

AN EPITHELIAL SCATTER FACTOR RELEASED BY EMBRYO FIBROBLASTS

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SUMMARY

Medium conditioned by human embryo fibroblasts breaks structural junctions between several types of epithelial cells, leading to separation and scattering of the cells. An assay developed in MDCK cells shows activity up to a dilution of at least 1 in 64, equivalent to less than 100 ng of total protein. The activity is non-dialysable, heat-labile, and sensitive to trypsin, and it is assumed to be due to one or more proteins. After addition of the factor, separation of MDCK cells begins in about 15 min and is complete in 10 h. It increases migration of MDCK cells into wounds, and causes collapse of domes. Locomotion of isolated cells is not enhanced, but cell shape is affected by local membrane movement. Under the culture conditions used the factor, or an associated protein, causes a weak inhibition of cell growth without cytotoxic activity. The scattering factor has not been purified, nor has a physiological role been identified, but it might be concerned in the mobilization of epithelial cells.

INTRODUCTION

In earlier publications we have shown that the junctional organization of human mammary epithelial cells is affected by the source of conditioned medium used for culture (Stoker & Perryman, 1982; Stoker, 1984). In medium conditioned by various fibroblastic cell lines, such as Nil8 hamster cells, a high proportion of clones comprised cells in contiguous sheets with well-developed desmosomal junctions. In medium conditioned by human embryo lung fibroblasts, however, most of the clones consisted of separated cells without desmosomal junctions. These effects were not due to selection, but to direct promotion or inhibition of junction formation.

In this paper we describe more detailed studies on the junction-inhibiting activity present in medium conditioned by the MRC5 strain of human embryo fibroblasts. We show that other epithelial cell types are sensitive, notably MDCK cells. This has permitted the development of an assay for the active agent, and preliminary investigation of its characteristics, its production by embryo fibroblasts, and its mode of action.

MATERIALS AND METHODS

Mammary epithelial cells from individual samples of human milk, the MRC5 strain of human embryo fibroblasts, kindly provided by Dr Robin Holiday, and the Nil8 line of hamster kidney cells, were propagated from frozen stocks as described previously (Stoker, 1984). The MDCK line of dog

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kidney cells, kindly provided by Dr Ky Symons, was propagated in Dulbecco's modified Eagle's medium (DME) with 10% foetal calf serum. The BSC1 line of monkey kidney cells was also propagated in DME with 10% foetal calf serum. Secondary cultures of human cervical keratinocytes on 3T3 feeder cells in DME with 10% foetal calf serum, 0.5 µg/ml hydrocortisone and 10 ng/ml EGF, were kindly provided by Dr Margaret Stanley.

Unless stated otherwise, conditioned medium (CM) was obtained by exposure of confluent sheets of MRC5 or Nil8 cells in 90 mm dishes to fresh medium for 48 h, with centrifugation before use to remove cells. For use with mammary epithelial cells the appropriate medium based on RPMI medium was used (Stoker, 1984). For all other cells the conditioned medium was DME with 10% foetal calf serum, or serum-free DME, obtained after washing the feeder cells twice with DME or phosphate-buffered saline (PBS).

Time-lapse cinematography was carried out with exposures at 90-s intervals using cultures in 30 mm dishes at 37°C in chambers flushed with 10% CO₂ in air. Colony areas and cell locomotion rates were calculated from tracings of projected films.

Assays of scattering activity were carried out in 96-well Titertek trays. Doubling dilutions of conditioned medium or derivatives were made from 1/2 to 1/256 or as required in 0.15 ml volumes of DME with 5% foetal calf serum. A total of 3000 MDCK cells in 0.15 ml DME with 5% foetal calf serum were then added and the trays incubated overnight at 37°C. The degree of cell scattering was then recorded after fixation and staining with Giemsa stain, using a dissecting microscope.

Guinea pig antiserum against large desmosomal proteins (desmoplakins) was kindly provided by Dr David Garrod and used as described previously (Stoker, 1984). Protein concentration in conditioned medium was measured by a modification of Lowry's method (Maxwell, Haas, Blicher & Tolbert, 1978) after desalting 2.0 ml samples of medium on 9.1 ml Sephadex G-25 columns (P-10 Pharmacia).

RESULTS

Further studies on mammary epithelial cells

Following the earlier observation that colonies of tightly joined cells were separated 4 days after exposure to MRC5 CM, the events following exposure were followed by time-lapse cinematography. Colonies of well-joined cells were grown in Nil8 CM. Single colonies were then filmed for 55 h, before changing to fresh Nil8 CM or MRC5 CM and continuation of filming for a further 25 h.

In the Nil8 CM individual cells remained in close apposition, with no separation of cells at the edge of the colonies. Cell numbers and colony area did not increase over the short period involved. Cell locomotion occurred in the form of mass migration of attached cells in different parts of the colony. Changing to fresh Nil8 CM did not affect these characteristics.

Replacement by MRC5 CM produced a rapid change (Fig. 1). Within 8 h the colony area increased, with a fall in cell density, and an increased rate of locomotion. By 30 h the cells had separated and the colony area had increased fourfold without significant increase in cell numbers. Since locomotion increased after exposure to MRC5 CM, the primary action could have been on cell motility resulting in cell separation and scattering as a secondary effect. Locomotion of freshly plated isolated cells was not affected, however, by either MRC5 or Nil8 CM, compared to locomotion in unconditioned medium (Fig. 2).

It is therefore unlikely that the primary effect is on cell motility. Nevertheless, a local agitation of the cell margin was observed in the presence of MRC5 CM, which will be mentioned again below.

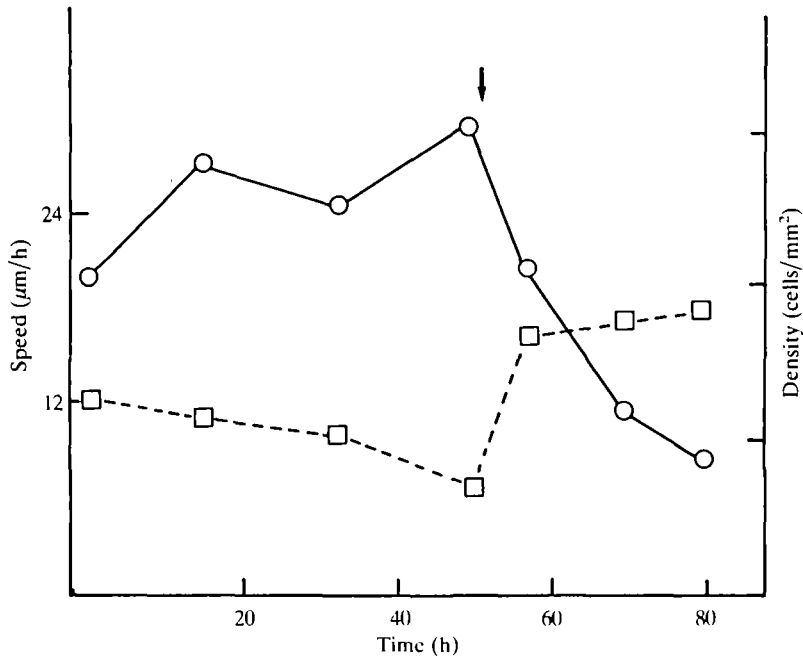


Fig. 1. Effect of scattering activity on density and movement of human mammary epithelial cells. Colonies, examined by time-lapse cinematography, were grown in Nil8 CM, which was then replaced with MRC5 CM, as indicated by arrow. Cell density (derived from colony area and cell number (○—○), and locomotion (□--□), were determined from tracings of the projected film.

These experiments on mammary epithelium showed that MRC5 CM had a rapid and direct action on the junctional relationships between the cells. However, freshly isolated mammary cells are heterogeneous, and not ideal for analysis. The susceptibility of other cells, perhaps more suitable for analysis, was therefore investigated.

Sensitivity of other types of cell

Because the effect of MRC5 CM on mammary cells was easily detected by time-lapse filming, the effects on other cells were examined in the same way. The medium used for conditioning by MRC5 cells was that appropriate for the cells concerned, normally DME with 10% foetal calf serum, instead of the RPMI-based medium used for mammary cells. Previously established colonies of the target cells were filmed for a period in unconditioned medium, then 20% MRC5 CM was added and filming was continued.

Rapid expansion of colony area with corresponding loss of cell density, and varying degrees of cell separation were found after adding MRC5 CM to human cervical keratinocytes, the BSC1 line of monkey cells, and the MDCK line of dog kidney epithelium. Addition of unconditioned medium had no effect. The effect of MRC5 CM on separation of fibroblasts was difficult to examine because they do not form continuously joined cell sheets, but no change was found in density, emigration or locomotion of 3T3 cells, either isolated, or in colonies or islands.

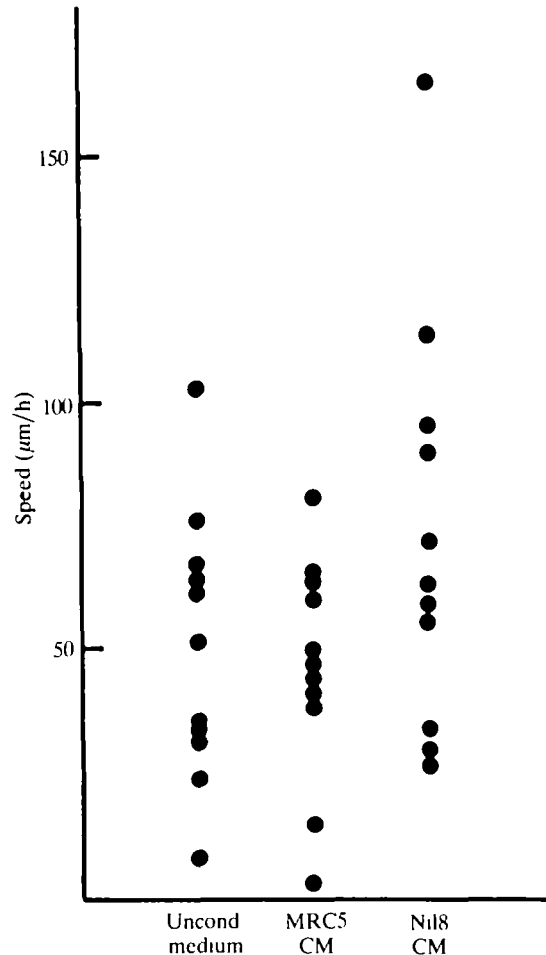


Fig. 2. Rate of movement of isolated mammary epithelial cells in MRC5 and Nil8 CM, and unconditioned medium. Measurements were made from tracing tracks of individual cells over 2.5 h, each spot representing one cell. Periods during which cells were attached to one another were omitted.

MDCK cells showed the most pronounced change after addition of MRC5 CM, and these cells were chosen as model target cells for further study of the activity, and for the development of an assay.

Assay of activity using MDCK cells

Assays were first set up by adding varying concentrations of MRC5 CM in DME with 5% foetal calf serum to established islands of MDCK cells in dishes or Titertek plates, with subsequent examination for scattering 24 or 48 h later. This method was superseded by a simpler and quicker assay in which suspensions of cells were added directly to doubling dilutions of conditioned medium in Titertek plates, followed by examination for scattering on the following day (see Materials and Methods).

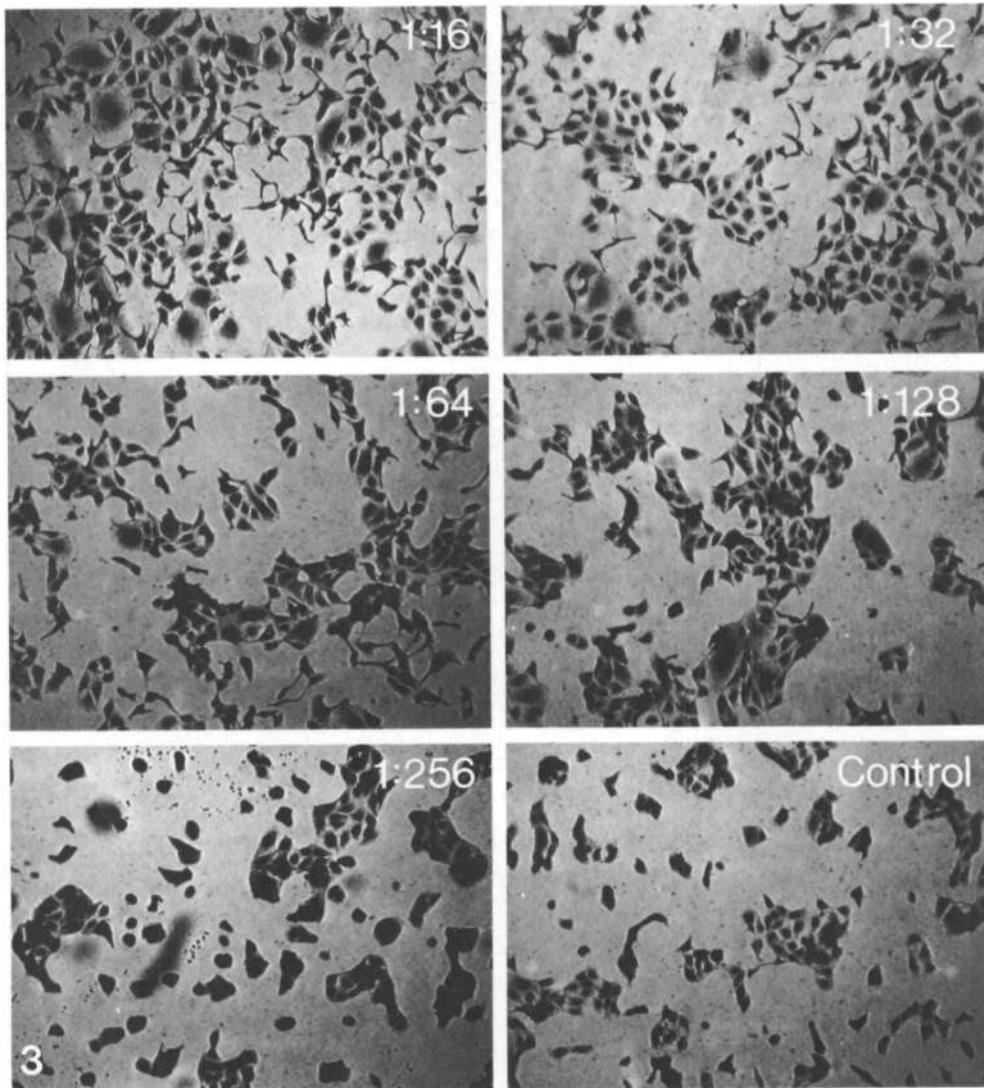


Fig. 3. Assay of activity of MRC5 CM. Plates show wells in Titertek plates with 3000 MDCK cells after 24 h in the presence of conditioned medium at the dilutions shown (top right). The limit of activity is 1 in 128. $\times 120$.

Time-lapse films had shown that MDCK cells freshly plated in DME with 5% foetal calf serum, rapidly attach and aggregate into tight and stable clusters of 2–10 cells as a result of random collisions. In the presence of MRC5 CM the random movement was not affected but the collisions did not result in clusters and the cells remained separated, and easily distinguished from the controls. Towards the limit of activity at the higher dilutions, cell separation was not complete but the islands were more spread and there was a higher proportion of free cells, which permitted

distinction from controls. The titre was taken as the highest dilution that allowed a clear distinction and to avoid subjective error the assay was normally read 'blind', without knowledge of the sample. Fig. 3 shows a typical assay.

Standard preparations of conditioned medium were generally active up to a dilution of at least 1 in 64 (the titre). A unit of activity was taken as the amount present at the limit of activity. An undiluted standard preparation would therefore have 64 units in the assay volume of 0.3 ml or 210 units per ml. The level of activity in a sample could vary by one or two dilutions in separate assays, and a standard control sample was always included to assess sensitivity.

Release of activity by MRC5 cells

The medium used in the original experiments was the growth medium required by the target cells that included serum. It was found, however, that omission of serum did not affect the release of activity by the MRC5 cells. Confluent cultures, washed to remove residual serum from the growth medium, and then exposed to DME, produced approximately the same levels of activity whether or not serum was present. In subsequent experiments described here the conditioned medium was serum-free DME exposed to MRC5 cells. It should be noted, however, that to assay the activity in MDCK cells 5% foetal calf serum was required.

In the experiment shown in Fig. 4, confluent replicate cultures of 5×10^6 MRC5 cells in 90 mm dishes, were exposed to 5, 10 or 20 ml of DME and sampled daily for 4 days. Total activity and total protein released are shown with allowance for volume of medium.

The activity in the medium increased for 3 days but with a reduced yield in the largest volume. Since the activity is stable at 37°C (see below) this suggests a fairly constant rate of production. There was no reduction of overall activity released into the smallest volume that showed the highest titre, so there is no evidence that, within these levels, release is regulated by a feedback mechanism.

In contrast, the final protein concentration was approximately equal whatever the volume, and so the total released varied, being greatest in the largest volume. As a result the specific activity in terms of protein was highest if the volume of medium was low (Fig. 3B). For attempts to purify the active material, therefore, the optimum release occurred with 5 ml of medium left for 3 or 4 days, yielding about 14 units per μg .

The MRC5 cells were used regularly between passages 26 and 28. Similar activity was obtained from MRC5 cells at different passage levels, and from WI38 cells.

Characterization of activity

Table 1 shows the effects of various treatments on the titre of activity in fresh MRC5 CM. The agent responsible is very stable at temperatures up to 37°C, and is partially inactivated by 30 min at 50°C. However, exposure of small volumes of serum-free CM to large areas of untreated plastic (e.g. culture dishes) for 24 h at 37°C, reduced the activity by 75–90%, presumably by absorption. This loss was

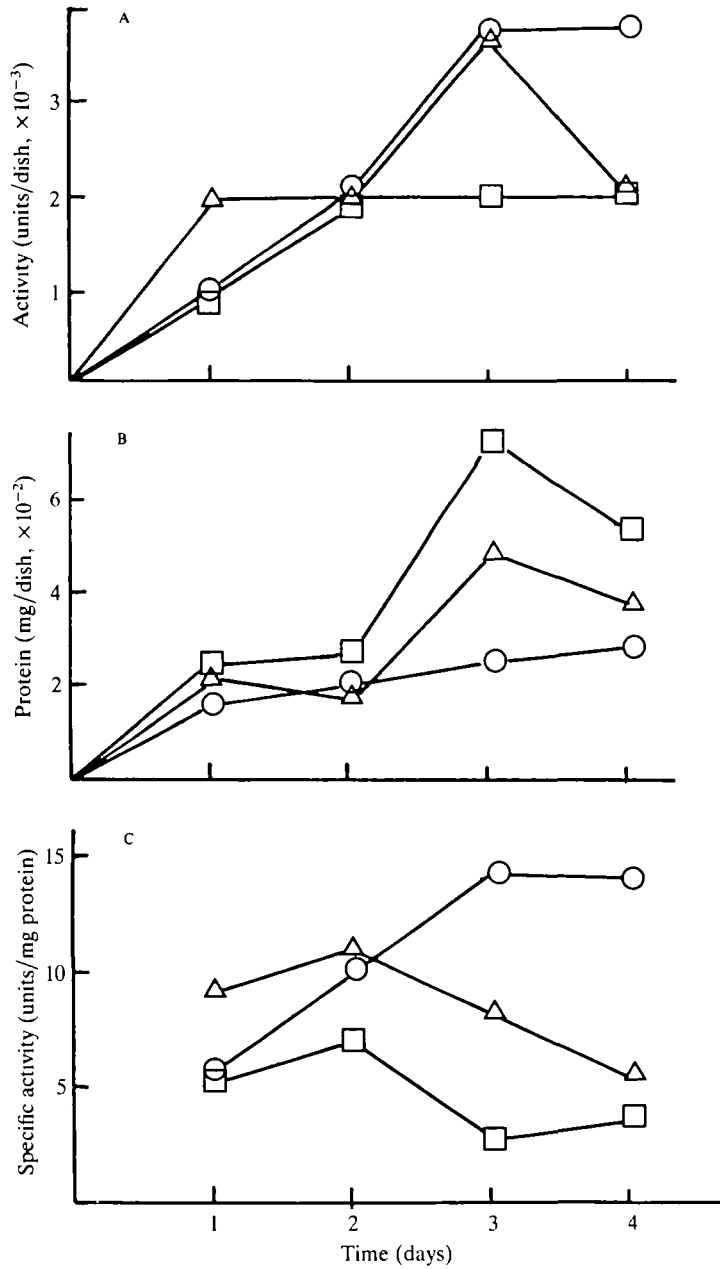


Fig. 4.A. Release of scattering factor by MRC5 cells. Confluent layers of 5×10^6 cells in 90 mm dishes were exposed to 5 ml (○), 10 ml (△) or 20 ml (□) of medium without serum. Activity at successive times was assayed in MDCK cells. B. Release of total protein from MRC5 cells as in A. C. Scattering activity released expressed as units per μg of protein.

Table 1. *Effect of various treatments on scattering activity released by MRC5 cells*

Treatment		% Activity remaining
70°C	30 min	<1
60°C	30 min	<2.5
50°C	30 min	12
40°C	30 min	100
37°C	30 min	100
37°C	10 days	100
4°C	70 days	100
-20°C	70 days	100
Trypsin (0.2 mg/ml), 37°C	5 min	12
Trypsin (0.2 mg/ml)	30 min	<3
Trypsin with 10% serum	30 min	100
pH 8*	120 min	100
pH 9*	120 min	100
Dialysis, dist. water		100
Dialysis, phosphate-buffered saline		100

Serum-free conditioned DME medium was assayed by titration on MDCK cells after treatment indicated.

*Serum-free HEPES medium adjusted with NaOH.

prevented by the presence of 10 % serum. The agent is non-dialysable and stable in glass-distilled water and phosphate-buffered saline. Activity released into HEPES-buffered DME is also stable up to pH 9; this is of possible importance because of occasional increase in pH in standard DME through loss of CO₂. Finally, the activity is destroyed by trypsin. We therefore conclude that the agent responsible is a protein factor or factors released by MRC5 cells.

Response of MDCK cells

Scattering. Fig. 5A,B shows an analysis by time-lapse cinematography of the effect of adding 20 % serum-free MRC5 CM or unconditioned DME to colonies of MDCK cells. The cultures had been established 2 days previously, and filming was started 2 h before the addition. Fig. 6 shows reproductions from the film, after 1 h (before addition of CM), 5 h, 12 h and 16 h of filming.

Fig. 5A shows an increase in the area of the field occupied by colonies of cells, and in total cell numbers. In the controls the cell numbers and occupied area rose steadily, as the growth continued over 16 h (mean intermitotic interval 11 h). Addition of MRC5 CM, however, caused a rapid increase in the colony area without an equivalent increase in cell numbers (see Fig. 6 at 5 h). This was similar to the effect on mammary epithelium and keratinocytes, but faster and more marked. The expansion in area could be detected by direct observation within the first four frames, i.e. by 15 min, and could be measured for a further 4.5 h, after which the cells began to separate.

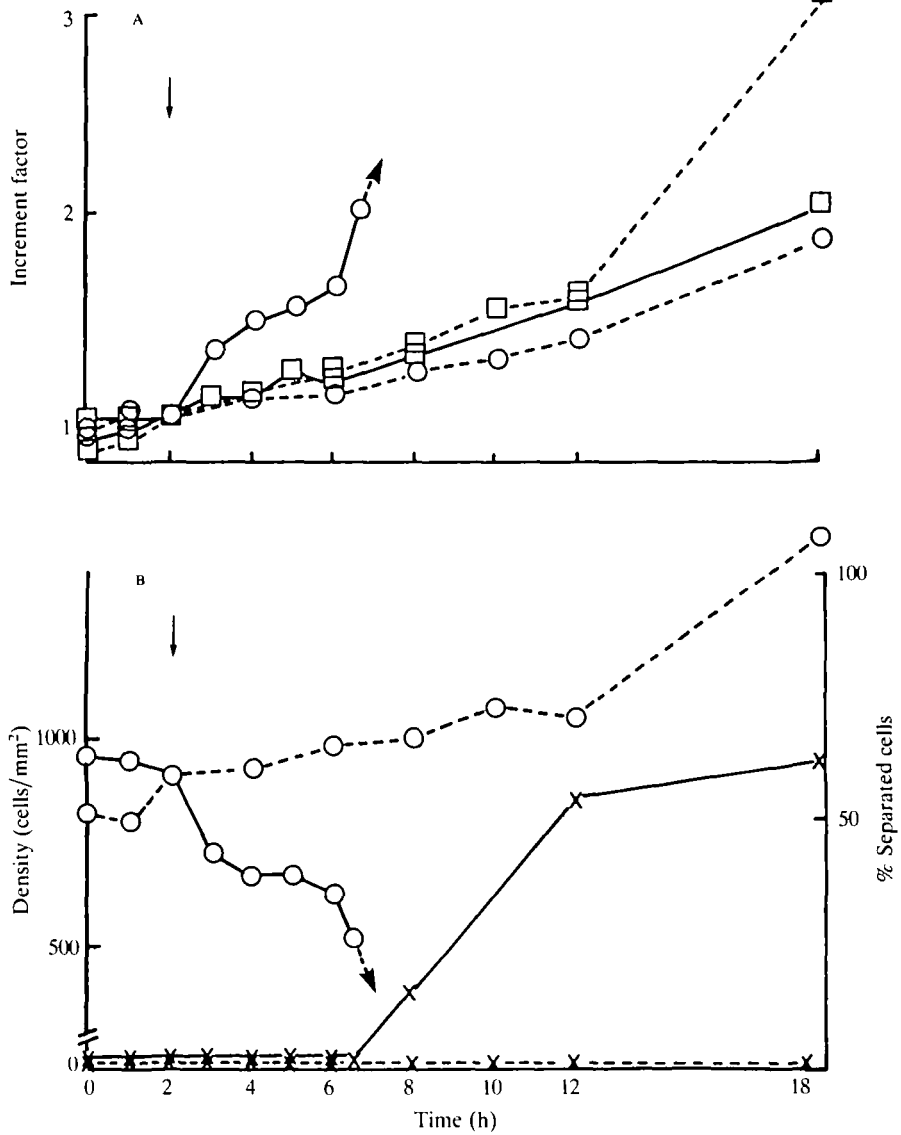


Fig. 5.A. Effect of MRC5 CM on colony area and cell number in cultures of MDCK cells. Colonies were grown in medium with 10% foetal calf serum. MRC5 CM 20% (—) or unconditioned medium (- - - -) was added as indicated by the arrow during time-lapse cinematography. Total colony area (○) and cell numbers (□) were measured with tracings of projected films. B. Effect on cell density (○-○) derived from A, and proportion of cells separated from neighbours (×-×).

Fig. 5B shows the change in terms of cell density in the colonies. It shows the gradual increase in density as cell numbers rose in the controls and, in contrast, the sudden drop in density after addition of MRC5 CM. Fig. 5B also gives the percentage of isolated cells separating from the colonies. These were uniformly low throughout in the controls, and also until 4.5 h after addition of MRC5 CM, but then the numbers

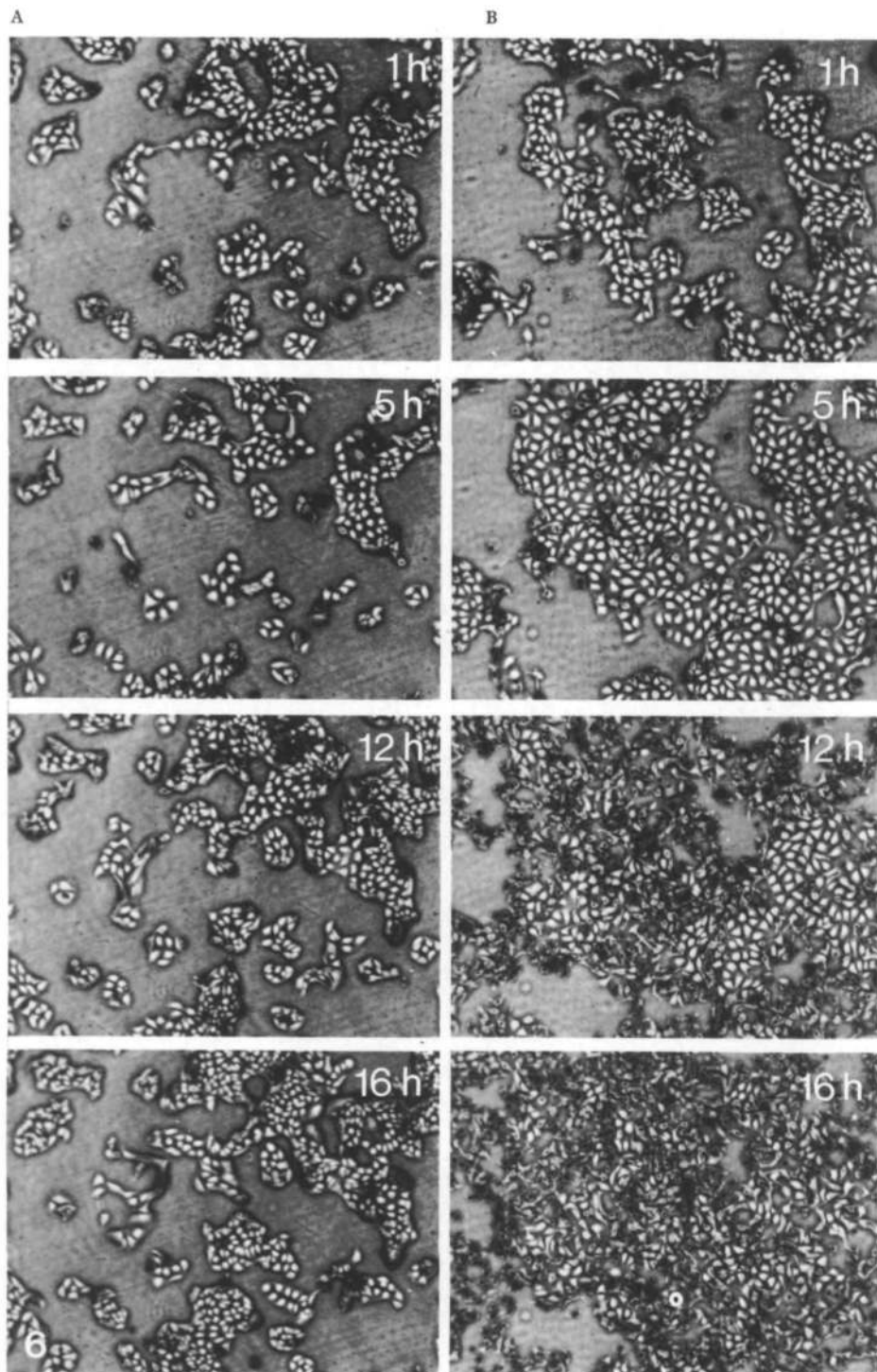


Fig. 6. Frames from time-lapse film shown in Fig. 5 at times given, showing expansion of colony area, reduced density and cell dispersal. A. Control; B, 20% MRC5 CM. $\times 45$.

of isolated cells rose sharply with the sudden disintegration of the cell sheets. The separation of individual cells is seen more easily by analysis of the moving film.

Thus the effect of the factor(s) in MRC5 CM is to cause the epithelial cells to scatter; this occurs in two stages. First, they spring away from one another without complete separation from their neighbours, either by increase in cell area or by maintaining point contacts only. Second, they separate completely and move away from one another independently. It is of interest that the MDCK cells at the limiting dilutions in the assay do not seem to progress beyond the first stage.

When MDCK cells were left for more than one or two days in MRC5 CM, they gradually reformed islands and continuity was restored. Since the factor is stable at 37°C, this suggests that the target cells themselves inactivate or inhibit the activity.

Growth inhibition. It will be seen from the cell counts in Fig. 5A that growth of MDCK cells is inhibited by MRC5 CM relative to growth in the controls. This was confirmed in several experiments by total cell counts in replicate cultures of MDCK cells over 48 h in MRC5 CM and unconditioned medium. An example is given in Table 2. Incorporation of [³H]thymidine was also moderately reduced in cultures exposed to MRC5 CM (data not shown). Since growth was inhibited even when conditioned medium was diluted 20% in fresh medium, the effect is not caused by depletion of constituents in the medium, and is presumably due to an inhibitor.

Local cell motility. As already mentioned, MRC5 CM does not affect the locomotion of individual mammary epithelial cells, and the same applies to MDCK cells. However, MDCK cells like mammary cells develop the jitters, i.e. they show an increase in local movement or 'running on the spot', without a change in the rate of transposition from one location to another. This results in a subtle alteration of shape, which is difficult to define.

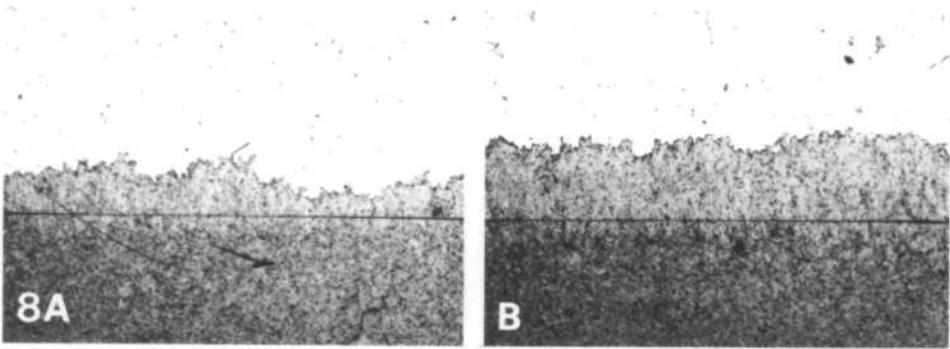
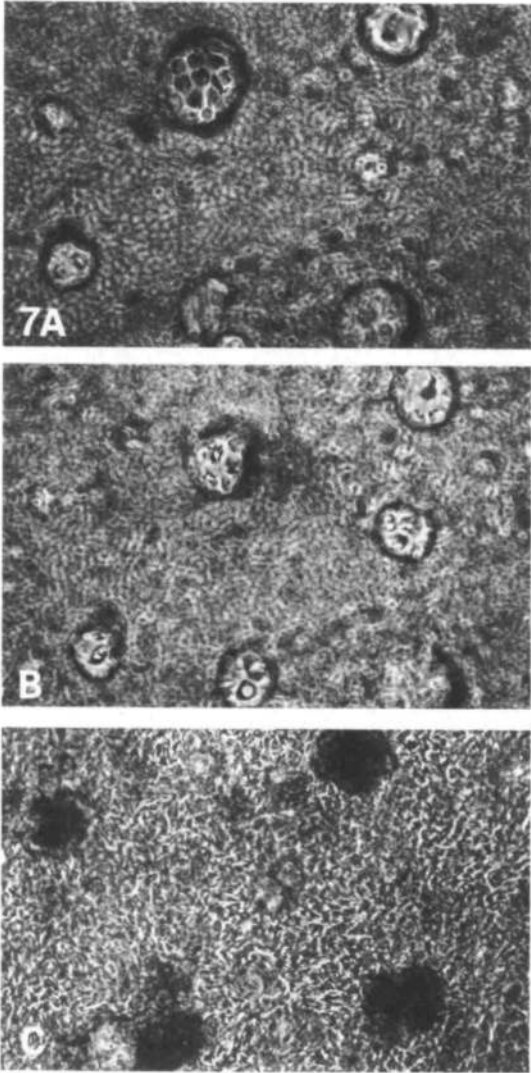
Disruption of domes. Confluent sheets of MDCK cells form local secretory domes. This is particularly marked when exposed to medium conditioned by the Nil8 cell line, perhaps due to dome inducers (Lever, 1979). When a domed sheet in Nil8 CM is then exposed to MRC5 CM there is a rapid collapse of domes that is complete in 7 h (Fig. 7). This is followed by abortive attempts at dome formation at the original patch of collapsed dome cells, i.e. the dome begins to form at the original site but

Table 2. *Growth of MDCK cells in the presence and absence of factor(s) in medium conditioned by MRC5 cells*

Before addition	Hours after addition			
	Conditioned medium		Unconditioned medium	
	6	48	6	48
10.3 (1.3)*	12.2 (0.9)	59.1 (2.0)	11.6 (0.4)	77.0 (2.3)

Sets of triplicate cultures were suspended and counted, before and at given times after addition of 20% MRC5 conditioned or unconditioned medium.

*Mean cells per culture (standard error).



Figs 7 and 8

collapses immediately. We assume that MRC5 CM prevents the maintenance of tight junctions necessary for the establishment of domes.

Wound healing in vitro. Confluent sheets of MDCK cells were wounded with a razor by Burk's (1973) method, then washed, and the medium was replaced by either MRC5 CM or unconditioned medium. After 48 h, the migration of cells into the wound area was much more marked in the conditioned medium than in the controls (Fig. 8), though by this time the continuity of the sheet was restored, as already noted.

Effect on desmosomal junctions. Detection of high molecular weight plaque proteins (desmoplakins) by immunofluorescence allows visualization of desmosomal junctions without the sampling problems of electron microscopy (Franke *et al.* 1981; Cowin & Garrod, 1983). MDCK cells show large numbers of fluorescent spots along contiguous cell boundaries. Fig. 9 shows the effect of MRC5 CM on these plaque constituents at successive intervals during the scattering process. It will be noticed

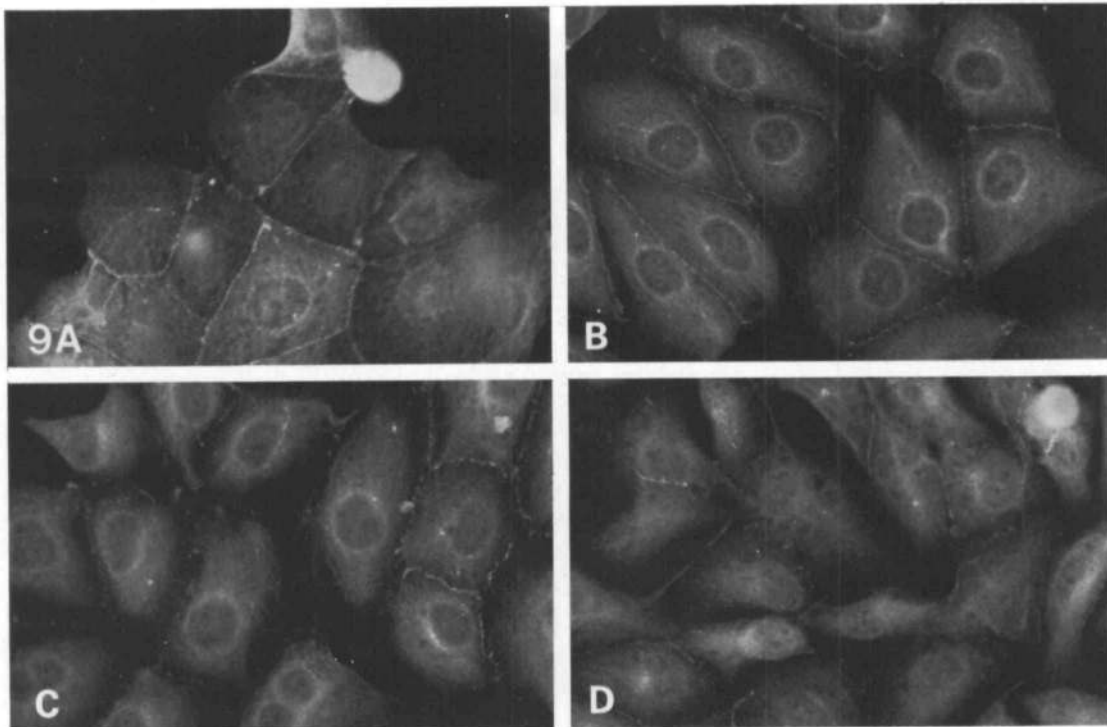


Fig. 9. Indirect immunofluorescent staining of MDCK cells using antibody against desmoplakin before (A), and 1 h (B), 6 h (C) and 24 h (D) after exposure to 20% MRC5 CM. $\times 300$.

Fig. 7. MDCK cells grown in Nil8 CM to form domes before (A) and 2.5 h (B) and 7 h (C) after replacement by MRC5 CM. $\times 120$.

Fig. 8. Migration of MDCK cells from original edge of wound (dark line) after 48 h. A. Control; B, 20% MRC5 CM. Stained with Giemsa $\times 5$.

that there is no rapid disappearance at an early stage, and plaques are retained even when separation is advanced. This indicates that the plaque component of the desmosome is unlikely to be the primary target of the factor. In this connection it is of particular interest that we have been unable to detect desmoplakin in the BSC1 line of epithelial cells, but, as noted above, they are sensitive to the scattering activity of MRC5 CM. Parallel experiments with anti-keratin serum (not shown) did not reveal any significant alteration in the filament pattern.

DISCUSSION

It is apparent that the MRC5 strain of human embryo lung fibroblasts releases biologically active material, which can be detected by its ability to disrupt the structural junctions of certain epithelial cells in culture, and cause them to scatter. The activity is non-dialysable and protease-sensitive, and so we assume that it is due to one or more proteins.

The experiments described in this paper were all carried out with the factor obtained from medium exposed to human embryo fibroblasts of the MRC5 strain as donors, and cells of various passage numbers and sources yielded similar levels of activity. The Nil8 line of hamster fibroblasts, on the other hand, yielded no activity detectable with the target cells used.

The action of the factor was not confined to mammary epithelium, in which it was first observed, but extended at least to cervical keratinocytes, and two lines of epithelial cells, BSC1 and MDCK. It was difficult to demonstrate a similar scattering activity on fibroblast cultures, because they do not form connected sheets of cells, but we have not so far observed any change in behaviour in 3T3 cells exposed to the factor.

Further investigation of the range of cell types that can release the scattering activity, and of those that can respond to it, should be of considerable interest and is being actively pursued, because of the implications for a possible role for the activity *in vivo*. But purification and characterization are essential to determine whether the various effects observed, for example, scattering, local movement and growth inhibition, are due to one or several types of molecule.

Fortunately, the sensitivity of MDCK cells has permitted the development of a relatively simple and rapid assay, which is now being used to monitor the concentration and purification of the factor. It is stable on storage at 4°C, and is released into serum-free medium, containing 50–100 µg of total protein per ml. Activity is detectable in the assay with less than 100 ng of total protein, so the purified factor may be active at low, potentially physiological, concentrations of protein.

An understanding of the mechanism of action that leads to cell separation will also be helped by isolation of the protein responsible. Desmosomal plaques, although they are finally lost after full separation, do not disappear early during scattering. Possibly, the primary targets are intermembrane glycoproteins (Gorbsky & Steinberg, 1981), or other structures such as the intermediate junctions or tight junctions. Hennings & Holbrook (1983) have shown that maintenance of desmosomal junctions requires Ca^{2+} . It has already been shown that MRC5 CM shows no depletion of total Ca^{2+}

(Stoker, 1984), and the low protein concentration at which activity occurs does not suggest that the scattering could be due to lack of availability of free Ca^{2+} . Finally, despite the lack of effect on the keratin pattern, a primary action on the cytoskeleton associated with the increased local movement might lead to loss of junctional integrity. The effect on communicating junctions has not yet been investigated.

Most of the known factors released by cultured cells have a primary effect on cell growth, and we cannot exclude the possibility that the factor may have already been identified using another assay. It would be interesting, for example, to compare the dispersal agent recently described by De Larco (1985).

Speculation as to the role of the factor *in vivo* is premature and requires a knowledge of the sites of syntheses and range of target cells, and possible changes in sensitivity of epithelial cells during differentiation. At this stage it is sufficient to note the possibility that the factor might be involved in some form of epithelial cell mobilization, for example, during embryonic development, or in wound healing.

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