Rabbit corneal epithelial cells grown in vitro without serum. MARIAN I. ALLMAN, ROBERT A. HARPER, MYRON YANOFF, LESLIE J. CURFMAN, J. DOUGLAS CAMERON, AND B. ALLEN FLAXMAN.

Primary outgrowth cultures of normal rabbit corneal epithelium can be initiated and propagated in vitro up to 6 days in serum-free medium. By the eighth day the majority of cells have ceased to divide. Epithelial cells grown without serum show DNA synthetic activity at a level comparable to control cultures grown with added serum.

Numerous studies have shown that some continuous or transformed cell lines can be cultivated in vitro in serum-free media of defined composition.1-2 This property has been compared with the well-known serum requirements for normal diploid cells grown in vitro, and it has been suggested that there may be a relationship between reduced serum requirements and biological behavior. However, the real significance of this property is still unclear because there is much variation in serum requirements between different types of transformed cells. The present communication reports an instance of limited, but striking proliferation of normal cells in serum-free medium.

Materials and methods. Adult albino rabbits weighing approximately 2 kilograms were killed by fracture of the cervical spine. The eyes were enucleated and corneas excised according to methods previously described.3-5 Corneal tissue was placed in a 60 mm. Petri dish with Eagle’s minimal essential medium, MEM (Grand Island Biological Co.), containing 100 U. per milliliter of penicillin, streptomycin, and mycostatin, without serum. After being placed epithelial side down on the bottom of the dish, small buttons were removed using a 2 mm. diameter trephine. The buttons were then placed in a 35 mm. diameter Petri dish (Falcon) containing MEM, where they were floated freely for 1 hour at room temperature in order to allow stromal swelling. The epithelial side of each button was then separated from the mass of underlying stroma by scissors and the final explants consisted of epithelium and minimal stroma. Three explants (each approximately 0.2 mm. thick) were placed epithelial side up on a glass cover-slip lying in the bottom of a 35 mm. Petri dish. A drop of MEM was added and a second cover-slip was placed on top of the first in order to hold the explants in place. Then 2.5 ml. of culture fluid (either MEM alone or MEM with 4 per cent fetal calf serum) was added and the cultures maintained at 37° in a humidified 95 per cent
Table I. Comparison of uptake of $^3$H-thymidine by rabbit corneal epithelial cells grown in MEM without serum (0 per cent) and with serum (4 per cent)

<table>
<thead>
<tr>
<th>No. of days in culture</th>
<th>LI* ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>100.0 ± 8.0</td>
</tr>
<tr>
<td>4</td>
<td>146.0 ± 6.2</td>
</tr>
<tr>
<td>5</td>
<td>101.8 ± 11.5</td>
</tr>
<tr>
<td>6</td>
<td>30.3 ± 1.4</td>
</tr>
<tr>
<td>8</td>
<td>26.7 ± 24.1</td>
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*Labeling index (LI) is the number of labeled nuclei per 1,000 cells.

air–5 per cent CO$_2$ atmosphere. After 24 hours in culture, the top coverslips were removed. At this point the explants were firmly attached to the bottom coverslip and showed outward movement of cells. Culture fluid was not changed throughout the entire experiment. The labeling index (LI) was determined by the addition of $^3$H-thymidine, 2 $\mu$Ci per milliliter (sp. act. 15 $\mu$Ci/mM) for 1 hour followed by fixation in 10 per cent buffered formalin. Following fixation, cultures were air dried. The explants were removed to facilitate permanent mounting of the epithelial cells on the coverslip onto glass slides. Coverslips were dipped in Kodak NTB-2 emulsion, exposed for 2 weeks at 4° C, developed, and stained with acid hematoxylin. The number of labeled cells per 1,000 nuclei was determined by random counting of three areas in each outgrowth for approximately 10 outgrowths.

Results. Corneal epithelium showed rapid outward growth from the explant in medium without serum (Fig. 1). The cells adhered readily to the glass surface. In general, the cells were compact and ovoid, without extensive cytoplasmic processes and were cohesive, giving rise to a coherent epithelium arranged as a multilayer close to the explant and a monolayer at the periphery. The advancing edge tended to be smooth except for the presence of irregularly spaced advancing protoplasmic spikes. Mitoses were numerous and were found throughout the outgrowth. Following exposure to $^3$H-thymidine, labeled nuclei were readily detected (Fig. 2) and, as shown in Table I, the LI was quite high through the sixth day in vitro. By the eighth day, the LI had decreased dramatically and by the twelfth day, cells had begun to detach from the dish. Days 1 and 2 were not included in Table I because not enough cells were present for analysis. The pattern of growth was not altered by changing the culture fluid every third day of the growth period.

Initially, different concentrations of fetal calf serum up to 10 per cent were tested for purposes of comparison. At a concentration of 4 per cent the cells appeared healthiest and showed the least amount of vacuolization. Corneal epithelial cells grown in 4 per cent serum produced outgrowths larger than did cells propagated without serum. This was due in part to the larger size of the individual cells, a greater tendency toward monolayering, lack of cell-to-cell cohesiveness, and exaggeration of cytoplasmic processes. Proliferative behavior as measured by the LI, however, was similar to that of cells grown without serum (Table I).

Discussion. Current methods of obtaining outgrowth cultures of corneal epithelium generally call for the use of a clot (plasma, clotted by chick embryo extract) to initiate cultures and serum (usually 5 to 20 per cent) to promote growth (proliferation).$^{10}$ Serum contains many unknown factors, both growth-promoting and growth-inhibiting, presenting investigators with a number of undetermined variables that are...
undesirable in performing highly critical work. There is a need, therefore, to develop a method of growing normal cells under defined conditions. The present report describes how this can be accomplished using rabbit corneal epithelium, and some interesting observations that resulted.

The ability of rabbit corneal epithelial cells in primary culture to adhere to the substratum and proliferate for up to 6 days in the absence of serum is impressive. The limited life span of these cells to survive past 8 days cannot be ascribed solely to serum deficiency. The limited life span of these epithelial cells must be related to other factors, as yet unknown.

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Key words: corneal epithelial cells, in vitro culture.

REFERENCES

The measurement of ocular transmittance and irradiation distribution in argon laser irradiated rabbit eyes. THOMAS P. KIDWELL, LESLIE A. PRIEBE, AND ASHLEY J. WELCH.

The transmittance of the ocular media has been measured in vivo in rabbit eyes using an argon laser light source and microthermocouple sensor. The intensity profiles at the cornea and retina were measured with the thermocouple and the ratio of the power associated with the intensity profiles indicated the fraction of light power reaching the retina. Measurements from 10 rabbits indicated a transmittance coefficient of 0.85 for wavelengths between 488 and 514.5 nm.

The fraction of radiant power incident on the cornea which reaches the retina is called the transmittance of the ocular media (TOM). A portion of the light incident on the cornea is reflected because of the difference in the index of refraction between air and the cornea. The remainder of the light is absorbed, scattered, and reflected at each interface and within the various ocular media.

The importance of measuring the transmittance of the ocular media was first stated by Ludvig and McCarthy in 1938. They reasoned such a measurement would permit correction of ocular visibility curves, comparison of the absorption of rhodopsin with the sensitivity of the eye, and the evaluation of theories concerning vision. The possible hazard of intense light sources such as the laser has added further impetus to accurately determine the fraction of light reaching the retina.

The papers of Geeraets and associates, Geeraets and Berry, and Boettner and Wolter have been the most quoted sources for the values of the transmittance of the ocular media as a function of wavelength. Both groups used a spectrophotometer to measure the light passing through the ocular media of an excised eye. Boettner and Wolter published values for direct transmittance and total transmittance. The direct transmittance measurements included an aperture in front of the detector to limit the divergence of the exit light from the eye to one degree; the total transmittance measurement included forward scattering of light up to 170 degrees. Geeraets