Effect of oxygen partial pressure on production of animal virus (VSV)

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Abstract

The effect of oxygen partial pressure on viral replication was investigated with Vero/VSV system. At 10% oxygen partial pressure in spinner culture, VSV titer was significantly increased 130 fold compared to that obtained at 21%. A similar result was obtained for viral production in 1 liter bioreactor. This implies that oxygen partial pressure during viral production has to be low. In low oxygen partial pressure, malondialdehyde concentration was decreased about 5 fold. Thus, low oxygen partial pressure allowed the reduced reactive oxygen species to be evolved, possibly by decreasing the random oxidation of the produced viral protein and membrane from the host cell.

Abbreviations: DO – dissolved oxygen; MOI – multiplicity of index; pfu – plaque forming unit; VSV – vesicular stomatitis virus

Introduction

Viral production systems need living cells as hosts. The tools available to grow and study viruses were animals and embryonic eggs, but this needs a large space and is high cost. Therefore, efficient large-scale cell culture systems are necessary for an economical production of the virus.

Recently, mass production of anchorage-dependent animal cells for virus and other proteins was achieved by using microcarriers instead of roller bottles and tissue culture flasks (Cosgrove et al., 1995; Mendonca et al., 1994). But, solid microcarriers have a relatively low surface area per unit volume. To overcome this problem, macroporous microcarriers (K-beads, Lim et al., 1992, 1995) were applied to high cell density culture of Vero and HepG2 cells. The process of viral production significantly depends on many environmental conditions. Specifically, the virus attachment onto host cells is affected by initial pH, multiplicity of infection, serum concentration, medium composition, etc (Viti et al., 1987; Spier, 1976).

Viral production using cell lines can be divided

into two steps. First is to obtain the high cell density. Second is the viral production step, which can be sub-divided into viral attachment stage onto host cells and viral replication stage. A high cell density system has already been developed in our laboratory. In this report, we focused on the second step, especially the viral replication step. Virus needs oxygen supply for its replication. However, virus infection decreases the physiological activity of host cells. This may cause the unbalanced respiration of host cells. It can affect the viral activity in medium during and after viral replication. So, the object of this report is to investigate the effect of oxygen partial pressure on viral productivity during and after viral replication and investigate whether this can be used to control the viral production.

Materials and methods

Cell lines and cell maintenance

Vero (African green monkey kidney) cells were ori-

Figure 1. Bioreactor system for animal cell culture.

Figure 2. Growth kinetics of Vero cells on macroporous gelatin microcarriers. Each O_2 partial pressure in chamber was fixed at 1 (●), 5 (○), 10 (▼), 15 (■), and 21% (□) after VSV infection (↑). 50% of culture medium was exchanged with fresh medium (↓).

ginally obtained from ATCC and VSV (Vesicular stomatitis virus) was the gift of Wei S. Hu, Univ. of Minnesota, Minneapolis, U.S.A. Vero cells were grown with DME medium (Gibco Laboratories, U.S.A.) supplemented with 10% fetal bovine containing streptomycin sulfate (100 μ g/ml, Sigma Chemical Company, U.S.A.) and penicillin G (100 U/ml, Sigma Chemical Company, U.S.A.).

Culture matrices

Culture matrices used were macroporous gelatin microcarriers (K-beads, Lim et al., 1992, 3 g/l) and tissue culture flask (25 cm²). For macroporous beads, the spinner flask (Wilbur scientific Co., U.S.A.) were equipped with two Teflon-coated 45° pitched blades (W 1.7 cm $\times$ H 2.3 cm) attached to the impeller suspended approximately 1.5 cm from the bottom of the vessel. The agitation rate was 60 rpm and working volume 100 ml. During cell growth, every other day, 50% of culture medium was exchanged with fresh medium for optimal pH (6.8–7.4) maintenance and for the supply of nutrients. A 1 liter bioreactor (Figure 1, Korea Fermentation Co., Korea) for the mass production of virus was operated with 600 ml working volume. Temperature was controlled at 37 °C and the dissolved oxygen concentration during host cell growth was at 30% with air/nitrogen/oxygen/carbon dioxide mixture. The impeller was Teflon-coated 45° pitched blades (W 7 cm $\times$ H 3.5 cm). 2 m of silicone rubber tubing (0.058 in (i.d.), 0.077 in (o.d.), Silastic tubing, Dow Corning Co., U.S.A.) was placed in the reactor to increase the oxygen transfer rate and agitation speed was maintained at 30 rpm. During cell growth, every other day, 50% of culture medium was exchanged with fresh medium for optimal pH (6.8–7.4) maintenance and for the supply of nutrients.

Cell enumeration

For cell enumeration, 0.5 ml of sample containing the beads was transferred into a centrifuge tube. After the beads settled by gravity, the supernatant was removed and 0.5 ml of 0.1% (w/v) crystal violet (Sigma Chemical Company, U.S.A.) in 0.1 M citric acid solution was added. The sample was incubated at 37 °C for 60 min, and then the suspension was sheared with a pasteur pipette in order to detach the nuclei of cells from the microcarriers. The nuclei were then counted on a hemacytometer.

Viral production

The virus production system is shown elsewhere (Spier, 1976). When the cell density in the vessels reached the late exponential phase, the agitation was stopped and microcarriers were allowed to settle out. The supernatant was removed and microcarriers were washed with fresh medium. Culture volume just before virus infection was adjusted to 1/4 of total culture volume. Virus suspension (0.1 MOI) was then added to the vessels. After 1 h infection, the culture volume was adjusted to the original volume, and the culture was resumed.

Viral plaque assay

Virus titer was determined by standard plaque assay method (Burleson et al., 1992). The procedure was as follows. 0.1 ml of 10-fold serially diluted sample was poured into 30 mm petri-dish covered by monolayer of host cells, and incubated at 37 °C for 1 h with occasional tilting. Each dish was overlaid with 2.0 ml of a 3:1 mixture of 1.5% Noble agar and 4-fold concentrated DMEM (supplemented with final 10% FBS). After 48 h, the agar overlay was removed and the dish was stained with 0.5% (w/v) crystal violet in PBS containing 40% (v/v) ethanol. Plaques were counted with naked eye to estimate the virus concentration in pfu (plaque forming unit)/ml.

Measurement of malondialdehyde concentration

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids and some proteins, serves as a convenient index for evaluating the extent of the peroxidation reaction. Malondialdehyde has been identified as the product of lipid and protein peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535 nm (Bird et al., 1984).

The stock TCA-TBA-HCl reagent was 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N hydrochloric acid. This solution was mildly heated to assist in the dissolution of thiobarbituric acid. 1.0 ml of culture medium containing cell and virus mixture, and 2.0 ml of TCA-TBA-HCl were thoroughly mixed. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the sample was determined at 535 nm against a blank that contains all the reagents minus the biological sample. The malondialdehyde concentration of the sample was calculated us-

Table 1. Effect of O₂ partial pressure on VSV production in Vero cells grown in spinner flasks

O ₂ Partial pressure (%)	Maximal virus titer	
	($\times 10^{13}$ pfu/ml)	($\times 10^{13}$ pfu/ 10^6 cells)
1	2.5 $\pm$ 0.3	0.50 $\pm$ 0.1
5	260.0 $\pm$ 80	51.00 $\pm$ 15.7
10	780.0 $\pm$ 100	152.90 $\pm$ 19.6
15	91.0 $\pm$ 7.0	17.80 $\pm$ 1.4
21	6.0 $\pm$ 0.9	1.18 $\pm$ 0.2

Table 2. Malondialdehyde concentration under various oxygen partial pressure. Cells were grown in tissue culture flasks (25 cm²)

Oxygen partial pressure (%)	Malondialdehyde (nM)
1	77.0
5	63.7
10	64.1
21	306.0

ing an extinction coefficient of $1.53 \times 10^5 \text{ m}^{-1} \text{ cm}^{-1}$ (Esterbauer et al., 1984). Biological samples were prepared from culture medium containing cell and virus mixture collected after 2 days incubation with VSV.

Results and discussion

Effect of O₂ partial pressure on viral production

The yield of many biological products depends upon cell density. For the mass production of host cells, we used macroporous microcarriers (K-beads) prepared in our laboratory. After 6 d culture (Figure 2), final cell density reached $5\text{--}6 \times 10^6$ cells/ml. This is 2–3 times

Table 3. Effect of O₂ partial pressure on VSV production in Vero cells grown in 1-liter bioreactor

O ₂ Partial pressure (%)	Maximal virus titre	
	($\times 10^{13}$ pfu/ml)	($\times 10^{13}$ pfu/ 10^6 cells)
10 (0%)	400.0 $\pm$ 8.0	85.1 $\pm$ 1.7
21 (3–5%)	11.0 $\pm$ 0.5	2.3 $\pm$ 0.1
(30%)	0.8 $\pm$ 0.1	0.2 $\pm$ 0.0

() = Dissolved oxygen concentration.

higher than that on commercial solid microcarriers ($2\text{--}3 \times 10^6$ cells/ml, Lim et al., 1995). By the use of this technique, virus was produced in two steps. First stage was to obtain the high cell density. Second stage was viral production step. We focused on the viral replication stage to obtain the increased virus titer.

Generally, *in vitro* studies of viral production were conventionally carried out with the cells exposed to oxygen tension close to that of ambient air. However, the physiological oxygen tension in the organ may be lower than that of ambient air. So, we searched for the optimal range of O₂ partial pressure during viral replication and release into the medium. After 6 d culture, 0.1 MOI of virus was introduced into host cell suspension. Culture vessel containing virus and cell suspension was incubated for 1 h at 37 °C for viral attachment onto host cells with intermittent stirring. After that, the culture vessels were subjected to different O₂ partial pressure conditions, such as 1, 5, 10, 15, and 21% and agitation speed was resumed to 60 rpm. Maximal virus titers are shown in Table 1. The virus titer at 10% O₂ partial pressure was approximately 130 times higher than that of the control (21% of O₂ partial pressure). This difference between oxygen partial pressure in ambient air and reduced oxygen partial pressure may play an important role for the mass production of VSV and other viruses. VSV consists of an inner nucleocapsid surrounded by a membranous envelope covered with spikes formed by one viral protein, G protein (glycoprotein). This protein plays an essential role in infectivity, haemagglutination, haemolysis and cell fusion of the virus. Treatment of this molecule with enzyme capable of removing polar groups destroyed their ability to interact with virus (Viti et al., 1987). When VSV is released from host cells, it is encapsulated by host cell membrane. So, it is postulated that superoxide ions evolved in culture system may affect the virus stability or virus production because of random oxidation of viral protein and host-originated membrane. Oxygen radical is a term currently used in the biochemical literature to indicate the reactive oxygen species which are generated as intermediates in redox processes leading from oxygen to water by several enzyme such as, oxidase, superoxide dismutase, catalase, and cytochrome oxidase, etc.

Partial reduction of an oxygen molecule yields extremely toxic products. Reduction by a single electron yields the superoxide radical, whereas reduction by two electrons yields hydrogen peroxide. Both products are highly reactive and potentially destructive to certain types of functional groups present in biomolecules

Figure 3. Growth kinetics of Vero cells in 1 liter bioreactor. Each O_2 partial pressure in bioreactor was fixed at 10 (●) and 21% (○) after VSV infection (↑). And 30% of DO was maintained after VSV infection (▼) 50% of culture medium was exchanged with fresh medium (↓).

(Peterhans, 1997). Also, viral infection causes the inhibition of host cell DNA, RNA, and protein synthesis. It generates many reactive oxygen species by incomplete reduction of oxygen and by inability of neutralization of reactive oxygen species. Therefore, virus and other cellular materials may be inactivated by the attack of reactive oxygen species.

Based on this assumption, the quantitative analysis of superoxide ions in viral production system was determined.

Measurement of malondialdehyde concentration

To quantitative analysis of superoxide ions, malondialdehyde concentration of culture medium containing cell and virus mixture collected after 2 d incubation with VSV was analyzed by TBA method (Table 2). Malondialdehyde was generated from lipid peroxidation and fragmentation of proteins by reactive oxygen species. Its concentration was decreased at reduced oxygen partial pressure. Consequently, viral infection caused the unstable cellular metabolism, and host cells cannot neutralize the reactive oxygen species, and thereby the reactive oxygen species caused lower viral production at ambient condition. Therefore, the re-

duced oxygen partial pressure during viral production period will promise the maximal virus titer.

VSV production in 1 liter bioreactor

Viral production was carried out in the more controlled bioreactor system. In order to control the dissolved oxygen at 30% during cell growth, air/nitrogen/oxygen/carbon dioxide mixed gas flowed into the culture vessel. pH was adjusted to 6.8–7.4 by intermittent medium exchange (Figure 3). After 6 d, final cell density reached $5\text{--}6 \times 10^6$ cells/ml. Oxygen partial pressure was adjusted at 21% (5% of DO) and 10% (0% of DO) after viral infection. Maximal virus titers were 1×10^{14} pfu/ml at 21% and 4×10^{15} pfu/ml at 10% (Table 3). When DO was adjusted to 30% during viral production period, virus titer was significantly decreased to 7.2×10^{12} pfu/ml. These results mean that we have to control the DO to a very low tension during the viral production stage. It evolves the reduced superoxide ion species and provides the high stability of produced virus. And also, the use of macroporous microcarriers increases the cell density and virus titer.

Conclusion

The use of macroporous microcarriers to produce the high cell density and the control of oxygen partial pressure during viral production to increase the virus titer has been demonstrated. Because the macroporous microcarrier can hold cells in its interior cavity (Lim et al., 1992), final cell density was increased 2–3 times higher than that with commercial microcarriers. Cells in the cavity are protected from mechanical damage and shear (Lim et al., 1995), so, it mimics small organs. Also, the physiological oxygen tension in the organ may be lower than that of ambient air. Therefore, our viral production strategy was to use a reduced oxygen partial pressure. This lowered superoxide ion species as determined by the estimation of malondialdehyde and the high virus titer. The reduced reactive oxygen species offered stability of produced virus by decreasing random oxidation. Therefore, the reduced oxygen partial pressure during viral production period promises the maximal virus production.

One liter culture allows the possibility of large-scale production of virus. We have to control the DO as 30% during cell growth for maximal cell density, and lower the DO during viral production to obtain a high virus titer.

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