Microcrystalline Collagen (Avitene) Film as a Substrate for Outgrowth of Primary Rabbit Kidney Fibroblasts (36230)

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(Introduced by C. M. Kagawa)
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The use of collagen as a substrate for the growth of tissue culture cells has been known since 1932, when Huzella and Lengyel (1) first studied the behavior of various cell types on fibrous collagen. Ehrmann and Gey (2), in 1956 studied the growth of 29 different cell strains on a transparent gel of reconstituted rat-tail collagen. They observed no lysis of the collagen gel and promotion of outgrowth without the retractions normally seen when cells are grown on glass. In 1958, Bronstein (3) used reconstituted rat-tail collagen as a substrate for tissue cultures in Maximow slides and roller tubes. Sanders and Smith (4) studied the effect of collagen and acidic polysaccharides on the growth of baby hamster kidney (BHK-21) cells in semisolid media. In the presence of native collagen, the colony forming capacity of the BHK-21 cells was enhanced 4–5 fold.

Ehrmann and Gey (2) knew that if the collagen gel was dried it would form a transparent film. It does not appear however, that they ever used the film as a substrate for cell culture. This study was undertaken to determine whether dried films prepared from microcrystalline collagen (Avitene) would be effective as a substrate for the growth of cell cultures.

Microcrystalline collagen is a new physical state of collagen prepared from bovine corium, as a stable acid salt (5).

Materials and Methods. Avitene microcrystalline collagen, a stable partial hydrochloride salt of bovine collagen, was obtained as a sterile fibrous white flour from Avicon, Inc., Fort Worth TX. Approximately 2 g of the Avitene flour was blended with 400 ml of sterile glass distilled water in a sterile dry Waring Blender. The blender was controlled with a rheostat; the blending sequence included blending at a rheostat setting of 30 for 2 min, followed by a setting of 100 for another 2 to 3 min. The temperature of the gel was not allowed to rise above 35°. The application of shear from the blending operation produces a 0.5% collagen gel with a viscosity of 10,000 ± 2000 cps, pH 3.2 ± 0.2. Twenty milliliters of the gel was pipetted into each sterile plastic 100 × 20 mm petri plate using a wide mouth 25 ml pipette. The plates were allowed to dry for 1 week at room temperature with the lids in place; they were then incubated in a walk-in, forced-air incubator at 35° until no moisture condensed on the lids. The plates were then transferred to a second forced-air incubator for 24 hr at 45°. At this time, the plates were considered ready for use. The collagen gel had formed a tough adherent transparent film.

Several other plates were dried at a faster rate than that described above by drying without the lids and then they were sterilized with ethylene oxide gas (12% ethylene oxide–88% Freon for a 12 hr cycle).

The Avitene coated plates and uncoated plate controls were seeded with primary rabbit kidney cells suspension at 2.0×10^5 cells/ml in the outgrowth media (Eagle's basal medium (Gibco, cat. No. G-11) plus 5% newborn calf sera buffered with 8% Na_2HCO_3), and 10 ml were applied to each plate.

The medium on the cells was changed on the fourth day after seeding. On day 5, after the cells had been plated, the uptake of neutral red dye by viable cells was measured. The outgrowth medium was removed from a group of plates and replaced by 10 ml of a 1:100 dilution in phosphate buffered saline

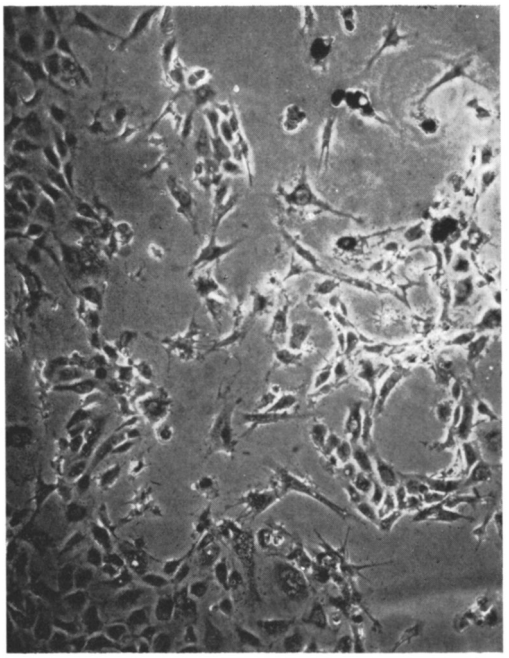

FIG. 1. Normal 5-day growth of primary rabbit kidney fibroblasts on a regular plastic tissue culture plate stained with 0.01% neutral red; 75×.

(PBS) of 1% neutral red. After 2 hr of incubation at 35°, the dye solution was decanted and the cell sheet was rinsed twice, or until the rinse was clear, with 1X phosphate buffered saline. In order to assess the amount of dye uptake, the cells were extracted with 95% ethanol–buffered Sorensen citrate buffer, pH 4.2 (1:1). This treatment develops the full color intensity of the dye (7). The extracted dye samples were diluted 1:20 with water and the optical density was measured at 540 mμ.

Avitene coated and uncoated control plates, containing 7-day-old confluent monolayers of rabbit kidney fibroblasts were inoculated with 40–60 plaque-forming units of vesicular stomatitis virus. The virus was absorbed for 1 hr at 34° in a humidified atmosphere of 2.5% CO_2 in air. After the attachment period and without removing the inoculum, the plates were overlaid with 10 ml of 1% agar medium. The agar medium was a modified Eagles medium containing Hanks' basal salt solution, 3% newborn calf serum, and 1% agar. The agar was allowed to solidify before the plates were inverted and incubated in humidfied 2.5% CO_2–air atmosphere for 48 hr. The cell monolayer was stained by adding 10 ml of a 1:100 dilution in PBS of 1% neutral red. After 2 hr of incubation at 35°, the dye solution was decanted and the number of plaques in the monolayers grown on the microcrystalline collagen coated plates were compared with numbers of plaques on the uncoated plates.

Results. Under blind conditions, three individuals observed, using an inverted light microscope, the cell growth on the Avitene coated and the uncoated plastic plates. Each individual noted the visible difference between the growth on the two sets of plates. Between the middle of day 4, and early in day 5, there was a usable monolayer on the Avitene coated plates while the monolayers on the control plates were not ready for use until day 7, after seeding (Figs. 1, 2, and 3). On day 7 there was still a visible difference microscopically in the coated and the control plates. On the Avitene plates, the cells were a very tightly packed monolayer, while on the

FIG. 2. Normal 5-day growth of primary rabbit kidney fibroblasts on Avitene coated tissue culture plate stained with 0.01% neutral red; 75×.

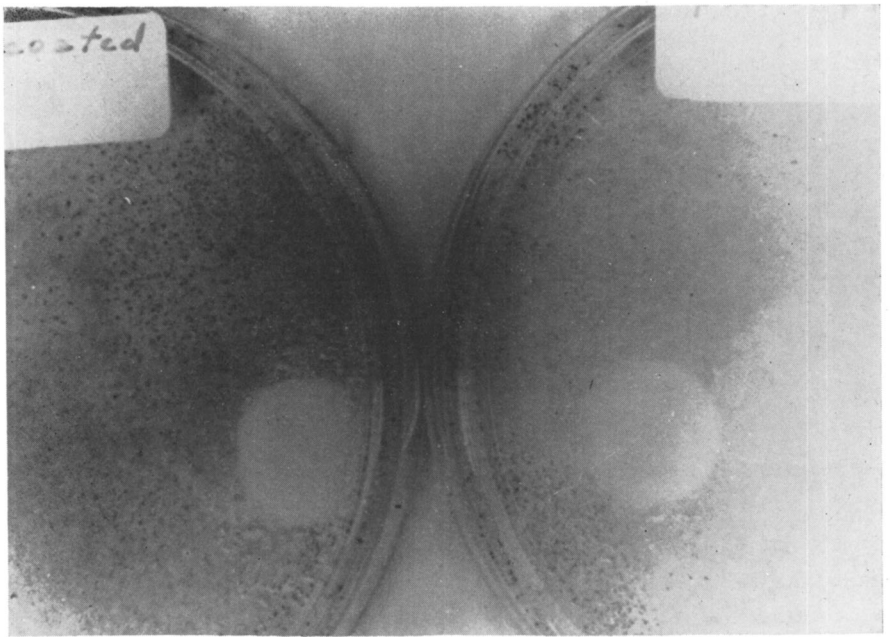

FIG. 3. Visual comparison of dye intensity on Avitene coated plate (left) and uncoated plate (right) 5 days after primary rabbit kidney fibroblasts have been seeded.

control plates, the cells formed the loose monolayer with tiny spaces between groups of cells usually seen. The results of the dye uptake experiments are shown inTable I. The data indicate that there was approximately twice as much dye taken up by the monolayer on the collagen coated plates as was taken up by the cell monolayer on the uncoated plastic plates. The amount of dye uptake is proportional to the number of viable cells (7). Ten milliliters of the outgrowth media containing no kidney cells were added to several collagen coated plates at the time of the seeding of the test plates. These plates were then treated exactly as test plates, including media changes, addition of dye and dye extraction. Even though the collagen film stained a very pale pink, no dye could be extracted from it.

TABLE I. Neutral Red Uptake by Viable Rabbit Fibroblast Cells Growing on Avitene Coated and Uncoated Plates.

Dye uptake optical density at 540 mμ[a]	
Avitene coated plates	Uncoated plates
0.80	0.54
0.85	0.51
0.90	0.63

[a] Each sample was diluted 1:20 in order to obtain a reading.

The plates inoculated with VSV virus were observed for plaque characteristics. The plaques on the collagen coated plates were the same size and shape as those on the control plates. There were no significant differences in the number of plaques per plate.

The data in Table II indicate that it takes a holding time of longer than 6 weeks for the cytotoxic effect of the residual ethylene oxide or ethylene chlorohydren from the gas sterilization procedure to dissipate to the point where the cells grow more rapidly on the Avitene film than on the uncoated plastic plates.

Discussion. It appears, from the data shown, that the microcrystalline collagen film is also an effective substrate for rapid cell growth. Our results agree with the studies [Ehrmann and Gey (2)] that were done using the gel form of a collagen material; the cells grew more rapidly on the collagen than on the glass used in prior studies or plastic

TABLE II. Effect of Ethylene Oxide Sterilization on Cell Growth.

Time held after sterilization	Cell growth	
	Avitene coated	Uncoated
1 day	—	—
3 days	—	±[a]
1 week	—	+
4 weeks	±[a]	+
6 weeks	±[a]	+

[a] Cells grew but a confluent monolayer was not obtained in the normal 7-day period.

used in these studies. They appear normal when observed through a light microscope; and they appear to be physiologically normal in their ability to support virus growth.

This *in vitro* data may be one of the possible explanations for the more rapid wound healing seen when microcrystalline collagen lyophilized mats are applied to rabbits ear skin defects. The normal fibroblasts in the wound bed spread more rapidly in the presence of the collagen, thus hastening the regranulation and healing which is observed *in vivo*. (8).

The microcrystalline collagen film may also be useful in experiments in which the presence of collagenase is being looked for in tissue explants; for example in burn damaged corneal tissue.

The Avitene coated plates prepared using aseptic techniques were sterile and did not need terminal (ethylene oxide) sterilization.

In order to use ethylene oxide sterilization, further studies would have to be done to find an efficient method for ridding the Avitene film of the residual gas.

Summary. Microcrystalline collagen (Avitene) when used to coat petri plates forms a tough transparent adherent film. This microcrystalline collagen film is an effective substrate for rapid growth of primary rabbit kidney cells. The cells grew out more rapidly than the cells grown on uncoated plates; the cells appear normal under the microscope and are normal in their ability to support virus growth.

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