maintained in Media of Various NaCl Concentrations.

NaCl concn of medium (% of physiologic concn)	Amount of virus[b]		
		DEN-1	
	CHIK virus at 10 hr incubation	At 24 hr incubation	At 72 hr incubation
10	12,000	77,000	13,000
30	nt[c]	4000	1900
40	7700	nt	nt
50	nt	310	150
60	1600	10	610
80	47	71	81
90	66	1	69
110	1	12	2
130	93	1	92
160	57	2	21
190	20	1	15

[a] Monolayer cells were infected with virus of m.o.i. 30–40 PFU and incubated at 37° for 10 hr (for CHIK) and 24 or 72 hr (for DEN-1). Unadsorbed viruses were washed out.

[b] Reciprocal of relative titers when virus in medium of the physiologic NaCl concentration is 1.

[c] Not tested.

ble I shows that low NaCl concentrations of media resulted in a marked decrease of CHIK and DEN-1 virus yields. High NaCl concentrations brought about similar but much less effects. It is to be mentioned that the cells maintained in extremely low (0.14% or less; 20% or less of the physiologic concentration) NaCl media exhibit certain degenerative alterations under an ordinary light microscope. However, moderate concentrations of NaCl (0.27 or 0.95%; 40 or 140% of the physiologic concentration) exerted few effects on the cell morphology at least under the conditions studied. In later experiments, the medium containing 0.27% NaCl was routinely used as the L-medium. Infectivity of virus suspended in L- or N-medium for 10 hr at 37° was reduced to about 90% of the original titers, however very few differences were noted between the reduction rates in L-medium and those in N-medium.

Reversiblility of low NaCl concentration effect. Virus-infected cultures were first incubated with L-medium which later was replaced by N-medium. At intervals thereafter, titers of virus present in the medium were measured. Figure 1 shows that cultures maintained in L-medium for 4 to 24 hr released the virus within 1 hr after being put in N-medium. Actually the release of virus after replacement of media took place very rapidly, for instance, within 30 sec as shown in Fig. 2. When the medium was again changed from N to L, the virus release was suppressed also within short periods of time. Thus the inhibition and recovery of virus release could be repeated practically indefinitely in a particular culture (Fig. 3).

Dependence of virus release on ionic strength. A series of experiments was designed to determine whether the release of virus depends upon the osmotic pressure or ionic strength of medium. Infected cells incubated in L-medium for 10 hr were put in media of various osmolarities or ionic strengths and titers of virus released were

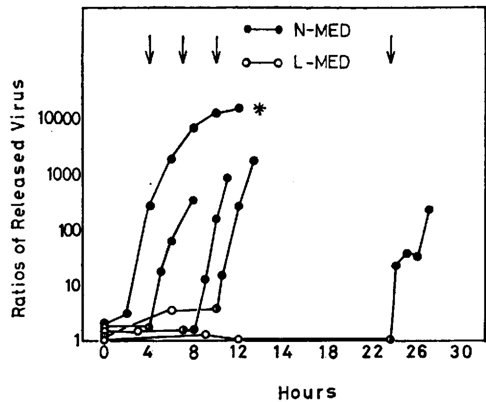

FIG. 1. Reversible inhibition of CHIK virus reproduction by changing culture media from L to N. Monolayer cells were inoculated with virus as described in Table I. After the virus was washed out, L-medium was added to the cultures which were then incubated at 37°. At the time indicated by arrows, the culture fluids were changed into N-medium and virus titers thereof were assayed by the plaque method; (●) virus titers in cultures maintained by N-medium, and (O) those by L-medium. Ordinate indicates ratios of virus titer at given time to that at "0" time. Abscissa indicates period of incubation (hr); (*) a control experiment in which cultures were maintained in N-medium throughout.

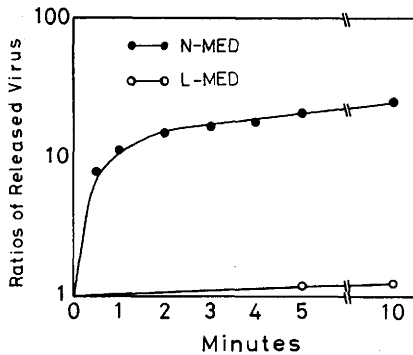

FIG. 2. Release of virus after changing culture media from L to N. Infected monolayer cells were incubated for 5 hr in L-medium which was then replaced by N-medium. At given time, the whole medium was changed and virus titers of the removed fluid were titrated (●). Control cultures were incubated continuously in L-medium and measured for virus titers (O). Ordinate indicates ratios of virus titer at given time to that at "0" time.

determined. The results obtained are summarized in Table II. It is clear from the data that the ionic strength of medium was a critical factor for the inhibitory effect. The effect of osmolarity was equivocal, although higher osmolarity in N-medium caused a slight inhibition upon virus release. No other substances such as amino acids or constituents of media seemed to be related to this effect as shown in Table III.

Electron microscopic observations. Ten hours after CHIK virus infection of BHK cells incubated in N-medium, a number of electron-dense spherical particles, about 30 nm in diameter and possessing no limiting membrane, were revealed in the cytoplasm (Fig. 4, insert). Numerous vacuoles were found, the majority of which were closely surrounded by a single layer of the dense particles. These particles appeared to be identical with "precursor particles" previously described in CHIK virus-infected Vero cells (8). Closely attached to the cell surface membrane were a number of particles which were regarded to be in the "budding process" (Fig. 4A). The budding particles could be easily distinguished from the cytoplasmic projections by the electron-density, shape and size. The envelopes of the particles were seen to be outer layer of the cell membrane (Fig. 4B); apparently the particles acquired the envelope from the cell membrane during the budding.

The same cells infected with CHIK virus and cultured in L-medium showed pictures quite different from the above mentioned. Beneath the cell surface membrane were particles of irregular electron density, which were of almost the same size of the electron-dense particles as shown in the N-medium cultured cells but which were clearly distinct from the ribosomes in size and shape (Fig. 5). In the cytoplasm, there were vacuoles, surrounding which particles similar as shown in Fig. 4 were observed. However, no "budding particles" adjacent to the cell surface membrane were found. The data indicated that the low ionic strength medium apparently influenced the budding process.

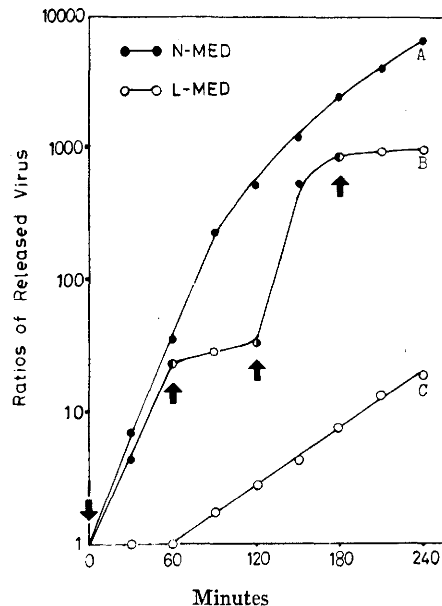

FIG. 3. Repetition of the inhibitory effect upon CHIK virus reproduction of low NaCl concentration by replacing culture medium. Monolayer cells were inoculated with virus as stated in Table II. Five hours after starting incubation in L-medium, the medium change was done as follows: (A) replaced by N-medium; (B) replaced by N- and L-medium, alternatively, at given times indicated by arrows; and (C) replaced by L-medium. Ordinate indicates ratios of accumulative virus titer at given time to that at "0" time.

TABLE II. Effect of Various Ionic Strengths or Osmotic Pressures upon Release of CHIK Virus from Cells[a] Cultured in L-Medium.

Medium	Ionic strength	Osmolarity[b] (Osm/liter)	Released virus ($\times 10^4$ PFU/ml) Before medium change	1 hr after medium change
N-Medium	0.16	0.27	1.0	345.0
L-Medium[c]	0.09	0.15	0.8	0.4
N-Medium + glucose, 0.10 *M*	0.16	0.38	1.2	381.0
0.20 *M*	0.16	0.47	0.8	121.0
L-Medium + glucose, 0.10 *M*	0.09	0.27	1.1	0.7
0.23 *M*	0.09	0.38	0.6	0.5
N-Medium + sucrose, 0.10 *M*	0.16	0.39	1.2	419.0
0.20 *M*	0.16	0.50	1.3	148.0
L-Medium + sucrose, 0.10 *M*	0.09	0.29	1.9	0.9
0.21 *M*	0.09	0.39	2.6	1.5

[a] Monolayer BHK-21 cells cultivated in L-medium for 10 hr were washed 3 times with L-medium and exposed to each of the indicated media for 1 hr; after that virus titers of the medium were measured.
[b] Measured by a Knauer Halbmikro-osmometer.
[c] Medium containing 0.27% NaCl (40% of the physiologic concentration).

Discussion and Summary. The present study indicates that production of CHIK and DEN-1 viruses in BHK-21 cells were inhibited by lowering NaCl concentrations of culture media. The inhibitory effect was reversed rapidly (within 30 sec) by placing the cells in normal medium. By changing the media from normal to low NaCl concentrations, the inhibition of virus release again took place within short periods of time. In this manner, the inhibition of virus release and its recovery could be repeated practically indefinitely in a particular culture. The effect was due to the ionic strength rather than osmolarity of media. No substances other than the salt such as amino acids or culture medium ingredients were regarded to be involved.

While the present data are compatible with those previously reported with viruses of

TABLE III. Effect of Various Solutions upon Release of CHIK Virus from Cells[a] Cultured in L-Medium.

Medium	Ionic strength	Osmolarity (Osm/liter)	Released virus ($\times 10^3$ PFU/ml) Before medium change	1 hr after medium change
N-Medium	0.16	0.23	11.1	541.0
L-Medium	0.10	0.15	6.4	9.9
Phosphate buffered saline[b]	0.17	0.25	3.7	225.0
Hanks' buffered saline	0.15	0.21	6.1	294.0
Physiologic saline[c]	0.15	0.25	6.0	185.0

[a] Monolayer BHK-21 cells cultivated in L-medium for 10 hr were washed 3 times with L-medium and exposed to each of the indicated media for 1 hr; after that virus titers of the medium were measured. Other legends are the same as stated in Table II.
[b] Dulbecco, R., and Vogt, M., J. Exp. Med. **99**, 167 (1954).
[c] NaCl (0.9%) in distilled water.

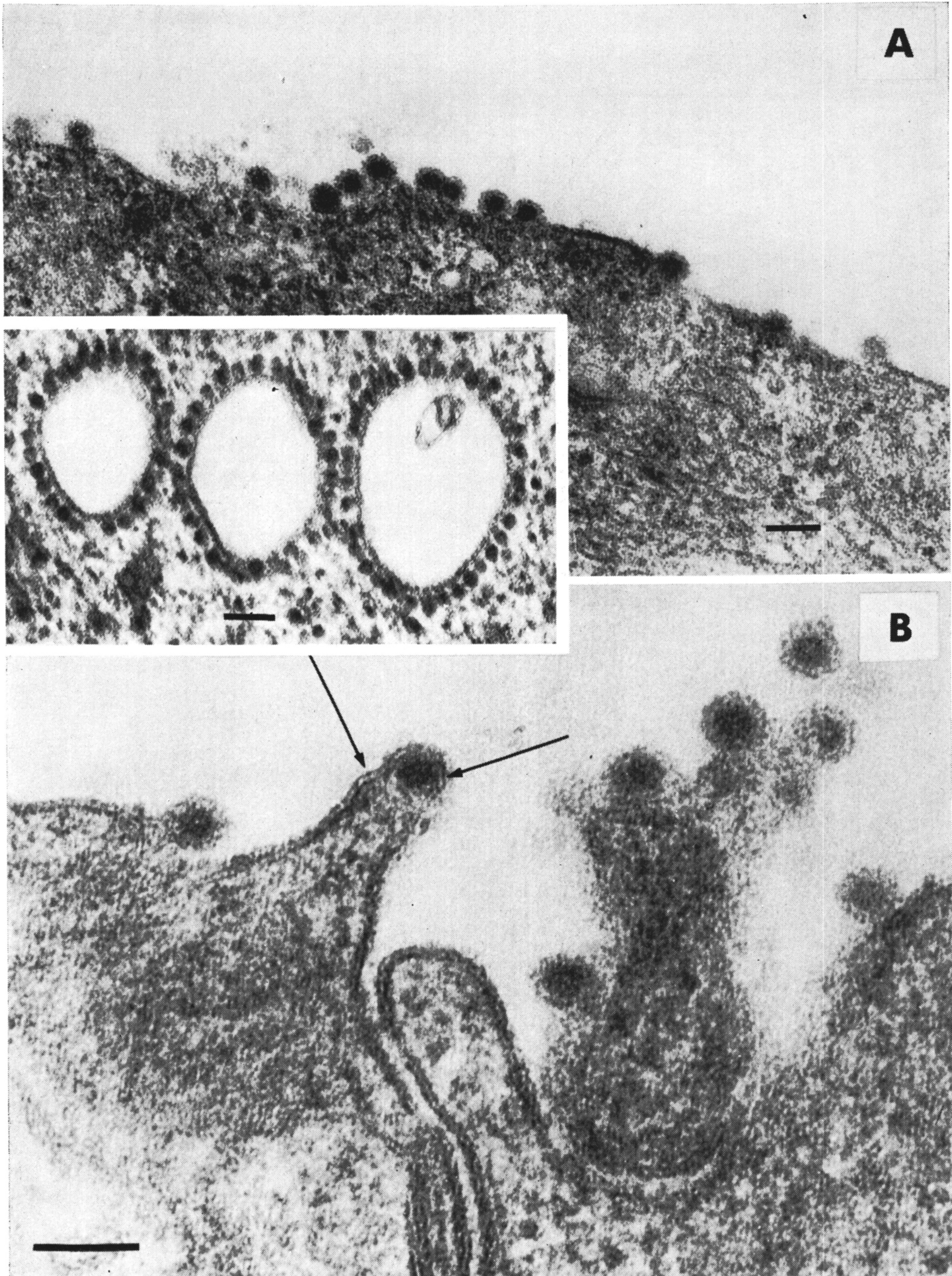

FIG. 4. Portions of a cell infected with CHIK virus and cultures in N-medium; 10 hr after the beginning of incubation. (A) Virus particles are budding from the surface of the cytoplasmic membrane; (insert) three vacuoles surrounded by a single layer of "precursor particles" are seen. Each particle, approximately 30 nm in diameter, possesses no limiting membrane. Bar represents 100 nm. (B) Budding particles on the cytoplasmic membrane. The triple layered unit membrane, visible at the tip of a projection of cell, shows an apparent continuation with the outer layer of a particle (arrows).

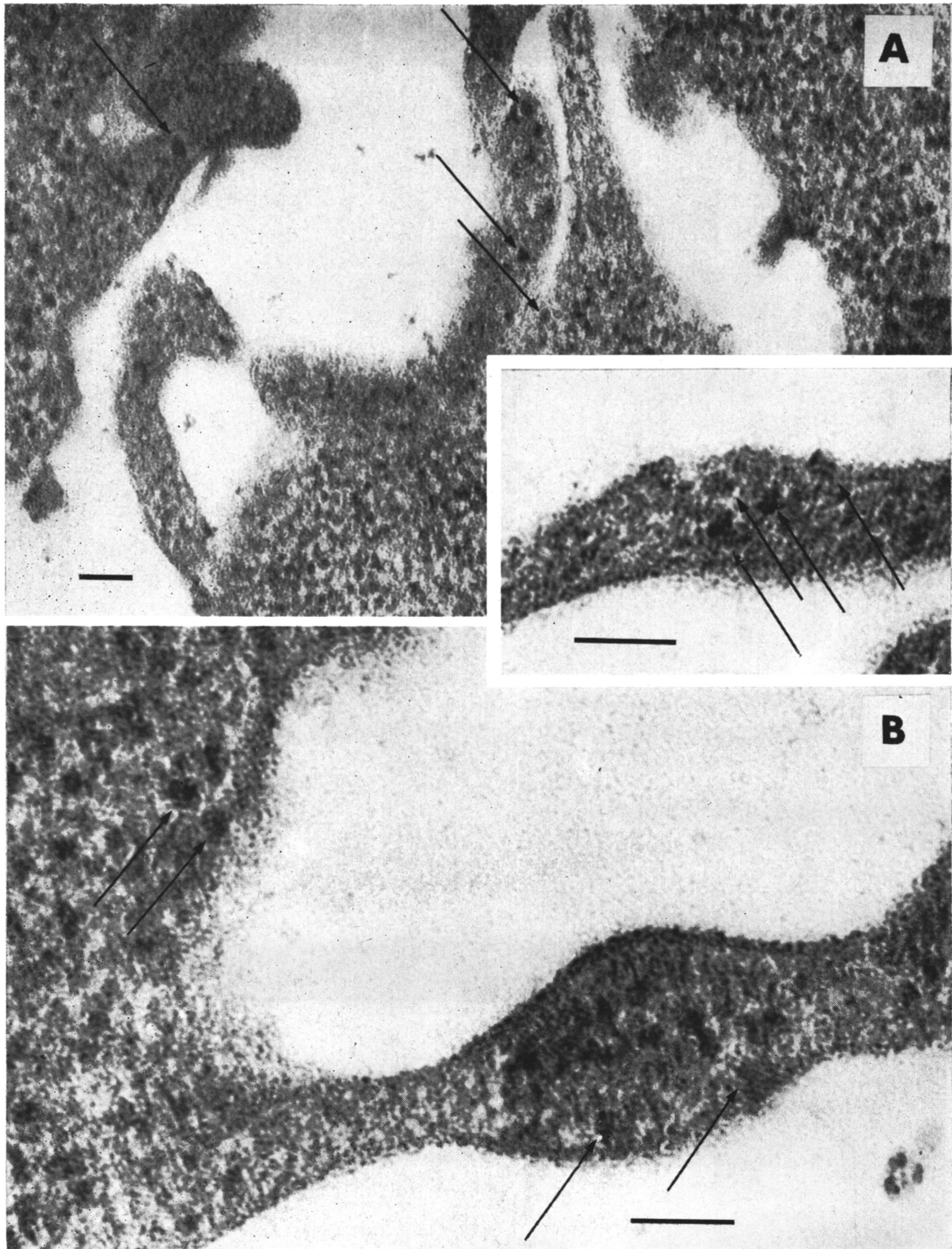

FIG. 5. (A, B) Portions of a cell infected with CHIK virus and cultured in L-medium; 10 hr after the beginning of incubation. The budding particles as characteristically shown in Fig. 4 are absent. Note that structures which can be regarded as "precursor particles" of virus are seen just beneath the cell membrane or within the cytoplasm (arrows). Bar represents 100 nm.

Sindbis (3) and polio (2), some unique findings were obtained in our electron microscopic studies. The cells cultured in low ionic strength media did not reveal characteristic pictures of viral budding from the cell surface membrane as commonly seen in cells cultured in normal media. In spite of it, the "precursor particles" (8, 9) were observed in the cells cultured in low ionic strength media as in normally cultured cells. As generally accepted (7–12), the group A arboviruses acquire their outer coat from the cell surface membrane of host cells during the budding stage. The ionic strength seems to influence this process, altering certain biochemical and/or biophysical conditions of the cell membrane structures. This may have a significant relation with the mechanism of virus release from host cells such as observed with Western equine encephalitis virus in chick embryo cells (13) or Venezuelan equine encephalitis virus in KB cells (14). Further investigations are in progress to elucidate factors related to the phenomena and will be presented in later reports.

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