Journal of Bioscience and Bioengineering
VOL. 111 No. 5, 600–604, 2011

www.elsevier.com/locate/jbiosc

Growth and poliovirus production of Vero cells on a novel microcarrier with artificial cell adhesive protein under serum-free conditions

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Received 3 September 2010; accepted 16 December 2010
Available online 23 January 2011

A microcarrier is used for the three-dimensional (3D) culture of adhesion-dependent mammalian cells. We developed a novel microcarrier by binding ProNectin F, an artificial cell adhesive protein synthesized by genetically engineered *Escherichia coli* to a polyacrylic superabsorbent polymer. The microcarrier is characterized by containing no animal-derived components. The serum-free culture of Vero cells for vaccine production using the microcarrier increased the number of Vero cells by approximately 30% compared with the existing dextran beads coated with porcine Type I collagen, which resulted in approximately a 30% to 40% increase in the infectivity titer of the Sabin 2 strain of poliovirus. These results suggested that the developed microcarrier should be unprecedented in permitting high-yield vaccine production by means of a serum-free culture.

[**Key words:** Microcarrier; Superabsorbent polymer; ProNectin F; Vero; Poliovirus; Serum-free culture]

Van Wezel developed a method to grow adhesion-dependent cells on microparticles and established the concept of a microcarrier culture (1). With a larger culture surface area per volume, a microcarrier is appropriate for the efficient culture of adhesion-dependent cells.

The materials used for a microcarrier include dextran and polystyrene (2). Dextran is swollen from water absorption and is highly transparent, allowing visual observation of cells adhered to the surface. In other words, cells can be observed with a typical inverted microscope without the need for staining, permitting convenient and rapid observation of cells (3). A microcarrier must have adequate dimensions to accommodate the efficient growth of cells. In general, a microcarrier should preferably have an average particle size around 150 to 250 μm in the culture medium (4).

Medical materials swollen from water absorption with an average particle size around 150 to 250 μm in the culture medium include microparticles of a superabsorbent polymer using acrylic sodium salt as raw materials. This polyacrylic superabsorbent polymer is used for disposable diapers and other hygienic goods and has recently attracted attention as safe in vivo microparticles used for embolization (5–7).

Diethyl amino ethyl (DEAE) and collagen are well known as surface-modifying substances for microcarriers. It was reported that the surface of a positively charged microcarrier with DEAE attracts cells, but such a microcarrier was inferior to a microcarrier with collagen in terms of cell growth (2,8,9). One study reported that both cell attraction by a positive charge and cell adhesion/growth by collagen were satisfied by coating the surface of a microcarrier with trimethylamine and collagen (2). However, collagen is an animal-derived substance, and thus the possibility of contamination from pathogens cannot be excluded.

Meanwhile, non-animal-derived cell adhesive proteins include recombinant proteins, such as ProNectin F (PnF), Retronectin, and Attachin. Plates coated with these proteins were useful for growing cells in a serum-free culture, and PnF was especially superior to other recombinant proteins for human umbilical vein endothelial cell (HUVEC) (10,11).

We developed a novel microcarrier by using a polyacrylic superabsorbent polymer as a microcarrier and binding PnF, an artificial cell adhesive protein synthesized by genetically engineered *Escherichia coli*.

MATERIALS AND METHODS

Preparation of microcarriers PnF was used for the culture substrate because of the foregoing performance and stability, that is, it was autoclavable and stable for more than two years at room temperature. PnF (Sanyo Chemical Industries, Kyoto, Japan) is an artificial cell adhesive protein synthesized by genetically engineered *E. coli*. PnF comprises 980 amino acids and contains 13 arginine–glycine–aspartic acid (RGD) sequences interspersed between repeated glycine–alanine–glycine–alanine–glycine–serine (GAGAGS) structural peptide segments derived from silk β-sheet structures (12). Based on the sequence, the molecular weight of PnF is estimated to be 72,728 (12).

Ten grams of a pearl-like (average particle size is 175 μm in 0.9% NaCl) polyacrylic superabsorbent polymer (Sanyo Chemical Industries, Kyoto, Japan) was added to 100 mL of solution containing 1 M of 2-dimethylaminoethyl chloride hydrochloride (DEAE) (Wako Pure Chemical Industries, Osaka, Japan) and 240 mM of sodium hydroxide, stirred at 60°C, and allowed to react for 5 h. The reaction mixture was removed by aspiration. The polymer was then added to 1 L of ion-exchanged water and stirred for 1 h, followed by removal by aspiration. The rinsing procedure was repeated 5 times in total. Then, the polymer was dried under reduced pressure for 12 h using a

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doi:10.1016/j.jbiosc.2010.12.018

reduced-pressure dryer (40°C, 0.1 kPa or less) to obtain the DEAE-bound polyacrylic superabsorbent polymer (hereinafter called "DSAP") for the cell culture.

Ten grams of DSAP was added to 100 mL of PBS solution (0.02 M, pH 5.2) containing 60 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Wako Pure Chemical Industries, Osaka, Japan), stirred at 25°C, and allowed to react for 0.5 h (suspension A). In order to obtain five kinds of PnF-bound superabsorbent polymer (PnF-DSAP), 10 mL of PBS solution (0.3 M, pH 7.4) containing PnF at 0.5, 2, 10, 100, or 1000 mg was added to suspension A, respectively, stirred at 25°C, and allowed to react for 2 h. Then, the reaction mixture was removed by aspiration, followed by the addition of 400 mL of PBS for rinsing. The rinsing procedure was repeated 5 times in total. Furthermore, 400 mL of ion-exchanged water was added and removed by aspiration. The rinsing procedure with ion-exchanged water was repeated 2 times in total. Subsequently, the solution was dried under reduced pressure for 12 h using a reduced-pressure dryer (40°C, 0.1 kPa or less) to obtain 5 kinds of PnF-DSAP (P1, P2, P3, P4, and P5).

Cytodex 3 (GE Healthcare Bio-Sciences, Uppsala, Sweden) composed of dextran beads coated with porcine Type I collagen was used as the control microcarrier.

Characterization of microcarrier The physical properties of DSAP, PnF-DSAP, and Cytodex 3 were evaluated in a saline solution (0.9% NaCl) because the diameters of the microcarriers were affected by the NaCl concentration (5). Particle size and morphology were confirmed by an inverted microscope, IX71 (Olympus, Tokyo, Japan). For the positive charge of the microcarrier, zeta potential was measured using a zeta potential meter, Mutek SZP-06 (BTG, Herrsching, Germany). The absorption volume of the microcarrier for the saline solution was determined by immersing 1 g of a microcarrier in excessive saline solution for 60 min, pouring the solution onto a nylon net, draining for 10 min, and subtracting 1 g from the resultant weight.

PnF bound to DSAP was detected by the immunofluorescent antibody technique, and DSAP was used as the control. Briefly, PnF-DSAP was treated with PBS solution (0.02 M, pH 7.2) containing 1% BSA. Then, anti-PnF monoclonal antibody (Sanyo Chemical Industries, Kyoto, Japan) was added. Following incubation at 37°C for 1 h, FITC-labeled polyclonal rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) was added. After rinsing with PBS, the presence of PnF in PnF-DSAP was observed with a fluorescence microscope IX71 with U-LH100HGAPO (Olympus, Tokyo, Japan).

The amount of PnF bound to DSAP (PnF density) was quantified with the Micro BCA Protein Assay Kit (Thermo Scientific, IL, USA).

Cell, virus, and medium Vero cells (CCL-81) were purchased from American Type Culture Collection (VA, USA). For the cell culture and virus production, VP-SFM (Invitrogen, CA, USA) supplemented with 4 mM L-glutamine was used as serum-free, non-animal-derived nutrient medium. Vero cells were grown through several passages using VP-SFM to adapt the medium before use, and the cells were used for an experiment at passage number of 130 to 140. The Sabin 2 strain of poliovirus (Iwate Medical University, Iwate, Japan) was adapted for use in the Vero cells.

Cell culture and virus production A total of 0.3 g of DSAP or PnF-DSAP (equivalent to the surface area of 1.4×10^3 cm^2 after swollen with 0.9% NaCl) and 0.5 g of Cytodex 3 (equivalent to the surface area of 1.4×10^3 cm^2 after swollen with 0.9% NaCl) were introduced into a silicon-coated spinner flask with a volume of 250 mL (Bellco, NJ, USA), added to a saline solution at 10 mL per flask, and sterilized in an autoclave (121°C, 20 min). The saline solution was removed by aspiration. VP-SFM was added at 50 mL per flask and allowed to stand for one hour. The medium was removed from a spinner flask by aspiration. VP-SFM was added again at 100 mL per flask. A spinner flask was allowed to stand for 2 h in a CO_2 incubator at 37°C. Then, Vero cells were resuspended in the medium so that the number of cells was 2×10^7 cells per flask. Subsequently, cells were incubated in a CO_2 incubator at 37°C while stirring at 30 rpm for 8 days. On day 6 of the culture, half the media was exchanged with new media. On days 2, 4, 6, and 8, cells were sampled for microscopic observation of cell conditions on microcarriers and cell number determination.

For cell number determination, the microcarriers with cells were washed with PBS once, then re-suspended in 0.1 M of citric acid containing 0.1% (w/v) crystal violet (Wako Pure Chemical Industries, Osaka, Japan) and incubated at 37°C for 1 h. The mixture was then gently pipetted, and the cell number was determined by counting the released-nuclei with a hemocytometer (13). The cell numbers from the crystal violet nuclei-staining method (CV) were confirmed through correlation with the conventional trypan blue method (TB) before use. For the trypan blue method, the microcarriers were trypsinized with 0.25% trypsin with EDTA·4Na (Invitrogen), and the cells detached from the microcarriers were dyed with trypan blue (Invitrogen) and counted hemocytometrically.

On day 8 of incubation, the poliovirus was inoculated at MOI = 0.001. Incubation was continued in a CO_2 incubator at 37°C while stirring at 30 rpm. On days 12 and 16 of incubation, the culture solution was aliquoted and stored at −80°C until required.

For measuring the infectivity titer of the virus, a total of 0.1 mL of culture media diluted in 10^{-1} to 10^{-8} with DMEM (2%FBS) was inoculated into Vero cells on the 96-well culture plate. Incubation was continued for 7 days in a CO_2 incubator at 37°C. The infectivity titer (TCID 50/0.1 mL) was calculated based on the judgment of the cytopathic effect (CPE) observation on day 7 according to the Reed and Muench method (14).

RESULTS

Cell counting Vero cell numbers using the crystal violet method and trypan blue method were measured with Cytodex 3 on days 1–6 of cultivation. Vero cell numbers were exactly the same for both methods as shown in Fig. 1. The crystal violet method was a simple procedure without the trypsin pre-treatment and was used subsequently.

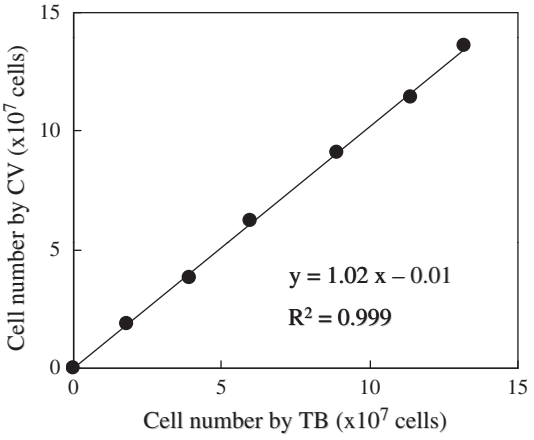

FIG. 1. Correlation of Vero cell number on microcarriers between crystal violet nuclei-staining method (CV) and trypan blue method (TB).

Optimization of PnF density Vero cell numbers were measured after 6-day incubation using DSAP (no PnF binding) and PnF-DSAP (P1, P2, P3, P4, and P5) in which the amount of PnF bound to microcarriers was 0, 0.01 (P1), 0.04 (P2), 0.2 (P3), 1 (P4), and 7 (P5) μg cm^{-2} each (Fig. 2). As shown in Fig. 2, the cell numbers tended to increase with the increase in the PnF modification of microcarriers. Also, the PnF modification reached a plateau around 1 μg cm^{-2}. Consequently, PnF-DSAP (P4) with a modification of 1 μg cm^{-2} was used for subsequent examinations.

Characterization of microcarriers The physical properties of DSAP and PnF-DSAP (P4) were evaluated. Cytodex 3 was used as the control microcarrier. The results are shown in Table 1. Particle density, diameter, and zeta potential were similar among all microcarriers examined. On the other hand, the absorption volume of 0.9% saline solution was higher with DSAP and PnF-DSAP than with Cytodex 3. DSAP and PnF-DSAP were made from a polyacrylic superabsorbent polymer as the major component; however, Cytodex 3 was made from dextran as the major component.

Moreover, the presence of PnF in PnF-DSAP was investigated with the fluorescent antibody method using anti-PnF monoclonal antibody. As shown in Fig. 3, PnF-DSAP generated fluorescence on fluoroscopic

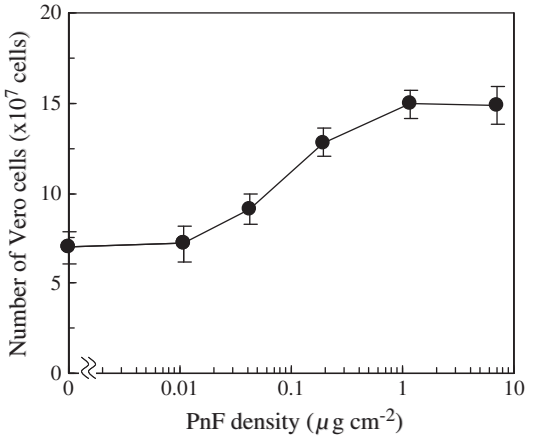

FIG. 2. The relationship between PnF density and growth of Vero cells on microcarriers in VP-SFM. PnF density at 0 μg cm^{-2} is DSAP.