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Human Serum and Epithelial Spread in Tissue Culture

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Summary. The dependence of epithelial spread on human serum in culture has been studied. Using measured pieces of mouse ear skin epithelial outgrowth about floating explants (epiboly) and from adherent explants was studied. Where compared directly the two systems show similar results. Because of its ease of quantitation, the adherent explant culture was studied in greater detail. In this system in the presence of serum appreciable spread was found only after 48 h but spread continued for at least the next 4 days. In the absence of serum only minimal epithelial spread occurred. Adding serum to deficient media enhanced spreading and removing serum from media depressed spreading. The extent of spread appeared independent of mouse age for the first 4–10 weeks though 2-week-old mouse skin showed quantitatively greater spreading.

The activity in human serum responsible for epithelial spread acts under conditions of minimal DNA synthesis and is not reproduced by bovine serum albumin, fetuin, or serum that had been exposed to 100° C for 5 min. The activity is not dialyzable and it is resistant to the protease inhibitors DFP and PMSF. These studies suggest that a specific serum component(s) serves to support epithelial spread in vitro.

Key words: Epithelium – Serum

Zusammenfassung. Die Abhängigkeit von menschlichem Serum für den Auswuchs von Epithelzellen wurde in Gewebekultur an gemessenen Stücken von der Haut von Mausohren studiert. Ein direkter Vergleich von schwebenden und von anhaftenden Gewebestücken gab ähnliche Resultate. Das System mit anhaftenden Gewebestücken wurde ausführlicher studiert, da es einfache meßbare Daten gab. In diesem System wurde gefunden, daß Auswuchs von Epithelzellen in größerem Auswuchs nur nach 48 h erfolgt und zumindest für die nächsten 4 Tage anhielt. In Abwesenheit von Serum wurde geringerer Auswuchs beobachtet. Zugabe von Serum zur Gewebekultur erhöhte den Auswuchs, und Entfernung von Serum unterdrückte den Auswuchs der Epithelzellen. Das Ausmaß dieses Auswuchses war unabhängig vom Alter der

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Mäuse in den ersten 4–10 Wochen, obwohl Mäusehaut von 2 Wochen alten Tieren größeren Auswuchs zeigte. Die aktive Komponente in menschlichem Serum kann nicht ersetzt werden durch Rinderserum-Albumin und Fetuin, und wird zerstört durch Erhitzen für 5 min bei 100° C. Die aktive Serum-Komponente ist nicht dialysierbar, ist resistent für die Enzyminhibitoren DFP und PMSF und wirkt trotz geringer DNS-Synthese. Diese Studie deutet darauf hin, daß ein oder mehrere spezifische Serum-Komponenten den Auswuchs von Epithelzellen in Gewebekultur verursachen.

Schlüsselwörter: Epithelzellen – Serum

Epithelial cell spreading plays important roles in the processes of wound closure, morphogenetic movements, and neoplastic invasion [1]. While the study of these processes has been pursued in vivo most approaches have been descriptive. Analytical studies of epithelial cell movement have been made in several in vitro systems utilizing dissociated epithelial cells [2,3] epithelial cell spread about explants (epiboly) [4–9] and epithelial cell outgrowth from explants [10–13]. In all these studies the explant preparation has been grown on a solid substratum in the presence of serum. The role of the substratum has been studied [14,15] but few studies have focused on the role of serum. Levine [16] found that no epithelial movement occurred in the absence of serum and concluded that the serum factor was of a large molecular weight since it was non-dialyzable. In contrast, Coombs et al. [17] found that the serum factor was dialyzable and heat stable. Since the role of serum appears to be very important in epithelial movement, as it has been demonstrated in vitro, and since its role remains poorly defined, we undertook the present study. In this report, using floating (epiboly) and adherent (surface spreading) measured mouse skin explants as assays, we describe several properties of the human serum component which supports epithelial spread. A preliminary report of studies with cow serum has been made [18].

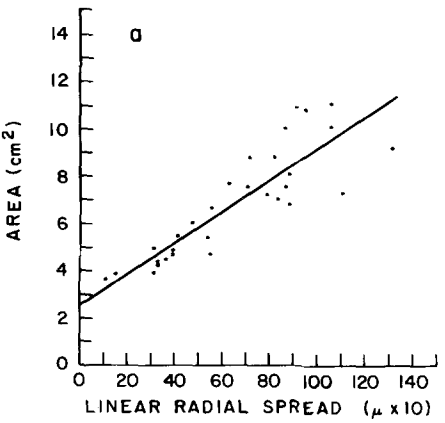
Materials and Methods

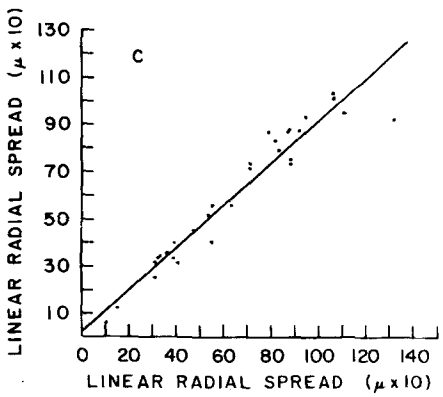
Tissue Culture

In all experiments, unless otherwise stated, the ear skin from 6 to 10-week-old Swiss male mice (CDl Charles River Breeding Lab., Wilmington, Mass.) was removed after cervical fracture. The skin was clipped of hair, rinsed in 70% alcohol containing 100 units/ml penicillin (Sigma Chem. Corp., St. Louis, Mo.), 100 µmg/ml streptomycin (Sigma Chem. Corp., St. Louis, Mo.), 0.25 µgm/ml Nystatin (Sigma Chem. Corp., St. Louis, Mo.) and rinsed in Ca-Mg free Tyrode's solution containing the same antibiotic concentrations (CMF Tyrode).

Using a dermal punch (G. Tiemann, Long Island City, N.Y., 0.2 cm diameter) ear skin disks were removed. The skin of both sides was separated at the cartilaginous plate with forceps and, for the adherent explants, laid dermal side down in plastic Petri dishes (34 × 10 mm, Falcon Plastics, Oxnard, Cal.). It was found both skin sides grew equally well over the plastic surface. The epiboly experiments were conducted according to Medawar [4] by lightly coating the explants with vaseline and floating them on media. For these experiments, only the skin half containing the cartilaginous plate was used.

Throughout these experiments Dulbecco's modified Eagle's medium (DME) (Grand Island Biological, Grand Island, N.Y.) containing antibiotics (as above for CMF tyrode) was used. In the epiboly experiments the explants floated on the surface of the medium. For the spreading experiments





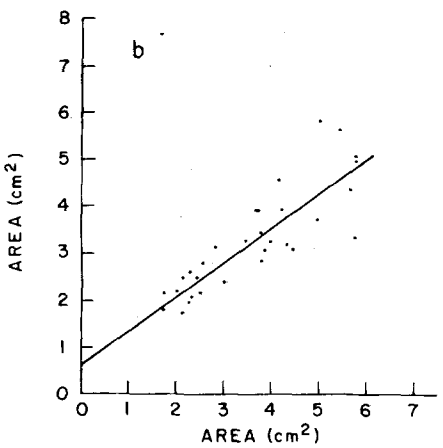


Fig. 1a–c. Scatter diagrams testing the correlation of area versus linear radial measurement in quantitating explant outgrowth after 3 days in culture (DME + HS). **a** represents the scatter diagram of 30 explant outgrowths. The straight line of best fit was derived from a least squares linear regression method and has an equation of $y = 2.529 + 0.66\ x$. **b** and **c** test the internal consistency of both methods of measurement. In these experiments explants were divided at random through the center and the areas (b) and linear radial outgrowth (c) of both parts were compared. The straight line of best fit had equations $y = 0.641 + 0.713\ x$ (b) and $y = 2.16 + 0.89\ x$ (c). Each dot represents one explant experiment

the explants were held down by layering each with bovine fibrinogen (90% clottable Miles Lab. Inc., Kankakee, Ill.), 4 mg/ml DME and then a few drops of bovine thrombin (Parke Davis and Co., Detroit, Mich. 10 units/ml; thrombin units were assumed as specified on the preparation) in CMF tyrode. After six disks of skin were placed in one dish, the dish was filled with 3 – 4 ml of fresh DME and incubated at 35 – 38° C (Model 28, Thelco, Chicago, Ill.) in 95% air plus 5% CO_2, water-saturated environment. After 1 h this medium was replaced by the test medium; thereafter, the medium was changed daily. Unless otherwise indicated, the serum concentration of the medium was 5 mg/ml of DME. The medium was sterilized by Millipore filtration (0.45 μ or 0.80 μ Millipore Corp., Bedford, Mass.).

After the specified time of incubation, the floating explants were fixed in Zenker's solution and prepared for hematoxylin and eosin histology by methods of Putt [19]. After sectioning and staining, epiboly was measured by light microscopy with the aid of an eye piece calibrated by a stage micrometer (AZI Corp., Dedham, Mass.). Epithelial spread was measured from the biopsy edge as determined from the sharply-cut lateral keratin of the explant border. The epithelial front was taken as the furthest epithelial cytoplasmic edge. The adherent explants were fixed and stained in Giemsa stain (Fisher Sci. Co., Pittsburgh, Pa.) [19]. Epithelial spread on the petri dish was measured by maximal linear radial outgrowth (using calibrated microscopic eye piece) and by area.

Epithelial spread on a Petri dish can be quantitated either by measuring the maximal linear radial outgrowth as Karasek [11] did, or by measuring total outgrowth area. Linear radial growth was determined by measuring with the calibrated eye piece of a light microscope the distance of maximal epithelial spread starting at the explant edge. Area was determined by projecting (Prado Universal Slide

Projector, E. Leitz, Wetzlar) on to paper and weighing the cutouts (Model H20, Mettler Instrument Corp., Highstown, N.J.). One square centimeter of this paper weighed 32.3 ± 0.2 [9] mg [average ± 1 S.D. (no. period observations)] and the initial time 0.2 cm diameter skin disk projected as a cutout of 3.78 ± 0.16 [9] cm^2. To test the correlation between the two methods of measurement a scatter diagram was prepared (Fig. 1). In Fig. 1a the two methods are compared and found to be strongly correlated (correlation coefficient = 0.86). To test the internal correlation of each method of measurement individual outgrowths were divided at random through the center and compared (Fig. 1b, Fig. 1c). In each case the correlation was good: for outgrowth area the correlation coefficient was 0.85, for linear radial outgrowth the correlation coefficient was 0.96. In the experiments reported here only outgrowth area was recorded though it is clear radial outgrowth measurements would have been equivalent. Autoradiography was conducted as previously described [20]; the tissue was pulsed with 2.2 μCi of [methyl ^{3}H] thymidine (46 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.) for 2 h and developed for 1 week at 4° C in the dark. As previously described [20], the labeling index was recorded as the number of positive cells per 100 cells and determined by counting the number of positive cells in the outgrowth of the adherent explant or the number of labeled basal cells in histological sections of the floating explants per 500 – 1000 cells. Significant statistical differences between values in all experiments were established by the Student's *t*-test.

Serum Preparation

Human serum (HS) was prepared from blood bank plasma by making the citrated plasma 0.05 M in $CaCl_2$ with 0.2 M $CaCl_2$. The resultant clot was pressed through cheese cloth and the expressed serum poured into dialysis bags [Fisher Sci. Co., prepared as previously described [21]) and dialyzed against 30 volumes of 10^{-4} M Tris HCl pH 7.4 for 48 h at 4° C. The serum was then cleared by centrifugation (3000 × g, 15 min, 4° C) and the supernatant lyophilized and stored at −14° C until used. Bovine serum albumin (BSA) (A-4378), human serum albumin (HSA) (A-9511), fetuin (F 9002), diisopropylfluorophosphate (DFP) and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma Chem. Co. All chemical and protein concentrations were established gravimetrically (Mettler H20, Mettler Instr. Corp., Hightstown, N.Y.).

Radiation Experiments

For radiation experiments skin disks were placed in 5 ml of CMF tyrode solution and exposed to 6,000 (or 12,000) rads of X-ray (Siemens Stabilipan 250; no added filter) which were delivered in 113 (or 226) s.

Results

Epiboly Experiments

Floating explants were used in the initial studies. The morphology of the epidermal spread was very similar to that recorded by Marks et al. [6] in their studies of human skin epiboly. Initially, epidermal tongues extended from the lower epidermis out over the cut edges of the explant. Eventually, the epithelium completely enclosed the explant. In the presence of serum complete epiboly occurred by 48 – 72 h. The spreading cells and their nuclei were flatter than the epidermal cells and the outgrowth did not show keratinization. As Marks et al. [6] reported, PAS stain showed a well-defined basement membrane under the original epidermis but none under the spreading epithelium. The influence of different media on epiboly is recorded in Table 1 using nonirradiated and irradiated mouse ear skin disks. It is seen that minimal epiboly was found at one day in serum but by 2 days on the average 80 % of the explant was covered by epithelium. At the same time there was

Table 1. Data are recorded as the percent [± 1 standard error (no. of observations)] of the explant dermal surface covered by epithelium. The values were derived by measuring the actual epithelial outgrowth (see Methods) from the explant edges over the dermal surface of the explant and dividing that distance by the complete linear distance about the lower explant starting at the cut edges. The irradiated skin disks had received 6×10^3 rads (see Methods)

Epithelial outgrowth about floating explants		
	Nonirradiated	Irradiated
DME alone 2 days	13 ± 4 (10)	6 ± 2 (10)
DME + BSA (1 mg/ml) 2 days	16 ± 3 (10)	13 ± 2 (12)
DME + HS (1 mg/ml) 1 day	14 ± 2 (5)	6 ± 1 (5)
DME + HS (1 mg/ml) 2 days	80 ± 12 (16)	72 ± 12 (9)
DME + heated HS (100° C for 5 min) 2 days	18 ± 4 (13)	4 ± 1 (11)

far less spread in those explants grown in DME alone, DME plus BSA and DME plus heated HS.

The epithelial spreading values for the irradiated and nonirradiated explants grown in the presence of serum were not significantly different. It is important that even though the labeling index for the nonirradiated explant (DME + HS) at 48 h was 37 ± 5% while that for the irradiated explant (6×10^3 rads) was 11 ± 5% (a 70% reduction of labeling index), the outgrowths were statistically identical. So, it would appear that DNA synthesis is not important to the mechanism of epiboly – at least for the first few days. This conclusion was also drawn by others [22]. The outgrowths of the irradiated and nonirradiated explants in DME + BSA were also essentially equal. The discrepancy between the outgrowth of the irradiated and nonirradiated explants in the presence of DME and DME plus heated HS is not explained.

Because the epiboly approach proved to be more difficult to prepare and quantitate, all the remaining experiments were conducted with the explant outgrowth assay.

Explant Outgrowth Assay

As described, the explant adherent tissue culture method employed is quick, easy, quantifiable, and reproducible. Using the fibrinogen-thrombin system to hold the explants down greater than 90% of the tissues showed some degree of outgrowth. The cellular outgrowths were characterized as epithelium morphologically in that the cells always moved as a sheet and identical outgrowths revealed tonofilaments and desmosomes by electron microscopy [18]. In the presence of serum the first epithelial spurs were seen at about 28 h while in the absence of serum very few epithelial cells were found at the explant margin even after 48 h. In Fig. 2 is pictured the daily epithelial outgrowths from explants incubated for 6 days in DME and

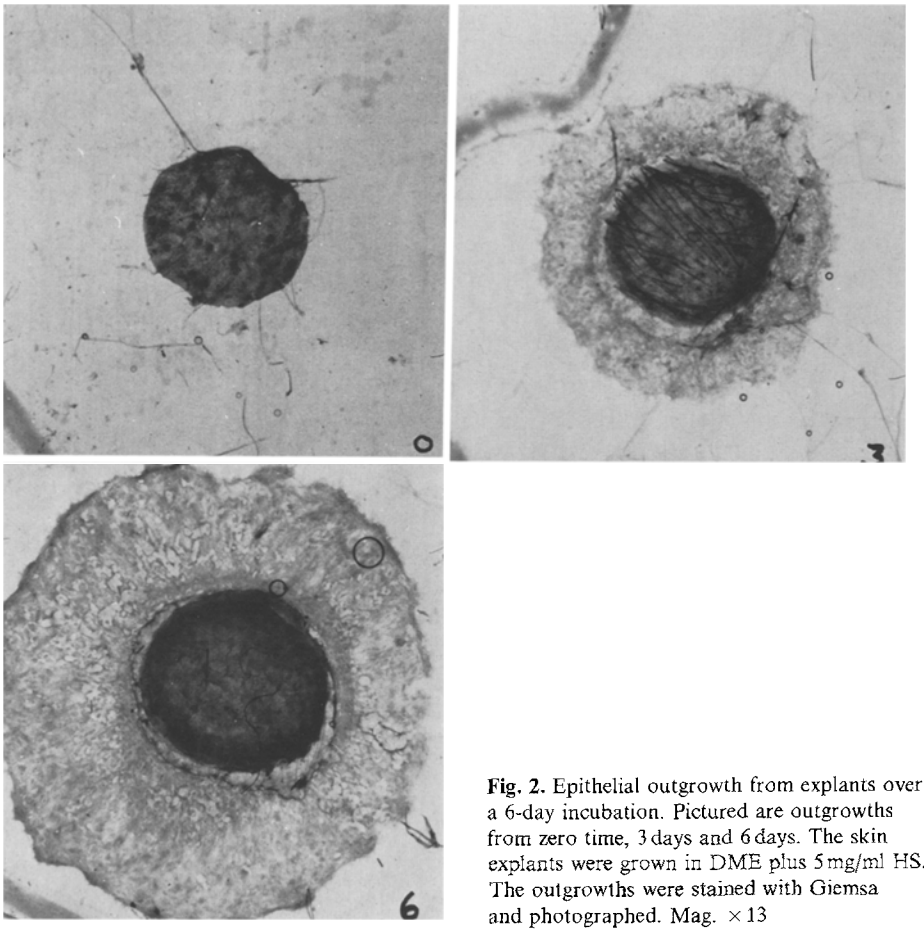


Fig. 2. Epithelial outgrowth from explants over a 6-day incubation. Pictured are outgrowths from zero time, 3 days and 6 days. The skin explants were grown in DME plus 5 mg/ml HS. The outgrowths were stained with Giemsa and photographed. Mag. $\times 13$

serum. Outgrowth was appreciable by day 3 and prominent by day 6. Although the cell layer was single at the periphery of the outgrowth, the centripetal portion of the outgrowth in older cultures showed some epithelial cell layering as seen by direct light microscopy.

Effect of Skin Age on Extent of Outgrowth

Experiments testing the effect of skin age on the extent of epithelial cell outgrowth is recorded in Table 2. The area of epithelial outgrowth from an adherent explant occurring after 3 days in the presence and absence of serum was measured using ear skin from mice 2 – 10 weeks of age. In Table 2 it is seen that in the absence of serum there is no significant difference in outgrowth between 2, 6, 8, and 10 weeks; however, the outgrowth of 4-week skin is significantly lower than that of the other skins tested ($P < 0.05$). The explanation for this discrepancy is not obvious at this time. In the presence of serum statistically equivalent outgrowth occurred at 4, 6, 8,

Table 2. The skin explants were handled as described under Methods. Measurements were taken after 3 days. The data are recorded as the average area of outgrowth (cm^2) ± 1 S.D. (no. of observations)

Epithelial outgrowth by 3 days from skin of varying age		
Age of mice (weeks)	Outgrowth area (cm^2)	
	DME alone	DME ± 5 mg/ml HS
2	3.46 ± 0.35 (11)	8.04 ± 1.06 (11)
4	2.91 ± 0.20 (11)	5.72 ± 0.58 (9)
6	3.44 ± 0.45 (12)	6.32 ± 1.28 (8)
8	3.36 ± 0.62 (9)	6.37 ± 1.47 (11)
10	3.23 ± 0.34 (9)	5.24 ± 0.69 (7)

and 10 weeks. The outgrowth of 2-week skin is significantly greater than the other skins tested ($P < 0.001$). In the subsequent experiments only ear skin from animals 6–10 weeks was used.

Rate of Epithelial Outgrowth and Serum Effect

Figure 3a shows plots of the area of epithelial outgrowth in different media over a 6 day period. In DME alone, that is, in the absence of serum, minimal to no outgrowth occurred even after 6 days. An identical pattern was seen in the presence of bovine serum albumin (5 mg/ml) and human serum albumin (5 mg/ml not shown). Since the protein concentrations were the same in the whole serum and the serum albumin experiments, it appears unlikely that the epithelial outgrowth effect of serum is a nonspecific effect of protein. Since in all experiments the explants were exposed to thrombin and fibrinogen at the beginning (to stick the explant to the dish), these two blood proteins or their products do not appear to serve the spreading role of serum although diffusible products would have been removed in the DME wash at the outset (see Methods).

In the presence of serum the epithelial outgrowth area increased linearly with time (DME and HS curve of Fig 3a) after the first day. Indeed, at 24 h only rarely were a few cell spurs seen at the explant edge. By 6 days, however, there was an impressive outgrowth of epithelium.

If serum were omitted from the growth medium for 1, 2 (not shown), or 3 days (Fig. 3b) and then added, the resultant outgrowth by 24 h equalled that of the experiment in which serum had been present from the beginning (compare Fig. 3b with the DME plus HS curve of Fig. 3a). This burst of cell spreading occurred as if the explant responded to the duration in culture as well as to the duration of serum exposure. The fact that epithelial cells were able to spread after as much as 3 days in culture with DME alone (Fig. 3b) indicates that the absence of cell movement in DME alone (Fig. 3a) is not due to cell death.

The converse of the experiments shown in Fig. 3b are shown in Fig. 3c. Here experiments are plotted which were initiated in the presence of serum but after 1 and 2 days, respectively, washed and exposed to DME alone. In both cases after removing serum, the rate of outgrowth slowed and by 6 days had not attained the

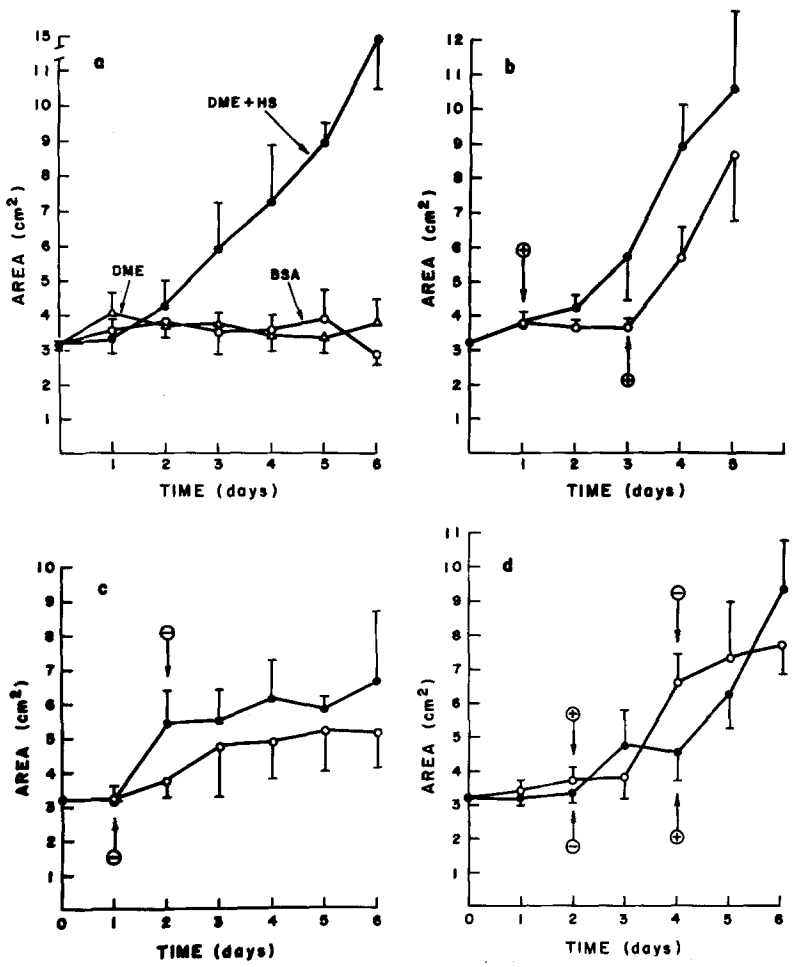


Fig. 3a–d. Epithelial outgrowth with time in different media. In **a** the area of epithelial outgrowth was followed with time for 6 days in DME alone (△), in DME plus 5 mg/ml BSA (○) and in DME plus 5 mg/ml HS (●). Each point is the average of 6–18 determination ± 1 S.D. In **b** the effect of adding serum to the explant growth medium after 1 (●) and 3 (○) days in DME alone is recorded. Serum at 5 mg/ml was added at the time indicated by the arrow. Each point is the average of 6 determination ± 1 S.D. In **c** the effect of removing serum from the growth medium after 1 (○) and 2 (●) days is recorded. The initial serum concentration of 5 mg/ml was replaced (as indicated by the arrows) by DME after twice rinsing with DME. Each point is the average of 5–22 determinations ± 1 S.D. In **d** the serum content of the medium was changed every 2 days over a 6 day period. The experiment represented by open circles show the course of the outgrowth in the absence of serum for 2 days, presence of serum (5 mg/ml) for 2 days and absence of serum for the final 2 days. The closed circles represent the outgrowth in the presence of serum (5 mg/ml) for 2 days, in the absence of serum for 2 days and in the presence of serum (5 mg/ml) for the final 2 days. The arrows indicate the time of serum addition or removal. Each point is the average of 5–12 experiments ± 1 S.D.

extent of growth found when serum was present throughout the experiment (compare to Fig. 3a). This experiment indicates that for continuous outgrowth serum is needed constantly. Next, explants were exposed alternatively to medium with serum, medium without serum, and then medium with serum or conversely to

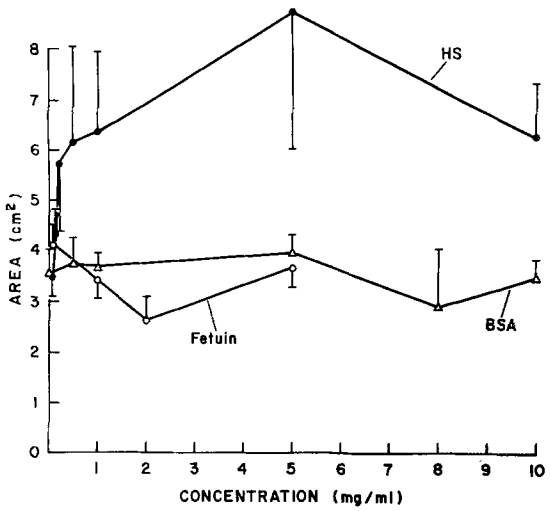


Fig. 4. Epithelial outgrowth after a 3 day incubation in media of various protein concentrations. Each point represents the average of 5–17 determinations ± 1 S.D.

medium without serum, medium with serum, and medium without serum for 2-day periods over a 6-day experiment (Fig. 3 d). As one would expect from Fig. 3 b and c, outgrowth could be manipulated by altering the serum content of the medium. The overall pattern then is that good outgrowth occurs in the presence of serum and diminished outgrowth occurs in its absence

Outgrowth Versus Protein Concentration of the Medium

Epithelial outgrowth in various serum concentrations was studied after a 3-day incubation. As shown in Fig. 4 maximal outgrowth occurred in the presence of 5 mg of serum per ml of medium. At 10 mg/ml serum there was some outgrowth inhibition. The difference between the outgrowth at 5 mg/ml and 10 mg/ml was significant at $P < 0.05$ and the outgrowth reduction was approximately 26%. Equivalent concentrations of BSA or fetuin in the medium produced minimal to no epithelial outgrowth.

Outgrowth from Irradiated Explants

Skin disks irradiated with 6,000 rads and 12,000 rads of X-ray were incubated continuously with serum and the outgrowth followed with time (Fig 5). The outgrowths in both cases were statistically equivalent. To test the efficacy of irradiation, tritiated-thymidine autoradiography was performed. The labeling index in nonirradiated skin outgrowths varied in different experiments from 26–48% while that of irradiated skin outgrowths varied from 1–4%. Irradiation, therefore, depressed the thymidine incorporation of epithelial outgrowth by 85–98%. In contrast to DNA synthesis, the area of outgrowth of the irradiated explants was, during the first 5 days in culture, very close to the non-irradiated controls (i.e., at least 65% of the control; compare Fig. 5 to Fig. 3 a curve DME and HS at 5 days). This experiment supports the conclusions of others [13, 22, 23] that epithelium can

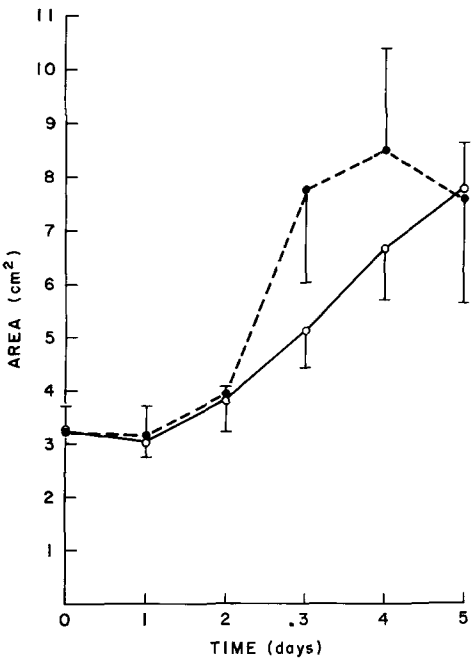


Fig. 5. Area of epithelial outgrowth from irradiated explants during a 5 day incubation (DME plus 5 mg/ml HS). Each point represents the outgrowth area from explants which had received 6×10^3 rads (●) or 12×10^3 rads (○) and is an average of 5–18 experiments ± 1 S.D.

Table 3. In these experiments serum was added to a final concentration of 1 mg/ml. The media were made by adding 1 ml of a stock 10 mg/ml HS solution in 0.05 M Tris HCl pH 7.4 to 9 ml of DME. The stock solution was treated in the heating experiments as indicated. An aliquot of the 10 mg/ml HS solution was exposed separately to 10^{-4} DFP, and 10^{-4} PMSF for 2 h at room temperature and then dialyzed for 48 h against 0.05 M Tris HCl pH 7.4 at 4° C. The data are recorded as the average area of outgrowth (cm^2) ± S. D. (no. of observations)

Epithelial outgrowth by 3 days after various serum manipulations

Conditions of outgrowth	Epithelial outgrowth area
DME alone	3.57 ± 0.43 (17)
DME + HS	6.36 ± 1.56 (10)
DME + heated HS (56° C, 2 h)	5.82 ± 1.07 (16)
DME + heated HS (100° C, 5 min)	3.97 ± 0.61 (16)
DME + DFP treated HS	5.90 ± 1.17 (10)
DME + PMSF treated HS	6.11 ± 0.58 (10)

move in the absence of cell division; and, moreover, serum has its effect independent of DNA synthesis.

Further Properties of the Epithelial Spreading Activity of Serum

To establish additional physiochemical properties of the serum activity supporting epithelial spread further studies were conducted. Since the serum preparation used in these experiments was exhaustively dialyzed (see Methods), it is apparent the serum component which supports cell spreading is not dialyzable. As recorded in Table 3, the activity was preserved after heating for 2 h at 56° C but it was destroyed

after 5 min at 100° C. The activity was not inhibited by the irreversible protease inhibitors DFP and PMSF and thus an active seryl residue is probably not important to its mechanism of action as for many serum enzymes.

Discussion

We have demonstrated using two different in vitro measurements that there exists in human serum a non-dialyzable, heat sensitive component(s) which must be continuously present in order to observe epithelial spread. Although most of the studies were done with an epithelial outgrowth assay upon plastic tissue culture dishes, comparable results were found with epiboly. The obvious and important difference between the two assays is the substratum upon which the cells move – plastic surface in one and dermal collagen in the other. Since, in the presence of serum, the results of the two systems are qualitatively alike, it would appear that the substrata are equivalent.

In this report without defining them we have used the terms „outgrowth" and „spread" synonymously in referring to the area about the adherent explant covered by epithelial cells after a given period. The assay used quantitates the area covered by the explant cells per unit time and thus is a measure of rate. The irradiation experiment (Fig. 5) indicates that even if thymidine incorporation is reduced, the area covered is not markedly different from that observed in nonirradiated explants. From this observation it would appear that at least for the first few days in culture cell motility plays a greater role than cell division in covering the measured area. Therefore, the assay measures predominately cell motility though cell division probably also plays a contributing – albeit smaller – role.

These studies provide only a general idea of the serum component supporting epithelial spread. We know this component cannot be replaced by several recognized purified serum proteins, such as bovine serum albumin, and thus is not a nonspecific property of protein solutions. Fetuin, a protein reported to be necessary for cell attachment [24] did not replace the serum activity in these studies. Other reported growth factors such as the heat-resistant epidermal growth factor of Cohen [25–28] and the serine protease, thrombin (shown to be important to in vitro cell division by Chen and Buchanan [29]) have properties different from those described here.

Others have searched for soluble factors influencing cell motility; however, very few studies have focused on soluble factors supporting epithelial movement per se. The serum component supporting epithelial spread about skin explants has been searched for previously. Levine [16] found that the human serum component was non-dialyzable and thus, of large molecular weight. In contrast, Coombs et al. [17] found that the factor supporting epiboly in fetal calf serum was lost after dialysis. They also found that the serum activity was not destroyed by heating (65° C for 5 min, and 56° C for 20 min). Our findings support the temperature studies of Coombs et al. though our additional observations show that the activity is not stable at higher temperatures (100° C for 5 min). We cannot explain why Coombs et al. lost activity after dialysis while Levine [16] and we did not. Our studies do not exclude the possibility that there exists a second epithelial spreading factor which is

dialyzable or that different species have factors of varying size. The activity in cow serum appears to be similar to that in human serum [18].

Many investigators have recorded a lag phase, or latent period after placing an explant in tissue culture (e.g. [10, 22]) or after cutaneous wounding [23, 30, 31] during which one is unable to measure significant cell outgrowth and division. Such a lag period during which measureable outgrowth is inapparent occurs in these experiments also. As the data of Fig. 3 indicate the latent period is at least 24 h. Preliminary studies indicate that this period is made of at least two components which are functions of 1. the time in culture and 2. the time of serum exposure. It appears both components must be satisfied before spreading occurs. The very rapid outgrowth response observed here after 1 and 3 days of serum deprivation (Fig. 3b) may be understood in terms of this latent period. Establishing what cellular control factors play a role in each component of the lag phase are important problems currently under study.

The present study indicates that one or several specific molecular species are present in serum which support epithelial spread. Future studies on the molecular nature and mechanism of action of these species may offer insight into the epithelial phenomena of wound closure, morphogenetic movements, and neoplastic invasion.

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