

THE BEHAVIOR OF FIBROBLASTS FROM THE DEVELOPING AVIAN CORNEA

Morphology and Movement *In Situ* and In Vitro

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ABSTRACT

The early chick cornea is composed of an acellular collagenous stroma lined with an anterior epithelium and a posterior endothelium. At stage 27–28 of development (5½ days), this stroma swells so that the cornea is 75–120 μm thick. At the same time, fibroblasts that originate from the neural crest begin to invade this stroma. Using Nomarski light microscopy, we have compared the behavior of moving cells in isolated corneas with the migratory activities of the same cells in artificial collagen lattices and on glass. *In situ*, fibroblasts have cyclindrical bodies from which extend several thick pseudopodia and/or finer filopodia. Movement is accompanied by activity in these cytoplasmic processes. The flat ruffling lamellipodia that characterize these cells on glass are not seen *in situ*, but the general mechanism of cell movement seems to be the same as that observed in vitro: either gross contraction or recoil of the cell body (now pear shaped) into the forward cell process, or more subtle “flowing” of cytoplasm into the forward cell process without immediate loss of the trailing cell process. We filmed collisions between cells *in situ* and in three-dimensional collagen lattices. These fibroblasts show, in their pair-wise collisions, the classical contact inhibition of movement (CIM) exhibited in vitro even though they lack ruffled borders. On glass these cells multilayer, showing that, while CIM affects cell movement, fibroblasts can use one another as a substratum. Postmitotic cells show CIM in moving away from each other. Interestingly, dividing cells *in situ* do not exhibit surface blebbing, but do extend filopodia at telophase. The role of CIM in controlling cell movement in vivo and in vitro is stressed in the discussion.

Our knowledge of the mode of fibroblast movement is based almost completely on studies made in vitro. This is, of course, mainly due to the fact that the connective tissue *in situ* tends to be too thick for light microscope studies, whereas it is relatively easy to photograph monolayers of cells in vitro. Indeed, almost all intact tissues are

opaque, and so our knowledge of living cell morphology and movement in vivo comes from those few tissues which possess optical qualities that permit their component cells to be viewed in the light microscope. Examples of these are the sea urchin embryo (15, 16), the *Fundulus* blastula (33, 34), and the tadpole tail (29). In addition, the use

of chambers implanted in rabbits ears has made it possible to study living cells in adult material (36). More recently, Izzard (23) has studied moving cells in the tunic of the ascidian *Botryllus schlosseri*. Nevertheless, such tissues are the exception rather than the rule, and, in practice, provide information on cells of a different type than those normally studied in tissue culture.

A convenient opportunity to examine typical vertebrate fibroblasts *in vivo* is provided by the developing avian cornea. On the 6th day of incubation, fibroblasts migrate into a swollen, collagenous stroma which is initially devoid of cells. This stroma is bordered anteriorly by an epithelium and posteriorly by an endothelium, the whole being about 100 μm thick on the 6th day (21). This tissue is sufficiently transparent under Nomarski optics, with their optical-sectioning properties (5), that reasonable resolution of individual cell morphology is possible and so the invading fibroblasts can be directly observed in dissected anterior eye segments.

A further advantage of this tissue is that the fibroblasts can readily be removed by brief enzyme treatment from their natural milieu and grown on two-dimensional glass and plastic substrata or in artificial three-dimensional collagen lattices (11). It is, therefore, possible to compare the behavior of the same cells *in situ* and *in vitro* to see how realistic the observations made on the cells *in vitro* are.

MATERIALS AND METHODS

Chicken embryos were obtained from Spafas (Norwich, Conn.) and maintained at 37°C at 60% humidity. Embryos were staged according to the Hamburger-Hamilton series (17).

Culturing Intact Corneas

For simple observations, the cornea, with a small quantity of surrounding tissue, was placed in a Dvorak-Stotler (D-S) chamber (Nicholson Precision Instruments Inc., Bethesda, Md.). This consists of two circular 25-mm cover slips separated by a metal ring with inlet and outlet ports (10). The excised cornea was placed on a circular cover slip in a drop of medium (25) and covered with a cover slip rectangle approximately 10-mm long and 3-mm wide. Small pieces of adhesive tape on the lower surface of the rectangle kept the cornea from being crushed, and large straps of adhesive tape on the upper surface fixed the rectangle to the larger cover slip. Once the cornea was immobilized between the two pieces of glass, the circular slip was inverted and became the top

part of the chamber. The chamber was then filled with medium and placed on the stage of a Zeiss WL microscope fitted with Nomarski optics and a 40 \times , long-working-distance, oil-immersion lens. The stage was heated by a Sage air curtain (Sage Instruments, White Plains, N.Y.) whose temperature sensor was placed in the immersion oil.

It proved comparatively easy to maintain the intact cornea in a chamber for a short time so that high resolution light microscopy was possible, but rather harder to persuade the tissue actually to develop on the microscope stage. The epithelium and endothelium rapidly became swollen and the optical resolution declined with time. In order to permit a degree of development to occur *in vitro*, the cornea was maintained under conditions that were, optically, a little less satisfactory. The major part of the anterior half of the eye (without the lens) was excised and placed in the chamber which was filled with medium and sealed. Under these circumstances, the cornea was not maintained in a horizontal position and could buckle. The chamber was then perfused with medium on a simple gravity feed with flows of about 0.2 ml per min. Under such circumstances, eyes stayed clear for a few hours.

Photography with Nomarski optics requires an intense beam of light, as the image is formed by destructive interference. The risk of overheating and killing the specimen was minimized by the use of a heat filter. In addition, the specimens were illuminated minimally for focussing and highly for photography alone. At all other times, a shutter was placed between source and condenser. In the absence of an intermediate cover slip, it was difficult to maintain focus without continuous monitoring and its attendant risk of overheating. We were thus unable to make satisfactory time-lapsed movie films and decided instead routinely to photograph fields every 5 min by hand, using a Nikon AFX automatic 35-mm camera (Nikon Inc., Instrument Group, EP01, Garden City, N.Y.) and Pan X film.

Isolating the Stromal Fibroblasts

Stage 28–29 corneas were excised for most experiments, care being taken to exclude surrounding tissue. The tissue was incubated in 0.1% trypsin (Nutritional Biochemicals, Cleveland, Ohio) and 0.1% collagenase (Worthington Biochemical Corp., Freehold, N.J.) in calcium- and magnesium-free Hanks' solution (pH 7.0–7.6) for 7 min (25). This loosened the endothelium and the epithelium which were then scraped away from the stroma with needles. The stromal fragments which contained the fibroblasts were then washed three times in medium and cultured in collagen gels, on glass or on plastic. Occasionally, the stromal fragments were reincubated in the trypsin-collagenase solution for a further 30 min to prepare cell suspensions. In this case, the cells were then washed three times with medium and cultured directly on glass or plastic.

Glass Substrata

Circular cover slips were dipped in acetone-alcohol and boiling water to clean and sterilize them. They were then placed in plastic tissue culture dishes (3.5-cm diameter, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and washed with enough medium to wet the surface of the glass and leave a little medium in the dish. Fragments of corneal stroma were placed in the center of the cover slip and the dish was transferred to an incubator to permit adhesion. 1–2 h later, 5 ml of medium was pipetted into the dish with great care. The fibroblasts migrated out onto the glass. Occasionally, pieces of stroma were grown on plastic dishes rather than on glass cover slips and were examined with a Nikon Model MS inverted phase microscope.

Collagen Substrata

Collagen gels were prepared according to the method of Elsdale and Bard (11). Here, solutions of rat-tail

tendon collagen extracted with acetic acid and dialysed against F-12 medium were precipitated in the presence of 0.17 N sodium hydroxide, 10% serum, and 10% of stock $10 \times$ F-12 medium to make a lattice of collagen fibrils. These lattices contain only about 0.1% collagen, but are robust enough to permit cell extension and movement within them without collapsing (11).

A drop or two of the freshly made mixture was placed on a circular cover slip in a petri dish, and a fragment of stroma was placed in the solution before the gel had set. The dish was transferred to an incubator for 30 min and then 3 ml of medium was added. The cover slip could be transferred to the D-S chamber at any subsequent time.

RESULTS

The Development of the Cornea at Stage 27

The structure and development of the cornea at the time of fibroblast migration has been described

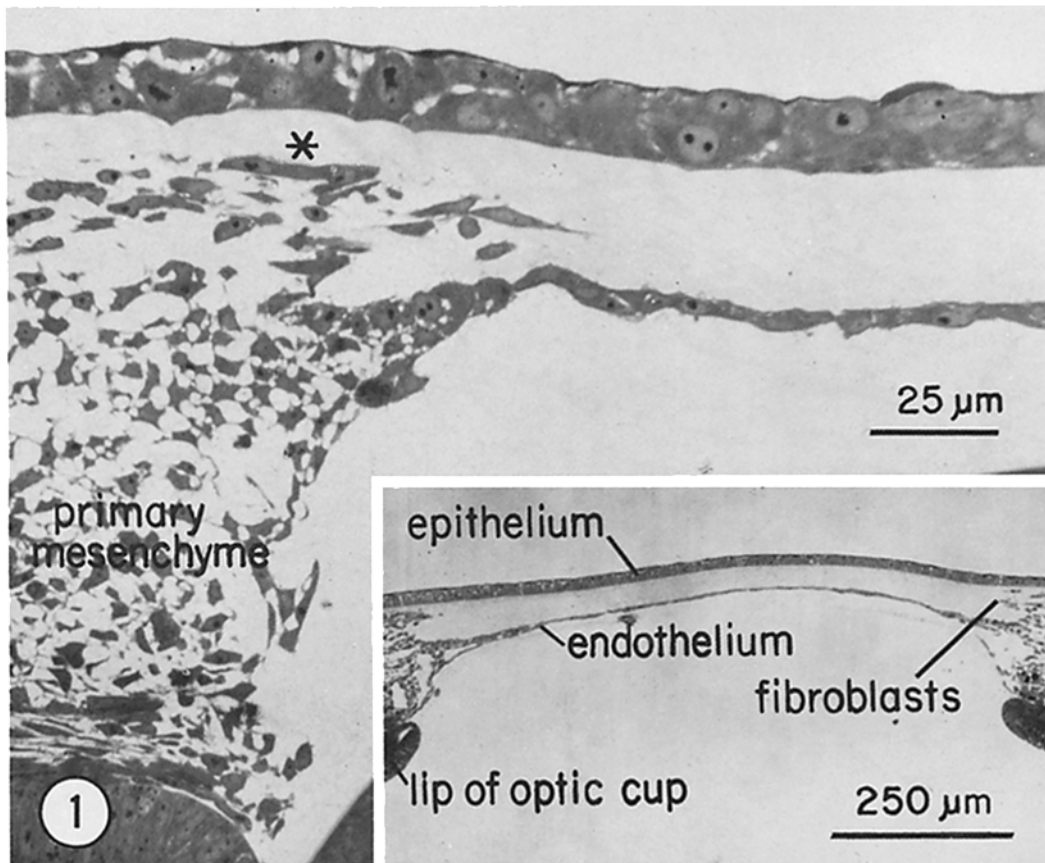


FIGURE 1 Light micrograph of a section of a stage 27 eye ($5\frac{1}{2}$ days) showing the initial invasion of fibroblasts into the cell-free stroma that lies between the corneal epithelium and endothelium. The migrating fibroblasts are elongated in shape and contain long filopodia. They are still dividing, and this is probably why they sometimes occur in pairs (*). Fibroblasts first migrate into the lower half of the stroma, which is rich in hyaluronate secreted by the endothelium. The inset shows the typical segment with lens removed that was cut out for study in the culture chamber. $\times 670$; inset, $\times 100$.

in thin sections viewed by light and electron microscopy (see reference 21 for review). At stage 27, the cornea consists of an acellular stroma sandwiched between an outer epithelium and inner endothelium (Fig. 1). The anterior cell layer is a thin periderm that is joined by cell junctions to the basal columnar cell layer of the corneal epithelium. This epithelium secretes the collagen and sulfated glycosaminoglycan comprising the primary stroma (9, 21, 25). By stage 27/28, the stroma is a highly hydrated mass of thin collagen fibrils of uniform thickness arranged in orthogonal array (21, 31), the whole forming a convoluted layer (7, 26). The posterior (inner) surface of this sandwich is a monolayered endothelium that faces the anterior chamber of the eye. At the side of the cornea, the epithelium is continuous with the surface ectoderm, and the endothelium merges with the vascular mesenchymal shelf destined later to become the uvea (primary mesenchyme, Fig. 1).

The fibroblasts that invade the stroma are

secondary mesenchymal cells (20, 21) from the neural crest (24). They arrive in the subectodermal stroma at the periphery of the cornea just as the stroma proper starts to swell. This swelling begins at exactly the time the endothelium is established as an intact sheet (21). It is not known how the cells from the neural crest are guided to the eye, but the stroma swelling undoubtedly facilitates their invasion into the cornea (Fig. 1). The stroma per se increases in thickness from $10\ \mu\text{m}$ at 5 days (stage 26) to $100\ \mu\text{m}$ at $5\frac{1}{2}$ days (stage 27/28) to $220\ \mu\text{m}$ at $8\frac{1}{2}$ days (21) and this growth is correlated especially with an increase in the amount of newly synthesized hyaluronic acid in the stroma (30). This glycosaminoglycan is produced by the endothelium (25, 32). It should be noted that the stroma not only has an unusually large amount of hyaluronic acid at this stage as compared to most connective tissue, but also contains chondroitin sulfate produced by the epithelium and surprisingly fine collagen fibrils: their diameter is

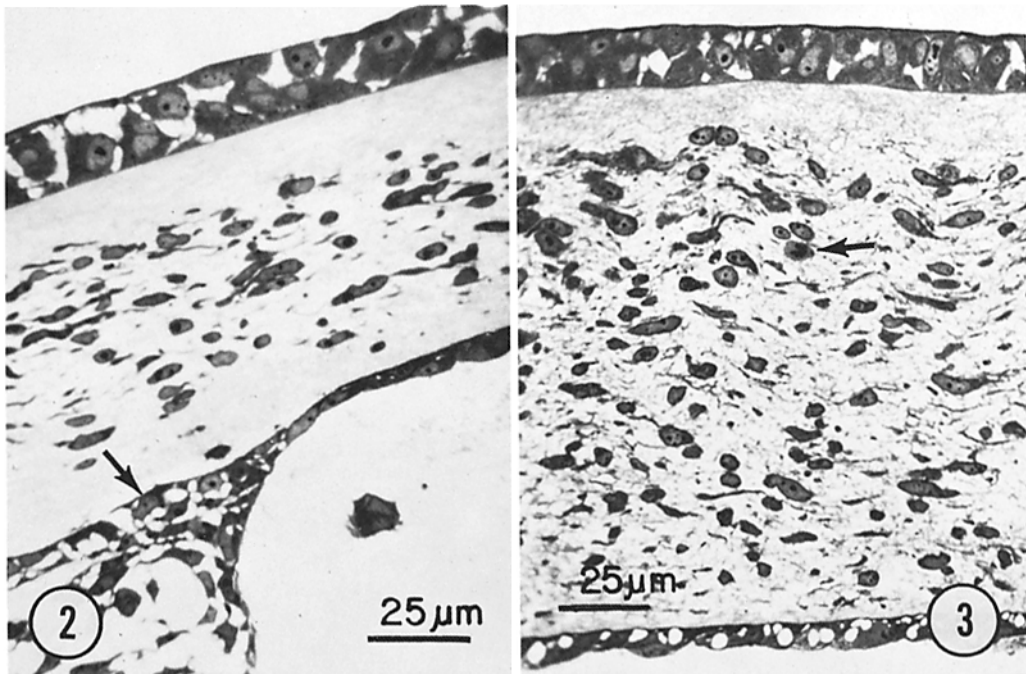
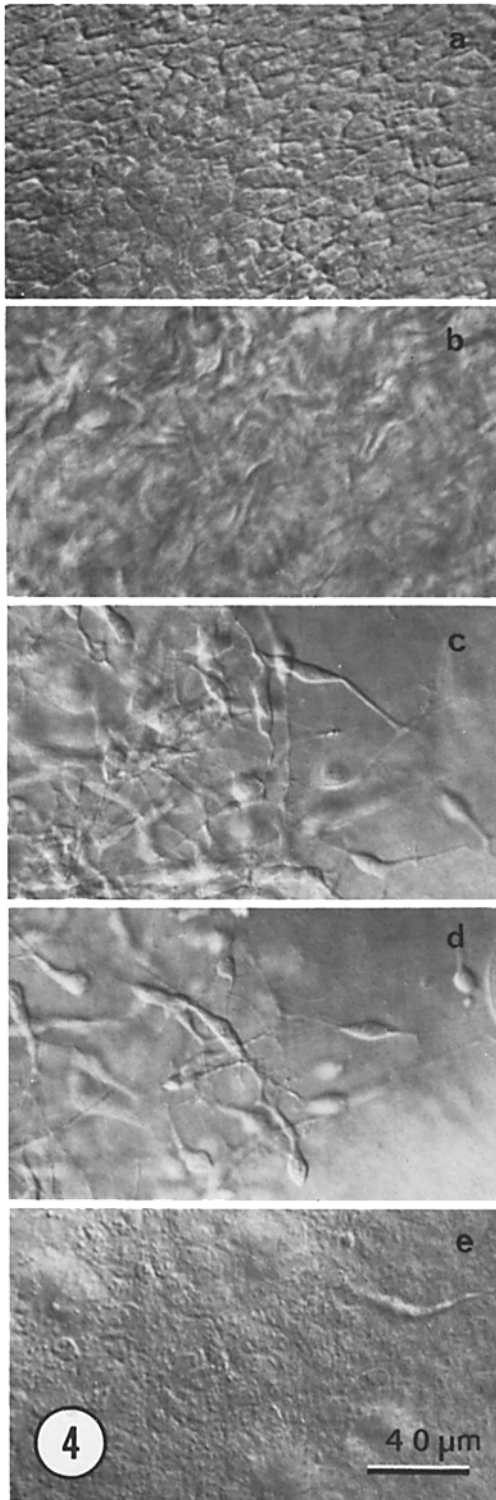


FIGURE 2 Light micrograph of a section of a stage 28 eye ($5\frac{1}{2}$ -6 days) showing a more advanced stage of fibroblast invasion into the swollen corneal stroma. The arrow calls attention to the sharp demarcation between the vascular (primary) mesenchyme below and the secondary (fibroblast) mesenchyme above. $\times 550$.

FIGURE 3 Light micrograph of a section of a stage 30 eye (7 days) showing a still more advanced stage of fibroblast invasion into the swollen corneal stroma. The arrow calls attention to the sharp demarcation stage 40 (14 days) and about 25 layers form; this is approximately the same number as there were layers of primary collagen fibrils. Mitosis also continues until stage 40. Half of a telophase appears at the arrow. $\times 475$.



~250 Å as opposed to the 1,000-Å diameter fibril normally encountered in connective tissue (21, 31).

The neural crest cells (fibroblasts) which appear at late stage 27 at the side of the eye in the stroma under the ectoderm (24) differ in their morphology from the existing mesenchyme (Figs. 1 and 2). The primary mesenchymal cells which will form the uvea are stellate in shape at this stage, whereas the fibroblasts (secondary mesenchyme) tend to be elongated in a plane parallel to the ectoderm. These fibroblasts are seen only in the stroma and do not appear to mix with the stellate cells of the mesenchymal shelf (arrow, Fig. 2). Interestingly, mitosis continues in the migrating fibroblast population (arrow, Fig. 3) up to stage 40, after which time cell migration and cell division cease, hyaluronic acid disappears, and the stroma condenses into its highly transparent adult form (21, 31).

Observations on Living Material

The morphology of the living cornea at stage light microscope with Nomarski optics (Fig. 4). If the specimen (inset, Fig. 1) is placed in the chamber epithelium side up, the epithelial cells are seen to form a cellular layer with a streamed appearance due to the slightly elliptical shape of the basal cells (Fig. 4 *a*). The long axis of these cells is perpendicular to the choroid fissure (31). It is difficult to see the very thin peridermal cells. Directly under the epithelium is an area of stroma which remains condensed (Fig. 4 *b*). This stroma is not colonized by fibroblasts and will eventually become Bowman's membrane (21). Fibroblasts can be seen directly beneath this layer. The optical sectioning ability of the Nomarski microscope is displayed to good advantage here, for the fibroblasts present in the periphery of the stroma at this time do not interfere optically with one another (Figs. 4 *c* and *d*). This narrow depth of focus is a mixed blessing, as it makes it difficult to see all the processes of a cell in a single field and renders it extremely hard to study more than one cell at a time. With appropriate focusing, the endothelium some 50–100 μm below the epithelium can be

FIGURE 4 A series of Nomarski optical sections of a living stage 27 cornea: (*a*) the epithelium, (*b*) acellular collagenous stroma, (*c* and *d*) fibroblasts in swollen stroma, and (*e*) the endothelium. The specimen was placed epithelium side up in the chamber and is about 75 μm thick. × 310.

clearly seen (Fig. 4 *e*). The cells are closely pushed together in the endothelial monolayer and it is difficult to make out the cell boundaries, even if the specimen is placed endothelium side up in the chamber. Interestingly, the living cornea is about 25% thicker as measured by Nomarski optics than the fixed and sectioned cornea.

The fibroblasts migrate in from all sides of the cornea, although they appear to come in first from the ventral surface near the choroid fissure (arrow, Fig. 5 *a*). They migrate in a sufficiently synchronized manner to give the impression of colonizing the stroma much as an iris diaphragm closes (Fig. 5 *b*), but in fact the individual cells are moving in a radial direction (Figs. 1 and 2). By stage 29–30, the cornea is so filled with fibroblasts (Fig. 3) that it is no longer possible to see the cells clearly in the Nomarski microscope. The large amount of matrix and number of cells present cause the light to be scattered, and the interference between the two polarized beams responsible for the Nomarski effect is no longer controllable. The fibroblasts do not remain passively in the cornea, but begin to build onto the complex lamellae of orthogonally arranged collagen fibrils that will comprise the adult stroma.

Corneal Fibroblast Morphology and Movement In Situ

Living fibroblasts in the stroma observed with Nomarski microscopy present a range of morphologies. Most are elongated and have extended cytoplasmic processes on their leading edges. These processes may be one or two substantial pseudopodia or several finer filopodia; in the former case the process may arborize into a number of fine filopodia.¹ The posterior end of the

¹ Our terminology for cell processes is based on morphology and largely follows that of Trinkaus (34). Pseudopodia and filopodia refer to cylindrical processes that may be found on either the leading or trailing edge of a cell. If there is a clear major process, this will be called a pseudopodium; the term filopodium is reserved for smaller, thin processes that usually sprout from a well-defined pseudopodium although they may arise directly from the cell body. A lamellipodium is a flattened cell process that can range in size; it may arise directly from the cell body and be a major feature of the cell or it may be only a little larger (wider) than a filopodium. These flat cell processes may or may not ruffle at their leading borders. A ruffle is a piece of cytoplasm, attached to the cell and enclosed by plasmalemma, that rises up away from the substratum. Trailing

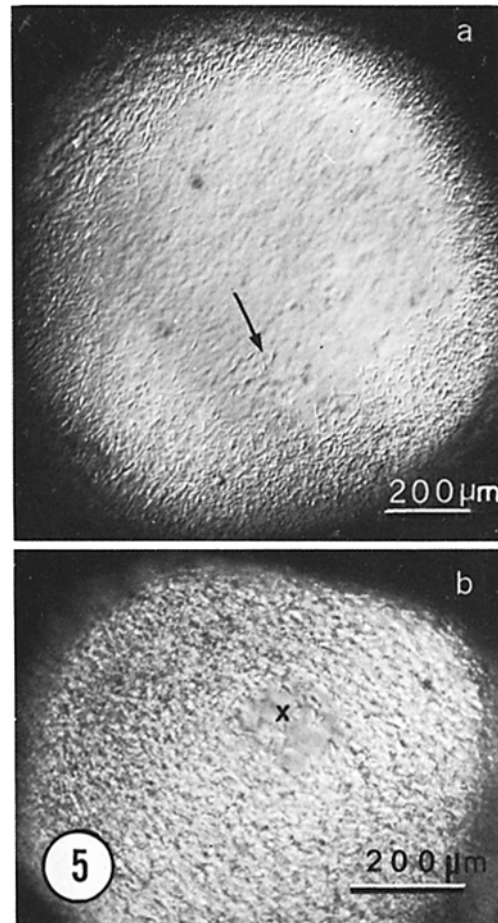
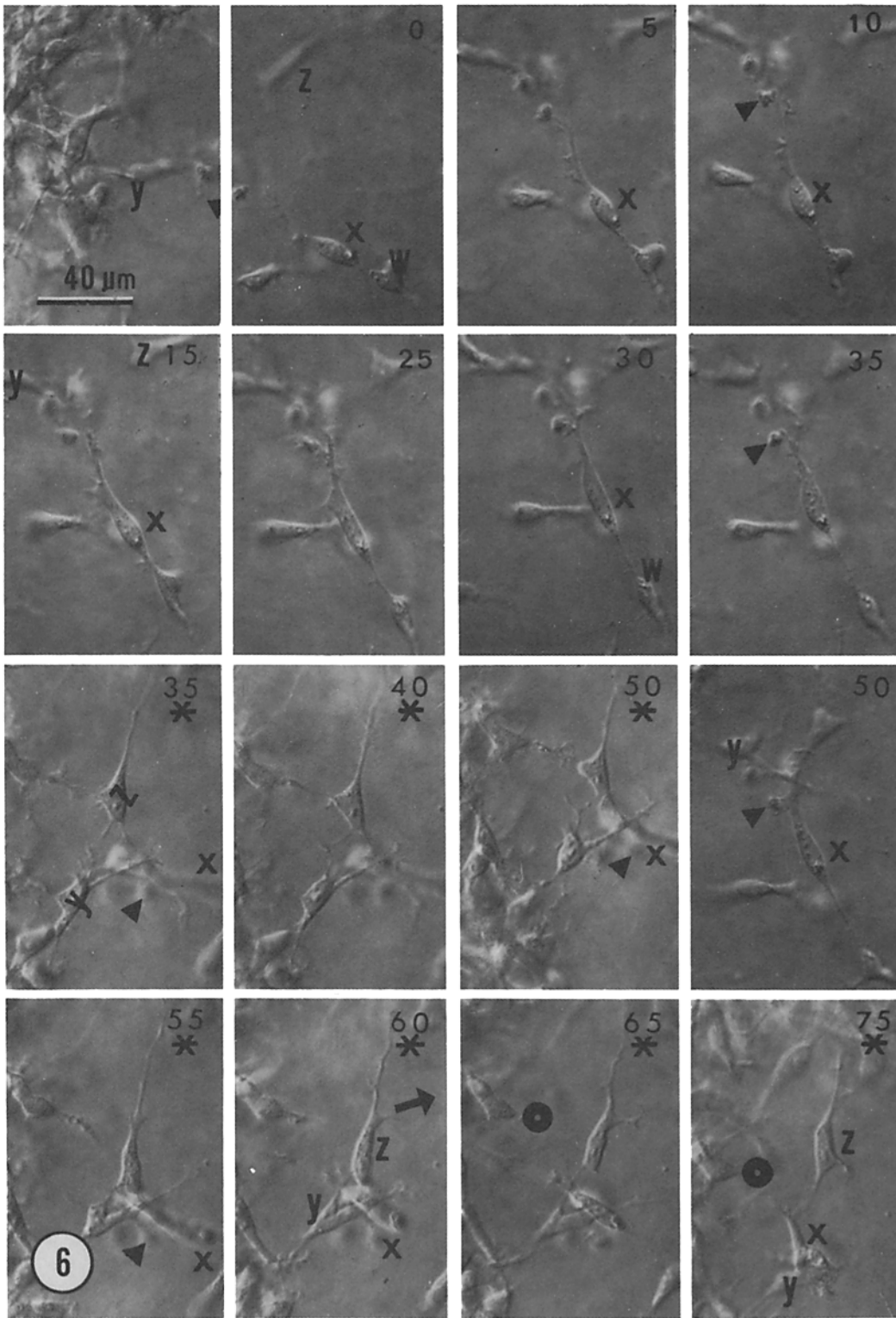


FIGURE 5 Low power Nomarski light micrographs of the corneal stroma at stage 27 (*a*) and stage 28 (*b*) showing the fibroblasts migrating in. In both cases, the eyes were oriented so that the choroid fissure is at the base of the picture. These specimens are too large for the microscope tube, and so not all of the cornea can be seen. The cells migrate in radially from all points of the corneal circumference, but the choroid fissure area provides the first cells (arrow). By the end of stage 28, the whole stroma is colonized except for the small area just above the center of the eye (*x*), but cells are still actively entering from the periphery. Fig. 5 *a*, $\times 56$; Fig. 5 *b*, $\times 76$.

cell processes are cytoplasmic extensions on the rear ends of cells. They retain residual adhesions to the substratum and may break, leaving pieces of cytoplasm behind. They can be identified unequivocally only by observing the direction in which a cell is moving. Blebs (18) are hemispherical herniations that can arise on any part of the cell membrane; they have diameters ranging from 2 to 10 μm .



cell often has a trailing cell process; it tends to be thinner and less arborized than leading processes. If there is no trailing process, the cell body is pear shaped (Fig. 6, time 0, cell *x*).

The range of morphologies reflects the dynamics of cell movement, for there is nothing to suggest that the population of fibroblasts is itself not homogeneous (21). Indeed, a single cell in the stroma may, in turn, take up any shape (Fig. 6, cell *x*, times 5–35 min). A cell process extends forward, makes an adhesion, and the cell body moves forward into the process (compare the position of the nucleolus labeled *x* in frame 15, Fig. 6, with its

position in frame 30 in relation to the cell traveling transversely across the field). Cells moving at this slow rate (the cell body moved 10 μm in the 15-min sequence just referred to, and the leading cell process advanced 10 μm in the same period) have a tapered trailing end. A second, more rapid type of movement of the cell body is observed which is accompanied by retraction of the trailing cell process and rounding up of the rear end of the cell (the cell body *x* moved 20 μm between time 55* and 60*, Fig. 6).

The leading processes of the moving cells seem to extend themselves in several ways: the broad

FIGURE 6 This plate shows a sequence of photographs of fibroblasts in a stage 28 cornea *in situ*, taken at 5-min intervals at two planes of focus (the second focal plane is marked by an asterisk). The first sequence of pictures (without the asterisk) shows the movement (to the left) of a cell (labeled *x* in frame 0). The parent of this cell divided some 25 min earlier into cells *x* and *w*. The second sequence of pictures (with the asterisk) shows two cells (*y* and *z*), which are out of focus in the initial picture, moving toward each other eventually to collide and show contact inhibition. The small protuberances on the cell bodies are probably cell organelles rather than blebs on the cell surface, as their position barely alters. Nucleoli tend to be well defined. Nomarski optics give a three-dimensional characterization to local changes of refractive index, whether they occur inside or on the surface of the cell.

In the first sequence of pictures, it can be seen that cell *x* initially has a pear-shaped body with a single long process extending in front of it (time 0). This anterior process on the leading edge is opposite the point where the fibroblast (*x*) is in contact with its sister cell (*w*). The anterior process elongates, developing many small filopodia along its lateral as well as leading edge (time 5). During the next 30 min, the cell body moves up (flows) into this process leaving a trailing cell process between it and cell *w*; cell *w* is moving in the opposite direction. By comparing the location of the stromal mass labeled by the triangle in frames 10 and 35, it can be seen that the cell body advances 10 μm in this time. During this period, the leading edge advanced continually and branched at one point (time 15); the branch withdrew (times 25–30), possibly as a result of a collision with a cell in the stroma below the plane of focus.

The second set of photographs (*) starts 35 min after time 0. In this plane of focus (times 35*–75*), the photographs have been rotated about 70° counterclockwise to fit the pictures onto the plate (compare location of cells *x*, *y*, and *z* at times 15 and 35*). Between time 0 and time 15, *y* and *z* travel towards each other, and between time 25 and 35, they meet. Both cells (*y* and *z*, time 35*) have on their leading edges several long thin filopodia a few microns in diameter and 5–25 μm in length. Each cell also has a process on its trailing edge. After 5 min (time 40*), the major set of leading cell processes on the left meet and touch. The cells which once were moving rapidly toward each other (time 0–35) stop moving toward each other (time 35*–75*). 10 min after contact (time 50*), it can be seen that the left-hand processes are still touching, whereas the right-hand leading process of cell *y* and its cell body have extended to the right past the particle labeled by the triangle. This process continues to extend to the right, pulling the body of cell *y* to the level of the triangle (time 55*). The left-hand cell processes remain in contact until about frame 60*, when the contraction of cell *z* to the upper right pulls it away from cell *y*, leaving a trailing cell process behind (time 65*). In frame 35*, cell *z* was moving toward cell *y*; in frame 65*, it is moving away from cell *y*, having developed an additional cell process on its new leading edge (arrow, 60*). At time 75*, cell *z* has moved dramatically into the leading cell body past the cell labeled by the circle, and the trailing cell process that was attached to cell *y* has probably broken.

In the meantime, cell *x* has continued to move past particle labeled by the triangle towards cells *y* and *z* (see 35 and 35*). Its leading edge passes under the immobilized left-hand processes of cells *y* and *z* (time 55*) and seems to attach to the stroma to the left of them. Between time 55* and 60*, the rear of the cell labeled *x* contracts to the level of the mass labeled by the triangle, probably because the trailing cell process broke away from the substratum. After the last photograph, the tissue (which was photographed over 2 h *in situ*) began to die. The center of the cornea is to the right. Time in minutes in upper right of each frame. $\times 370$.

pseudopodium may increase in length and arborize; alternatively, the filopod simply extends, at a rate of 1–2 μm per min. It is of interest that, at the resolution of these micrographs (Fig. 6), there was no evidence of the flattened ruffling lamellipodium (ruffled border phenomenon) that characterizes fibroblast morphology on plastic.

We were fortunate to be able to observe in good focus a direct collision between two cells within the three-dimensional corneal stroma illustrated in Fig. 6 and could thus see whether or not contact inhibition of movement (CIM) occurred *in situ* as reported in vitro (3). In this case (and in collisions observed in artificial collagen lattices), the cells met and touched filopodia (see cells *y* and *z*, Fig. 6,

times 35*–50*). The filopodia stopped moving, and the touching processes seemed to form a temporarily stable adhesion (see legend of Fig. 6 for detailed description). After this cessation of movement, other cell processes became active: in one cell, the trailing process started to move and branch (cell *z*, times 35*, 60*, Fig. 6) and the cell reversed its direction of migration; in the other cell (*y*, times 35*, 60*), the fibroblast cell body moved away from the inhibited process into a second leading filopodium to the right. The conclusion to be drawn from this set of observations is that CIM can occur *in situ* in collisions between two migrating cells.

A further comment on contact phenomena oc-

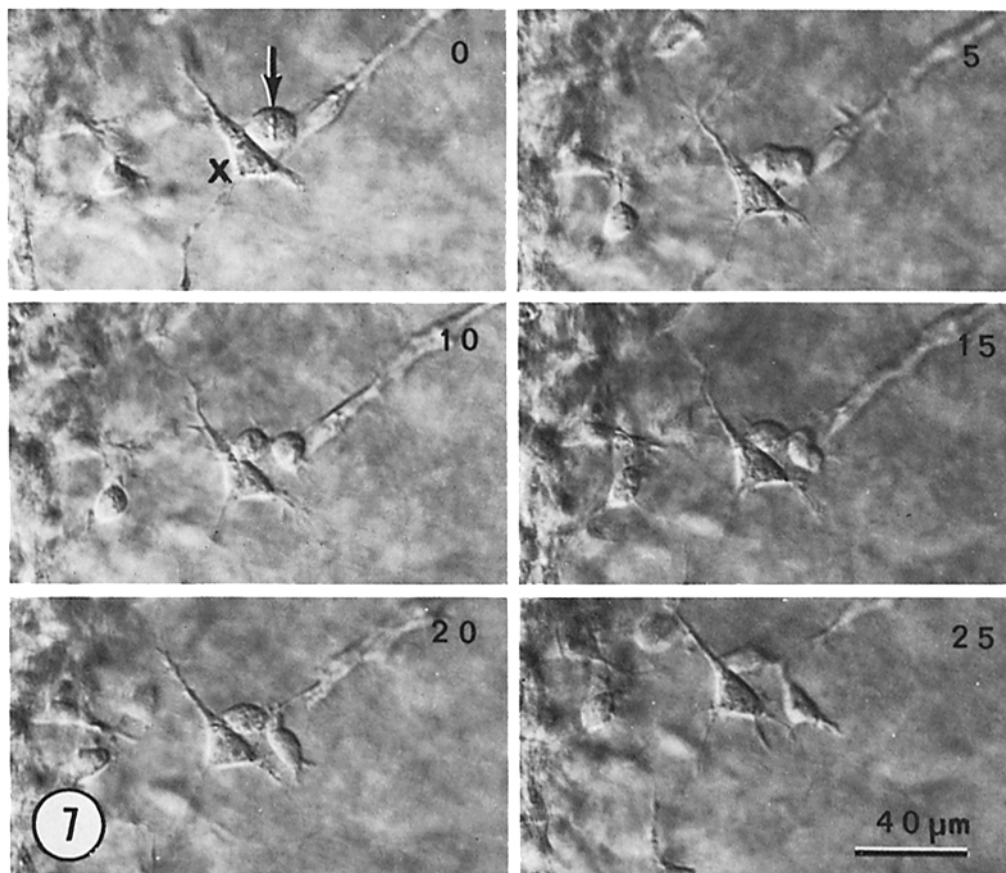


FIGURE 7 A rounded-up cell in the corneal stroma (stage 28) undergoing mitosis *in situ* (metaphase plate at the arrow, time 0). Between 5 and 10 min, the cells separate and put out fine filopodia opposite each other. During the next 15 min, the cells change their shape several times and start to spread. Note that there are no blebs *in situ* of the type that would be visible in vitro at this end stage of mitosis. The overlying cell (*x*, time 0) has the typical morphology of fibroblasts in the stroma *in situ*: there are several pseudopodia extending from the cell body, and from these small filopodia extend and withdraw. This cell does not move at all, but we watched an underlying cell (which is out of focus in these photographs) move its body some 20 μm over the period under observation. Nomarski optics. Time in minutes in upper right of each frame. $\times 370$.

curring between cells *in situ* is provided by looking at mitosis. In the course of scanning the stroma, one occasionally comes across pairs of rounded-up cells that have just divided; these cells eventually move away from each other. An example was seen in the previous figure: cells *x* and *w*, which divided 25 min before time 0 (Fig. 6), moved away from each other. In the course of photographing a fibroblast (cell *x*, Fig. 7) which did not move, we happened to film a rounded-up cell undergoing cytokinesis over a period of about 20 min. The metaphase plate (arrow, Fig. 7, time 0) and its subsequent separation into two sets of chromosomes could be seen. The separate cells put out filopodia at points of the cell surface almost diametrically opposed to the region where the cells separated (Fig. 7, times 10–20) and moved away from the region where they once contacted each

other. The cells underwent several changes of shape during the period of observation, but at the resolution of the photographs, interestingly, there were no blebs to be seen on the surfaces of the dividing cells. This contrasts sharply to *in vitro* observations, where gross blebbing of the cell surface is a striking component of the end stage of mitosis.

Corneal Fibroblast Morphology and Movement *In Vitro*

ARTIFICIAL COLLAGEN LATTICES: 24 h after a living stromal fragment has been placed within a tendon collagen gel, fibroblasts have migrated out from the tissue to a distance of about 2 mm from the fragment, suggesting that the maximum rate of movement is about 1 μm per

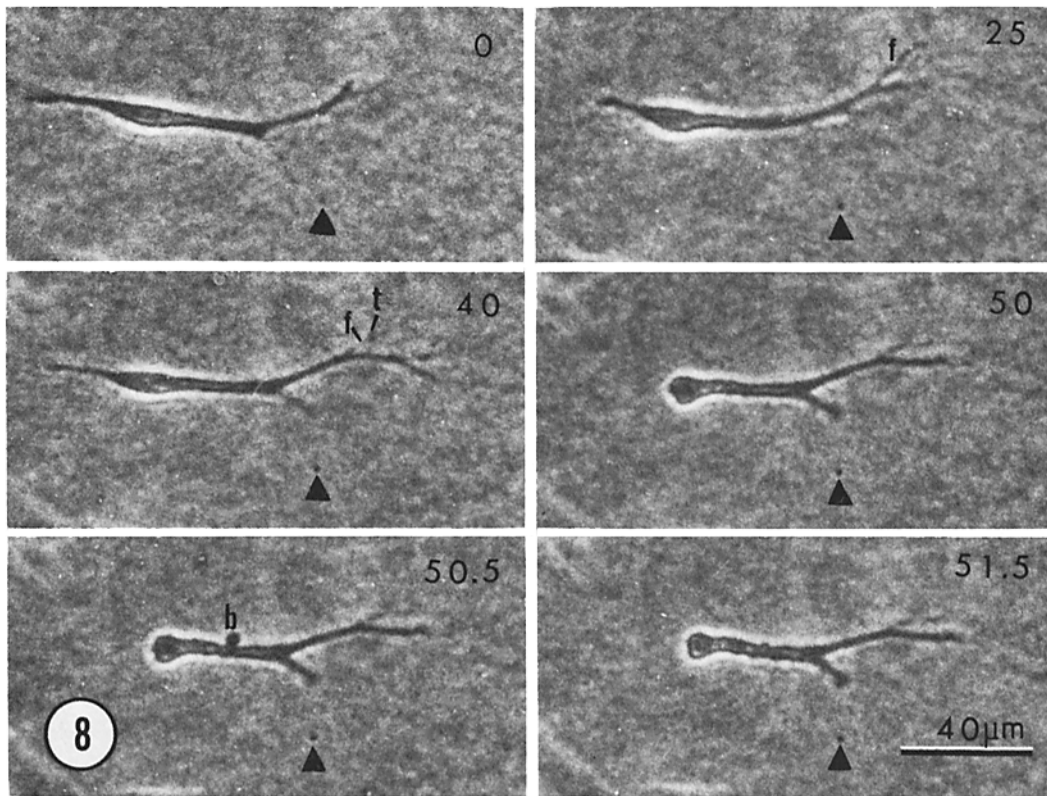
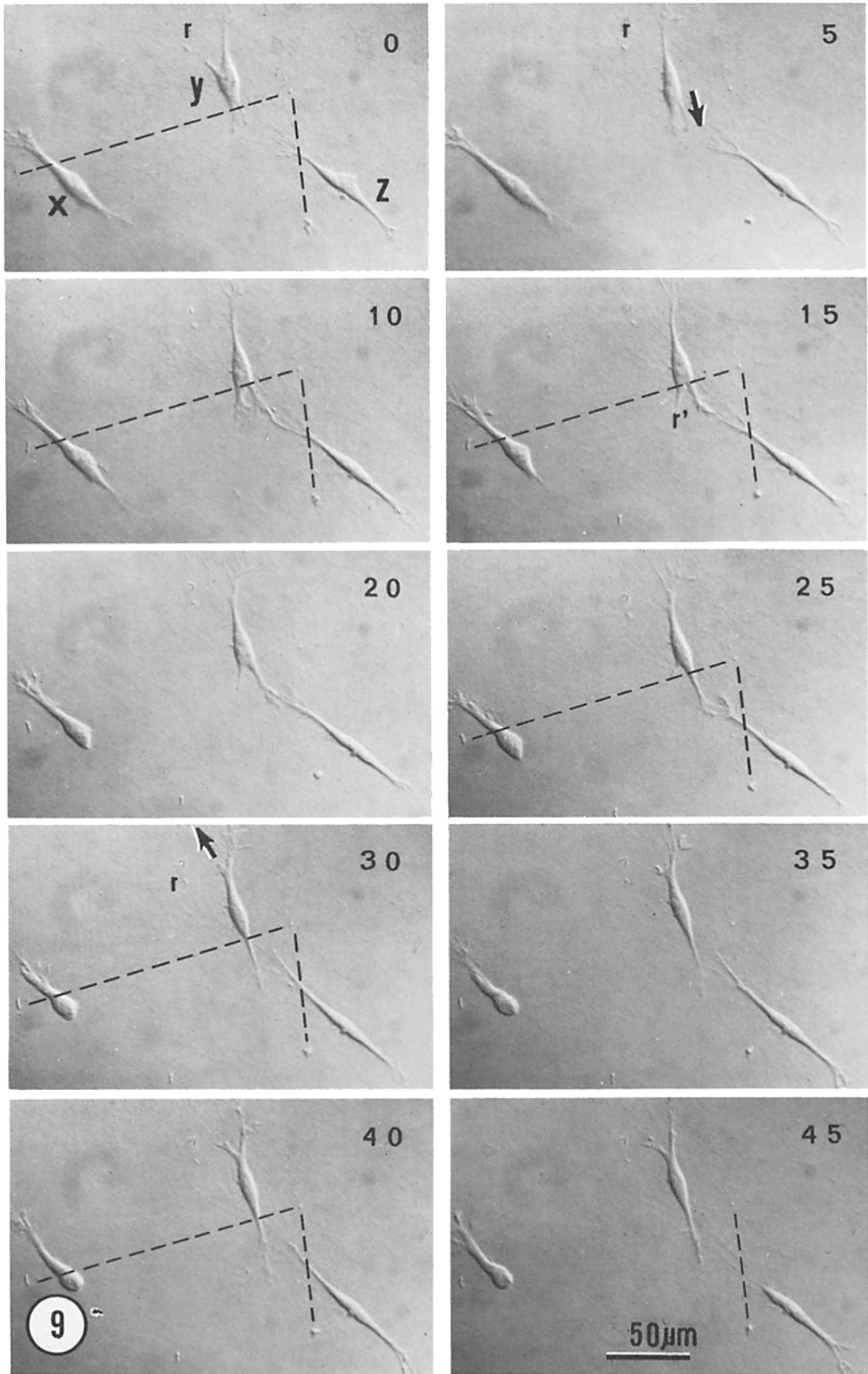


FIGURE 8 A series of phase contrast micrographs of a corneal fibroblast moving in an artificial collagen lattice (an *in vitro* collagen gel). The photographs are taken from a 16-mm film (lapse rate: 2.5 s). The sequence shows the essential characteristics of cell movement in the lattice as this fibroblast passes the particle in the gel labeled by the triangle. Slow pseudopodial extension (time 0 and 25) is followed by relatively rapid cell body contraction (recoil) after withdrawal of the trailing process (times 40–50). The small subsidiary processes (filopodia) that project from the leading pseudopodium can be very fine (*f*, time 25), changing in thickness as at *f* and *t*, time 40. During contraction of the cell body, a bleb (*b*) is put out and, within 1.5 min, retracted again. Time in minutes in upper right of each frame. $\times 470$.



min. The migrant cells tend to disperse, although occasional pairs of cells are seen which probably represent recent cell divisions.

Corneal fibroblasts are similar in appearance to the human embryonic lung fibroblasts described in a similar culture system (11). The cells are usually bipolar with long pseudopodia at either end (Figs. 8 and 9), although sometimes there is a third or even a fourth process branching out from the base of a pseudopodium. Cell processes are broad near the cell body, but split into a number of finer filopodia towards the ends. Time-lapse films (lapse rates of 2–5 s) show that the cell processes are in motion: they can extend and then contract pulling the cell forward, or they may merely move to and fro (*f.* Fig. 8). We saw no ruffling, that is, upward movement of cell processes away from the substratum; since the cells are completely surrounded by substratum, it would be difficult by definition¹ for ruffling to occur.

As a consequence of the extending and retracting of cell processes, small blobs of cytoplasm may be left behind (*r*, times 0, 5, Fig. 9). These probably result from firm attachment of the tip of a trailing process to the collagen fibrils. Even *in situ*, pieces of cytoplasm seem to be broken off here and there (triangle, Fig. 6).

We have not observed significant differences between the mode of movement of fibroblasts in the stroma *in situ* and that of the same cells in the artificial collagen lattices, but in general it is easier

to photograph fine cell processes and their movement in collagen gels than in the corneal stroma itself. Bipolar cells with single active anterior cell processes move by extending the pseudopodium away from the cell body (times 0, 25, Fig. 8); the cell body then seems to start flowing towards the end of the pseudopodium as observed *in situ*. Both ends of the bipolar cell may show activity. Thus, movement in a particular direction appears to result from the dominance of one end over the other.

As is the case *in situ*, the posterior part of the cell can also move forward accompanying a rapid contraction of the cell into the forward cell process (times 40, 50, Fig. 8). It is easy to see in the cultures that the posterior cell process retracts before or at the same time as the cell body moves forward. Thus, the apparent contraction of the cell body may be a recoil. During the recoil, interestingly, there is little or no forward extension of the leading pseudopodium (Fig. 8; cell *x*, Fig. 9).

We photographed several collisions between cells with bipolar morphology in collagen gels. The fibroblasts touched their leading cell processes, and the cells withdrew and changed direction completely. Filopodia in contact with each other stopped moving, but the total effect was slowing rather than complete cessation of movement along the cell border in contact (cells *y*, *z*, Fig. 9). The posterior trailing processes became anterior ones, and vice versa. Indeed, one cell (lower right, time

FIGURE 9 Nomarski light micrographs of corneal fibroblasts (from a stage 28 cornea) moving in a collagen gel. They were photographed at 5-min intervals, as indicated in the upper right of each frame. The sequence shows the morphology and movement of a single cell on the left and contract inhibition between a pair of cells on the right. The single cell (*x*) is bipolar at the start (time 0), but between time 10 and 15, it begins to retract its trailing process and in the next 5 min changes from a bipolar to a pear-shaped morphology. In the next 20 min, there is a slight additional contraction of the cell body (compare relation of cell *x* to the horizontal line in frames 0, 25, and 40). In the hour following the end of this sequence, cell *x* became bipolar and started to move more actively.

The pair of cells (*y* and *z*, time 0) were initially moving rapidly towards the center of the frame, and they have very active processes on their leading edges. Between time 0 and time 5, a trailing process on cell *y* contracts leaving its tip behind (*r*). At time 10, a cell process (arrow, time 5) put out by cell *y* touches the leading process of cell *z*, and the cells stop moving (compare times 10 and 15). By time 25, cell *y* has retracted one of its formerly leading processes (*r'*, time 15). The opposite ends of the cells now become more active. Cell *y* produces a new cell process (arrow, time 30) on its former trailing edge. This edge is now the leading edge (compare its relation to the particle labeled *r* in frames 0 and 30). 35 min after the collision, cell *z* (lower right, frame 45) contracts in a direction opposite the contact zone. Note that the structure of the collagen is just resolvable and that these fibrils appear to align up along the cell processes. This is presumably a result of the stress that the filopodia exert on the matrix and is most clearly shown to the left of the dotted line in the last photograph. Time in minutes. The dotted lines have been drawn between stationary particles; these particles are undoubtedly pieces of cytoplasm left attached to collagen after the cell moved by (e.g., *r*, times 0 and 5). $\times 254$.

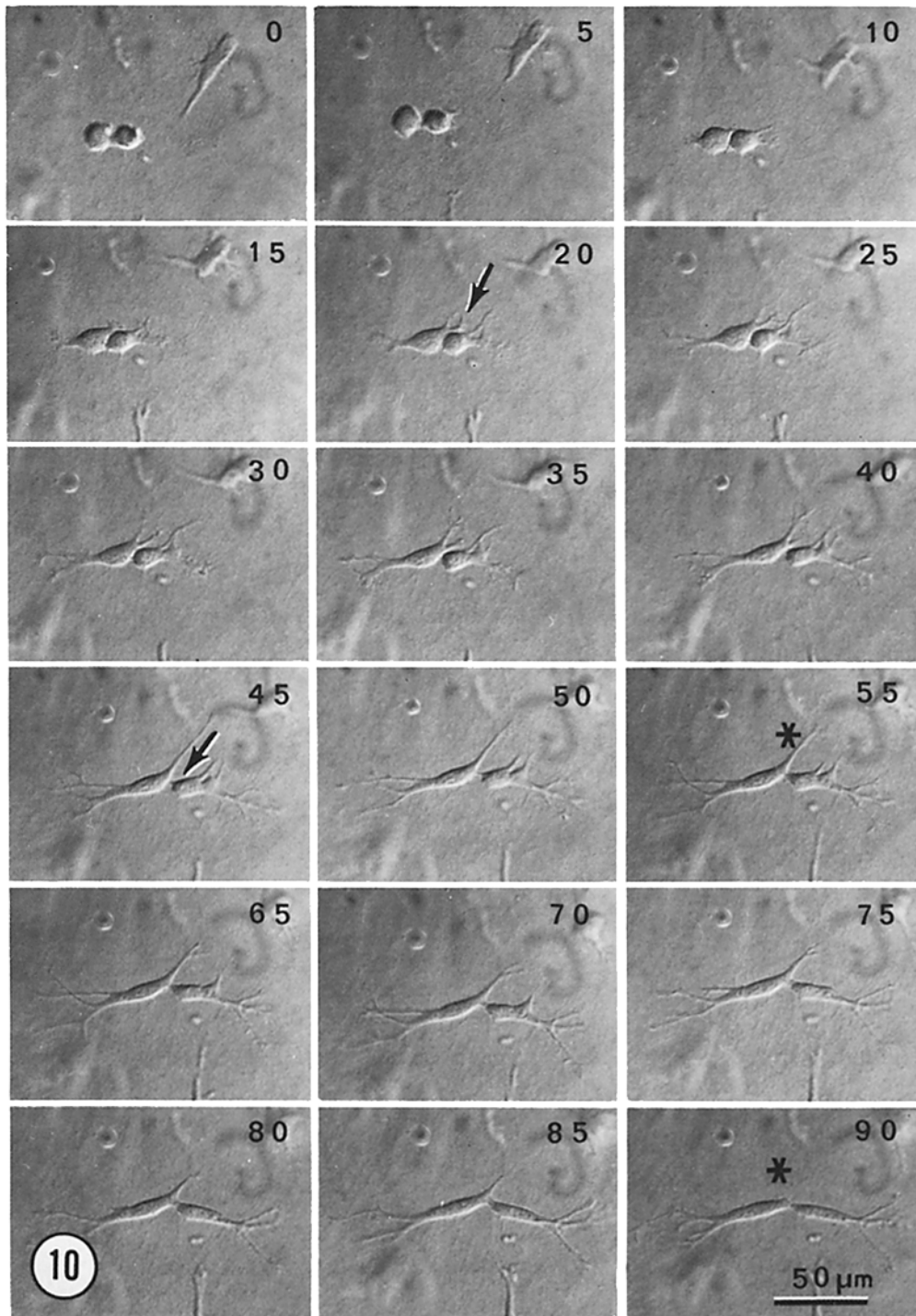


FIGURE 10 A sequence of Nomarski light micrographs of two fibroblasts in collagen gel that have just been through mitosis and are initially still rounded up (left center, time 0). The cells extend long thin filopodia and stretch out but, over a period of 90 min, do not move apart. At time 20, the cell on the left puts out a filopodium (arrow) just next to the other cell, but later withdraws it (arrow, time 45). It also withdraws a second cell process (*time 55-90) put out in that direction. The approximate rate of filopodium extension is a little over a micron a minute. Time in minutes in upper right of each frame. $\times 254$.

45, Fig. 9) withdrew its former leading process. We have not seen a collision where one of the cells was pear shaped at the start, probably because the major event occurring while the cell is pear shaped is recoil of the rear end, not movement of the anterior cell process forward.

These collisions demonstrate that the CIM that sparse fibroblasts display on glass occurs between isolated cells in collagen even though the cell morphology is different in the two situations. A further perspective on this phenomenon is provided by a chance observation on a dividing cell in a collagen gel (Fig. 10). The rounded-up cells in anaphase put out filopodia that, in main, were on the side opposite the cleavage furrow. As division progressed, one cell also put out a long thin process just by the newly formed second cell (arrow, time 20, Fig. 10), but, 25 min later, this process near the zone of contact of the two cells had withdrawn (arrow, time 45, Fig. 10). Subsequently, a second cell process (asterisk, time 55, Fig. 10), projecting from the first cell toward the second, is also withdrawn (asterisk, time 90). An additional phenomenon that we occasionally saw is also displayed by these two cells: although the major cell processes were very active, the cell bodies did not immediately draw apart.

GLASS SUBSTRATA: Fibroblasts migrate out rapidly from stromal fragments placed on glass cover slips. The appearance of these cells is markedly different from that shown by the same cells in collagen. The fibroblasts are now greatly flattened and have wide lamellipodia on their leading edges. Cell movement (Fig. 11) is accompanied by the extension and activity of the lamellipodium. Ruffling activity along the leading edges of these lamellipodia is a conspicuous feature in these cultures.

The cell body seems to flow into the lamellipodium at the same rate as into a leading pseudopodium (the spot labeled *x* in Fig. 11 moved 25 μm to the right in 35 min). The lamellipodium, however, may move faster (the leading edge at time 35, Fig. 11, is 50 μm in front of its location at time 0). The cells on glass also exhibit the rapid contraction of cell bodies seen *in situ*. A chance observation suggests that this contraction is secondary to loss of adhesion of the trailing cell process with its substratum. At time 10 (Fig. 11), the cell on glass has two trailing processes; the lower one (*r'*, time 10) loses its attachment and quickly disappears (time 15).

Stromal fibroblasts initially colonize available

substrata rather than overgrowing one area and thus form a monolayer. However, corneal fibroblasts on glass do not cease dividing at confluence, but form the orthogonal overgrowth patterns (Fig. 12) that are characteristic of other fibroblasts grown beyond confluence (12, 28).

A further observation is of interest. Immediately after transfer of the cover slips from petri dishes to the D-S chamber, transient blebbing of the fibroblast membranes was seen. These herniations on flattened fibroblasts were up to 5 μm in diameter, but rounded cells had even larger blebs. After an hour or so, this activity subsided. This behavior contrasted with that of the cells in collagen lattices where blebbing was rarely seen.

DISCUSSION

We report in this paper a study by Nomarski optics of the migration of corneal fibroblasts within their natural stroma and observations on the same cells in artificial collagen lattices and on glass substrata. We have found that cell behavior in the original stroma and in the artificial collagen lattices is very similar in spite of the known differences in glycosaminoglycan content and collagen organization between the two matrices. For the purpose of discussion, therefore, we group observations on fibroblasts in these two environments in order to compare them with observations made on the same cells on glass substrata.

A major reason for studying such interactions is to obtain insight into how cells move in normal tissue. We confirm the fact (7, 11-14, 26, 27) that pronounced ruffled borders² are, for mesenchymal cells, an artifact of flat *in vitro* substrata. We describe two types of fibroblast movement, one in which a bipolar configuration is maintained, and the other, a unipolar configuration due to contraction of the whole cell into the leading process. The most interesting new observation that we have made is that fibroblasts in three-dimensional collagenous matrices can show CIM of the same general type that fibroblasts show on glass and plastic. It is a limitation of the optics of this system that only pair-wise collisions between cells can be studied. In order to understand how cells behave in dense tissues, one must extrapolate from such limited data as can be obtained from studies *in vivo* and from information derived from mass cultures *in vitro*. We conclude the paper by discussing the possible roles that CIM can play in morphogenetic movements *in vivo* and *in vitro*.

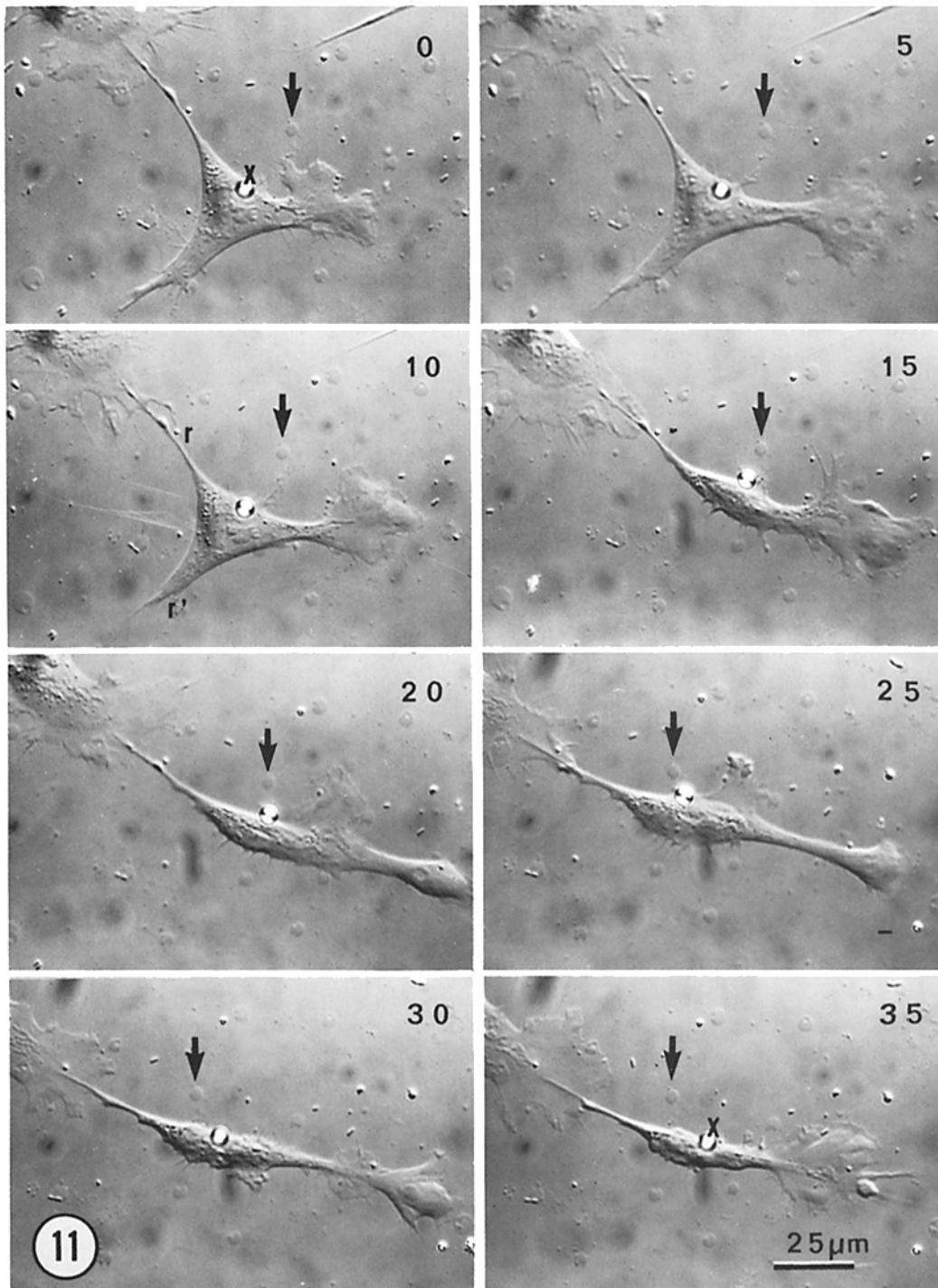


FIGURE 11 A sequence of Nomarski light micrographs of a stromal fibroblast moving on a glass cover slip past a particle on the substratum (marked by the arrow). The rate of movement past the particle can be appreciated by comparing, in frames 0 and 35, the relation to the arrow of a bubble (x) that was adherent to the cell surface. The black and white markings in the bubble are an artifact of Nomarski optics. The anterior part of the cell contains a wide lamellipodium which shows ruffling activity on its leading edge. In the course of the sequence, various parts of this lamellipodium ruffle and/or extend filopodia as the process moves to the right. Between time 10 and 15, a trailing cell process, *r'*, detaches from the substratum, and this part of the cell contracts (recoils) into the cell body. The trailing process labeled *r* is thus brought closer to the particle (arrow). The strong contraction of the cell body forward between frames 20 and 30 is not a recoil; it must be an active movement because trailing process *r* does not detach. Between frames 30 and 35, the leading cell process seems principally to be engaged in ruffling rather than forward extension, although the cell body itself is still moving to the right. Time in minutes in upper right of each frame. $\times 420$.

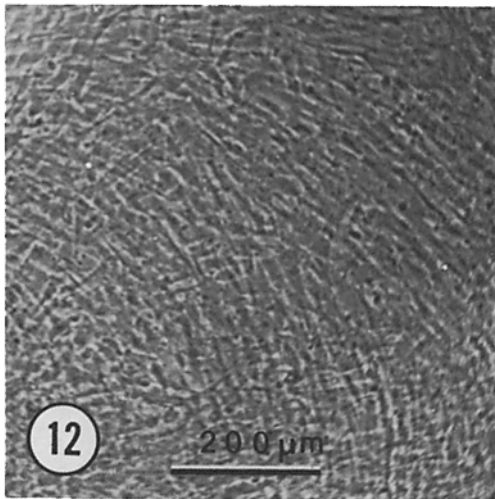


FIGURE 12 A low power phase-contrast light micrograph of stromal fibroblasts on plastic demonstrating that these cells do not cease growth at confluence but form orthogonal overlays. $\times 100$.

Fibroblast Morphology and Movement

The results show that corneal fibroblasts in situ or in artificial collagen gels usually have one or two long pseudopodia which end in multiple fine processes or filopodia. On glass, in contrast, these cells are flattened and have broad leading lamellipodia that ruffle. These differences can best be understood as deriving from the cell's adaptation to different environments. The glass or plastic substratum is flat and highly charged, encouraging the cell to adhere to it to the greatest possible extent. In addition, the plastic substratum is featureless; cell movement is characterized by a leading edge that rises up and away from the flat surface (22). In the collagen matrix, however, the environment is very different: the cell is embedded in a three-dimensional jungle of fine collagen fibrils (with strong electrostatic charges) and its morphology possibly reflects the equal pull in all directions by the surrounding substratum. The long, thin filopodia probably adhere directly to the collagen fibrils, for the moving cell can be seen exerting a stress on the lattice. In addition, there is no other source of physical support for the moving cell. The nature of the adhesion to the collagen is still obscure though some insight is to be obtained from the fact that these adhesions are not trypsin-sensitive as are the cells' adhesions to plastic (11).

In spite of the differences between cell morphol-

ogy in collagen and that on glass, there are similarities in the movement. Both in the elongated (generally bipolar) cell in collagen and in the flattened cell on glass with its ruffling lamellipodium, the leading process extends, makes a new adhesion, and then the cell body "flows" into it (contracts toward it). The posterior end of the cell, in both cases, moves forward most dramatically when the trailing cell process breaks its adhesion to the substratum; when this happens, the cell body jerks forward. In this, the behavior is similar to that of the ascidian tunic cells (23). This manner of movement suggests that the adhesions of the leading edge to the environment can be considerably stronger than those of the trailing edge.

In some cases, cell processes may break when they retract, leaving cytoplasm behind in the stroma. Depending on the number and strength of the adhesions constantly being broken and remade, movement may be slow and smooth, or abrupt, in the direction of the leading edge.

The most important similarity between the cells that overrides the morphological difference in their leading edge is in their contact interactions. When one cell meets another in an isolated collision, leading cell processes stop moving and cell processes distal to the point of collision become motile, causing the cells to move off in new directions.

Contact Interactions

In the few collisions between cells in a three-dimensional matrix that we were able to watch, the touching filopodia of the contacting pseudopodia stopped moving and other pseudopodia took over as organs of locomotion. These cells thus showed CIM in the sense that contact inhibited their movement (2), but not in the sense that the cells were unable to use one another as a substratum (1, 4); this distinction will be seen to be significant. There do appear to be slight differences between CIM in collagen and that on glass: after collision in three dimensions in collagen, trailing processes become leading ones whereas following contact between cells on glass, there is no movement until an apparently random portion of the cell membrane develops ruffling activity (2). Nevertheless, it is clear that the morphological differences between cells on glass and in collagen do not affect the functional ability of cells to show CIM in the two environments.

Further insight into the contact interactions between cells comes from observations on dividing

cells. The persisting cell contact after cytokinesis tended to inhibit not only filopodial activity, but also filopodial formation itself. In the divisions that we observed, cells put out processes that were, in the main, opposite the region of cleavage. This suggests again that intercellular contact inhibits cytoplasmic movement. Interestingly, in some cases, touching cell processes seemed to form temporary adhesions with the surrounding collagen that tended to inhibit the cells from drawing apart. Moreover, we did not observe in anaphase and telophase *in situ* the dramatic blebbing of the cell surface visualized *in vitro*.

Interactions between only two isolated cells *in vivo* are rare. Normally a cell is in contact with many neighbors, and in order to understand cooperative interactions between many cells we must extrapolate beyond the data reported here. We now try to show how CIM can help explain some of the mass properties of morphogenetic movements *in vivo* and *in vitro*.

CIM

A principal aspect of fibroblast behavior *in vitro* for which CIM is classically introduced as an explanation is monolayering, that is, the tendency of cells to colonize available substratum rather than to overgrow themselves. The argument is based on the classic observation (1, 4) that, in pair-wise collisions, a cell tends to change its direction of movement rather than migrate over a second cell. This, in turn, has been interpreted to mean that one cell will not use a second as a substratum. The fact that corneal fibroblasts can multilayer on glass as well as *in situ* may be due in part to the secretion of a thin coat of extracellular material (ECM) on their surfaces with time *in vitro*, so that contact inhibition is prevented (20). In lung fibroblast cultures, also, cells initially monolayer (11, 12). Supernumerary cells have been shown to use one another as substrata for adhesion and spreading (13) and they form orthogonal patterns (12) like those demonstrated here with corneal fibroblasts. Carter has suggested (8) that the initial monolayering in such cultures is due not to CIM, but to preferential adhesion of the cells to the plastic. Harris has, however, disputed this (18, 19). It may be that more attention needs to be paid to the presence or absence of ECM on cell surfaces *in vitro* (20).

Contact inhibition of movement is also used as

an explanation of the direction of migration taken by cells moving *in vitro* or *in vivo*. Thus, cells show a radial outgrowth from the original explant *in vitro* (4, 35) and move away from the crowded primitive streak *in vivo* (20). Within the crowded tissue, cells may show some degree of movement, but this is slight compared to the peripheral movement. The results of Armstrong and Armstrong (6) suggest a net maximum directed displacement of $\sim 100 \mu\text{m}$ in 3 days within mesonephric mesenchyme. This is to be contrasted with a maximum movement of $\sim 50 \mu\text{m/h}$ in cell-sparse collagen gels *in vitro*.

There is, then, little doubt that cells on an unrestricted periphery move much more readily than cells in the center of an explant (3). Abercrombie's suggestion (1) that CIM will encourage cells to colonize empty matrix seems borne out by the present study of corneal fibroblasts moving in collagen gels and *in situ*. What is seen is that cells in contact tend to repulse each other, colonizing the gel at different levels, but always moving away from the densely populated explant.

In the context of the cornea *in situ*, the observation of CIM predicts well the movement inwards of fibroblasts as they invade the cell-bare stroma. In the *in vivo* situation, however, a number of additional questions arise in terms of the problems facing the corneal fibroblasts as they migrate from the neural crest. Do they show CIM in their initial journey? If so, why do they reach the cornea as a group of cells rather than as widely separated individuals? If not, do they change cell surface properties on reaching the swollen cornea at stage 27/28 and are such changes due to the changing glycosaminoglycan make-up of the ECM? Clearly, we need to take into account numerous factors that may be operating together in the intact embryo. Nevertheless, the present paper does establish for the first time the existence of the phenomenon of CIM among migrating fibroblasts in the intact corneal stroma; the phenomenon can be invoked with some assurance to explain certain aspects of cell movement *in vivo*.

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