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PURE GELATIN MICROCARRIERS: SYNTHESIS AND USE IN CELL ATTACHMENT AND GROWTH OF FIBROBLAST AND ENDOTHELIAL CELLS

KIMBERLY W. WISSEMANN AND BRUCE S. JACOBSON

Department of Biochemistry, University of Massachusetts, Amherst, MA 01003

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SUMMARY

A new type of microcarrier was described using bead emulsion-polymerization techniques. An aqueous solution of gelatin and glutaraldehyde was dispersed in a hydrophobic phase of mineral oil, using Triton X-114 as an emulsifier, and polymerization was initiated. The resultant spherical beads, composed entirely of gelatin, showed excellent mechanical stability to ethanol drying, sterilization, and long-term use in microcarrier spinner cultures. The solid gelatin microcarriers supported the growth of L-929 fibroblast, swine aorta endothelial, human umbilical endothelial, and HeLa-S_3 cultures with no adverse effects on cell morphology or growth. The beads were transparent in growth medium and attached cells were clearly visualized without staining. The beads were also compatible with techniques for scanning electron microscopy. Collagenase could be used to entirely digest the gelatin beads, leaving the cells free from microcarriers and suspended in solution while retaining 98% cell viability. The results further showed that after collagenase treatment the cells would populate fresh gelatin microcarriers and grow to confluence. Cell attachment kinetics revealed that the endothelial cells attached to the gelatin beads at the same rate as to tissue culture plates, whereas the fibroblast cells attached to the beads more slowly. However, once the fibroblast cells were attached to the gelatin microcarriers they spread and grew normally.

Key words: gelatin; microcarriers; emulsion-polymerization; fibroblast cells; swine endothelial cells; cell attachment.

INTRODUCTION

The use of microcarriers for cell culture was first described in 1967 by van Wezel (27). Since then, growing anchorage-dependent cells on microcarriers has increased in popularity due to a better understanding of the technology and an increased demand for high cell density cultures. To meet this demand several types of microcarriers have been developed including those based on dextran (5,15,19,27), cellulose (23), acrylamide (22), fluorocarbon-polylysine (11), and polystyrene (3,9,10,14).

Recently the technique of coating beads with a layer of gelatin (denatured collagen) has been used (5,9,19). The role of collagen in cell attachment and growth has been well studied (12,13,16) and the use of gelatin has become a popular alternative to the use of native collagen. Early work by Traub and Piez (26) indicated that as denatured collagen cools, tripeptide structures of native collagen are reformed, leading to the theory that collagen and gelatin may have similar sites for cellular attachment. Goldberg (6) has also shown that denatured fragments of collagen will bind to a variety of cells. Charge interactions between gelatin and cells will also lead to cellular attachment. The exact nature of crosslinked gelatin is not known, but several researchers have studied gelatin as a substratum for cell growth and have indicated that gelatin is a valuable cell culture substrate.

The purpose of this study was to produce a microcarrier made entirely out of gelatin and to test cellular attachment and growth on its surface. The gelatin beads were created by an emulsion-polymerization technique that has been used to prepare polyacrylamide (22) and liquid (11) microcarriers. We report here a reliable procedure to produce, dry, sterilize, and use pure gelatin beads in microcarrier culture.

MATERIALS AND METHODS

Synthesis of microcarriers. All glassware used in this procedure was presiliconized by treatment with 1% dimethyldichlorosilane in toluene. The gelatin microcarriers were prepared by an emulsion-polymerization technique outlined in Fig. 1. One hundred and eighty milliliters of mineral oil (Saybolt viscosity 340 to 360, Marathon Morco, Dikinson, TX) was placed in a 2-liter round bottom flask, fitted with a glass stir paddle, and heated. When the temperature of the mineral oil reached 40° C, 1.8 ml 1% Triton X-114 (vol/vol in water, Sigma

Chemical Co., St. Louis, MO) was added and stirred evenly at 200 rpm for 10 min. The aqueous phase was prepared by dissolving 3 g swine skin gelatin (300 bloom, Sigma Chemical Co., St. Louis, MO) in 20 ml of 20m*M* sodium phosphate buffer, pH 5.5, in a 60° C water bath. Two hundred and twenty-five microliters of 8% electron microscope (EM) grade glutaraldehyde (Polysciences, Inc., Warrington, PA) was added to 18 ml of the 15% gelatin solution, and the resultant solution was added immediately to the mineral oil stirred at 200 rpm. It was essential to add the glutaraldehyde/gelatin solution quickly, before the aqueous solution polymerized. After 15 min, 500 ml of 20 m*M* sodium phosphate buffer, pH 5.5, was added slowly and the emulsion was stirred for an additional 10 min.

The beads were allowed to settle (ca. 1 h) out of the oil phase, transferred to an Erlenmeyer flask, and washed three times with 300 ml 20 m*M* sodium phosphate buffer, pH 5.5. The beads were then suspended in 300 ml of the same buffer, the pH was adjusted to 7 with saturated sodium tetraborate, 2.4 ml 8% EM grade glutaraldehyde was added, and the solution was placed on a rotary shaker (ca. 200 rpm) for 1 h. To stabilize the beads they were washed once with 20 m*M* phosphate buffer, pH 5.5, resuspended in 300 ml of the buffer, adjusted to pH 9.3 with saturated sodium tetraborate, and 0.06 g sodium borohydride was added. The solution was left on the rotary shaker overnight at 200 rpm and then washed twice with distilled water, twice with 20 m*M* sodium phosphate buffer, pH 5.5, and once with 60% ethanol. Forty-three percent of the initial gelatin (dry weight) was converted into gelatin beads.

The beads were suspended in 60% ethanol and sized by sieving through a polyethylene mesh screen (Spectramesh, Fisher Chemical Co., Pittsburgh, PA).

Handling of microcarriers. The gelatin microcarriers were stored in 60 or 70% ethanol or were dried and stored in sterile vials. Ethanol dehydration was used to dry the beads. The beads were placed in 60% ethanol in a siliconized flask, swirled, allowed to settle (ca. 10 min), and the ethanol above the beads was removed. This process was repeated with five times the settled volume of the beads with 70, 80, 90, 95%, and absolute ethanol. The ethanol-dried beads were transferred to a petri dish with a small amount of absolute ethanol and air dried in a sterile hood.

The sterile, dried beads were rehydrated by swelling them in sterile phosphate buffered saline (PBS, 140 m*M* NaCl, 15 m*M* K_2HPO_4, pH 7.2) at a ratio of 0.5 g beads to 25 ml PBS, in 37° C waterbath for 4 h. The PBS was removed, the beads were washed once with PBS (no incubation), three times with medium plus calf serum (0.5 g beads/25 ml medium), and were ready to use.

The gelatin microcarriers that were stored in 60 or 70% ethanol were prepared for cell culture by removing the ethanol and resuspending the beads in three volumes of sterile medium plus calf serum. The bead-medium suspension was swirled, the beads allowed to settle, the medium removed, and the process repeated two more times. The beads were finally suspended in the desired volume of sterile medium to obtain a known bead per milliliter concentration. This stock solution of beads was stored at 4° C for up to 2 wk. Before use, this stock solution was incubated in a 37° C water bath. The gelatin beads were always handled under sterile conditions because they were susceptible to bacterial proteases.

The beads used in these experiments were 150 to 44 μm with a mean diameter of 300 μm. To determine the number of beads per milliliter, several aliquots of suspended beads were stained with trypan blue and counted. Once a packed volume of beads per milliliter was established, this was the measurement used to quantitate bead density.

Cell lines and media. Cell cultures included: mouse fibroblast (L-929), human cervical carcinoma (HeLa-S_3), and endothelial cells from swine aorta and human umbilical veins. The L-929 cells were obtained as an established cell line from Dr. C. Woodcock of the Department of Zoology, University of Massachusetts, Amherst. They were grown in tissue culture flasks (80-cm^2 flasks) containing RPMI 1640 (KC Biologicals, Inc., Lexena, KS) supplemented with 5% calf serum (GIBCO, Grand Island, NY) and additions (3 mg/ml $NaHCO_3$, 100 U penicillin G/ml, 100 μg/ml dihydroxystreptomycin, and 2 μg/ml butyl *p*-hydroxybenzoate). Confluent cultures were subcultured by decanting the growth media, washing twice with PBS plus 1 m*M* EDTA, and incubating at 37° C in 0.5 ml of PBS/EDTA until the cells detached from the surface of the flask. The detached cells were suspended in growth media and added to fresh T-flasks.

The HeLa-S_3 cells were obtained in growth phase from Dr. D. J. Giard of the Massachusetts Institute of Technology Cell Culture Center. The cells were grown in suspension culture in RPMI 1640 medium plus 5% calf serum and the above additions. They were maintained in mid-log phase (3 to 5 × 10^5 cells/ml) in Bellco spinner flasks.

Swine aorta endothelial cells were isolated by established techniques (4,17) and obtained from Drs. L. L. Slakey and L. W. Hayes (Department of Biochemistry, University of Massachusetts, Amherst). The cells were grown in Dulbecco-Vogt modified Eagle's medium (DMEM) (GIBCO) plus additions (17) in T-75 flasks. Human umbilical vein endothelial cells were isolated from fresh human umbilical veins and were grown in T-75 flasks in Medium 199 (GIBCO) supplemented with 20% fetal bovine serum (FBS), 90 μg/ml heparin, 90 μg/ml endothelial cell growth factor, 4 m*M* glutamine, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. Removal of endothelial cells from the surface of the T-flasks was effected by treatment with 0.05% trypsin (Difco Laboratories, Detroit, MI). All cultures were grown at 37° C in a humidified 5% CO_2-air incubator.

Microcarrier spinner culture. Microcarrier spinner culture was initiated by combining the cells and beads in a sterile, siliconized microcarrier spinner flask (Bellco Glass, Inc., Vineland, NJ) with a working volume of 100 ml. A small volume of media (ca. 40 ml) and the appropriate number of media-washed microcarriers were preincubated in the spinner flask for 30 min. The number of beads used was varied depending on the cell density desired; for L-929 cells 8000 beads/ml would yield approximately 4 to 5 × 10^6 cells/ml. To

the beads plus media either L-929 or HeLa cells were added to a final concentration of 5×10^4 cells/ml and allowed to attach for 3 h without spinning in the incubator. Every 30 min the suspension was stirred at 30 rpm for 2 min (19). After the attachment phase the cultures were allowed to spin continuously at 35 to 40 rpm on a Bellco microcarrier magnetic stirrer.

To ensure growth to high cell densities it was necessary to replenish the medium daily. The microcarriers with attached cells settled to the bottom of the culture vessel, the medium was then poured off and replaced with an equal volume of fresh media. Alternatively, when the culture was being maintained at a very high cell density the procedure of Crespi and Tilly (2) was used for bead-to-bead cell transfer. The culture was swirled to get an even suspension and 50% of the total volume of beads, cells, and media was removed. The remaining beads with cells were allowed to settle, the medium removed, and the culture was diluted with fresh microcarriers and medium. After addition of fresh beads to established cultures it was not necessary to repeat the attachment procedure as described above.

Static cell culture. Endothelial and L-929 cells were grown on the gelatin beads under static cell culture conditions as described by Davies (3). The microcarriers were prepared for tissue culture use as described above and plated in 24-well (17 mm) tissue culture plates, the surface of which had been treated with the inert polymer hydroxyethyl methacrylate (Hydron Laboratories, New Brunswick, NJ) to prevent cells from attaching to the surface of the plate. Eight to ten drops of a 1.2% solution of hydroxyethyl methacrylate in 95% ethanol was added to each well to completely cover the surface and allowed to dry in a sterile hood. Each well was rinsed with 1 ml of fresh media before use. This polymer prevented the attachment of the cells to the tissue culture dish, but had no adverse effect on the growth of the cells on the beads. To each polymer-coated 17-mm well was added 1 ml media, 10 000 beads, and cells to a final concentration of 0.5 to 1×10^5 cells/ml. The wells were then filled completely with media and fed daily by aspirating off the media above the beads and replacing it with fresh media. The swine endothelial cells were always plated onto the beads in DMEM + 20% FBS and changed to DMEM + 20% swine plasma-derived serum (4) the following day.

Growth determination. Cells in microcarrier cultures were enumerated by counting cell nuclei (25). A 1-ml sample from the spinner cultures was centrifuged at 1450 $\times g$ for 5 min and the growth medium was removed. Cells and microcarriers comprising the pellet were then washed in 1 ml of cold PBS and recentrifuged. The washed pellet was resuspended in 1 ml of 0.1% crystal violet in 0.1 *M* citric acid, vortexed, and incubated for 1 h at 37° C. The solution was then vortexed thoroughly and the number of released nuclei counted in a hemocytometer. The gelatin beads did not interfere with the assay.

To determine the cell density in the 24-well tissue culture plates the entire content of the well was harvested and the well was rinsed twice with 1 ml cold PBS. After centrifuging at 1450 $\times g$ for 5 min the pellet was washed in 1 ml cold PBS, recentrifuged, and treated with crystal violet as described above. The trypan blue exclusion method (20) was used to determine the number and viability of unattached cells in the microcarrier cultures.

Collagenase treatment. Experiments were conducted to show that collagenase could be used to release the cells from the gelatin beads and leave the cells free in suspension. L-929 fibroblast cells were grown on gelatin microcarriers in both spinner and static culture. Confluent microcarriers (40 000 beads) were harvested from culture, transferred to a sterile siliconized test tube, and allowed to settle. The medium was removed, the beads were rinsed three times with 5 ml sterile cold PBS to remove serum, and then incubated with 2 ml 500 μg/ml collagenase (CLS II, Worthington Biochemical Co., Freehold, NJ) in PBS. The suspension was incubated at 37° C for 10 to 15 min with occasional agitation. When the cells were released or the beads were digested, 10 ml of warm (37° C) media plus calf serum was added and mixed thoroughly. An aliquot of the cells was removed for cell counting by hemocytometry and the remaining cells were plated onto new gelatin beads (10 000 beads/well) in polymer coated, 24-well tissue culture plates at a concentration of 2×10^4 cells/ml. The cells were fed daily. The cells and microcarriers from two 17-mm wells were harvested daily and the cells were counted by the crystal violet assay.

Cell morphology. The gelatin microcarriers are transparent in growth medium, so the morphology of the attached cells could be monitored directly via a light microscope. A Leitz Dialux 20 microscope with an attached Orthomat W camera was used for all of the light photomicrographs. For scanning electron microscopy (SEM), L-929 cells on beads were harvested from spinner culture, washed three times with cold PBS, centrifuged at 300 $\times g$ for 5 min, and suspended in 3% glutaraldehyde in PBS. The microcarrier suspension was allowed to fix on ice for 1 h with occasional agitation before it was mounted onto gelatin coated glass cover slips in 8% glutaraldehyde. After 1 h on ice the samples were dehydrated in a graded series of ethanol washes and critical point dried in a Polaron critical point dryer model E3000. A 360 Å layer of gold was applied to the samples with a Polaron specimen coater model E5000 and viewed in a Jeol JSM-25S scanning electron microscope.

Kinetics of cell attachment. The rate of cell attachment to two different substratums, gelatin microcarriers and tissue culture plates, was studied for the swine endothelial and L-929 cell lines. A modification of the procedure by Reuveny et al. (23) was used. The endothelial cells used for this experiment had been passaged seven times. Duplicate 24-well tissue culture plates were prepared with 12 of the wells coated with the Hydron polymer to inhibit cellular attachment to the plate, and 12 of the wells left uncoated. All wells had 1.5×10^5 cells in a total volume of 1.5 ml/well. In addition, the coated wells contained 20 000 gelatin microcarriers. The dishes were incubated at 37° C and at the specified time the wells were harvested with a pipetman by carefully mixing to suspend the unattached cells and then

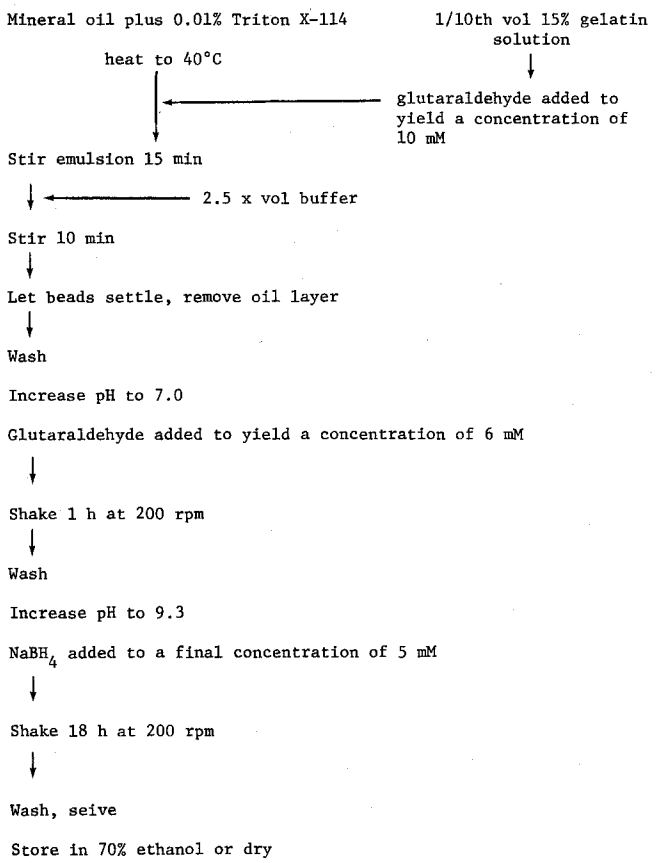


FIG. 1. Preparation of the gelatin microcarriers.

removing a 450-μl sample to determine the number of cells remaining in the supernatant fluid (23). All attachment experiments were based on the percentage of unattached live cells determined by trypan blue exclusion.

RESULTS

Preparation of the gelatin beads. The gelatin beads were prepared as described in Materials and Methods and outlined in Fig. 1. Several factors were important to ensure the success of the emulsion polymerization procedure. These included the quality of the gelatin, mineral oil, and glutaraldehyde, the type and concentration of detergent, the pH of the wash buffer, and the stirring speed. Before the other factors could be evaluated it was necessary to have an apparatus that was able to stir the emulsion evenly and smoothly. The most reliable results were obtained with a Dyna-mix stirrer purchased from Fisher Chemical Co. with an attached glass stirring rod and paddle. This unit was able to maintain a stirring speed of 200 rpm for extended periods of time without fluctuations.

Four types of gelatin were tested: swine skin (300 Bloom), swine skin (175 bloom), calf skin (225 Bloom), and calf skin (60 Bloom). Bloom number is an indication of the strength of gels produced, with higher Bloom numbers designating stronger gels. The two types of calf skin gelatin resulted in beads that were dark brown. Thus, the calf skin gelatin was not used because of the unappealing color and the possibility of impurities. For the production of the gelatin microcarriers the swine skin, 300 Bloom, was found to yield the most stable beads. The color of these beads was a pale yellow. The pH of the wash buffer was kept at 5.5 (below the isoelectric point of the swine skin gelatin) to avoid clumping of the beads during their preparation.

A series of detergents were evaluated to determine the most suitable emulsifier, based on the criteria that the beads have a smooth and uniform surface. The Triton X series of detergents which range in hydrophile-lipophile balance (HLB) values from 7.8 to 18.7 were chosen for evaluation. The resultant beads were examined microscopically and graded on a scale of 1 to 5 with 5 representing the best-shaped beads. Triton X-114, the type of detergent that had a significant effect on the quality of the beads, consistently resulted in high yields of excellent beads (Fig. 2). Triton X-100 and Triton X-102 could also be used; however, beads produced with these detergents gave a large variation in the size of the beads within one batch and the overall quality was not consistent between batches. The remaining detergents in the Triton X series were evaluated as poor or unacceptable because they would not allow the beads to settle out of the oil layer,

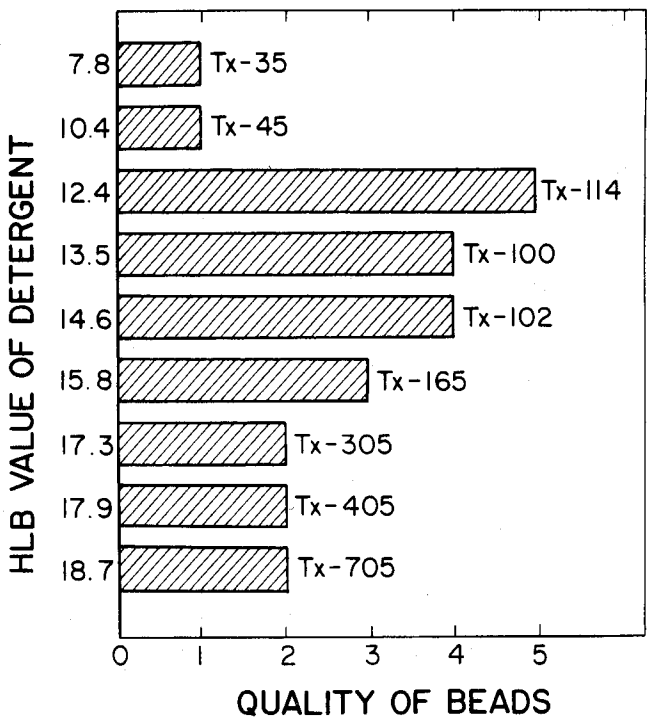


FIG. 2. Effect of various detergents used in the emulsion-polymerization procedure on the quality of the gelatin beads. *HLB* is the hydrophile-lipophile balance of the individual detergents. The quality of the resultant beads was rated: 1. Unacceptable—beads remained in the oil layer, massive clumping. 2. Poor—odd-shaped beads, low yield, significant clumping. 3. Fair—good bead shape, lots of size variation, beads stayed in oil layer longer. 4. Good—some size variation, quality not consistent. 5. Excellent—uniform size, consistent results, high yield.

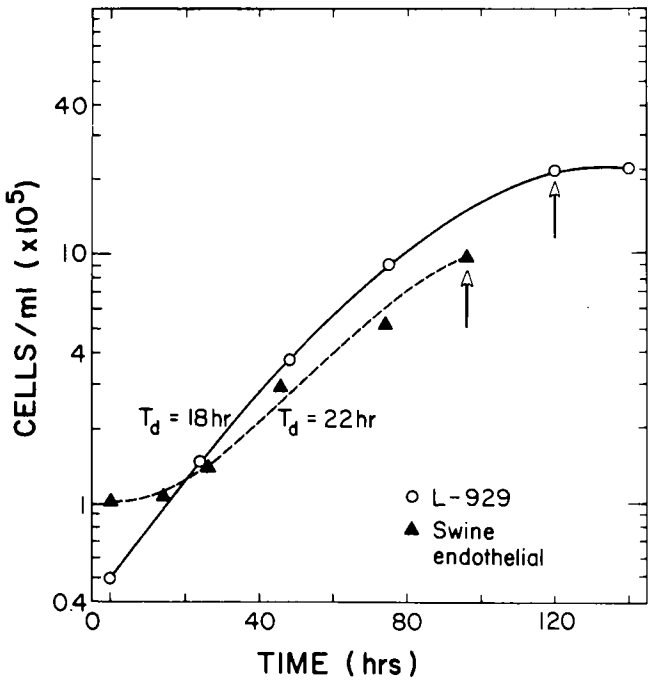


FIG. 3. Growth of L-929 (O---O) and swine aorta endothelial (▲---▲) cells on gelatin microcarriers. Both cell lines were inoculated onto 10 000 gelatin beads in polymer-coated, 17-mm tissue culture wells and grown under static conditions. The medium was replaced daily. *Arrows* indicate when the cultures became confluent.

caused extensive clumping, odd-shaped beads, and low yields. Tween 20, Tween 80, and sorbitan monoleate were also unacceptable emulsifiers for bead preparation. Several different detergent concentrations were tried for TX-114, 100, and 102. The results indicated that a final concentration of 0.01% was the most effective. In our hands, it was not possible to significantly change the mean diameter of the beads by variations in detergent concentration or stirring speed or both as reported by Reuveny et al. (22) with polyacrylamide-based microcarriers; therefore beads of the desired size were obtained by seiving.

The final procedure for bead production included a second treatment with glutaraldehyde to help stabilize the beads. Initially, stabilization was attempted by doubling the glutaraldehyde concentration present in the first step; however the gelatin polymerized so quickly at this glutaraldehyde concentration (20 m*M*) that the beads were not formed properly. By decreasing the glutaraldehyde concentration to 10 m*M* in the first step there was enough time to mix the gelatin and glutaraldehyde solutions together, pour the mixture into the stirring mineral oil, and allow the gelatin to coalesce into a uniform bead shape before the cross-linking was complete. To ensure that all of the gelatin was cross-linked the formed beads were then placed in a solution of 6 m*M* glutaraldehyde. The procedure worked best with electron microscope grade glutaraldehyde.

It was also possible to incorporate other macromolecules into the matrix of the bead by including them in the aqueous phase with the gelatin. The glycosaminoglycan, chondroitin sulfate, was incorporated successfully into the gelatin beads at a concentration of 0.1% wt/vol in the initial 15% gelatin solution. However, no studies were done on cell growth on the gelatin/chrondroitin sulfate beads.

Sterilization of gelatin microcarriers. Autoclaving of the gelatin microcarriers swollen in PBS, 20 m*M* sodium phosphate, pH 5.5, 100 m*M* sodium phosphate, pH 5.5, 7.0, or 9.0 were all found to cause degradation of the beads. However, the gelatin beads could be stored in 70% ethanol with no adverse effects on their integrity or the subsequent cell attachment and growth on their surface. Beads stored for up to 8 mo. in ethanol behaved identically to fresh beads. The gelatin microcarriers sterilized in alcohol could be used for long-term continuous culture without causing contamination of the culture. Drying the beads with a graded series of ethanol washes and placing them in sterile vials provided an equally effective method of storing beads. The dried beads were rehydrated at 37° C in PBS.

Cell growth on gelatin microcarriers. Growth of L-929 and swine aorta endothelial cells was monitored on the newly synthesized gelatin microcarriers. Figure 3 illustrates their growth pattern in stationary culture on 10 000 beads/17-mm well. Cells were seeded at 0.5×10^5 and 1×10^5 cells/ml, respectively, for the L-929 and endothelial cells. There was a 24-h lag phase after attachment for the primary endothelial cell cultures, similar to the lag observed when the cells were grown on

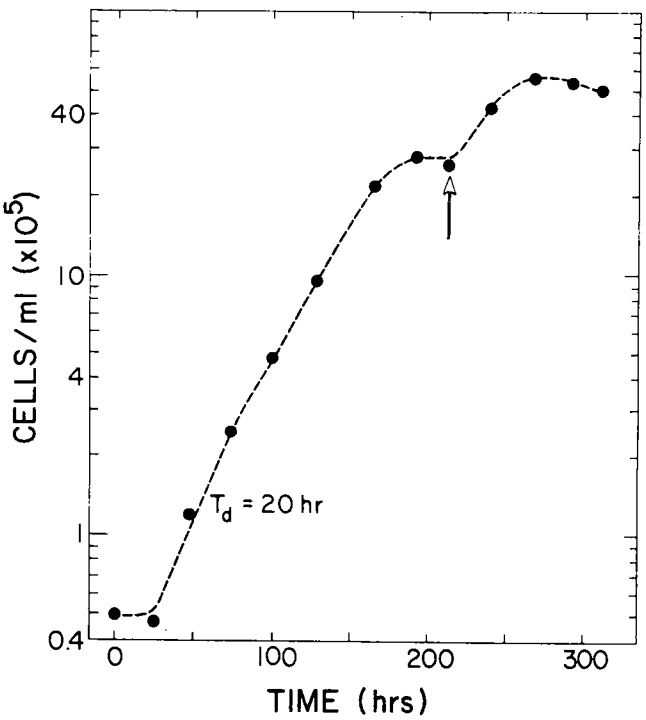


FIG. 4. Effect of bead concentration on the growth of L-929 cells on gelatin microcarriers in spinner culture. L-929 cells were planted onto 4000 beads/ml in 100 ml of culture medium. At confluence, *arrow*, the microcarrier concentration was doubled to 8000 beads/ml. Fifty percent of the growth media was replaced daily.

T-flasks, after which they readily grew to confluence with a doubling time of 22 h. The fibroblast cells did not exhibit the lag phase after planting on the beads and grew well on the beads with a doubling time of 18 h. Both of these values are typical doubling times for the respective cell lines.

Figure 4 is a growth curve for the L-929 cells grown on gelatin microcarriers in spinner culture. The culture was initiated with 4000 beads/ml and 0.5×10^4 cells/ml. After a lag phase of 24 h the cells doubled every 20 h until all of the beads were confluent, a final concentration of 3×10^6 cells/ml. It was shown that if fresh beads were added to double the bead concentration (8000 beads/ml) the cells would populate the new beads and double their density to 6×10^6 cells/ml. The culture could be maintained at this level as long as the beads and media

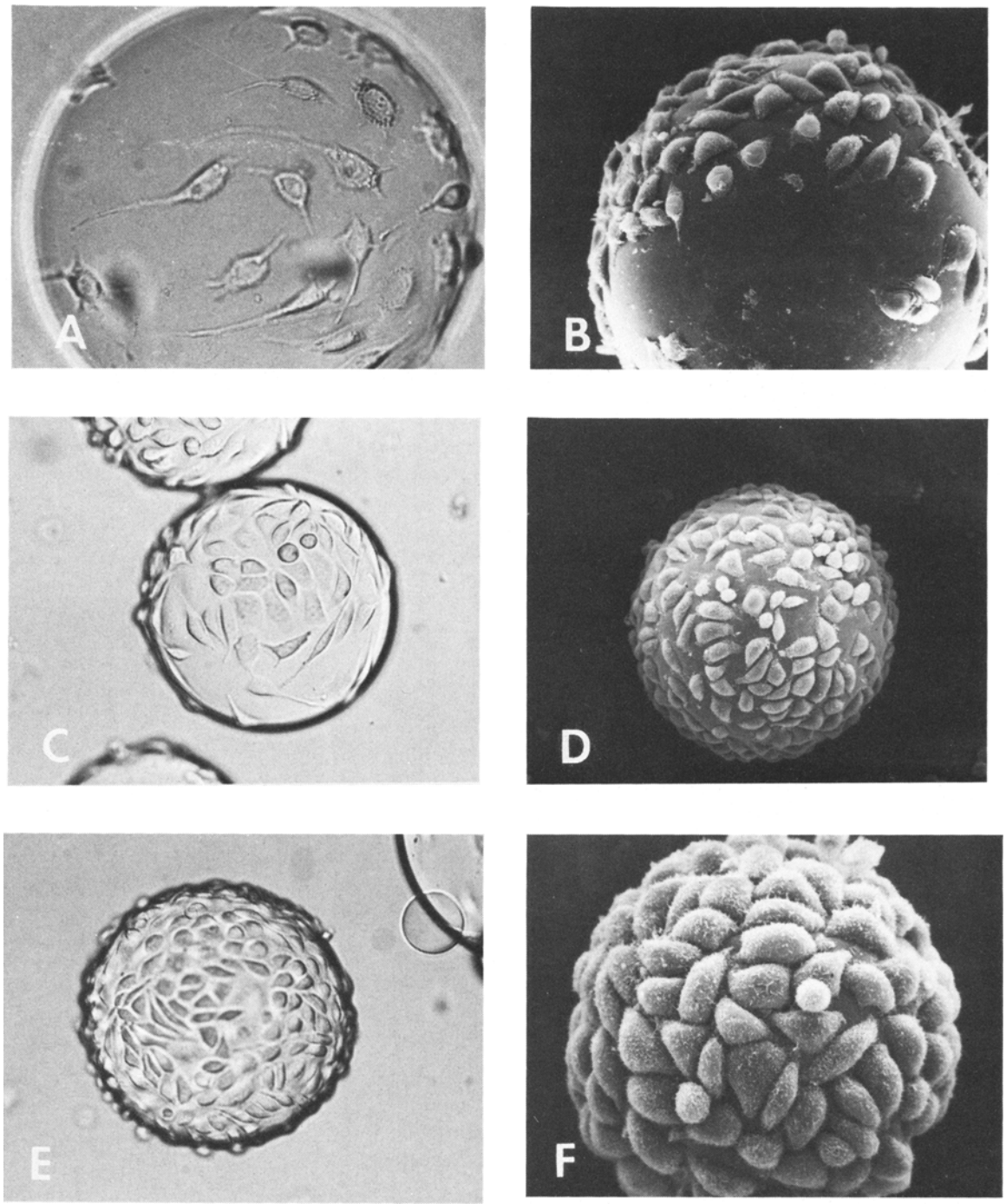


FIG. 5. Growth of L-929 cells on gelatin microcarriers in spinner culture. Twenty-four hours after cell seeding: *A*. Light micrograph. ×205. *B*. SEM. ×388. Below confluence: *C*. Light micrograph. ×160. *D*. SEM. ×257. Confluent microcarriers: *E*. Light micrograph. ×160. *F*. SEM. ×599.

were replaced with fresh microcarriers and growth media daily. A spinner culture of L-929 cells was maintained at 3 × 10^5 cells/ml using the bead-to-bead cell transfer method (2) for 3 mo. on 8000 gelatin microcarriers/ml with no adverse effects on cell morphology or growth.

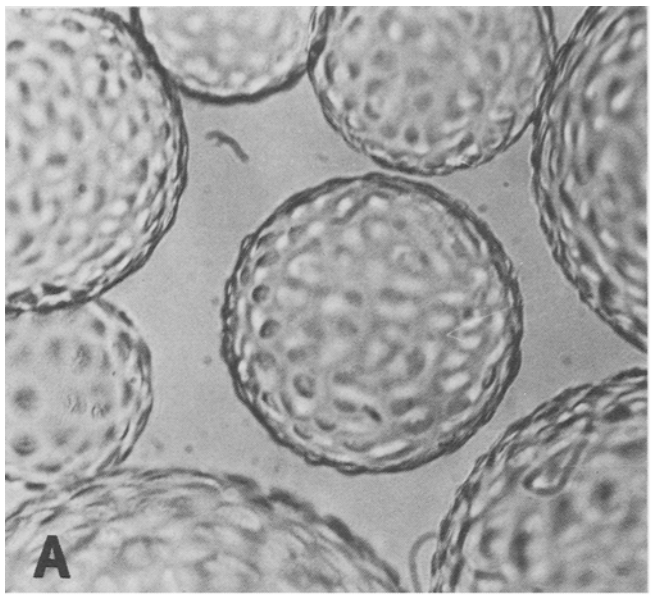
The morphology of the L-929 cells grown on the gelatin beads in spinner culture was observed using both SEM and light microscopy (Fig. 5). Figure 5 *A,C,E* are photomicrographs taken with bright field illumination of the L-929 cells on gelatin beads 24 h after inoculation (Fig. 5 *A*), below confluency (Fig. 5 *C*), and at confluency (Fig. 5 *E*). Shown next to each micrograph is the corresponding SEM photo for the three respective stages of cell growth (Fig. 5 *B,D,F*). The results showed that the L-929 cells grown on the gelatin microcarriers in spinner culture exhibited the same cell morphologies as were normally observed when the cells were grown in monolayers of T-flasks. At low cell density, 24 h after inoculation, the cells were attached and well spread. The long, thin fibroblast morphology was evident (Fig. 5 *A*). The cells were less elongated as the culture became more dense and confluent patches started to appear (Fig. 5 *C,D*). Once the cells reached confluence (Fig. 5 *E,F*), they had assembled into a tight mosaic pattern typical of confluent monolayers of L-929.

The gelatin microcarriers are transparent so it was not necessary to stain the cells to see them clearly under a light microscope. It was also possible to observe the cells growing both on the top and bottom of the bead by simply changing the field of focus. The dark spots in the lower corner of Fig. 5 *A* are two cells growing on the bottom side of the bead and are thus out of focus.

Scanning electron microscopy was facilitated by gelatin beads that shrink and swell with the cells during the sample preparation. Thus, common dehydration artifacts such as cell cracking and breakages were absent from the gelatin microcarrier samples.

Other cell types were tested for their growth on the gelatin microcarriers and photographs are shown in Fig. 6. Fig. 6 *A* is a light micrograph of swine endothelial cells grown on gelatin beads in static culture. At confluence these cultures exhibited the typical "cobblestone" appearance of a confluent monolayer of endothelial cells (4). The cells covered the beads in a polygonal, tightly monolayered pattern free from cells of different morphologies. Endothelial cells from umbilical veins had a similar morphology. Figure 6 *B* is a light micrograph of HeLa-S_3 cells at confluence grown on gelatin beads in spinner culture. The HeLa cells grew rapidly on the gelatin beads. However, there was always a significant number of unattached cells in these cultures.

Collagenase digestion of gelatin microcarriers. The most effective method for removing cells from the gelatin microcarriers was by treatment with collagenase. The microcarriers with attached cells were harvested from culture and treated with a sterile 500 μg/ml collagenase solution prepared in PBS. Figure 7 is an illustration of the effect of collagenase on L cells grown on gelatin microcarriers. Figure 7 *A* is a photograph of the freshly harvested culture after several washes with PBS and immediately before treatment with collagenase. After 5 and 7 min at 37° C in the collagenase solution the cells rounded up and started to detach from the microcarrier surface (Fig. 7 *B, C*). Figure 7 *D* shows that after 10 min virtually all of the cells were free of the beads and floating in suspension. After 10 min it was difficult to focus on the beads because they were starting to dissolve. Between 15 and 20 min after the suspension had been treated with the collagenase, the gelatin beads were completely digested leaving the cells free in suspension. Figure 7 *E* is a



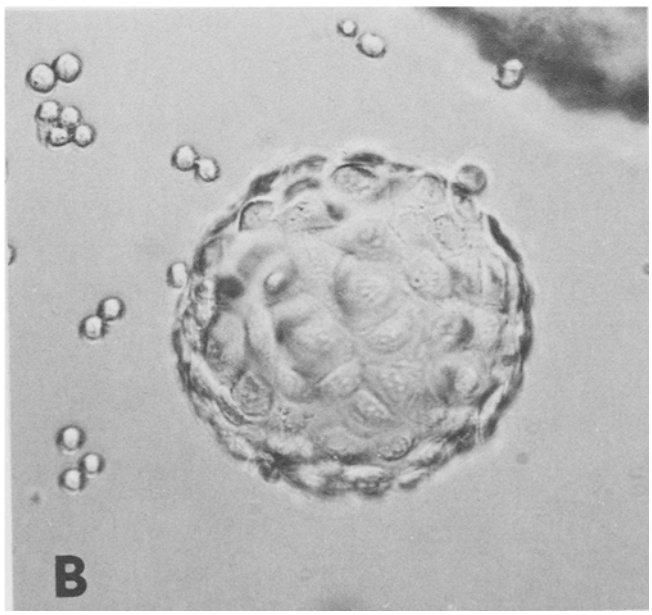


FIG. 6. Cells at confluence grown on gelatin microcarriers. *A.* Swine aorta endothelial cells in static culture. ×142. *B.* HeLa-S_3 cells in spinner culture. ×181.

unique photograph of a cluster of cells in which the bead had disappeared leaving the cells still adhering to each other.

After a 15-min incubation with collagenase, warm media plus calf serum was added to the cells and they

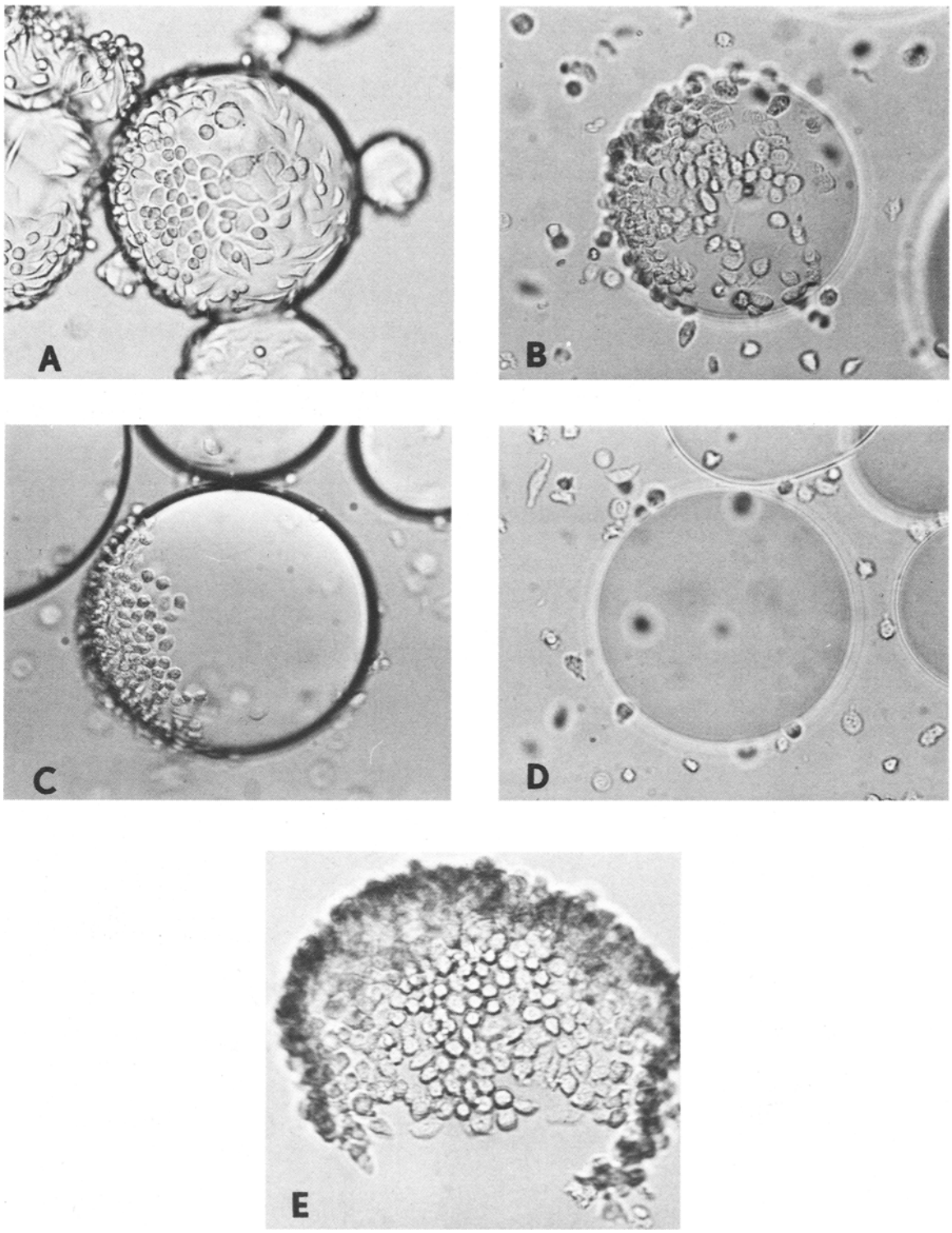


FIG. 7. Treatment of gelatin microcarriers plus attached L-929 cells with collagenase. Effect of collagenase over time: *A*. Time zero. *B*. 5 min. *C*. 7 min. *D*. 10 min. *E*. 20 min. Magnification of photos *A-D* is ×154, and ×198 for *E*. *A-D* were incubated at 37° C with occasional agitation. *E* was incubated at 37° C without shaking during the collagenase treatment.

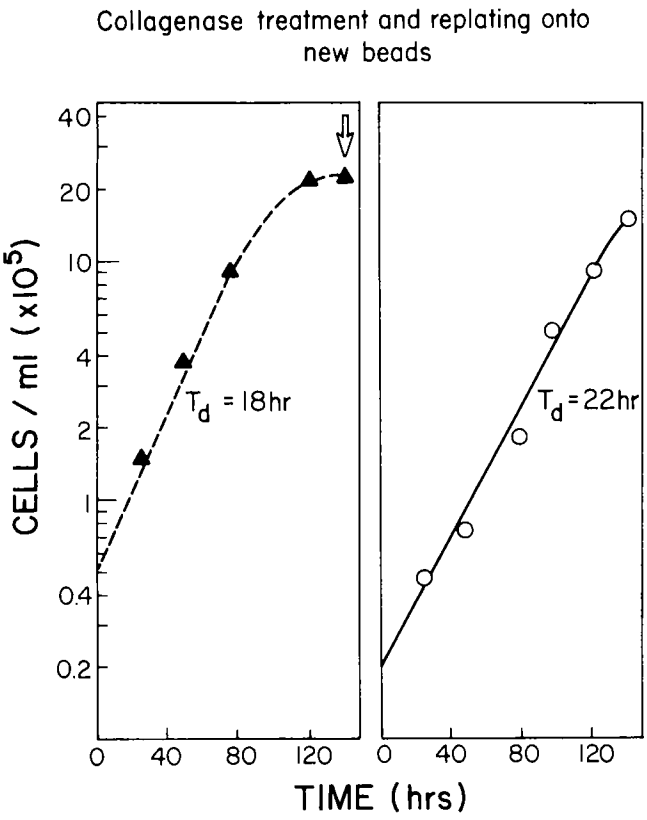


FIG. 8. Growth of L-929 cells on gelatin microcarriers before (▲---▲) and after (O---O) treatment with collagenase. The *arrow* indicates where the microcarrier culture was harvested, treated with collagenase, and replated onto fresh beads.

were replated onto fresh beads. The viability of the released cells was excellent (98% by trypan blue exclusion) and Fig. 8 illustrates that they grew well on the new beads, eventually reaching the same cell density (2×10^6 cells/ml) as before the treatment.

Endothelial cells grown on the gelatin microcarriers could also be released with collagenase and replated onto fresh beads with similar results (not shown). However, due to the tenacious attachment of these cells to the gelatin beads a longer collagenase incubation was required to release the cells. Usually it was necessary to wait until all of the beads had dissolved (20 min) before the endothelial cells were free in suspension.

Trypsin (Difco Laboratories) was also used to release the cells from the gelatin microcarriers, but the results were not as satisfactory as with the collagenase. At low cell densities (below 1×10^6 cells/ml) trypsinization released uniform suspensions of cells but at higher cell concentrations clumping of the cells resulted, making it difficult to resuspend the cells. This clumping may have resulted from strands of undigested gelatin that aggregated the released cells. There was a more complete digestion of the gelatin when collagenase was used.

Cell attachment kinetics. Figure 9 is a comparison of the attachment kinetics of swine endothelial and L-929 cells on the gelatin microcarriers and tissue culture plates. The results indicated that the swine endothelial cells attached twice as fast to the gelatin beads as did the L-929 cells. It took only 15 min for 30% of the endothelial cells to attach whereas it took 30 min for 30% of the L-929 cells to attach. Moreover, 80% of the endothelial cells were attached within 60 min vs. 120 min for the L-929 cells. The attachment kinetics for the swine endothelial cells revealed that they attached at the same rate to the tissue culture plate as to the gelatin beads. However, it was observed that the endothelial cells would attach to the gelatin beads even in the absence of serum (results not shown), whereas serum was necessary for endothelial cell attachment onto the tissue culture plate. The L-929 cells did not attach as rapidly to the gelatin beads as they did to the tissue culture plastic. These cells also never reached 100% cell attachment onto the gelatin beads even though 100% attachment was achieved on the tissue culture plates. However, once the fibroblast cells attached to the beads, they spread and grew normally as shown in Figs. 3, 4.

DISCUSSION

The purpose of this study was to create a microcarrier made enitrely out of gelatin and to evaluate its suitability for cell attachment and growth. Gelatin beads were prepared successfully by an emulsion-polymerization

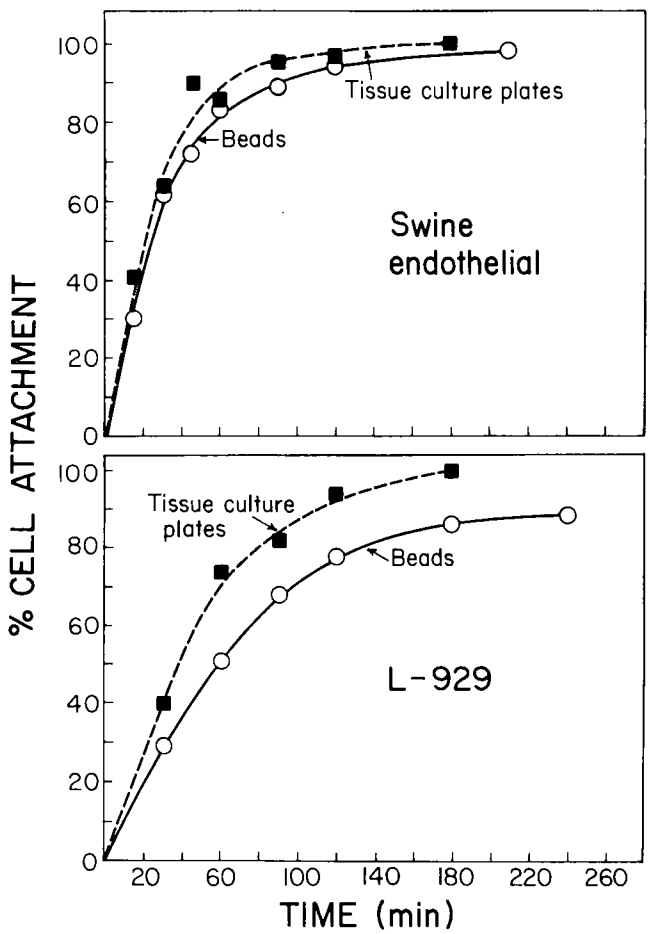


FIG. 9. Cell attachment kinetics on gelatin microcarriers (O---O) and on tissue culture plates (□---□) for swine endothelial and L-929 cells. The points represent the average of four experiments with duplicate cell counts per experiment.

technique. This method has been used recently for the production of polyacrylamide (22) and fluorocarbon-polylysine (11) microcarriers. A water-in-oil emulsion was produced using a 15% gelatin solution in the aqueous phase with glutaraldehyde as the crosslinker, mineral oil as the hydrophobic phase, and Triton X-114 as the emulsifier. Several factors were evaluated for their effect on the bead-emulsion process before a combination was found that consistently resulted in high yields of uniform, well-shaped spheres. The method reported here is also flexible enough to allow for the incorporation of other macromolecules in the bead matrix. In this study it was found that the extracellular matrix component, chondroitin sulfate, could be added in with the gelatin with no decrease in bead quality. Therefore, it should be possible to modify the procedure to include other physiologic molecules, such as fibronectin or laminin (29). This flexibility would make it possible to design and prepare specialized microcarriers to test the role of specific molecules in cell adhesion. The solid gelatin beads satisfy all of the microcarrier requirements set forth by van Wezel (28), which include optimal surface properties, density, size, optical properties, nonrigidity, and nontoxicity.

Sterilization procedures for the gelatin beads were important both for tissue culture work and for the stability of the beads. The beads were susceptible to bacterial proteases and therefore it was necessary to handle them under sterile conditions after they were produced. Although they could be dried and stored in sterile vials, it was just as effective to wash and store the gelatin beads in 60 or 70% ethanol. Seventy percent ethanol is the acceptable standard for killing contaminating microorganisms and the ethanol would penetrate the matrix of the bead even if a contaminant were trapped within the bead.

Three cell lines were evaluated for growth on the newly synthesized microcarriers. HeLa-S_3 is an established, hardy cell line that is not anchorage-dependent and grows well in suspension culture. L-929 fibroblast cells grow best if they are planted onto a solid substratum, but some lines can grow in suspension without microcarriers (7). Swine aorta endothelial cells are anchorage-dependent primary cells.

The fibroblast cells did not attach to the gelatin microcarriers as fast as they did to the tissue culture plates (Fig. 9). However, once the cells were attached to the beads they spread and grew rapidly (Figs. 3-5). Excellent cell growth (up to 6×10^6 cells/ml) was obtained on the gelatin beads with the L-929 cells. Our results indicated that in spinner culture if the concentration of beads was increased the cell density would increase proportionately (Fig. 4). At a concentration of 8000 beads/ml it was possible to continuously maintain a microcarrier culture of fibroblast cells for 3 mo. at a cell density of 2 to 3×10^6 cells/ml with the bead-to-bead transfer technique (2). The size of the cell inoculum for the spinner and static cultures was between 6 and 12 cells/bead. Several researchers have reported that high cell inocula are necessary to obtain maximal cell growth (1,3,8). Butler and Thilly (1) and Davies (3) reported that an initial cell-to-bead ratio greater than 7 and 10, respectively, was required before the number of unoccupied beads became insignificant in culture.

The endothelial cells were only grown in stationary culture. The results revealed that this cell line attached rapidly to the gelatin microcarriers and, after a 24 h lag phase, grew to confluence with a doubling time of 22 h. In general, it was noticed that even if a culture did not look good on the tissue culture flasks they resumed their normal polygonal morphology upon growth on the gelatin beads. The endothelial cells seemed to find the gelatin microcarriers a more desirable surface to attach and grow inasmuch as it was noted that the cells would attach to the gelatin beads even in the absence of serum. This latter observation was never seen on tissue culture flasks. Davies (3) has had good success growing vascular endothelial cells on solid plastic (Biosilon) beads.

For both the endothelial and fibroblast cell cultures grown in tissue culture plates (17-mm wells) the cell densities never exceeded 3×10^6 cells/ml. As shown in Fig. 3, a bead concentration of 10 000 beads/17-mm well (3100 beads/ml) supported a cell density of 2.5×10^6 cells/ml. However, if the bead concentration were increased to 20 000 or 40 000 beads/well the cell density did not increase above 3×10^6 cells/ml. Presumably these results are due to the small volume in which the cells were allowed to grow, which prevented equilibration of CO_2, lowering the pH and inhibiting cell growth. In the spinner cultures there was better aeration and movement of cellular metabolites away from the surface of the cells.

The gelatin microcarriers supported the attachment, spreading, and growth of HeLa-S_3 cells, permitting growth of this cell line of gelatin beads for 1 mo. in spinner culture. Figure 6 *B* is a photograph of a bead covered with HeLa cells taken from that culture. Interestingly, cultures of HeLa cells on the gelatin microcarriers contained a large number of cells that grew in suspension even when the beads were not confluent; presumably, because the HeLa cell line used did not need to be attached to a surface for growth. Jacobson and Ryan (9) have shown that when gelatin-coated polystyrene microcarriers were used to grow HeLa cells, unattached cells were always present in the culture medium. However, when the polystyrene beads were coated with the positively charged polymer, polyethyleneimine, the HeLa cells only appeared in the medium after they were at confluence.

Figures 7 and 8 illustrate that the gelatin microcarriers could be dissolved completely in 15 to 20 min with a 0.05% collagenase solution. The cells were 95 to 98% viable after the procedure and would attach and grow to confluence on fresh beads. The results in Figures 7 and 8 are based on L-929 cells grown on gelatin beads, but similar results were also obtained with the endothelial cells on the gelatin microcarriers. The removal of cells from the surface of microcarriers and the separation of eluted cells from these spent microcarriers is necessary whenever the cells are used for biochemical studies in which the beads will interfere. Ryan and co-workers (24) have developed a technique to remove and separate cells from beads by vortexing an aliquot of cells on beads and then passing the suspension through a 44 μm filter fitted onto a syringe. The filter retains the beads and allows the cells to be collected in the filtrate. Manousos et al. (18) have outlined a

method using Ficoll gradients to separate released cells from spent microcarriers. The development of a microcarrier that can be completely dissolved by proteases leaving the cells viable and free in suspension has eliminated the problem of removing and separating the cells from the beads.

Corning Glass Works has recently indicated that they are developing a gelatin microcarrier (21). Their gelatin bead also dissolves completely in about 12 min using a 0.13 to 0.25% trypsin or a 0.4% dispase solution. The abstract indicated that their gelatin-derived microcarrier supports the growth of human fibroblast, MDCK, Vero, and swine testicle cell lines. More information on the synthesis of this microcarrier was not available from the abstract.

The results presented here have shown that a solid gelatin microcarrier was developed. The beads were prepared by an emulsion-polymerization technique designed with the flexibility to include other macromolecules. The microcarrier had good mechanical stability and supported excellent cell growth in stationary or spinner culture. The gelatin beads were transparent, compatible with SEM techniques, nonrigid, and nontoxic. Cells attached to the beads with attachment kinetics similar to those obtained with tissue culture plates, grew to confluence, and exhibited the characteristic morphology of all cell lines studied. Finally, the solid gelatin microcarriers could be completely dissolved with a collagenase solution that left the cells viable and free of microcarriers.

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