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EFFECT OF AMINO ACIDS ON GROWTHS OF SINGH'S *AEDES ALBOPICTUS* CELLS AND JAPANESE ENCEPHALITIS VIRUS

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SUMMARY "Non-essential" amino acids were found to stimulate the growth of Singh's *Aedes albopictus* (SA) cells and that of Japanese encephalitis virus (JEV) in SA cells. Serine and, to a lesser extent, proline were required for maximal growth of SA cells, when cultivated with Eagle's minimal essential medium (MEM) supplemented with 10% non-dialyzed calf serum. On the other hand, growth of JEV in SA cells was stimulated by proline and, to a lesser extent, by glycine in medium for virus growth (2% calf serum in MEM). Growth of JEV in BHK21 cells did not depend on these amino acids.

INTRODUCTION

Previously, we reported that a certain strain of Japanese encephalitis virus (JEV) can grow to high titer in Singh's *Aedes albopictus* cells (Igarashi et al., 1973b). This virus-cell combination seemed to offer a good system to study arbovirus growth in mosquito cells. The properties of the progeny JEV grown in the cells were also studied (Igarashi et al., 1973a). In the early work, following the procedure of Singh (1967), the cell line was cultivated with 20% fetal bovine serum in Mitsuhashi-Maramorosch's medium which contained lactalbumin hydrolysate and Yeastolate as nutrients (Mitsuhashi and Maramorosch, 1964). This complex medium containing natural ingredients made it difficult to study the events occurring in virus-infected cells at a molecular levels, by methods such as labeling precursors of macromolecules. Accordingly we attempted to adapt the cell line to media of much simpler and defined composition, such as Eagle's minimal essential medium. We also attempted to replace fetal bovine serum with calf serum which can be obtained more easily. During studies on this adaptation, we found that certain amino acids were required for growth of the cell line and for multiplication of JEV in infected cells.

MATERIALS AND METHODS

1. *Virus*

Japanese encephalitis virus (JEV), JaOH-0566 strain, was used. The origin, passage history and methods used to prepare seed virus and to measure virus activity were as reported previously (Igarashi et al., 1973a, b), except that the medium used for virus growth was 2% calf serum in Eagle's minimal essential medium (MEM, Eagle, 1959).

2. *Cells*

The origin of Singh's *Aedes albopictus* (SA) cells

and the origin and cultivation of BHK21 cells were reported previously (Igarashi et al., 1973b).

3. *Adaptation of SA cells to MEM*

SA cells were cultivated with 10% calf serum in a mixture of MEM and Mitsuhashi-Maramorosch (MM) medium (Mitsuhashi and Maramorosch, 1964). The proportion of MM medium was progressively reduced with increase in that of MEM once a week at the time of each cell transfer, starting with a 1 : 1 mixture of MEM and MM.

For cell transfer, the growth medium was removed and the cell sheets were washed twice with PBS (Dulbecco and Vogt, 1954) and once with 0.05% trypsin (Difco, 1 : 250) and 0.02% EDTA in PBS. Then the washing solutions were removed and bottles were kept for 15 min at room temp. Cells were resuspended by pipetting in fresh medium at a concentration of $1\text{–}2\times10^5$ cells/ml (4–8 fold dilution of the cells). Aliquots of 5 ml of the cell suspension were put into 2 oz, rubber-stoppered prescription bottles and incubated at 28 C.

4. *Measurement of cell growth*

SA cells were transferred at a concentration of 1.5×10^5 cells/ml to basal growth medium consisting of 10% calf serum in MEM with or without supplements. At each time of sampling measurements were made on at least triplicate cultures in 2 oz bottles. The growth medium in each bottle was discarded and the cell sheet was washed twice with 4 ml of PBS and then scraped into 3 ml of PBS using a rubber-policeman. Part of the cell suspension was mixed with an equal volume of 0.1% crystal violet in 0.1 M citric acid and the nuclear count was measured in a hemocytometer (Haff and Swim, 1957). Another part was used to measure protein content by the method of Lowry et al. (1951), with bovine serum albumin as standard. The rest of the cell suspension was used to measure turbidity, by reading the optical density at 550 mμ using a Karl-Zeiss spectrophotometer, type PMQII, with a 10 mm light path. Photomicrographs of intact cells were taken with a Nikon inverted microscope, type MD, with DDL 10 objective lens at a direct magnification of 100, using Fuji Minicopy film.

5. *Measurement of virus growth*

Growth medium was removed from confluent sheets of SA or BHK21 cells. Then they were inoculated with seed virus (0.2 ml/2 oz bottle) at an input multiplicity of 10 PFU/cell. During the adsorption period of 2 hr, at 28 C for SA cells and at 37 C for BHK21 cells, virus was spread every 30 min. Then, cell sheets were washed twice with 4 ml of PBS to remove unadsorbed virus, and were overlayed with medium for virus growth consisting of 2% calf serum in MEM with or without supplements. After incubation at 28 C for SA cells and 37 C for BHK21 cells, portions of infected culture fluid from duplicate cultures were pooled every day and assayed for JEV activity.

6. *Nutrients*

Heat-inactivated calf serum, lot No. 2, was obtained from the Tissue Culture Center, The Research Foundation of Microbial Diseases of Osaka University, and was used throughout this work without dialysis. Powdered MEM in Earle's balanced salt solution (Earle, 1943), lacking glutamine and $NaHCO_3$, and containing final concentrations of 0.64 mM succinic acid, 0.37 mM sodium succinate and 60 mg/liter of Kanamycin was supplied from Nissui Seiyaku Co. Ltd. Tokyo. The powder was dissolved in redistilled water as indicated and 0.3 g of L-glutamine and 1.2 g of $NaHCO_3$ per liter were added. Then it was sterilized by filtration. Lactalbumin hydrolysate and Yeastolate were products of Nutritional Biochem. Co. and Difco Lab., respectively. Amino acids were purchased as the L-isomers from Nakarai Chem. Co. Kyoto. Stock solutions of each amino acid were prepared in redistilled water at 100 fold the final concentrations required in the basal medium, and were sterilized by filtration through a Millipore HA filter or by autoclaving (110 C, 10 min).

RESULTS

1. *Effects of amino acids on growth of Singh's Aedes albopictus (SA) cells*

In the early stage of adaptation of SA cells to MEM, the growth rate was fairly good. However, after reducing the MM content to less than 10% of MEM, cell growth gradually decreased and with an MM content of less than 5% of MEM, cell growth almost stopped. So some components in lactalbumin hydrolysate or Yeastolate seemed necessary for growth of SA cells. As shown in Table 1,

TABLE 1. *Growth promoting effect of lactalbumin hydrolysate, Yeastolate and "non-essential" amino acids on the growth of Singh's Aedes albopictus cells*[a]

Addition to basal growth medium[b]	Turbidity OD_{550}	Nuclear count (10^5/0.5 ml)	Protein mg/ml
None	0.04±0.01	0.4±0.1	0.02±0.01
Lactalbumin hydrolysate, 0.8 mg/ml	0.23±0.06	3.8±1.2	0.15±0.03
Yeastolate, 0.6 mg/ml	0.16±0.09	2.5±1.5	0.11±0.06
"Non-essential" amino acids[c]	0.22±0.01	3.6±0.2	0.13±0.12

[a] Average of five replicate experiments with SE, sampled 7 days after cell transfer.
[b] 10% calf serum in MEM.
[c] Alanine, asparagine, aspartic acid, glutamic acid, glycine, proline and serine, at a final concentration of 0.1 mM.

TABLE 2. *Effects of omitting each of the 7 "non-essential" amino acids on the growth of Singh's Aedes albopictus cells*[a]

Amino acid omitted	Turbidity OD_{550}	Nuclear count (10^5/0.5 ml)	Protein mg/ml
None	0.23±0.01	3.8±0.2	0.10±0.00
All 7	0.09±0.03	0.9±0.3	0.04±0.02
Alanine	0.22±0.02	3.9±0.4	0.12±0.02
Asparagine	0.21±0.02	4.2±0.7	0.10±0.01
Aspartic acid	0.22±0.02	4.2±1.0	0.11±0.01
Glutamic acid	0.23±0.03	3.9±0.5	0.11±0.01
Glycine	0.23±0.03	3.9±1.1	0.10±0.01
Proline	0.18±0.01	3.5±0.4	0.09±0.00
Serine	0.11±0.02	1.1±0.3	0.05±0.01

[a] Average of triplicate experiments with SE, sampled 7 days after cell transfer in basal growth medium supplemented with 7 "non-essential" amino acids (0.1 mM each).

when the basal growth medium consisting of 10% calf serum in MEM was supplemented with lactalbumin hydrolysate, growth of SA cells was stimulated, while addition of Yeastolate was less effective. Addition of 7 "non-essential" amino acids (Eagle, 1959) was almost as effective as addition of lactalbumin hydrolysate. Thus some of the "non-essential" amino acids seemed to be required for growth of SA cells. Accordingly we prepared mixtures, each lacking one of the 7 "non-essential" amino acids, and tested their effects in stimulating growth of SA cells. Table 2 shows that omission of serine resulted in reduction of cell growth to almost the same level as that of the control without any supplements. Omission of proline resulted in slight decrease in the cell growth. Cell growth was not affected by omission of one of the other 5 amino acids (alanine, asparagine, aspartic acid, glutamic acid and glycine).

The effects of serine and proline on the growth of SA cells are shown in Fig. 1. For the first 5 days after cell transfer, the growth rates were similar in cultures supplemented with serine alone, serine and proline, and all 7 of "non-essential" amino acids, each at a final concentration of 0.1 mM. However, the growth rate decreased after 5 days in cultures supplemented with serine alone, but not with

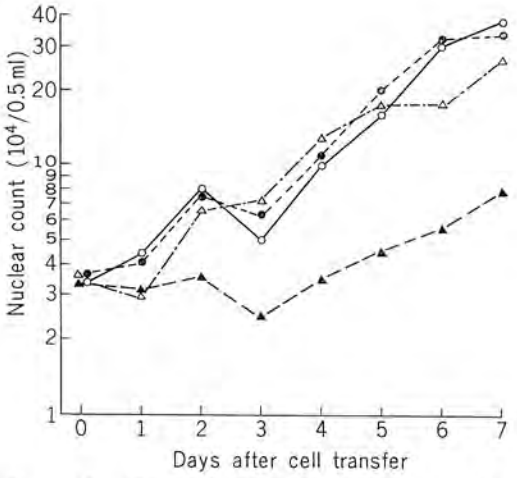

FIGURE 1. *Effects of serine and proline on growth of Singh's Aedes albopictus cells.*
Basal growth medium consisting of 10% calf serum in MEM was supplemented with 7 "non-essential" amino acids (●-----●) each 0.1 mM; with serine and proline, 0.1 mM each (○——○); with serine 0.1 mM (△-·-·-△); without any supplementation (▲----▲).

serine plus proline or all 7 "non-essential" amino acids. No significant stimulation of cell growth was observed on supplementation of proline only. After 7 days, the cells began to shrink and become detached from the glass surface in cultures without any supplements, or with proline or serine only. This was not observed in cultures supplemented with serine and proline or with all 7 "non-essential" amino acids. Cell growth was stimulated to almost the same extent by supplementation with 0.1–10 mM serine and 0.1–4 mM proline.

2. *Effects of amino acids on the growth of JEV*

Preliminary experiments indicated that 2% calf serum in MEM was not so effective for JEV production in SA cells as the 1 : 1 mixture of MEM and MM in previous experiments (Igarashi et al., 1973b). Since "non-essential" amino acids were found to stimulate growth of SA cells, their effects on the growth of JEV in SA cells were examined. Addition of 7 "non-essential" amino acids, each at a concentration of 0.1 mM, to the medium for

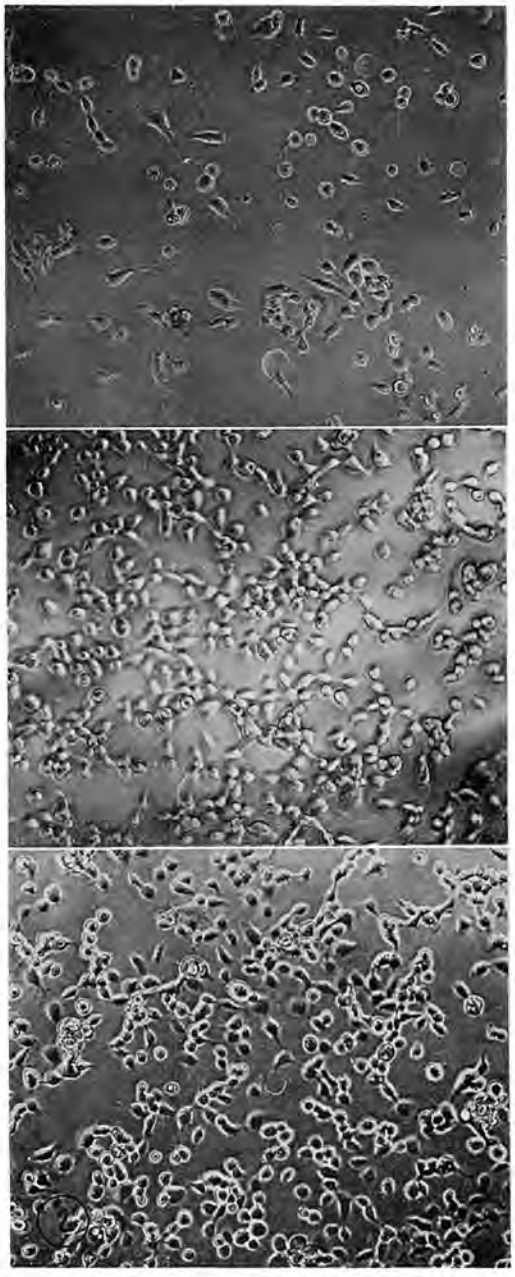

FIGURE 2. *Phase contrast micrographs of Singh's Aedes albopictus cells 5 days after cell transfer to basal growth medium consisting of 10% calf serum in MEM (upper), supplemented with 0.1 mM serine (middle), and supplemented with 0.1 mM each of serine and proline (lower). Magnification: ×440.*

TABLE 3. *Effects of omitting each of the 7 "non-essential" amino acids on the growth of JEV in Singh's Aedes albopictus cells*[a]

Amino acid omitted from medium for virus growth[b]	Maximum titer of JEV (10^8 PFU/ml)
None	4.69±0.56
All 7	0.53±0.16
Alanine	4.74±0.95
Asparagine	4.53±1.04
Aspartic acid	6.45±0.75
Glutamic acid	4.45±0.52
Glycine	3.25±0.50
Proline	0.54±0.10
Serine	4.25±0.70

[a] Average of triplicate experiments with SE, sampled until 7 days after virus inoculation. Incubated at 28C.
[b] 2% calf serum in MEM supplemented with 7 "non-essential" amino acids (0.1 mM each).

virus growth increased the virus yield (Table 3). Omission of proline reduced the yield of JEV to almost the level in control cultures without supplements, and omission of glycine slightly decreased the virus yield. On the other hand, omission of any one of the other 5 "non-essential" amino acids (alanine, asparagine, aspartic acid, glutamic acid and serine) did not decrease the yield of JEV in SA cells. The titers of JEV recovered by homogenizing the infected cells were lower than those of the infected culture fluids both with or without amino acid supplementation.

These results on the effects of "non-essential" amino acids on the yield of JEV were supported by results of experiments on the effects of adding the 7 "non-essential" amino acids to the medium for virus growth in SA cells (Table 4). Addition of proline greatly increased the yield of JEV and addition of glycine increased it slightly. When the medium for virus growth was supplemented with proline plus glycine, the virus titer approached that obtained on adding all 7 "non-essential" amino acids. None of the other

TABLE 4. *Effects of adding each of the 7 "non-essential" amino acids on the growth of JEV in Singh's Aedes albopictus cells and in BHK21 cells*[a]

Amino acid added to medium for virus growth[b]	Maximum titer of JEV (10^8 PFU/ml) grown in	
	SA	BHK21
None	1.13±0.08	0.72±0.13
Alanine	1.18±0.44	0.91±0.12
Asparagine	0.55±0.22	0.82±0.15
Aspartic acid	0.70±0.22	0.87±0.11
Glutamic acid	0.71±0.27	0.74±0.11
Glycine	1.45±0.44	0.82±0.17
Proline	7.14±0.68	0.75±0.10
Serine	1.15±0.04	0.65±0.08
All 7	15.3 ±4.1	0.54±0.07
Proline and glycine	13.6 ±0.3	NT[c]
Proline and serine	6.74±1.79	NT

[a] Average of triplicate experiments with SE, sampled until 7 days after infection at 28 C for SA cells, and until 3 days after infection at 37 C for BHK21 cells.
[b] 2% calf serum in MEM. Final concentration of each amino acid: 0.1 mM.
[c] Not tested.

5 "non-essential" amino acids caused any significant stimulation of growth of JEV in SA cells. So proline, and to a lesser extent, glycine seem necessary for multiplication of JEV in SA cells. Multiplication of JEV in BHK21 cells was not affected by addition of these "non-essential" amino acids (Table 4).

DISCUSSION

Serine and, to a lesser extent, proline were required for maximal growth of Singh's *Aedes albopictus* (SA) cells. The requirement for serine was observed even in medium containing 10% of non-dialyzed calf serum and a moderate sized inoculum of cells (1.5×10^5 cells/ml). The requirement for serine was also observed with several other lots of calf serum tested. This suggests that serine is strongly required for growth of SA cells.

There are several reports of the serine requirement of mammalian cells. When a relatively small inoculum of a human cell line, such as under cloning conditions, was tested with dialyzed serum, serine was required, but no significant serine requirement was observed with undialyzed serum (Lockart and Eagle, 1959; Eagle and Piez, 1962). For growth of rabbit fibroblasts the serine requirement was less specific than that of 13 essential amino acids (Haff and Saim, 1957). The growth of several stable lines and primary cultures of mammalian or avian cells were stimulated by serine and glycine (Neuman and Tyrell, 1960), but in some cases glycine was more effective than serine (McCoy, Maxwell and Kruse, 1959) or serine could be replaced by glycine (Herzenberg and Rosa, 1960). In the case of SA cells, glycine apparently cannot replace serine. Less attention has been paid to the serine requirement of invertebrate cells than that of mammalian or avian cells. In this connection it seemed interesting that Wyatt's medium for cultivation of cells of the silkworm, *Bombyx mori* L. (Wyatt, 1956), contained a high concentration of serine (10.5 mM). No growth stimulatory effect of proline has been reported for mammalian cell cultures (McCoy, 1960). However, Landureau and Jollès (1969) showed that proline was required for growth and survival of a cell line of the cockroach, *Periplaneta*.

In virus-infected mammalian cells, deficiency of a certain amino acid(s) has been found to inhibit propagation of viruses. Arginine deficiency inhibited the growth of DNA viruses, such as adeno (Rouse, Bonifas and Schlesigner, 1963), herpes (Tankersley, 1964), SV40 (Goldblum, Ravid and Becker, 1968) and polyoma (Winters and Consigli, 1969), as well as RNA containing Newcastle disease virus (Iinuma, Maeno and Matsumoto, 1973). Other amino acids were found to be required in certain cases, such as lysine for reovirus (Loh and Oie, 1969), serine for Newcastle disease virus (Ito et al., 1969) and phenylalanine for respiratory syncytial virus (Levine, Buthala and Hamilton, 1971). However, there is no report of proline requirement for growth of animal viruses. Moreover there are no reports of any amino acid requirements for growth of arboviruses.

The mechanism of the requirement of serine (and proline) for growth of SA cells and of proline (and glycine) for growth of JEV in SA cells are unknown. Glycine did not replace serine for growth of SA cells and serine did not replace glycine for growth of JEV in SA cells. This is in contrast to the case of vaccinia virus replication in L cells (Holterman, 1969), where the amino acids required for virus growth were the same as those required for growth of L cells themselves. Serine and glycine are known to be interconverted in mammalian cells by serine hydroxymethylase (Lembach and Charalampous, 1967), so this interconversion may be poor or absent in SA cells.

Proline had more effect on the growth of JEV in SA cells than on the growth of SA cells, and the growth of JEV in BHK21 cells did not depend on the presence of proline (and glycine). These findings suggest that proline is not synthesized so much in JEV-infected SA cells as in BHK21 cells, or that more proline is required in JEV-infected cells than uninfected SA cells.

The inhibitory effect of proline (or glycine) deficiency on growth of JEV in SA cells was not complete. This may be because the SA cells were not depleted of these amino acids before virus inoculation and because we did not use dialyzed serum. These problems require further study.

We are now able to propagate JEV in SA cells in semi-defined medium. We have also transferred SA cells in MEM supplemented with serine and proline and 10% calf serum for more than 10 generations.

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