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NUTRITION AND THE GROWTH OF HUMAN ADENOVIRUS TYPE 1 IN MONKEY CELL CULTURES: THE IMPORTANCE OF ARGININE*

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Summary. — When the interval between the last feeding of the eta line of rhesus monkey kidney cells and their inoculation with human adenovirus type 1 (HA1) was two or three days, the cells were markedly refractory to the cytopathic effects of the virus and supported only meagre virus multiplication (1% of the control); in contrast, control eta cell cultures fed fresh medium on the day of inoculation were rapidly destroyed by HA1 and supported virus multiplication well (titers of 10^7 to 10^8 PFU/ml) as expected. This contrast in the effect of HA1 was found to be due mainly to the alteration or conditioning of the medium by the eta cells during the 2 or 3 day pre-inoculation period. The addition of L-arginine to eta-conditioned medium completely, or nearly completely, restored the capacity of the medium to support HA1 cytopathic effects and multiplication on eta cells. Addition of the other medium components tested did not restore this capacity, except that calf serum had slight restorative activity, probably due to its small content of arginine. Thus, the eta cells apparently have a high capacity to deplete the medium of arginine, much higher than that of the KB line of human carcinoma cells. Some of the instances of poor growth of human adenoviruses on monkey cells may be due to unusually rapid depletion of arginine from the medium by the monkey cells.

Introduction

In the usual tests for viral cytopathic effects on vertebrate cells cultured under liquid medium, the cells are fed fresh medium on the day of their inoculation with virus. With such a feeding, cultures of the eta line of rhesus monkey kidney cells (Chapin and Dubes, 1964) are rapidly destroyed by human adenovirus type 1 (HA1). When, however, the inoculation with virus is delayed for two days after adding the fresh medium, and there is no further feeding with fresh medium, the eta cells are nearly completely refractory to the cytopathic effects of the virus, and yet are maintained in good con-

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dition by this medium for several days, usually 8 to 13 days, after inoculation, as judged by microscopic examination.

Thus, after two days, at 37° C, the medium or the cells, or both, are changed in some way which makes the culture unable to support the usual cytopathic effects of the virus. The purpose of this report is to show (a) that the major important change is in the medium and that this change is the depletion of arginine from the medium by the eta cells, and (b) that change in the cells is also important, but plays a distinctly minor role.

To report that adenoviruses require exogenous arginine is not new: in 1959, Bonifas and Schlesinger reported a high requirement for exogenous arginine for plaquing HA2 on the KB line of human carcinoma cells. It turned out, however, that their KB cultures were contaminated with *Mycoplasma*; nonetheless, when they used KB cultures freed of *Mycoplasma*, an absolute, though markedly lower, requirement for exogenous arginine was still found (Rouse *et al.*, 1963). The *Mycoplasma* had elevated the amount of exogenous arginine required by rapidly depleting it from the medium.

What is new in the present report is the stress on the importance of the kind of vertebrate cell used. We used two kinds of cells: eta and KB, neither contaminated with *Mycoplasma*; but only the eta cells showed the marked dependence of the adenovirus cytopathic effects on time of feeding, and the data suggest that the eta cells deplete arginine from the medium at a far greater rate than do the KB cells. We further emphasize the relevance to the design and interpretation of certain experiments of this competition between cell and virus for exogenous nutrilite.

Materials and Methods

Cells. The monkey cells used were the eta line (Chapin and Dubes, 1964) of rhesus monkey kidney cells. For virus titration and comparative purposes, the KB line of human carcinoma cells was used. The KB cells were calf serum-adapted cells obtained from the American Type Culture Collection (ATCC). Tests of both lines for *Mycoplasma* and of the eta line for infective simian virus 40 (SV40) were negative (Dubes *et al.*, 1968).

Media. Cells were grown and maintained under medium S (Chapin and Dubes, 1964), which differs from the basal medium of Eagle (BME) (1955) only in the following ways: Medium S contains 4% calf serum, L-cystine at four times its concentration in BME, and the balanced salt solution of Hanks and Wallace (1949) in place of the salt solution of BME, except that the concentration of $NaHCO_3$ is kept at 20 mM. For washing the cells and diluting the virus stocks, the phosphate-buffered saline (PBS) of Dulbecco and Vogt (1954) was used.

Viruses, plaquing, and multiplication experiments. The HA1 strain was Adenoid 71, which was obtained from the ATCC as a stock without history of passage of non-human cells. A rapid (*r*) mutant of Adenoid 71 was used in some experiments. Virus stocks of Adenoid 71 wild type and *r* mutant were found negative for *Mycoplasma*, using the Hayflick (1965) medium sensitive even to fastidious *Mycoplasma*; positive controls of the fastidious *Mycoplasma orale* inoculated in the same tests produced many colonies.

The plaquing of and multiplication experiments with these viruses were done essentially as described by Dubes *et al.* (1968). Infectivity was titrated by plaquing on KB cells, which are about twenty times as sensitive as eta cells to plaque-forming units (PFU) of HA1, either wild type or the *r* mutant. All references to titers or to numbers of PFU inoculated are based on such titrations on KB cells. Early harvests, i.e. those made from 1.5 to about 20 hours after inoculation, showed an essentially constant residuum of infectivity, often about 10^4 PFU/ml; most of this residuum is apparently adsorbed but uneclipsed inoculum virus.

Results

Dependence of viral cytopathic effects on time of feeding

Representative data showing the striking effect of time of feeding eta cells on HA1 cytopathology are shown in Table 1. For comparative purposes, analogous data obtained with KB cells in the same experiment are also shown in this table. With KB cells, the effect of time of feeding was of much lower magnitude than with eta cells.

Table 1. Dependence of HA1 cytopathic effects on time of feeding

Cell[1])	Interval[2])	Mean cytopathic score[3]) at day[4])						
		3	5	6	10	11	13	17
eta	0	0.1	0.9	2.0	4.0			
	1	0	0	0.1	1.0	1.1	1.1	1.9
	2	0	0	0	0	0	0	0.2
	3	0	0	0	0	0	0	0
	4	0.2	0.2	0.3	0.5	1.7	3.0	3.3
KB	0	0.2	3.4	4.0				
	1	0.2	2.1	2.8	4.0			
	2	0.2	0.6	1.2	4.0			
	3	0.4	2.5	3.1	4.0			
	4	0.5	0.9	1.9	4.0			

[1]) The cultures were started by delivering 1.5 ml of cell suspension in fresh medium per 16 × × 150 mm test tube 7 days before inoculation with virus. After starting, each culture was fed with fresh medium only once. This was done by discarding the used medium and adding 2 ml of fresh medium per tube.

[2]) Days between replacement of the culture medium and inoculation with virus.

[3]) Three or four tube cultures were scored for each interval for each kind of cell. The scoring was made on the scale: 0, no cytopathic effect detected; up to 4, complete destruction of the cells.

[4]) Number of days after inoculation with 10^5 PFU of wild-type HA1 in 0.05 ml PBS per tube.

The two factors accounting for this dependence

The effect of time of feeding on viral cytopathology could be mediated through changes in either the cell population or the medium, or both. We tested the two possibilities by inoculating (a) eta cultures which had been fed fresh medium three days before inoculation *and* on the day of inoculation (day i) and (b) eta cultures which were fed only on day i but with medium which had been "conditioned" by parallel eta cultures for three days. As compared with the usual eta cell system (fresh medium only on day i), system (a) thus had the same medium but a different cell-feeding history, whereas system (b) had the same cells but a medium which had been "conditioned". The results of a typical experiment of this kind are presented in Table 2. The data show that both the cell-feeding history and the conditioning of the medium are important factors, though the latter is by far the more important.

Table 2. The effects of cell feeding history and of conditioning the medium

Medium added at day[1])		Mean cytopathic score at day[1])		
i−3	i	i+5	i+7	i+11
None	Fresh	1.4	3.8	4.0
Fresh	None	0	0	0[3])
Fresh	Fresh	0.4	0.9	4.0
None	Conditioned[2])	0	0	0[3])

[1]) Day i is the day of inoculation with virus. The cultures were started on day i−9, and the procedures were as in Table 1.
[2]) Conditioned by parallel eta cultures for three days.
[3]) In some tests, cultures handled like these had fewer cells still adhering to the glass than in corresponding PBS-inoculated control cultures; thus in these cases, HA1 effected an attrition, without producing the characteristic adenovirus cytopathology.

By varying the pre-feeding history over a wider range, the effect of this history on HA1 cytopathology could be greatly amplified. An illustrative experiment is shown in Table 3. In this experiment the medium added on day i was a constant; that is, all the cultures received fresh medium on day i.

"Conditioning" the medium at 37° C in the CO_2 incubator in the absence of cells had no effect on the capacity of the medium to support HA1 cytopathology. Conditioning the medium by KB cells instead of eta had a small effect, as shown in Table 4, in which are presented the results of a crisscross test. Such crisscross tests were complicated by the fact that heterologous conditioned media provided inferior cell maintenance; this difficulty was acute in the case of trying to maintain KB cells under eta-

Table 3. Amplification of the effect of cell-feeding history

Medium added at day*				Mean cytopathic score at day						
i−12	i−9	i−6	i−3	i+2	i+4	i+6	i+8	i+11	i+12	i+13
None	None	None	None	1.8	2.2	3.9	4.0			
Fresh	None	None	None	0.2	0.8	3.0	4.0			
Fresh	Fresh	None	None	0.1	0.2	1.6	3.8	4.0		
Fresh	Fresh	Fresh	None	0	0.1	0.1	0.5	4.0		
Fresh	Fresh	Fresh	Fresh	0	0	0	0	0	0.6	1.1

* The cultures were started on day i−15, and the procedures were as in Table 1. All the cultures received fresh medium on day i.

conditioned medium. Nonetheless, the crisscross tests clearly showed that eta cells were superior "conditioners".

Effects of additions to eta-conditioned medium

The effect of conditioning the medium on its capacity to support HA 1 cytopathology could be mediated either through addition of something to the medium or of subtraction of something from the medium, or both. We

Table 4. Crisscross test

Cell	Medium*	Mean cytopathic score at day						
		i+2	i+5	i+6	i+8	i+9	i+12	i+13
eta	Fresh	0.2	0.9	1.3	2.9	3.9	4.0	
	eta-conditioned	0	0	0	0	0	0	0
	KB-conditioned	0	0.2	0.4	1.1	2.6	4.0	
KB	Fresh	0.1	3.0	4.0				
	eta-conditioned	0	0	unsc.**				
	KB-conditioned	0.1	1.2	4.0				

* Added at day i. The conditioning was for two days.
** Unscorable. The corresponding PBS-inoculated controls had degenerated.

tested the subtraction hypothesis by adding back to eta-conditioned medium each of the components of fresh medium S, except inorganic salts, phenol red, and antibiotics. The additions were made from concentrated solutions so that the dilution of the conditioned medium would be inappreciable. The amount added was such that after addition the concentration of the component would be the same as in fresh medium plus whatever was the con-

Table 5. Restorations to eta-conditioned medium

Medium*	Mean cytopathic score at day						
	i+3	i+6	i+8	i+10	i+12	i+14	i+17
Fresh	0.1	0.4	1.2	2.5	4.0		
Cond.	0	0	0	0	0	0	0
Cond. + L-cystine**							
Cond. + D-glucose	0	0	0	0	0	0	0
Cond. + EAA	0.1	0.2	2.2	3.0	4.0		
Cond. + vitamins	0	0	0	0	0	0	0
Cond. + L-glutamine	0	0	0	0	0	0	0
Cond. + calf serum	0	0	0.1	0.2	0.8	1.1	2.5
Cond. + all six	0	0	0.2	0.5	2.1	2.6	4.0

* Added on day i. Cond = 3-day eta-conditioned medium. EAA = the other eleven amino acids of BME. "All six" means that all 6 of the above additions were made to the same sample of conditioned medium.
** At this level, the L-cystine was cytotoxic. In other tests, lower concentrations were tested and the L-cystine was found not to restore the capacity of the conditioned medium to support HA1 cytopathology.

centration after conditioning. The effects of such additions on the capacity of the medium to support HA1 cytopathology are shown in Table 5. The concentrate of eleven amino acids (EAA) showed full activity, calf serum showed slight activity, and the complete restoration designated "all six" showed moderate activity though decidedly less than EAA alone, thus suggesting inhibition of the effect of EAA by one or more of the other five additions.

The eleven amino acids in EAA are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and tyrosine. Each of these amino acids was tested individually. L-Arginine was

Table 6. Virus multiplication under various media

Medium[1])	Log titer[2])					
	Experiment 1[3])			Experiment 2[3])		
	A	B	C	A	B	C
Fresh	8.0	8.2	8.0	7.2	6.2	5.9
Fresh-arginine[4])	5.0	5.1		4.7		
Cond.	5.7			5.0	4.7	
Cond. + EAA	7.4					
Cond. + L-arginine	7.5	7.6		6.7	5.9	
Cond. + OTAA	5.7	5.3		4.8	4.2	

[1]) Added at day i. Cond. = 3-day eta conditioned medium. EAA = eleven amino acids, as before. OTAA = EAA minus arginine.

[2]) Log_{10} (PFU/ml). Eta cultures were grown in 60 mm Petri dishes. The cultures for experiment 1 were started on day i—10 and were not fed fresh medium after starting and before inoculation; those for experiment 2 were started on day i-23 and had their medium replaced with fresh on days i—16, i—9, i—6 and i—3. In experiment 1, the confluent eta cell sheets were inoculated with 1.3×10^7 PFU of HA1 wild type, incubated at 37° C for 90 minutes, washed 5 times with PBS, fed with 5 ml medium, and harvested 2 days later; experiment 2 was the same except inoculum was 8.8×10^6 PFU, adsorption was for 20 minutes, the number of washes was 2, the volume of medium was 8 ml, and harvests were 6 days after inoculation.

[3]) Log titer of residuum in Exp. 1 was 4.1. In Exp. 2, residuum was not titrated. A, B and C refer to replicate harvests, where obtained.

[4]) Fresh medium minus arginine except for the small amount of arginine contributed by the 4% serum component.

found to restore completely the capacity of eta-conditioned medium to support HA1 cytopathology. None of the other ten showed any restorative activity; nor did the addition of all ten have any activity.

Correlation with virus multiplication

The virus might be multiplying normally in eta cells under eta-conditioned medium even though the viral cytopathology is nil or only slight, or in some cases of the atypical "attrition" type (see Table 2 legend). Total harvests were made from such cultures, from control cultures under fresh medium, and from cultures under conditioned medium plus restorative and non-restorative additions. Fresh medium minus arginine was also tested as an interesting reference. The harvests were titrated on KB cells; representative

results are presented in Table 6. These results on virus multiplication correlate well with those on viral cytopathology. The virus did multiply to a limited extent uder conditioned medium, the infective yield being roughly 1% of that under fresh medium and somewhat higher than that under fresh medium minus arginine. L-Arginine almost completely restored the capacity of the medium to support HA1 multiplication, whereas the mixture of the other ten amino acids exhibited no restorative activity.

Discussion

Our results with HA1 and arginine emphasize the importance of the nature of the host vertebrate cell being used. Repeated tests of the eta cells, and of the virus stocks used, revealed no *Mycoplasma*. It appears very likely that the eta cells themselves rapidly deplete the medium of arginine, and in so doing render the milieu unsuitable for good growth of HA1. The fate of the arginine removed from the medium by the eta cells is not known, though no doubt part of this arginine is incorporated into cell proteins, and probably not much of it is converted into argininosuccinate or citrulline and no further, since exogenous citrulline can substitute for arginine for adenovirus growth, whereas ornithine cannot (Rouse and Schlesinger, 1967).

How the eta cell line compares with other monkey kidney cell lines, and with different primary monkey kidney cell cultures, in capacity to deplete the medium of arginine is not known. Poor growth of human adenoviruses (in the absence of helper viruses) on monkey kidney cell cultures has been repeatedly observed; and that the growth may be poor, in some instances, because of relatively rapid depletion of arginine from the medium by the cells seems worth considering.

Though eta cells apparently have the capacity to deplete arginine from the medium rapidly, we would expect that the rate of this depletion would depend on the feeding history of the eta cell culture, through the effect of feeding history on the activities of relevant enzymes, for example arginase, arginine: glycine amidinotransferase, arginyl RNA synthetase, and argininosuccinase. This expectation may provide the explanation for data such as those presented in Table 3, where eta cell cultures which had been fed regularly every third day showed much greater refractoriness to the cytopathic effects of HA1 than did eta cultures which had fewer feedings, even though the cultures received fresh medium on the day of inoculation with virus.

We thus see the eta cells and the adenovirus as competing for the arginine, and the eta cells as competing well when they have been properly "prepared" by frequent feedings. Competition between cell and virus for a nutrilite should assume particular virological importance whenever (a) the virus is synthesized relatively slowly in the cell chosen and (b) the cells relatively rapidly deplete the medium of a component essential for virus synthesis. For such a situation, we would predict that the plateau yield of infective virus would be markedly dependent on the number of PFU inoculated, larger inocula giving progressively larger yields. Interestingly enough, Rapp *et al.* (1967), using HA7 and primary African green monkey kidney

cell cultures, have observed such a relationship between plateau yield and number of PFU inoculated; see their Fig. 2. Whether this observation is a reflection of the depletion of something from the medium, or is due to something else, is apparently not known as yet.

Schlesinger and co-workers, whose discovery (see introduction) of the arginine requirement of adenoviruses was confirmed by Philipson (1961), have recently further pursued their investigation, with interesting results. They have presented evidence which indicates that arginine is necessary for some late step in adenovirion biosynthesis (Rouse and Schlesinger, 1967).

The present report bears earmarks of unexpected and peculiar resemblance to features of a recent study by Henle and Henle (1968), though there are marked dissimilarities. Henle and Henle found that incubation of their medium *without cells* at 37° C for two days resulted in an *increase* in its capacity to support multiplication of the herpes-type virus, EB virus, in Burkitt tumor cells. They traced this to what was apparently a decrease in the arginine content of their medium, and found that the decrease was effected by the fetal calf serum also present in the medium. Thus, in striking contrast to adenoviruses, EB virus apparently multiplies better when the medium is deficient in arginine.

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