Application of a New Medium Supplement for Propagation and Storage of Human Cytomegalovirus

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Abstract A new medium supplement, NU-SERUM, was evaluated for cultivation of human embryonic lung fibroblasts (HEL) and for propagation and storage of human cytomegalovirus (HCMV). NU-SERUM was comparable to fetal bovine serum (FBS) in promoting rapid growth of HEL if they were seeded at a sufficient density. HCMV replicated quite satisfactorily in HEL cultured with media supplemented with NU-SERUM as well as FBS. Inactivation of HCMV at 37°C occurred similarly when the medium contained FBS or NU-SERUM. However, at -70°C, HCMV was less stable in NU-SERUM-containing medium than in FBS-containing medium. Sorbitol added to the NU-SERUM-containing medium improved the unstableness of HCMV at -70°C, and HCMV was storable with such medium. Thus, NU-SERUM is useful as an alternative to FBS not only for growth of HEL but also for propagation and storage of HCMV.

Human cytomegalovirus (HCMV) prefers human diploid cells for replication in vitro. The diploid cells grow best in media supplemented with fetal bovine serum (FBS). Thus, FBS has been indispensable in every experiment on HCMV. However, FBS is expensive, subject to market fluctuations and sometimes unobtainable due to the shortages. In addition, the quality of FBS may vary from lot to lot. A new medium supplement, NU-SERUM, was developed as an alternative to FBS and to make up for the shortcomings of FBS. NU-SERUM is characterized by a 25% FBS component and by 75% well-defined constituents, including epidermal growth factor, endothelial cell growth factor, transferrin, hormones, vitamins, glucose, and amino acids (manufacturer's information: NU-SERUM News). NU-SERUM seems to be advantageous over FBS as a growth medium supplement, but little work has been done on the substitution of NU-SERUM for replication of viruses. Its low protein content decreases interference in bioassays and increases facility of purification and filtration of the materials produced by the cells. However, this may affect stability of viruses released into the medium. In the present study, we compared the effect of NU-SERUM with that of FBS on growth of HEL, replication of human embryonic lung fibroblasts (HCMV) in HEL and stability of HCMV.
MATERIALS AND METHODS

Cells and medium. Human embryonic lung fibroblasts (HEL) cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FBS were used at the 16th passage level. Either FBS or NU-SERUM (Lot. Nos. 83–124. Collaborative Research, Inc., Lexington, Mass., U.S.A.), was used as the medium supplement to culture HEL and to propagate HCMV.

Virus. The AD 169 strain of HCMV was used throughout the experiments. HCMV-infected HEL were harvested when almost 100% of the cells showed cytopathic effects. Then the infected cells were disrupted ultrasonically and centrifuged at 2,500 rpm for 15 min. The supernatant fluid was dispensed in screw-capped vials and stored in a deep freezer, and infectious viruses were plaque-titrated with confluent HEL in 35 mm polystyrene dishes.

Growth curve of HEL. To estimate the population doubling time of HEL, kinetic studies were performed on the cultured cells. Two different concentrations of HEL, 1.0 × 10^5 or 1.0 × 10^4 cells in 5 ml of MEM supplemented with 10% NU-SERUM or 10% FBS, were seeded in 60 mm polystyrene dishes. The media were renewed every other day. The duplicated cultures were monitored daily for living cells by the dye exclusion method after trypsinization.

Size of plaques produced by HCMV in HEL monolayers. HCMV, 1.0 × 10^2 PFU per 0.1 ml, were inoculated into the confluent HEL in 35 mm polystyrene dishes and adsorbed at 37°C for 1 hr. The plates were overlaid with 2.5% methylcellulose in MEM supplemented with 5% FBS or 5% NU-SERUM. The plates received a second overlay 1 week later, and after 2 weeks, the overlay was removed and the cells were stained with methylene blue (0.03%). The major and minor axes of the plaques were measured using a projection microscope.

Replication of HCMV in HEL. HEL in culture tubes were infected with HCMV at a m.o.i. of 0.2 PFU per cell. After incubation for 1 hr at 37°C, the unadsorbed viruses were aspirated off and the cells were maintained with MEM containing 5% NU-SERUM or FBS. The duplicated tubes were harvested for each sample at 24 hr intervals after inoculation of virus except for the harvest at 12 hr. At the indicated time, infected cells were scraped off with a rubber policeman, disrupted ultrasonically and centrifuged at 2,500 rpm for 15 min. The supernatant fluids were stored at −70°C and plaque-titrated in HEL for infectious viruses.

Stability of HCMV. To exclude the effect of certain metabolites on the stability, the stock virus with an original titer of 7.0 × 10^6 PFU/ml was diluted 1:70 for a final titer of 1.0 × 10^5 in the test medium. Firstly, to examine stability of HCMV in MEM supplemented with 10% NU-SERUM or 10% FBS, HCMV (1.0 × 10^6 PFU) in the media were placed in thin-walled test tubes and allowed to stand in a water bath at 37°C. After 0, 1, 2, and 4 hr, duplicated tubes were pooled and the remaining infectivity was plaque-assayed immediately by inoculating aliquots of serial ten-fold dilutions into monolayers of HEL grown on 35 mm polystyrene dishes. Secondly, to examine the stability of HCMV at −70°C, HCMV (1.0 × 10^6 PFU) were suspended and frozen in four different storage media. They included
MEM supplemented with FBS (10\%) alone, MEM with FBS (5\%) and sorbitol (35\%), MEM with NU-SERUM (10\%) alone and MEM with NU-SERUM (5\%) and sorbitol (35\%). Each virus suspension was dispensed in screw-capped vials, and duplicated vials were pooled and titrated before and after freezing for various times, \textit{i.e.}, $-0$ hr (before freezing), $+0$ hr (immediately after freezing), 1 week, 2 weeks, and 4 weeks after freezing.

**RESULTS**

\textit{Growth Curve of HEL}

Growth characteristics of HEL in MEM supplemented with 10\% NU-SERUM or FBS are shown in Figs. 1 and 2. When $1.0 \times 10^5$ cells were seeded, HEL multiplied equally well when the supplement was FBS or NU-SERUM (Fig. 1). Under these conditions, the saturation density of HEL was $3.5 \times 10^4$ cells per cm$^2$ for NU-SERUM and $3.2 \times 10^4$ cells for FBS, respectively. In contrast, when $1.0 \times 10^4$ cells were seeded, multiplication of the cells was not sufficient and the cell sheets were not confluent in medium supplemented with 10\% NU-SERUM (Fig. 2). These cells remained in this state without showing any further multiplication. Thus, cell growth in MEM supplemented with NU-SERUM instead of FBS was dependent on the number of cells seeded.

![Growth curve of HEL](image)

\textit{Fig. 1. Growth curve of HEL (1).} A relatively high concentration of HEL, such as $1.0 \times 10^6$ cells, in 5 ml MEM supplemented with 10\% NU-SERUM or 10\% FBS was seeded in 60 mm polystyrene dishes. The duplicated cultures were monitored daily for living cells by the dye exclusion method after trypsinization.
Plaque Formation of HCMV in HEL

The effect of NU-SERUM on plaque formation was assessed by plaquing efficiency and plaque size in comparison with those obtained using FBS. There was no difference in each parameter of plaque formation between NU-SERUM and FBS (Table 1).

Replication of HCMV in HEL

Monolayers of HEL in tubes were infected with HCMV, AD 169 at an m.o.i. of 0.2 PFU per cell, and cultivated in MEM supplemented with 5% NU-SERUM
Fig. 3. Replication of HCMV in HEL. HEL in culture tubes infected with HCMV were maintained with MEM containing 5% NU-SERUM or 5% FBS. The duplicated tubes were harvested for each sample at the indicated times after inoculation of virus. Replicated HCMV in tubes were plaque-titrated in HEL and expressed as PFU per culture.

Fig. 4. Stability of HCMV at 37°C. HCMV (1.0 × 10^5 PFU) in MEM supplemented with 10% NU-SERUM or 10% FBS were placed in thin-walled test tubes which were allowed to stand in a water bath at 37°C. After 0, 1, 2, and 4 hr, remaining infectivity was plaque-assayed immediately by inoculating aliquots of serial ten-fold dilutions into monolayers of HEL grown on 35 mm polystyrene dishes.
or FBS. Titers of infectious viruses (cell-free and cell-associated) were determined at the indicated times (Fig. 3). The replication pattern and final yield of HCMV were almost identical under the two different conditions.

Stability of HCMV at 37°C

Infectious viruses in MEM supplemented with 10% NU-SERUM or 10% FBS at various sampling times were compared in Fig. 4. HCMV suspended in these two media showed thermal inactivation at a similar rate.

Stability of HCMV at -70°C

Stability of HCMV at -70°C in various stock solutions is illustrated in Fig. 5. HCMV in MEM containing 10% NU-SERUM alone or 10% FBS alone lost about 50% infectivity after only one cycle of freezing and thawing. The titers of HCMV decreased more rapidly in the former than in the latter (Fig. 3). However, addition of an equal volume of 70% sorbitol to 10% FBS or 10% NU-SERUM protected HCMV from such deterioration in the media without it. Thus, NU-SERUM-containing media with sorbitol can also be used for virus storage.
A NEW MEDIUM SUPPLEMENT FOR HUMAN CMV

DISCUSSION

A newly developed medium supplement, NU-SERUM, has been used in certain laboratories, (1, 2, 5, 9). In this paper, we evaluated NU-SERUM as a supplement for media to support cell growth and virus replication, and to store the virus at $-70\,\text{C}$.

Firstly, growth of serially passaged HEL in MEM with 10% NU-SERUM was comparable with that in MEM with 10% FBS if the cells were seeded at a sufficient density. NU-SERUM has already been used to cultivate various cells, especially to establish the primary culture for such cells as pigmented chicken melanocytes (1), skin fibroblast lines (5), rod cells from retina (9), and glomerular cells (2).

Secondly, replication of HCMV in HEL with NU-SERUM-containing medium was also comparable to that with FBS-containing medium. This was evidenced by the growth pattern, virus yield, plaquing efficiency and plaque size.

Thirdly, however, NU-SERUM was inferior to FBS in preserving infectivity of HCMV at $-70\,\text{C}$. The infectivity of herpesviruses has been stabilized by several additives besides serum (12). They include glycerol (3), dimethyl sulfoxide (10), gelatin (8), sucrose (4, 6), sorbitol (7, 11), and skim milk (8). Addition of sorbitol (final concentration of 35%) to NU-SERUM (final concentration of 5%)-containing medium remarkably improved the stability, and infectivity of HCMV in the presence of NU-SERUM with sorbitol remained at a higher level than that of HCMV in the presence of FBS without sorbitol.

In conclusion, NU-SERUM was practically substitutable for FBS as a medium supplement in propagating HCMV. However, care should be taken that the cells are split at a low ratio to make monolayer cultures and a stabilizer, such as sorbitol, is added to the medium for storage of the virus at $-70\,\text{C}$.

REFERENCES


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