J. gen. Virol. (1974), **22**, 115–128
Printed in Great Britain

Requirement of Arginine for the Replication of Marek's Disease Herpes Virus

By T. MIKAMI, M. ONUMA AND T. T. A. HAYASHI
Department of Microbiology, Sapporo Medical College, S1, W17, Sapporo 060 Japan

(*Accepted 3 September 1973*)

SUMMARY

The requirement of arginine for the replication of Marek's disease herpes virus (MDHV) was studied in Japanese quail embryo fibroblast cell (QEFC) cultures. Arginine was essential for the replication of MDHV in QEFC cultures. The plaque-forming activity of MDHV was inhibited by omission of arginine from the medium but resumed upon addition of arginine in arginine-deprived infections. The MDHV genome survived in a recoverable form in infected cultures kept in arginine-deprived medium for up to 10 days. Arginine was required around or after 12 h following inoculation. Omission of arginine from the culture medium did not affect virus adsorption, penetration, or the synthesis of virus DNA but did prevent the formation of virus structural protein which was detectable using immunofluorescent antibody techniques.

INTRODUCTION

Synthesis of adenovirus (Rouse & Schlesinger, 1967), polyoma virus (Winters & Consigli, 1971) and SV40 virus (Goldblum, Ravid & Becker, 1968) was inhibited to the greatest extent by omission of arginine from the maintenance medium. Since Tankersley (1964) demonstrated that the replication of herpes simplex virus was dependent upon the presence of arginine in the culture medium, a number of studies were performed with regard to the effect of arginine deprivation on various virus functions (Becker, Olshevsky & Levitt, 1967; Inglis, 1968; Spring, Roizman & Spear, 1969; Courtney, McCombs & Benyesh-Melnick, 1970, 1971; Olshevsky & Becker, 1970).

Arginine is not required for the adsorption, penetration and synthesis of DNA and many of the virus structural proteins of herpes simplex virus. However, it appears to be required for the synthesis of the protein essential for the initial encapsidation of the virus DNA genome (Olshevsky & Becker, 1970), or responsible for the migration of virus-specific proteins from their cytoplasmic site of synthesis to their site of assembly in the nucleus by an unknown mechanism (Courtney *et al.* 1970, 1971).

The effect of arginine on the replication of Marek's disease herpes virus (MDHV) or other cell-associated herpes virus had never been investigated, except for Epstein–Barr virus. In the case of Epstein–Barr virus, arginine-deficient media promoted virus reproduction in cell lines derived from Burkitt tumours (Henle & Henle, 1968). In the present communication we describe the effect of arginine deprivation on MDHV infection in Japanese quail embryo fibroblast cell (QEFC) cultures and discuss a possible mechanism of its effect.

METHODS

Virus. The JM strain of MDHV in infectious heparinized blood was obtained from Dr H. Sazawa of the National Veterinary Assay Laboratory, Tokyo, and was originally supplied by Dr B. Burmester of the Poultry Research Branch, U.S.D.A., East Lansing, Michigan, U.S.A. The virus was inoculated into 1-day-old chicks obtained from a resistance-inducing factor free and specific-pathogen free flock of the Nippon Institute for Biological Science, Tokyo. The cultures were prepared from kidneys of chickens showing clinical signs and lesions of Marek's disease as described previously (Mikami & Bankowski, 1970). The virus obtained from 10 infected monolayer cultures of chick kidney cells in 60 mm plastic plates with numerous plaques was propagated in QEFC cultures according to the method described by Onoda *et al.* (1970). In the present experiments, trypsinized cells of infected QEFC cultures (5 to 7th passage) having 2×10^3 to $1{\cdot}2 \times 10^5$ p.f.u./ml were used as virus stock inoculum. For the adsorption of the virus, inocula were introduced into QEFC cultures without removing tissue culture fluids. The medium was changed every 2 or 3 days. An assay of virus infectivity was done by a plaque count under a liquid medium and unless specified, the plaques were counted on the 4th day after inoculation.

Cell cultures. The QEFC cultures were prepared from decapitated 8- to 9-day-old Japanese quail embryos according to the method described by Onoda *et al.* (1970). The Japanese quails were kindly given by Dr K. Takaku of the Kanonji Institute of Foundation for Microbial Disease of Osaka University, Kanonji, Kagawa, and they were raised in our laboratory as a source of hatching eggs. Two kinds of cultures were prepared. First, cells (1×10^6/ml) were propagated in 35 mm plastic plates using Eagle's minimal essential medium (MEM) supplemented with 10 % undialysed calf serum, and the monolayers were washed three times with phosphate-buffered saline (PBS, pH 7·2) prior to virus inoculation. These monolayers will be referred to as regular monolayers. Secondly, cells (2×10^6/ml) were propagated in plates using Eagle's medium, lacking arginine, supplemented with 10 % undialysed calf serum for the first 1 or 2 days, and then the monolayers were maintained for an additional day by an arginine-deficient medium containing 10 % dialysed calf serum prior to virus inoculation. This period of arginine starvation was used in an attempt to reduce the cellular arginine pools. These monolayers will be referred to as Arg^- monolayers. The cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

Media. The Eagle's MEM used in the present studies was based on the formula described by Eagle (1959). The routine Eagle's MEM will be referred to as complete medium and that devoid of arginine will be referred to as Arg^- medium. The media were supplemented with 0·15 % (w/v) sodium bicarbonate and contained 200 units of penicillin, 200 μg of streptomycin, 100 μg of kanamycin and 2·5 μg of fungazon per ml. Unless specified, medium supplemented with 5 % undialysed calf serum was used for the maintenance medium.

Chemicals. 5-iodo-2'-deoxyuridine (IUdR, Wako Chemical Co., Osaka), at a concentration of 100 μg/ml in complete medium, was used to inhibit DNA synthesis. Puromycin dihydrochloride (Nutritional Biochemical Corporation, Cleveland, Ohio), at a concentration of 0·5 μg/ml in complete medium, was used to inhibit protein synthesis. L-Arginase (Fluka AG, Buchs SG, Switzerland), at different concentrations described in text, was added to complete medium to digest L-arginine in the medium in infected cultures.

Evidence of virus biosynthesis. Acridine orange (AO) staining was used as an indicator of virus DNA synthesis (Pollard & Starr, 1962). Monolayer cultures on coverslips were used for staining. Cells synthesizing virus DNA showed a yellow nuclear reaction, whereas

normal cells showed a green nuclear reaction. Cells were examined under oil immersion at a magnification of × 400 using a Chiyoda fluorescent microscope. The number of positive cells was counted on 50 different microscopical fields and expressed by positive cell per field.

Since Nazerian & Purchase (1970) found that cells containing immunofluorescent antigen also contained MDHV particles and conversely cells lacking this antigen lacked MDHV particles, fluorescent antibody (FA) staining was used as an indicator of virus structural protein synthesis. The monolayer cultures were trypsinized and the dispersed cells were washed twice with PBS. Then the cells were smeared on slide-glass, dried in air for 3 h at room temperature, fixed in cold acetone for 30 min and stained with fluorescein isothiocyanate-conjugated anti-MDHV chicken serum for 1 h at 37 °C in a humidified chamber. After staining, the preparations were washed 3 times with PBS, dried in air and mounted in 90 % (v/v) glycerol on slide-glass. The conjugated serum was kindly supplied by Dr S. Kato of the Research Institute for Microbial Disease, Osaka University, Osaka and was known to be highly specific (Naito *et al.* 1969). This serum was collected from chickens infected with Marek's disease. A minimum of 500 cells were examined for each preparation under oil immersion at a magnification of × 400 in a Chiyoda fluorescent microscope. Cells showing brilliant fluorescence were considered as positive and their numbers were expressed by percentage. The conjugated serum gave no staining at all with uninfected QEFC cultures.

A viable cell count was made by trypan blue staining (McLimans *et al.* 1957). The monolayer cultures were trypsinized and the dispersed cells were resuspended into 1 ml of 0·2 % (w/v) trypan blue; these cells were counted immediately and the number was expressed/ml.

RESULTS

The effect of individual amino acid omissions from the medium on the plaque-forming activity of MDHV

Amino acid requirements of MDHV replication were examined by omitting the individual amino acids from Eagle's MEM and observing the plaque-forming activity on the 5th day after inoculation. The virus was inoculated into regular monolayers which had been fed with the deficient media containing 5 % dialysed calf serum. The relative p.f.u. are given as a percentage of the control. Uninfected cultures maintained in the deficient media for 7 days were examined for cytopathic change and an estimate of the completeness of the monolayer was expressed in percent. Viable cell counts of uninfected cultures were made at the same time.

MDHV demonstrated a requirement for most of the individual amino acids of Eagle's MEM, but arginine, isoleucine and tyrosine deprivations inhibited plaque-forming activity to the greatest extent (over 90 % inhibition, Table 1). Since uninfected cultures could be maintained in media lacking all of the individual amino acids, except cystine and methionine, for 7 days without the appearance of cytopathic effects or a significant difference in cell counts, these inhibitions should not be considered a reflexion of host-cell death. In view of that, the effect of arginine deprivation on herpes simplex virus has been studied extensively; we have investigated the effect of arginine on the replication of MDHV belonging to cell-associated herpes virus.

Table 1. *Effect of individual amino acid deprivations on plaque-forming activity and growth of uninfected QEFC cultures in deficient media*

Amino acid deleted	Plaque counts (range %)	Completeness of monolayers (%)	Viable cell count × 10^{-4}/ml
None	100 (200 p.f.u./0·1 ml on the 5th day after inoculation)	100	128
Arginine	9·4	100	110
Cystine	0	0	2·5
Glutamine	68·3	100	103
Histidine	84·0	100	121
Isoleucine	3·2	100	100
Leucine	60·0	100	105
Lysine	33·0	100	117
Methionine	0	60	82
Phenylalanine	58·9	100	129
Threonine	21·1	100	126
Tryptophane	52·2	100	128
Tyrosine	0	95	110
Valine	10·5	100	108

The results are the average of three different trials.

Table 2. *Viable cell counts of QEFC cultures maintained in media containing or lacking arginine*

Age of cell (days)	No. of cell × 10^{-6}/ml of: Arg⁻ monolayers when maintained with Arg⁻ medium	Arg⁻ monolayers when maintained with complete medium	Regular monolayers when maintained with Arg⁻ medium	Regular monolayers when maintained with complete medium
4	1·3	1·3	1·5	1·7
6	1·2	1·8	1·6	1·9
8	1·3	1·8	1·6	2·2
10	1·2	1·8	1·6	2·2
12	1·1	1·8	1·4	2·3

The results are the average of two different trials. The growth media were replaced by the maintenance media on the 4th day for Arg⁻ monolayers and on the 3rd day for regular monolayers.

The requirement of arginine for MDHV plaque formation

The effect of arginine deficiency on the growth of QEFC cultures was examined by taking a viable cell count after trypan blue staining. Cell growth of Arg⁻ and regular monolayers in Arg⁻ medium was compared with growth in complete medium (Table 2). Up to 10 days, the cell count in cultures grown in Arg⁻ medium did not differ significantly from that grown in complete medium and the cells grown in this medium remained morphologically normal for at least 14 days. When Arg⁻ medium was supplemented by 5 % dialysed calf serum in another experiment, Arg⁻ monolayers remained morphologically normal for 8 days. In general, regular monolayers maintained in Arg⁻ medium remained morphologically normal for longer periods of time than Arg⁻ monolayers did.

The influence of different concentrations of dialysed or undialysed calf serum in the media on the plaque-forming activity of MDHV was examined (Table 3). When the virus

Table 3. *The number of plaques in QEFC cultures in media containing different concentrations of calf serum*

Concentration of calf serum (%)	No. of p.f.u./0·1 ml observed in: Arg⁻ monolayers (Expt. 1) when maintained with: Arg⁻ medium DS*	Arg⁻ medium US*	Complete medium DS	Complete medium US	Regular monolayers (Expt. 2) when maintained with: Arg⁻ medium DS	Arg⁻ medium US	Complete medium DS	Complete medium US
20	0	2	90	124	21	138	211	250
10	0	0	80	126	19	23	185	219
5	0	0	43	80	19	21	155	193
0	0	0	0	3	0	9	46	75

Both experiments were carried out at different times using virus having different titres and all results consist of the average of two trials.

* Containing dialysed (DS) or undialysed serum (US).

was inoculated into Arg⁻ monolayers, no plaque was observed in cultures maintained with Arg⁻ media containing even 20 % dialysed calf serum. A small number of plaques (2 p.f.u.) was observed in cultures maintained with Arg⁻ media supplemented by 20 % but not 10 % or less undialysed calf serum. Upon inoculation of the virus into regular monolayers, the p.f.u. increased as the concentration of calf serum was increased, except in Arg⁻ media containing 5 to 20 % dialysed calf serum. These results indicate that Arg⁻ media containing up to 10 % dialysed or undialysed calf serum were inadequate for plaque formation of MDHV in Arg⁻ monolayers. But when the same Arg⁻ media were used for regular monolayers, approx. one-tenth of the p.f.u. (e.g. 19 *vs.* 185, 23 *vs.* 219, etc.) was obtained as compared to that when complete media were used. However, these plaques in Arg⁻ media became smaller in size on the 7th day and disappeared from the monolayers upon further incubation (10th day). Therefore plaques formed on the 4th day appeared to be made by utilizing the pooled or free residual arginine in regular monolayer cells.

The effect of different concentrations of arginine in the media on MDHV plaque-forming activity was examined (Fig. 1). At least 10·5 mg/l of arginine was required to obtain a p.f.u. comparable to that obtained with complete medium (105 mg/l of arginine) for both Arg⁻ and regular monolayers. The p.f.u. decreased as the concentration of arginine in the media was increased from 10 to 50 times or decreased from 50 to 100 times. A great excess of arginine (100 times) resulted in a toxic effect on host cells.

The effect of different concentrations of arginase in the media on MDHV plaque-forming activity was examined (Table 4). Plaque-forming activity was completely inhibited when 100 μg/ml (2 units/ml) of arginase was added to complete medium following inoculation of the virus in regular monolayers. An increase in the amount of arginase (1000 μg/ml) in the medium showed a toxic effect on host cells. With a lesser amount (10 to 50 μg/ml), there was about 40 % reduction in the number of plaques when compared with that of inoculated cultures without arginase.

Survival of virus genome in an arginine-deprived infection

Experimentation was done to determine the maximal interval between infection in Arg⁻ medium and successful recovery of plaque-forming activity by adding arginine to the medium. The virus was inoculated into Arg⁻ or regular monolayers with the absence of

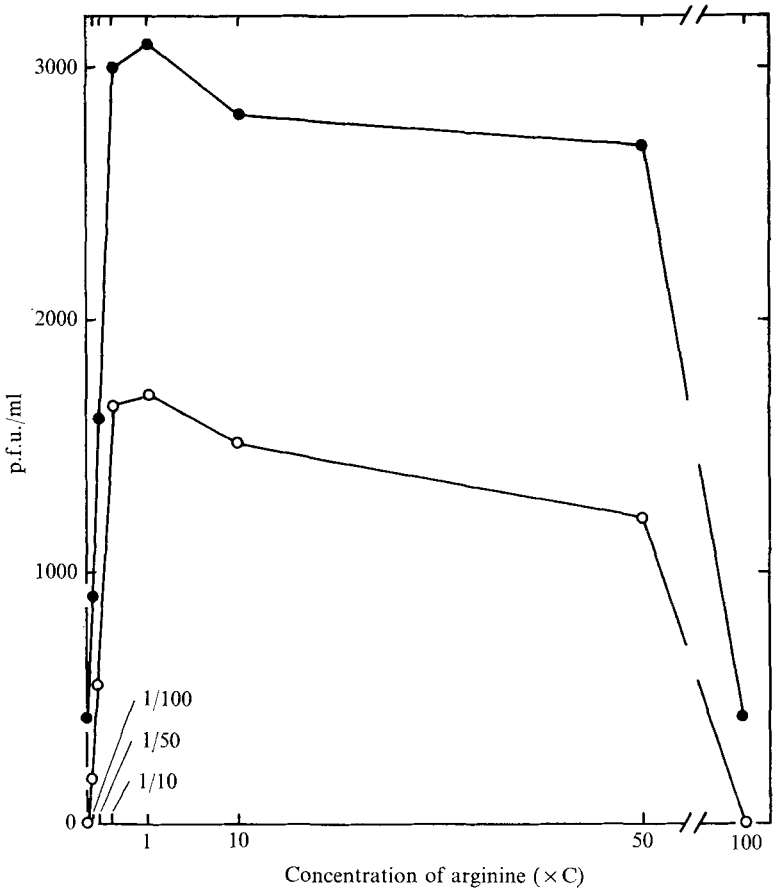

Fig. 1. The effect of different concentrations of arginine in the media on plaque-forming activity. The virus was inoculated into Arg$^-$ monolayers (○—○) or regular monolayers (●—●), and plaques were counted on the 4th day after inoculation. The concentration of arginine in complete medium (105 mg/l) was C; ten times this was 10C; one-tenth was $\frac{1}{10}$C, etc. Experiments using both monolayers were carried out at different times using virus having different titres. The results consist of the average of three trials.

arginine in the medium. After adsorption for 24 h, Arg$^-$ medium was exchanged for complete medium at various time intervals (Fig. 2). Arg$^-$ medium containing 5 % undialysed calf serum was used since this enhanced cell survival while remaining inadequate for plaque formation. The plaque counts were made on the 4th day after changing to complete medium.

The number of plaques decreased as the period of arginine deprivation was extended. For the first day of deprivation, the plaque-forming activity resumed without noticeable change. However, about a 50 % of plaque reduction was observed in cultures kept in Arg$^-$ medium for 2 to 3 days. On the 10th day, no plaques were observed in Arg$^-$ monolayers but a few plaques were counted in regular monolayers. These results indicate that the virus remained in a recoverable state in infected regular monolayers maintained in Arg$^-$ medium as long as 10 days, which was the maximum period of days of recovery examined.

Table 4. *Effect of different concentrations of arginase in the medium on plaque-forming activity*

Concentration of arginase (μg/ml)	No. of p.f.u./0·1 ml
1000	0*
100	0
50	480
10	570
5	820
1	840
0	820

* The monolayers were destroyed on the 3rd day after virus inoculation due to the toxic effect of the high concentration of arginase in the medium.

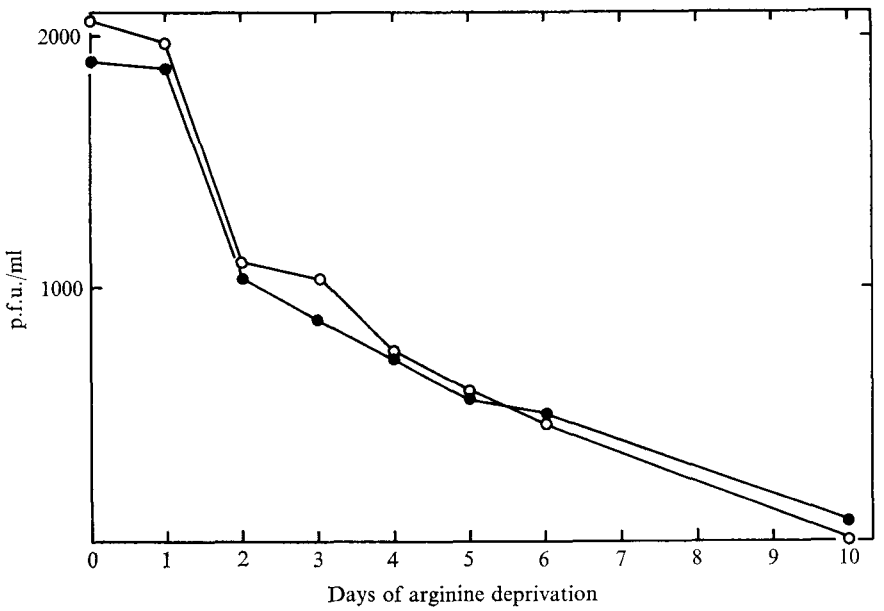

Fig. 2. The recovery of the plaque-forming activity in Arg⁻ monolayers (○—○) or regular monolayers (●—●) when Arg⁻ media were exchanged for complete media. Both monolayers were inoculated with the same amount of virus.

The effect of arginine on virus adsorption and penetration

To determine the effect of arginine on virus adsorption and penetration, Arg⁻ or regular monolayers were exposed to the virus with the absence of arginine in the medium for various periods of time. After adsorption, the monolayers were washed 3 times with PBS, placed in complete medium, and then the monolayers were observed. We examined the length of time necessary for the recovery of plaque-forming activity in the cultures which had been in Arg⁻ medium when compared with the number of plaques obtained in cultures exposed to the virus for the same periods of time with the presence of arginine in the medium (Table 5). Plaque counts were made up to 4 days after changing to complete medium.

When the cultures exposed to the virus for 2 to 24 h in the absence of arginine were

Table 5. *Comparison of the plaque-forming activity in cultures when the virus was adsorbed for different periods of time with the presence or absence of arginine in the media*

Time of virus adsorption in Arg⁻ medium (h)	No. of p.f.u./0·1 ml counted different days after virus inoculation					Time of virus adsorption in complete medium (h)	No. of p.f.u./0·1 ml counted on the 4th day after virus inoculation
	4	4·25	4·5	5	6		
2	220 (130)*					2	200 (135)
6	340 (240)	460 (290)				6	420 (280)
12	260 (180)	380 (210)	440 (300)			12	460 (330)
24	190 (150)	300 (250)	420 (300)	440 (330)		24	460 (300)
48	40 (40)	—	120 (60)	160 (70)	230 (120)	48	480 (300)

* Numbers without parentheses are the number of p.f.u. when Arg⁻ monolayers were used and those in parentheses are the number of p.f.u. when regular monolayers were used. Numbers underlined show the number of p.f.u. counted on the 4th day after changing to complete medium. Both monolayers were inoculated with the same amount of virus.

Table 6. *Determination of arginine-dependent periods during virus replication*

Times when Arg⁻ or complete media were exchanged for each other after inoculation (h)	Percentage of FA-positive cells at 48 h in cultures for which media were exchanged from	
	Complete medium to Arg⁻ medium (regular monolayer)	Arg⁻ medium to complete medium (Arg⁻ monolayer)
0 (exchanged at the time of virus inoculation)	2·4	21·8
4	5·7	20·8
8	6·8	19·3
12	10·3	21·0
16	10·3	12·3
20	9·7	13·0
24	9·5	10·0
36	10·1	4·1
48 (not exchanged)	10·6	1·4

Both monolayers were inoculated with the same amount of virus ($1{\cdot}2 \times 10^4$ p.f.u./0·1 ml) and adsorbed for 4 h.

compared with those exposed with the presence of arginine in the medium, there was no significant difference in the number of p.f.u. counted on the 4th day after changing to complete medium. These results suggest that the virus adsorbed and penetrated at the normal rate with the absence of arginine in the medium. However, the cultures that adsorbed the virus for 48 h in Arg⁻ medium showed about 40 to 48 % reduction in the number of plaques as compared with that adsorbed with the presence of arginine in the medium. Furthermore, when the number of p.f.u. counted on the 3·5th day after adding arginine to the cultures which were adsorbed with the virus for 24 h in Arg⁻ medium was compared to the number of p.f.u. counted on the 4th day after inoculation of cultures which were adsorbed with the virus for the same period of time in complete medium, the number of the former was approximately the same as that of the latter (over 90 % recovery in Arg⁻ monolayers and 100 % in regular monolayers). These results indicate that early recovery of plaque-forming activity was obtained upon addition of arginine to arginine-deprived infections and that arginine might be required for the late steps of virus synthesis.

The determination of an arginine-dependent period during virus replication

To determine an arginine-dependent period of the virus during replication, the same amount of the virus was inoculated into Arg⁻ monolayers in Arg⁻ medium or regular monolayers in complete medium and adsorbed for 4 h in both cultures. After adsorption, the monolayers were washed twice with PBS and maintained with the respective media for various periods, and then Arg⁻ medium was replaced by complete medium or vice versa (Table 6). In order to remove the residual arginine in regular monolayers that had been inoculated with the virus in complete medium, the monolayers were washed additionally twice with PBS prior to the exchange of the medium for Arg⁻ medium. Experimentation was terminated at 48 h after inoculation and the synthesis of virus structural protein was examined by FA staining.

In the cultures for which Arg⁻ medium was exchanged for complete medium, the rate of synthesis of the virus structural protein was normal as compared with the totally arginine system if the exchange was made in the first 12 h, but if it was later, a reduction resulted

which increased as the duration of arginine-deprivation increased. In the cultures for which complete medium was exchanged for Arg$^-$ medium, the rate of synthesis increased in the first 8 h as the duration of virus infection in complete medium increased. However, the rate of synthesis reached a plateau if the exchange was made as much as 12 h later. These results indicate that the arginine-dependent period for MDHV infection seems to be 12 h or later after virus inoculation.

The effect of arginine deprivation on virus DNA and protein synthesis

Tests were made to examine the effect of arginine deprivation on virus DNA and protein synthesis by using inhibitors (IUdR and puromycin) in the medium and staining techniques (AO and FA staining). As shown in Table 7, Arg$^-$ monolayers were inoculated with the virus, maintained for the first 24 h in one of the four different kinds of medium (Arg$^-$ medium, complete medium, complete medium containing either IUdR or puromycin), respectively, and then washed twice with PBS. After 24 h, some of the cultures were continued in their respective media (culture nos. 1, 2, 4 and 6) and others which had been maintained in Arg$^-$ medium were fed with one of the media: either complete medium (culture no. 3), complete medium with IUdR (culture no. 5) or complete medium with puromycin (culture no. 7). The viable cells were counted on the 2nd day and the plaques were counted on the 4th day after inoculation. All media but Arg$^-$ medium which was supplemented with 5 % dialysed calf serum were supplemented with 5 % undialysed calf serum.

Many cells showing virus-induced nuclear change after AO staining were observed in nos. 1, 2 and 3 cultures, whereas few cells showing a similar nuclear change were observed in nos. 4, 6 and 7 cultures. Nuclear change observed in cells of no. 5 culture may be due to the virus DNA formed under an arginine-deprived condition during the first 24 h. These results indicate that there was no significant effect of arginine-deprivation on virus DNA synthesis.

Upon FA staining of the dispersed cells from cultures nos. 2 and 3, more than 4 % of cells in one trial and 11 % of cells in other trial showed a positive reaction. In the culture (no. 5) which was fed with complete medium containing IUdR 24 h after virus inoculation to inhibit further DNA synthesis, 2 % of cells in one trial and 6·3 % of cells in the other trial showed a positive reaction. This result indicates that virus structural protein was synthesized during an additional 24 h incubation period by the previously formed DNA under the arginine-deprived condition. A few positive reactions were observed in cells from nos. 1, 4, 6 and 7 cultures. This might be due to the reaction of cells which synthesized virus structural protein by utilizing arginine originating either in the host cells or the inoculum (culture no. 1). As another possibility, this might be due to the reaction of cells from the inoculum remaining in the cultures in spite of washing three times after virus adsorption (culture nos. 1, 4, 6 and 7). Furthermore, the amount of inhibitors used in this experiment might not be sufficient to inhibit completely macromolecule synthesis (culture nos. 4, 6 and 7).

The number of viable cells in each culture showed no significant difference. Plaques were observed in both nos. 2 and 3 cultures which showed a high percentage of FA-positive cells, but not in no. 5 culture which showed a lesser extent of FA-positive cells. Since the former had repeated cycles of virus replication but the latter had only one cycle of replication during the 4-day incubation period, it was obvious that the latter formed no plaques.

Table 7. *Effect of arginine-deprived infection on virus DNA or structural protein synthesis*

Culture no.	Kinds of media used before and after 24 h following virus inoculation at 0 time		No. of AO-positive cells/field	Percentage of FA-positive cells	No. of cell count/ml ($\times 10^{-5}$)	No. of p.f.u./0·1 ml on the 4th day after virus inoculation
	Before	After				
1	Arg⁻ medium	Arg⁻ medium	40·2 (70·0)*	0·4 (1·7)	6·6 (10·0)	0 (0)
2	Complete medium	Complete medium	85·0 (175·0)	4·5 (16·3)	8·0 (10·5)	$1·8 \times 10^3$ ($1·2 \times 10^4$)
3	Arg⁻ medium	Complete medium	65·0 (105·0)	4·1 (11·9)	8·0 (9·0)	$9·0 \times 10^2$ ($4·8 \times 10^3$)
4	Complete medium with IUdR	Complete medium with IUdR	3·5 (6·2)	0·2 (1·6)	8·1 (9·0)	0 (0)
5	Arg⁻ medium	Complete medium with IUdR	11·2 (N.D.)†	2·0 (6·3)	6·7 (8·5)	0 (0)
6	Complete medium with puromycin	Complete medium with puromycin	3·5 (4·8)	0·4 (1·8)	8·1 (9·5)	0 (0)
7	Arg⁻ medium	Complete medium with puromycin	4·6 (N.D.)†	0·5 (1·7)	7·0 (9·5)	0 (0)

* Number of p.f.u. in parentheses indicates the results of a second trial.
† N.D. = not done.

DISCUSSION

The purpose of the present experiment was to study the arginine requirement for the replication of MDHV in the QEFC cultures. In view of the cell-associated nature of an infectious MDHV, this can only be investigated by using MDHV infected cells as the inoculum. Therefore, this has obvious limitations, because complete eradication of the pooled or free arginine in the infected culture system is impossible to achieve. The results are nevertheless informative and reflect on the requirement of arginine for the replication of MDHV. Inoculation of MDHV in Arg$^-$ monolayers resulted in complete inhibition of plaque-forming activity by omission of arginine from the maintenance medium. However, inoculation of the virus into regular monolayers resulted in about 10 % of plaque-forming activity in arginine-deprived medium when compared to that in complete medium. The appearance of a few plaques in regular monolayers might be due to free or pooled arginine originating in the host cells and/or the inoculum. Addition of arginase (2 units/ml) to complete medium in MDHV-infected cultures inhibited completely the plaque-forming activity indicating further that MDHV requires arginine for its replication.

Inglis (1968) reported that synthesis of herpes simplex virus in RK 13 cells increased when the concentration of arginine was increased to 2 times (210 mg/l) in routine Eagle's medium (105 mg/l), but further addition of arginine did not continue to improve the virus yield. Furthermore, the synthesis of virus was completely inhibited when the concentration of arginine was decreased to one-fifth or one-tenth. On examination of the effects of the different concentrations of arginine in the media on MDHV synthesis by observing plaque-forming activity, it was found that at least one-tenth of arginine concentration in routine Eagle's medium was adequate for obtaining a p.f.u. comparable to that obtained in routine Eagle's medium. Excess of arginine (10 to 50 times) or shortage of arginine (1/50 to 1/100) in the medium resulted in the decrease of p.f.u. ranging from 10 to 29 % and 48 to 90 %, respectively.

Inglis (1968) further reported that the addition of undialysed calf serum (up to 20 %) to Arg$^-$ medium was inadequate for herpes simplex virus replication when cells were infected at low input multiplicities. At high input multiplicities a small amount of virus was produced in deficient medium supplemented by 20 % but not 10 % undialysed calf serum. Present studies indicate that Arg$^-$ medium containing up to 10 % dialysed calf serum is inadequate for MDHV plaque formation in Arg$^-$ monolayers. However, when the same medium was used with regular monolayers, about one-tenth the number of the p.f.u. was obtained as compared to that obtained by using complete medium.

The MDHV genome could survive in a recoverable form in infected regular monolayers kept in Arg$^-$ medium up to 10 days. Inglis (1968) reported that herpes simplex virus genome could survive in a recoverable form in infected RK 13 cells maintained in Arg$^-$ medium up to 16 days. She suggested that the virus had remained in a recoverable state as long as the cells survived.

The effects of arginine deprivation on herpes simplex virus infection and the mechanisms for their effects have been studied in detail. Becker *et al.* (1967) and Olshevsky & Becker (1970) described that the synthesis of virus DNA and many of the virus structural proteins was not affected by the absence of arginine from cultures. Arginine might be required for the synthesis of the protein essential for the initial encapsidation of the virus DNA (Olshevsky & Becker, 1970), or responsible for the migration of virus-specific proteins from their cytoplasmic site to their site of assembly in the nucleus by unknown mechanisms (Courtney *et al.* 1970, 1971). The experiments described in the present paper indicate that DNA

synthesis of MDHV is also independent of the presence of arginine in the medium, but that the synthesis of virus structural protein which is detectable by FA staining is affected by the absence of arginine from the cultures. When the MDHV infection system was deprived of arginine for 24 h and then arginine was restored to the infection (Table 5), the number of p.f.u. counted on the 3·5th day after adding arginine was approximately the same as that of p.f.u. counted on the 4th day in cultures infected with MDHV using complete medium from the beginning, indicating that early recovery of plaque-forming activity was obtained upon addition of arginine to arginine-deprived infections, and thus arginine might be required for the late steps of virus synthesis. Furthermore, the synthesis of virus structural protein was not affected in arginine-deprived infections if the restoration of arginine to infected cultures was made within 12 h after inoculation (Table 6), indicating that arginine was required for MDHV replication around or after 12 h following virus inoculation. Roizman, Spring & Roane (1967) have suggested that arginine is not necessary in the first 4 h but is required after 6 h post-inoculation in herpes simplex virus infections. Recently, it was found that the synthesis of membrane antigens in MDHV-infected cells, which was known as an early virus function, was not affected by the absence of arginine in the medium; DNA inhibitor did not prevent its formation whereas protein inhibitor did so significantly (Mikami, Onuma & Hayashi, 1973). From these results, it is assumed that virus adsorption, penetration and other early virus functions of MDHV still occur even with the absence of arginine in the medium.

The percentage of FA positive cells in the cultures (Arg^- monolayers) in which Arg^- medium was replaced by complete medium up to 12 h after virus inoculation was about two times higher than that in cultures (regular monolayers) in which complete medium was replaced by Arg^- medium between 12 and 36 h after inoculation (Table 6). Similarly, when the same amount of virus was inoculated into Arg^- or regular monolayers in complete medium, the number of p.f.u. in Arg^- monolayers was always greater than that in regular monolayers (Fig. 2 and Table 5). These differences might be due to the different efficiencies of cells in MDHV uptake which depended on the stage of development of both monolayers, since it was found that chick kidney cell suspensions which were actively forming a monolayer produced more plaques upon MDHV inoculation than those which had already formed a complete monolayer did (Mikami & Bankowski, 1971). Although Arg^- monolayers were microscopically complete monolayers but were not densely formed cell sheets as seen in regular monolayers, these might become densely formed cell sheets if Arg^- medium were replaced by complete medium at the time of or after virus inoculation. For this reason, Arg^- monolayers might produce more FA-positive cells or plaques than regular monolayers did.

Experiments using a combination of DNA or protein inhibitors in the medium and FA or AO stainings of infected culture cells showed that inoculation and adsorption of MDHV for 24 h in Arg^- monolayers with the absence of arginine in the medium, and restoration of arginine to the cultures in the presence of an inhibitor for an additional 24 h incubation, resulted in the synthesis of virus structural protein in the presence of DNA inhibitor but not protein inhibitor. In the same experiments, the synthesis of virus DNA in cells examined by AO staining was not affected by the absence of arginine in the medium. These results indicate that the virus DNA which was synthesized in an arginine-deprived condition was translated into virus structural protein. It was reported that arginine deprivation of herpes simplex virus-infected cells had no effect on the synthesis of certain immunofluorescent virus antigens (Spring *et al.* 1969), of virus specific complement-fixing antigens (Courtney *et al.* 1970) and of some virus structural proteins (Olshevsky & Becker, 1970). In the case

of MDHV infection, however, the role of arginine in the synthesis of late protein other than virus structural protein which is detectable by FA staining remains to be determined. Nevertheless, it might be concluded that arginine is required for the synthesis of the late protein detected by FA staining for the replication of MDHV.

We are indebted to Dr S. Kato for supplying the conjugated anti-MDHV chicken serum, Dr K. Takaku for the Japanese quails and Dr H. Sazawa for the MDHV. We heartily express thanks for their kindness.

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(*Received* 3 *July* 1973)