Effect of Arginine-deficient Media on the Herpes-Type Virus Associated with Cultured Burkitt Tumor Cells

WERNER HENLE¹ AND GERTRUDE HENLE

Virus Laboratories, The Children's Hospital of Philadelphia, and The School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19146

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After incubation at 37 C for 2 or more days, Basal Medium Eagle (BME) supplemented with 25% fetal calf serum (FCS) was not able to support adequate growth of EB3 and other lines of Burkitt tumor cells. The medium did, however, increase, by a factor of about 10, the number of cells synthesizing herpes-type virus with which the cultures were persistently infected. Not every lot of FCS produced these effects, nor were these effects seen when BME and FCS were incubated separately for 7 days before the medium was completed. At 37 C, appropriate lots of FCS interacted with several of the amino acids present in BME; this interaction resulted in an inhibition of cellular growth, whereas interaction with arginine yielded the virus-enhancing effect. Arginine-free BME, supplemented with 25% FCS and used without prior incubation, prevented cellular replication and promoted viral infection to a similar extent as did preincubated complete medium. Replenishment of arginine reduced, but did not regularly abolish, the virus-enhancing activity of preincubated media. RPMI-1629 medium was less affected by preincubation with FCS because it contained twice the amount of arginine that BME contained. The FCS factors which act upon arginine and other amino acids are not dialyzable and are partially resistant to heating at 56 or 60 C for 30 to 60 min. Calf and horse sera appear to be devoid of these activities. The nature of these interactions, as well as the mechanism by which arginine deficiency enhanced the viral infection, remains to be ascertained.

A herpes-type virus has been observed in many continuous cell lines derived from Burkitt lymphomas (2, 14, 15, 17, 19), lymph nodes, or peripheral blood of patients with leukemias or other diseases (10, 13, 22), as well as from buffy coats of healthy donors (12; P. Gerber, *personal communication*). For convenience, this agent was termed EB virus (EBV) after the first cultures in which it was detected (2). Human serum surveys, which were performed by use of an indirect immunofluorescence test (4-6, 11), indicate that EBV has a worldwide distribution and represents an apparently new member of the herpes group (4, 5, 7). Study of the properties of EBV, especially its potential oncogenicity, has been hampered by two factors: (i) most of the virus particles seen in persistently infected cultures (by electron microscopy) are struc-

turally defective (9, 21), a circumstance which has conceivably prevented the transmission of the agent to host systems other than cultured leukocytes or EBV-free Burkitt cell lines (3; Horoszewicz, *personal communication*); and (ii) relatively few cells in carrier cultures produce virus particles, making the extraction, purification, and concentration of these particles a formidable task. Since the virus-producing cells tended to decline gradually when the cultures were maintained on media which insure vigorous growth (17; Epstein, *personal communication*), it seemed reasonable to assume that cultural conditions which reduce cellular replication would favor the virus. The validity of this approach is indicated by a recent report of Hinuma et al. (8), who observed that the HR-1 clone of the Jijoye line of Burkitt tumor cells succumbed to EBV infection when the cultures were maintained without feeding for more than 10 days. Our studies with the EB3 line showed that Basal Medium Eagle (BME),

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when incubated for several days with 25% fetal calf serum (FCS) at 37 C, may no longer support satisfactory growth of the cells, although it enhances the EBV infection. This phenomenon has been analyzed, and the results of this analysis form the basis of this report.

MATERIALS AND METHODS

Cells. The EB3 line of Burkitt tumor cells (2) was mainly employed. This line was repeatedly cultured for mycoplasmas with negative results.

In a few preliminary tests, we also used EB1, EB2, Kudi, and Ogun cells, as well as the HR-1-K clone of Jijoye cells (8). The EB lines were supplied by M. A. Epstein, Bland-Sutton Institute, Middlesex Hospital, London, England; the HR-1-K clone was provided by J. T. Grace, Jr., Roswell Park Memorial Institute, Buffalo, N. Y.; and the remaining cultures were supplied by R. A. Manaker, National Cancer Institute, Bethesda, Md. HR-1-K cultures were maintained on medium RPMI-1640 supplemented with 20% FCS; all other cultures were grown on medium RPMI-1629 supplemented with 10% FCS (1629-10). The media contained 100 units of penicillin and 100 μ g of streptomycin per ml. As a rule, the stock cultures were divided once a week by sedimentation of a sufficient number of viable cells (trypan blue exclusion) at $900 \times g$ for 10 min; the cells were then resuspended in fresh medium to yield 10^5 or 2×10^5 cells/ml. The cultures were incubated in Erlenmeyer flasks having an air space of 80% of the volume.

Media. RPMI 1629 and 1640 media were purchased from BBL or Grand Island Biologicals (Grand Island, N.Y.). BME or its components (100 $\times$ solutions of amino acids, vitamins, or glutamine), as well as fetal and other sera, were obtained from Microbiological Associates, Inc. (Bethesda, Md.). Experimental amino acid solutions (50 $\times$) were prepared by I. Zajac, Jefferson Medical College, Philadelphia, Pa., according to the procedure of Eagle et al. (1).

Experimental design. To preincubate individual medium components or combinations of components with FCS, the test materials (e.g., 100 $\times$ solutions of amino acids, vitamins, etc.) were diluted in Hanks' balanced salt solution (containing 0.0375% sodium bicarbonate and antibiotics) and were mixed with FCS; the volumes prescribed for complete BME were used and appropriate serum concentrations were chosen. After incubation of the mixtures for 7 days at 37 C and storage of the controls at -20 C, the remaining components were added to complete the medium.

Counted lots of viable EB3 cells from stock cultures on 1629-10 medium were sedimented, resuspended in the experimental media (usually to yield 2×10^5 cells/ml), and incubated at 37 C, unless otherwise stated. Total and viable cell counts were obtained at various intervals during incubation, and cell smears were prepared for determination of EBV antigen-producing cells by immunofluorescence. In some experiments, the cultures were maintained for up to 14 days without feeding. In others, the cul-

tures were fed on the 7th day by sedimentation of the cells and resuspension in the appropriate medium to yield 2×10^5 cells/ml. Other variations in the protocols are described in the Results.

Immunofluorescence tests. The techniques employed for preparation of cell smears and for fluorescent-antibody staining were fully described elsewhere (4). The direct technique, staining by fluorescein isothiocyanate-conjugated human γ globulins, was used primarily. To estimate the percentage of immunofluorescent (virus-producing) cells we selected microscopic fields (at 750 $\times$ magnification) in which the cells were evenly spread and their number was sufficiently low (100 to 200/field) to permit accurate counting of stained and unstained cells. Ten or more fields, covering various sections of the smear, were counted, and the average percentage of fluorescent cells was calculated.

RESULTS

Effect of preincubation of culture media at 37 C on cell growth and EBV infection. Sedimented EB3 cells from stock cultures maintained on medium RPMI-1629 and 10% fetal calf serum (FCS) were resuspended in preincubated (at 37 C for 7 days) BME and 25% FCS (BME-25). Cell counts revealed severe reductions in cellular growth and virus-specific immunofluorescence rapid increases in EB virus-producing cells (Fig. 1). A significant rise in dead cell counts did not occur. Upon electron microscopic examination, ultra-thin sections of centrifugal sediments from such cultures revealed many more cells which contained virus particles than were seen in similar preparations from stock cultures; no mycoplasmas were noted. Furthermore, virus particles could be extracted from such cell populations in sufficient quantities to be suitable for antibody coating and agglutination tests (7).

The described effects of preincubated BME-25 depended upon the lot of FCS employed (Fig. 2). Medium preincubated at 37 C for 7 days with FCS #1 supported cellular growth nearly as well as did the control medium kept at -20 C. The percentage of cells stainable by fluorescent antibodies (FA+ cells) showed only minor changes. The results obtained with FCS #2 represented the opposite extreme. When the preincubated medium was used, the cells hardly grew and the percentage and actual number of FA+ cells increased >10-fold. When the corresponding control medium was used, the increase in cells, by the 4th day, was low as compared to the first example, the number of viable cells declined thereafter, but no significant rise in the percentage of infected cells was noted. The two other lots of FCS yielded results intermediary between those just discussed.

Variation in the period of preincubation of

FIG. 1. Immunofluorescence elicited in EB3 cells 0, 2, 4, and 5 days (A-D) after the transfer of the cultures from medium 1629-10 to preincubated BME-25 (37 C for 7 days). $\times 1,800$.

BME-25 revealed (Fig. 3) that medium kept at 37 C for 2 days produced a marked reduction in cellular growth and a significant increase in EBV antigen-producing cells. These effects increased in intensity on further incubation of

the medium for a total of 8 days. When other lots of FCS were used, the effects were less pronounced after 2 days but were always maximal, or nearly so, after 7 days of preincubation of the medium.

FIG. 2. Differences in the effects of preincubated BME-25 (37°C, 7 days) on cultures of EB3 cells depending upon the lots of fetal calf serum (FCS) employed.

The effects of preincubated BME-25 depended to a considerable extent upon the age of the stock EB3 cultures at the time of their use (Fig. 4). Samples were removed from a stock culture 3, 4, 5, and 7 days after the last feeding with 1629-10 medium, and, after sedimentation, the cells were resuspended in preincubated BME-25. Cellular growth decreased successively, and maximal levels of FA+ cells were reached more rapidly as the age of the stock cultures increased from 3 to 7 days.

In several experiments, we compared preincubated BME and 1629 media supplemented with 10 or 25% FCS (Fig. 5). The reductions in cellular growth and the increases in immunofluorescent cells were generally more striking with preincubated BME than with 1629 medium, and the most striking effects were found with BME preincubated with 25% FCS. The results obtained with 1629-10 were usually indistinguishable, regardless of whether the medium was preincubated. Even with preincubated 1629-25, the reductions in cellular growth and the increases in FA+ cells were often only minor. However, preincubation of 1629 with 40% FCS produced effects which resembled those seen with BME-25. The evident spread of FA+ cell percentages obtained with preincubated BME-25 may be ascribed, at least in part, to

the extent of the infection in the stock EB3 line, which in the course of these experiments declined gradually from about 3% to <0.5%. Correspondingly, the higher values obtained when cells were transferred to preincubated BME-25 were noted in earlier experiments, whereas the lower levels were observed in later experiments. When we revived a stock EB3 culture which had been preserved in a liquid nitrogen freezer for 3 years, it revealed about 5% FA+ cells, and this percentage increased to >25% on transfer to preincubated BME-25.

The initial concentration of cells in experimental cultures, whether 10^6 , 5×10^5 , or 2×10^5 /ml, did not influence the virus-enhancing effect of preincubated BME-25 (Fig. 6). Cellular growth was increasingly more affected, however, and, as the initial cell concentration was raised, a considerable loss in cell number was noted. Control cultures on fresh 1629-10 medium showed steep and continuing increases in cells at the two lower starting levels, but significant changes in FA+ cells were not noted. Control cultures initiated with 10^6 cells/ml showed only slight growth in 4 days, followed by a rapid decline in cell number; the number of EBV antigen-containing cells did not increase.

In several experiments, cultures were set up on preincubated BME-25 and on fresh 1629-10,

FIG. 3. Effect of preincubated BME-25 (at 37 C for 0, 2, 3, 6, and 8 days) on cultures of EB3 cells. The control culture on 1629-10 medium is represented by an asterisk.

and these cultures were incubated at temperatures from 32 to 39 C. At 39 C, the cells died rapidly on both media. At 32, 35, and 37 C, the results obtained with preincubated BME-25 were indistinguishable from those just described, with the exception that, at 32 C, cell growth was even more reduced, and maximal levels of FA+ cells were attained later than at the higher temperatures. The control cultures on 1629-10 grew equally well at 35 and 37 C but somewhat less rapidly at 32 C; significant increases in immunofluorescent cells were not produced.

Interactions of fetal calf serum with components of BME. Separate preincubation of FCS, before the addition of fresh or preincubated BME, did not affect the EBV infection or cellular replication (Table 1). Thus, it was evident that these parameters were affected by an interaction between FCS and BME. To determine which constituents were participating in the

interaction with FCS, three groups of BME components (vitamins, glutamine, and amino acids) were incubated separately with FCS before appropriate completion of media. The results clearly showed that only the amino acids were involved. The 12 amino acids of BME, other than glutamine, were then divided, in alphabetical order, into four pools of three amino acids each, and these pools were exposed to FCS at 37 C for 7 days. After completion, three of these media reduced cellular growth but only one increased the percentage of FA+ cells. The components of this particular amino acid pool, that is, arginine, cysteine, and histidine, were then preincubated separately with FCS. All three of the completed media substantially inhibited cellular replication, but only the medium containing the preincubated arginine-FCS mixture produced a rise in immuno-

FIG. 4. Influence of the age of stock EB3 cultures on the effects of preincubated BME-25 (37 C, 7 days). Cells were used 3, 4, 5, and 7 days after the last 1629-10 medium renewal. The control culture continued on 1629-10 medium is represented by an asterisk.

FIG. 5. Comparison of the effects of BME and 1629 media (preincubated at 37 C for 7 days with 10 or 25% fetal calf serum) on cultures of EB3 cells.

FIG. 6. Comparison of the effects of preincubated BME-25 (37 C, 7 days) and fresh 1629-10 medium on EB3 cultures initiated at different cell concentrations.

TABLE 1. Effect of preincubation of various components of Basal Medium Eagle (BME) with fetal calf serum (FCS) on cultures of EB3 cells

Preincubation (37 C, 7 days)	Completion of medium	Results	
		Reduction in ^a cell growth	Increase in ^b FA ⁺ cells
BME + FCS ^c	—	+	+
BME	FCS	—	—
FCS	BME	—	—
Glutamine + FCS	Vitamins, amino acids	—	—
Vitamins + FCS	Glutamin, amino acids	—	—
Amino acids + FCS	Glutamine, vitamins	+	+
Amino acids	Missing amino acids, glu- tamine, vitamins	+	+
* 1-3 + FCS		+	—
* 4-6 + FCS		+	—
* 7-9 + FCS		—	—
* 10-12 + FCS	Missing amino acids, glu- tamine, vitamins	—	—
Arginine + FCS		+	+
Cysteine + FCS		+	—
Histidine + FCS		±	—

^a Symbols: +, >67%; ±, 30 to 67%; —, <30% reduction.
^b Symbols: +, fivefold or greater; ±, two- to fourfold; —, <twofold increase.
^c The serum concentration in all completed media was 25%.

TABLE 2. *Effect of BME containing preincubated mixtures of arginine and FCS, at varying concentrations, on cultures of EB3 cells*

Fetal calf serum ^a (%)	Arginine concn ^a							
	0.1 mM		0.2 mM		0.4 mM		0.8 mM	
	Viable cells	FA+ cells	Viable cells	FA+ cells	Viable cells	FA+ cells	Viable cells	FA+ cells
10	0.9×	4×	2.1×	1×	2.5×	1×	0.7×	1×
20	0.8×	10×	1.0×	7×	2.3×	4×	0.7×	2×
40	1.0×	19×	—	—	2.4×	8×	1.5×	9×

^a Concentrations in completed media.

^b Maximal increase in number of viable cells or percentage of immunofluorescent cells after 4 to 7 days of incubation.

fluorescent cells. When multiples (1, 2, 4, and 8 times) of the quantity of arginine present in BME were preincubated with varying concentrations of FCS before completion of the media, the results (Table 2) revealed that, to obtain a reduction in cellular replication and an increase in FA+ cells, increasingly more FCS is required as the amount of arginine is increased.

Since it is probable that the interaction between arginine and FCS results in a deficiency of this amino acid, we prepared six lots of arginineless BME supplemented with 25% FCS. To these six lots, we either did not add arginine or we added concentrations of the amino acid ranging from 0.01 to 1× the standard amount (0.1 mM). These media were used without prior incubation at 37 C. The results (Fig. 7) revealed that media without arginine or with only 1 to 3% of the usual amount of arginine prevented cellular growth and increased FA+ cells to a similar extent as did preincubated complete BME-25 or media containing preincubated arginine-FCS mixtures. As the amount of arginine was increased, the effects on cellular replication and the EBV infection became successively less pronounced.

In further experiments, three types of BME medium were prepared: (i) complete, (ii) without arginine, and (iii) with no amino acids except arginine and glutamine. All media were supplemented with 25% FCS; one part of each medium was incubated at 37 C for 7 days, whereas the other part was kept at -20 C. After the addition of the remaining 11 amino acids to (iii), the media were divided into two lots, and a standard amount of arginine was added to the second of each. The effects of these media on cultures of EB3 cells are summarized in Table 3. The three preincubated media lacking

arginine yielded similar results as to reduction of cellular growth and increase in FA+ cells. In the case of complete or arginineless BME, addition of arginine after preincubation did not reverse the inhibition of cellular replication, but it reduced, or abolished, the increase in immunofluorescent cells. In the third type of medium, containing arginine and glutamine but lacking the remaining 11 amino acids during preincubation, replenishment of arginine reversed the inhibition of cellular growth only partially and some increase in FA+ cells was not prevented. Of the media kept at -20 C, only the arginineless BME-25 showed, as expected, effects on cell replication and the EBV infection; cell replication and EBV infection were completely abolished by the addition of arginine.

The results of several experiments indicate that the FCS factors which interact with the amino acids, especially arginine, are at least

FIG. 7. *Effects of arginineless BME-25 or BME-25 containing 0.01 to 1× the standard amount of arginine (0.1 mM) on cultures of EB3 cells. The control culture on 1629-25 medium is represented by an asterisk.*

TABLE 3. *Effects of arginine replenishment on three types of preincubated BME-25 media: (i) complete, (ii) without arginine, (iii) with no amino acids except arginine and glutamine*

Preincubation with 25% FCS for 7 days		No arginine added		Arginine added	
BME	Temp (C)	Reduction in cell growth ^a	Increase in FA ⁺ cells ^b	Reduction in cell growth	Increase in FA ⁺ cells
Complete	37	+	+	+	—
	—20	—	—	nd ^c	nd
No arginine	37	+	+	+	— or ±
	—20	+	+	—	—
No amino acids except arginine and glutamine ^d	37	+	+	±	±
	—20	—	—	—	—

^a Symbols: +, >67%; ±, 33–67%; —, < 33% reduction.

^b Symbols: +, fivefold or greater; ±, two- to fourfold; —, < twofold increase.

^c Not done.

^d Remaining amino acids added after preincubation.

partially resistant to heating at 56 and 60 C for 30 to 60 min and are not dialyzable. However, some lots of dialyzed FCS prevented cellular growth in control cultures on medium which had not been preincubated. In these instances, the cells died rapidly without enhanced immunofluorescence. When BME was supplemented with horse or calf sera instead of FCS and was preincubated at 37 C for 7 days, the media did not induce an increase in the number of FA⁺ cells. In the presence of calf serum, cellular growth was often reduced, regardless of whether the media were preincubated.

In a few preliminary experiments, we noted that the effects of preincubated BME-25 or of arginineless BME-25 were not restricted to EB3 cultures but extended to other Burkitt tumor cell lines as well. Significant increases in FA⁺ cells were seen in EB1, EB2, and Kudi lines and in the HR-1-K clone of Jijoye cells.

DISCUSSION

Our experiments have shown that BME is rendered unsatisfactory for growth of EB3 cultures and other cell lines derived from Burkitt tumors when preincubated with certain lots of FCS at 37 C. This effect was found to result from an interaction between FCS and five to possibly nine of the amino acids present in BME. In addition, the preincubated media increased the number of cells infected with the herpes-type virus. This particular effect was not merely due to inhibition of cellular growth, which conceivably could favor replication of the indigenous virus; it was specifically caused by an interaction of FCS with arginine. Indeed, arginineless BME supplemented with FCS

produced, without prior incubation at 37 C, similar effects on cellular and viral replication. The addition of fresh arginine to media containing preincubated FCS-arginine mixtures reduced, but did not regularly abolish, the virus-enhancing activity of these media. This suggested that the products of the interaction between FCS and arginine may augment the effect of arginine deficiency. This possibility remains to be explored.

Preincubation of medium RPMI-1629 with concentrations of FCS similar to those used for supplementation of BME generally had less or no effect on cell growth and EB virus infection. This difference is readily explained by the fact that RPMI-1629 medium contained twice the amount of arginine that was present in BME. When concentrations of arginine (from one to eight times that present in BME) were preincubated with FCS, increasing amounts of serum were required to yield media with virus-enhancing activity. When 1629 medium was preincubated with 40% FCS, we obtained results similar to those observed with BME-25.

A lack of essential amino acids explains the reduction in cellular replication, but the mechanism by which arginine deficiency promotes viral reproduction is not yet understood. It has been reported that arginine is required for replication of herpes simplex virus (16, 20), and this may also be true for other members of the herpes group. Therefore, the arginine-deficient media may promote the EBV infection indirectly, possibly by a reduction in intracellular inhibitors. Although sufficient arginine remained available for viral replication in infected cells, it is likely that viral replication was activated only in in-

vaded cells where viral synthesis was held in check by unknown factors. This suggestion is based on two observations: (i) maximal increases in FA + cells usually occurred within 4 to 6 days after transfer of the cultures to a deficient medium, and the infected cells then degenerated and partially disappeared; and (ii) the relative increases in immunofluorescent cells were generally about 10-fold, but, since FA + cells in the stock EB3 line decreased in the course of these experiments from about 3 to <0.5%, the maximal percentages of infected cells attained after transfer to arginine-deficient media declined from 20 to 30% in early experiments to about 5 to 10% in later tests. The fact that there was no further spread of the infection on prolonged incubation of the cultures may denote that, in time, the deficient media rendered the uninfected cells unsuitable for viral replication.

Arginine and other amino acid deficiencies apparently develop in the course of cultivation of EB3 cells with fresh BME-25. Cellular growth often ceased abruptly on the fourth day of incubation, but a significant increase in FA + cells usually did not become apparent within 3 days of further incubation. The results obtained by Hinuma et al. (8) with the HR-1 clone of the Jijoye line of Burkitt tumor cells could conceivably be due to the gradual development of an arginine deficiency. When cultures of these cells were maintained on 1640-20 medium for more than 10 days without feeding, they succumbed and >50% of the cells contained EB viral antigen. In preliminary experiments, the HR-1 clone, as well as EB1, EB2, and Kudi cultures, was grown on arginine-deficient BME, and increases in FA + cells comparable to those seen with the EB3 line were observed.

Stock and experimental EB3 cultures were fed media supplemented with FCS from the same sources and often from the same lot. Culturing or electron microscopy was used to check these cultures for the presence of mycoplasmas; the results were negative. Although mycoplasmas can cause an arginine deficiency (18), they were excluded as causative agents in the present study. The FCS factors which are required for the creation of amino acid deficiencies remain to be identified; however, it is probable that these factors are enzymes.

These observations may have practical applications. On the one hand, it is expected, and indeed has already been shown (7), that cultures grown on arginine-deficient media provide a richer source for the extraction and purification of EB virus particles than do stock cultures.

This finding should facilitate further analysis of the properties of the virus and its potential oncogenicity. On the other hand, there have been considerable variations in the suitability of FCS lots for various types of cell cultures. Preincubation of FCS with amino acids before completion of culture media would provide a simple screening test for the elimination of unsatisfactory lots.

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