CLONED PIGMENTED RETINAL EPITHELIUM

The Role of Microfilaments in the Differentiation of Cell Shape

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ABSTRACT

3-wk-old clones of pigmented epithelial cells from chick retina can be divided into four zones on the basis of cellular morphology and pigmentation. These zones appear to represent different stages in the re-expression of differentiation: those cells with essentially no differentiated characteristics are at the outer edge and those with the greatest number are at the center. Cells of the colony exhibit three different types of movement when analyzed by time-lapse cinemicrography: focal contractions, extension and retraction of apical protrusions, and undulations of the lateral membranes. All the cells of the colony contain microfilaments, 4–7 nm in diameter, which are primarily arranged as apical and basal webs. In addition, less well defined filamentous networks are found in the apical protrusions and lateral interdigitations. When colonies are treated with 10 μg/ml of the drug cytochalasin B (CCB), the apical microfilament arrays are disrupted and movement stops. Both phenomena are reversible upon removal of the drug.

During the process of redifferentiation, the cells change their shape from squamous to cuboidal, and the greatest change is found where the colony exhibits the greatest number of focal contractions. The evidence suggests that the apical microfilament arrays are directly responsible for the observed movements, particularly the focal contractions, and that focal contractions contribute to the development of the differentiated cellular shape. Possible roles for the other movements are discussed.

KEY WORDS pigmented epithelium · cell differentiation · clonal culture · cytomorphology

Single cells dissociated from 8-d-old chick pigmented retinal epithelium can be grown in clonal culture under conditions which permit the expression of their differentiated characteristics (8, 9, 13, 27, 28). Under the culture conditions described in Materials and Methods, the cells attach to the culture dish, lose their pigment and characteristic polygonal shape, and proliferate rapidly to form small disk-shaped colonies of squamous cells. Over the course of several weeks, the cells in the center of the colony regain their characteristic differentiated cuboidal shape and begin to synthesize large amounts of pigment while those at the outer edge remain squamous in shape and unpigmented (8, 13–15). After 3 wk in culture, these colonies are ~10 mm across, and four distinct zones can be distinguished. From the outside to the center these are squamous, strati-
fied, intermediate and pigmented cuboidal zones, respectively (15). These zones appear to represent different stages in the re-expression of differentiation, and the most differentiated cells are found in the center of the colony (14).

One of the most striking aspects of the redifferentiation process is the development and maintenance in vitro of the cellular shape characteristic of differentiated pigmented epithelial cells in vivo. Previously, electron microscopy, time-lapse cinephotomicroscopy, and the drug cytochalasin B (CCB) were employed to demonstrate the presence of arrays of thin (4-7 nm) microfilaments in the cells of the pigmented cuboidal zone, and to determine their role in maintaining the shape of these cells (15).

The present paper describes the movements and the morphological changes of cells in the periphery of the colony during redifferentiation. It further details the relationships between the arrays of microfilaments and the observed cellular movements to determine the role of the microfilaments and movements in the development of the differentiated cellular shape.

MATERIALS AND METHODS

Culture Technique

Sheets of pigmented epithelium isolated from 8-d-old embryonic chick eyes were dissociated into single cells and grown in Ham's F10g medium containing 4% fetal calf serum and 1% Sephadex G-25 excluded fraction of embryo extract (F10g H0.1) by the method described previously (8, 15). Colonies for transmission electron microscopy (TEM) were grown directly on 100-mm Falcon plastic tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). Those for time-lapse cinephotomicrography were grown on sterile washed coverslips in 35-mm nontissue culture dishes which were inoculated with 1.0 × 10^4 cells in 1 ml of medium, rather than 2.0 × 10^3 cells, because the plating efficiency was lower on the glass coverslips.

CCB Studies

The ultrastructural effects of CCB on cells in 3-wk- old colonies were studied by treating six tissue culture dishes, each of which contained between 6 and 15 colonies, as follows: two dishes were incubated in normal growth medium (F10g H0.1), two dishes were incubated in growth medium plus 1% Dimethylsulfoxide (DMSO), two dishes were incubated in medium plus 1% DMSO and 10 μg/ml CCB. After 12 h, the dishes were removed from the incubator and rinsed twice with saline G. One dish from each group was fixed and embedded for TEM by the methods described previously (15). The others were fed growth medium and returned to the incubator for a further 12 h and again washed with saline G, fixed, and embedded for TEM.

Cinephotomicrography

Suitable chambers for cinephotomicrographic studies which contained no heavy metals were constructed from 60-mm Falcon plastic Cooper dishes as follows: To provide a nonpolarizing light path and a specimen-to-coverslip distance suitable for use with Nomarski interference phase-contrast microscopy, the sides of the plastic dishes were shortened to 11.5 mm and the bottom of the lower half of the dish was replaced with either a glass slide or a coverslip. These were cemented to the bottom of the dish with Epon 812 (24). To complete a glass optical path, a window was also cut in the depression in the upper half of the dish, and a coverslip of the desired thickness and size was mounted inside. To maintain the correct temperature, pH, and humidity, the edges of the chamber were sealed into a plastic bag which was continuously gassed with humidified CO_2 and air. The entire assembly was mounted on a thermoelectric stage (12) set to regulate at 37°C. One side of the bag was opened to allow access to the chamber and escape of the gas mixture. Chambers were sterilized for 2-4 h in 70% ethanol. Longer exposure to the ethanol solution was avoided because it softened the Epon.

After sterilization, the chamber was washed twice with sterile saline and once with growth medium to remove all traces of ethanol. Coverslips containing colonies of the desired age were placed in the chambers with 2 ml of growth medium and photographed at various speeds with a Kodak Reflex S camera, controlled with an intervalometer, mounted on a Zeiss Universal microscope (Carl Zeiss, Inc., New York) with Nomarski optics. A Chadwick-Helmuth strobe (Chadwick-Helmuth Co., Inc., Monrovia, Calif.) synchronized with the camera was mounted in place of the regular illumination source after it was discovered that the cells did not survive well under continuous illumination. The cine films were processed commercially.

The number and timing of the events in individual focal contractions in each of the outer zones of three different colonies were determined over a 24-h period from films taken at one frame/10 s. After the initial analysis, the stratified zone was divided into two zones, because there were large differences in the number of contractions occurring in the different regions of this zone.

The effects of CCB on cell movements were followed cinematographically by treating the clones sequentially with the following solutions while filming at a speed of one frame/10 s: (a) Normal growth medium (Ham's F10g H0.1) for 3 h; (b) Growth medium containing 1% DMSO for 1 h; (c) Growth medium containing 1% DMSO and 10 mg/ml CCB for 2 h at one frame/5 s followed by one frame/min overnight; (d') Three washes
Analysis of Cellular Dimensions

Cellular dimensions in different zones of the colony were measured as follows: 1-μm serial sections were cut parallel to the colony surface through an entire colony radius. For each of the four zones of the colony, outlines of 10 cells sectioned through the apical junctional region were traced on graph paper with a camera lucida. The cell outlines were cut out and weighed on a Mettler H16 balance (Mettler Instrument Corp., Hightstown, N.J.), and the planar surface areas of the cells from each zone were calculated by dividing the average weight by the weight of a 10-μm² surface area of the paper. Cellular heights were measured directly from 1-μm cross sections.

RESULTS

Cellular Movements in a 3-wk-old Pigmented Epithelial Clone

Time-lapse films revealed three classes of movements in pigmented retinal colonies: focal contractions, rapid pulsations of the apical protrusions, and undulations of the lateral membranes (15).

Focal contractions occurred primarily in cells of the stratified and intermediate zones of the colony (Table I). These movements involved a slow drawing together of groups of 10-100 cells towards 1 or 2 focal cells located in the center of the groups, followed by a slow relaxation. During contraction, the cells appeared to become rigid, and all apical and lateral movements ceased. During relaxation, the cells lost their stiff appearance and expanded slightly, although relaxation never seemed to be complete. Attempts to obtain detailed measurements of these movements failed because of the size of the image and the shadow effect of the Nomarski optics. The time required for contraction was relatively constant both within a colony and among different colonies (t = 55 s, σ = 9). Recovery times were slower and more variable than contraction times (t = 173 s, σ = 36).

Focal contractions tended to be grouped spatially and temporally. They generally had different focal cells, although on occasion two contractions were observed which centered on the same cells. In some cases, contractions on adjacent foci overlapped. Where the overlap was only spatial, there was no effect on the timing of the contractile events. If both spatial and temporal overlap occurred, one or both of the contractions were prolonged. During a contraction, cells between the center of focus and the outside of the colony usually underwent more movement than their counterparts towards the center.

In addition to the contractile activity described above, intermediate-sized apical protrusions found on cells in the middle two regions of the colony were seen to extend and retract, the entire process requiring ~1-3 min. Cells exhibiting this type of apical activity were usually found in small groups, often forming the center of focal contractions. Just before a contraction, the surface protrusions of the central cells became more active. During the contraction itself, the protrusions ceased their movements and extended stiffly from the apical surface. Movement resumed as relaxation began.

Small apical protrusions in the intermediate and pigmented regions of the colony appeared to exhibit movements when viewed at 220 × normal speed. Detailed analysis of these movements was difficult because of the small size of protrusions.

Arrangement of Microfilaments in Cells of 3-wk-old Colonies

Bundles of microfilaments, 4–7 nm in Diam, were found in all cells of the colony. In cells of the Squamous Zone, the bundles extend from short junctional regions (Figs. 1–3) and from the base of the cells to form an incomplete sack which enclosed the majority of cellular organelles. There were distinct parts to the sack. In the basal cytoplasm, bundles of thin filaments extended between large, amorphous dense areas and formed a basal web (Fig. 4) similar to that described in cultured rat embryo and 3T3 cells (21, 22).
Microfilaments from both the lateral basal attachments and the apical junctional complexes extended around the lateral margins of the cells (Fig. 3). Other bundles of microfilaments arched across the cellular apex. The apical bundles often passed through indentations in the nuclei.

Where junctions were lacking, apical microfilaments were associated with the inner aspect of the cellular membrane opposite concentrations of extracellular material (Figs. 1 and 5). This material stained with Alcian Blue and Hales colloidal iron showed β-metachromasia with toluidine blue, and stained lightly with the periodic acid-Schiff technique (13, 14), suggesting that it consisted of glycosaminoglycans (acid mucopolysaccharides). It was identical in staining properties with material found between cells of the stratified zone (Fig. 6).

In cells at the outer edge of the squamous zone, almost all of the apical microfilament bundles extended completely across the cell. In cells found at the middle of this zone, a few microfilaments curved around to make contact with adjacent junctional areas. Where this occurred, the lateral elements of the sack were not present. A few regions of density (dense bodies) were associated with the microfilament bundles of this zone, and microfilaments were anchored at the membranes in small amounts of dense material.

In cells of the Stratified Zone, microfilaments were localized in the basal and apical regions of the upper cells (those with a free apical surface) (Figs. 6 and 7). The majority of apical bundles of microfilaments were anchored in regions of dense material at the apical junctions and extended across the cells at this level. They formed a peripheral band of thin microfilaments which ran parallel to the lateral cell membranes in close association with the apical junctions (Fig. 7). This arrangement was similar to the purse string filaments described in neurulating amphibian cells (1, 5, 20, 37), developing pancreas (46), developing lens (50), and ovalbumin gland (49). Scattered bundles of thin microfilaments which extended across the apex, branching and anastomosing, formed widely spaced apical webs (Fig. 7). Dense bodies were associated with bundles of microfilaments, particularly at points of branching and anastomosis (Fig. 7).

Bundles of thin microfilaments also arched across the cellular apex just below the membrane and extended into the apical protrusions. Where the apical surface was convex, these filaments were separated from the lower components of the apical web. If the cell surface was flat, the two groups of microfilaments ran together forming an apical web similar to that described in the intermediate zone.

In cells from the outer portion of the zone, the peripheral band was broad and elements of the apical web were widely spaced. In cells from the inner part of the zone, the peripheral band was more condensed and the web contained more filaments and dense bodies.

The basal web was composed of broad bundles of microfilaments and associated dense bodies which traversed the base of the cell. These were embedded in dense material associated with desmosomelike junctions found on the lower lateral cell surfaces.

Thin filaments and dense bodies occurred throughout the cytoplasm of cells in the lower layers of the stratified zone. They inserted at...
**Figure 4** Section parallel to the plate surface through cells of the squamous zone of a colony showing extensive basal web of microfilaments (BW). The dense areas (DA) represent intracellular points of microfilament attachment. These are found opposite points of cell-substrate attachment. × 5,300.

**Figure 5** A section parallel to the apical surface of cells in the stratified zone demonstrating the attachment of intracellular thin microfilaments (MF) to the membrane opposite extracellular material (EM). × 25,000.
scattered adherens junctions. Discrete apical webs and peripheral bands were not apparent in these cells.

The Intermediate Zone cells were polygonal in surface view and cuboidal in profile (Figs. 8 and 9). The basal cell surfaces rested on a basal lamina underlaid by a reticular lamina containing collagen (Fig. 8), similar to that described previously (15, 28). Microfilaments in the apex of these cells formed a well developed apical web and peripheral band (Fig. 9). These were similar in structure to those described in the stratified zone, but the thin filaments were more densely packed. Dense bodies (Fig. 10) were found at points of branching and anastomosis. Less organized microfilament networks appeared to extend into the apical protrusions from the apical web.

The basal web of filaments and associated dense bodies was less extensive than that found in cells at the outer two zones of the colony and consisted of narrow bands which ran in more or less parallel arrays across the base of the cell (Fig. 11). These bands of microfilaments terminated in plaques of intracellular dense material associated with punctate junctions located towards the base of the cells (Fig. 11, inset). Although the junctions resembled desmosomes (16), they did not exhibit the intercellular dense lines characteristic of these structures.

Except for the cells at the outer edge of the squamous zone, the bundles of microfilaments forming the apical and basal webs were generally arranged so that the longitudinal axis of the majority of the bundles was in one direction (Figs. 7, 9, and 11). This longitudinal axis usually lay along the radius of the colony.

The Pigmented Cuboidal Zone occupied the center of the colony and has been described in detail in a previous publication (15). The cells were more densely pigmented, with melanin granules similar in structure to those described in the mouse (25, 26), and the apical microfilament webs were denser than those in the cells of the outer zones. The basal webs were reduced to a few scattered bundles similar to those described in the intermediate zone.

Microtubules and thick microfilaments (~10 nm in Diam) paralleled the thin filaments in all zones of the colony (Figs. 10 and 14).

**Effect of CCB on Microfilaments and Cellular Movements**

Application of media containing 10 g/ml CCB to 2- to 3-wk-old pigmented clones caused cessation of cellular movements and retraction of apical protrusions in all cells of the colony within 3–4 min. Over the next 2 h, the cells lost their characteristic shape, became arborized as described by Sanger and Holtzer (32), and drew together into large groups.

Recovery was rapid upon removal of the drug. The apical protrusions reappeared and movement began within 2–3 min. The cells expanded laterally over the next 1–5 h, made contact with each other, and re-established their polygonal shape. Focal contractions were first noted 3–4 h after removal of the drug.

TEM examinations of cells from the outer three zones of the colony which had been treated with 10 g/ml CCB for 12 h showed changes similar to those described for cells of the pigmented cuboidal zone (15). The apical surface of the cells was smooth and convex (Fig. 12). In the majority of cells, the nuclei remained roughly in the center. However, in some cells in the stratified zone the nuclei occupied blebs of cytoplasm situated above the main mass of the cell and connected to it by a thin stalk of cytoplasm, a configuration similar to that described in cells of the pigmented cuboidal zone of the colony after 12 h of treatment with 1 g/ml CCB (15).

Detailed examination of the region formerly occupied by the apical web showed that the majority of thin filaments and associated dense bodies were no longer present (Fig. 12). Their former position was occupied by scattered cellular organelles and patches of feltlike material (CB bodies). The latter were more numerous adjacent to junctional areas of the membrane. Occasionally, single skeins of intact microfilaments with their dense bodies were also found in this region. They were almost always adjacent to the junctional membranes in the position occupied by the peripheral band in the more differentiated cells, a structure which has been shown to be more resistant to CCB than the web itself (15). The adherens junctions were less distinct. This appeared to be because of a loss of the dense material in which the filaments were formerly embedded.

The basal web was less affected by the drug. The arrays of microfilaments and their associated dense bodies remained intact but appeared less distinct (Figs. 13 and 14). The adherens junctions also remained intact. CB bodies appeared in the basal region of the colony, but it was impossible to determine whether they represented disrupted...
arrays of microfilaments from the basal web or those displaced from the apical web.

Thick microfilaments remained intact in all regions of the cells. Indeed, there appeared to be more of them after treatment with the drug, although this may have been because of better visualization of these structures after dispersal of the thin filaments.

12 h after removal of the drug, the apical web had returned to its former position, and the lateral cell borders were again joined by extensive adherens junctions.

Cell Shape Changes

In general, the cells underwent an average decrease in planar surface area from the squamous to the pigmented cuboidal zone of the colony. Cell height increased from the squamous to the intermediate zones of the colony. A decrease in height as well as a decrease in planar surface area was noted between cells of the intermediate and pigmented cuboidal zones. This suggests that some cell mass may be lost during this stage. These results are summarized in Table I.

DISCUSSION

There is increasing evidence of the presence in all cells of contractile proteins similar to those found in muscle (30). Such proteins are thought to be responsible for cellular motility and appear to be represented by arrays of microfilaments 4–7 nm in Diam which are found in almost all motile cells. These arrays of microfilaments are arranged so that contraction of them would bring about the movements observed in the cells. In addition, they bind heavy meromyosin, suggesting that they are actinlike in structure (2, 19, 23, 40, 42, 43).

Further evidence that the microfilaments are involved in cellular movements is derived from studies involving the drug CCB which reversibly disrupts both movements and microfilament arrays (10, 15, 36–39, 42, 47, 48, 52).

Cells in a 3-wk-old pigmented epithelial clone undergo three types of movements. Arrays of CCB-sensitive microfilaments 4–7 nm in Diam form apical webs in all cells of the colony. In addition, loose networks of similar CCB-sensitive filaments are sometimes found in the apical protrusions of these cells. Disruption of these microfilament arrays with CCB occurs concomitantly with loss of all cellular movements. It appears that these arrays of microfilaments are responsible for the observed movements.

The thin basal webs of microfilaments and dense bodies in squamous and stratified zone cells resemble the sheath in cultured cardiac myoblasts (42, 44) and the web of microfilaments found in the base of cultured 16-d-old rat embryo cells (29) which resist disruption by CCB. Microfilaments and dense bodies in the basal web of both the stratified and squamous zone cells also resist disruption by 10 μg/ml CCB.

Does the lack of sensitivity mean that the basal webs are not directly involved with the cellular movement or, as suggested by Spooner (43), is the structural integrity of this web alone insufficient for movement, and is the presence of the apical web also required? Not all microfilament arrays in the same cell are equally sensitive to the drug (29). The apical web of cells from the pigmented center of the colony is sensitive to 1 μg/ml CCB, whereas it requires 10 μg/ml to disrupt the peripheral band (15). It is possible that the basal webs of the cultured pigmented epithelial cells are indeed affected by the drug but are less sensitive to it, requiring >10 μg/ml CCB to be disrupted. Further experimentation is needed to provide answers to these questions.

The dense material associated with the microfil-

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**Figure 6** Cross section through cells of the stratified zone showing the cellular shape and arrangements of the organelles. Extracellular mucopolysaccharides (glycosaminoglycans) (EM) are present between the cell layers. Bundles of microfilaments (MF) with their associated dense bodies (DB) are associated with the junctional areas (J) in the apex of the cells of the upper layer. Scattered bundles of microfilaments can also be seen in cells of the lower layers which exhibit shallow interdigitations with their neighbors (arrows). G, Golgi bodies. N, nucleus. ×4,500.

**Figure 7** Section parallel to the surface of a colony through the apical junctions (J) of the upper layer of cells in the stratified zone. The microfilaments (MF) and the associated dense bodies (DB) form an incomplete apical web and a broad peripheral band (PB). Note the bundles of thin microfilaments passing through a complex indentation in the nucleus (N). G, Golgi bodies. ×6,100.
FIGURE 11  Section in a plane similar to that of Fig. 4 through the base of cells of the intermediate zone. The basal web has been reduced to skeins of microfilaments (MF) running across the base of the cell. These skeins are associated with desmosomes (D) rather than with regions of cell-substrate attachment in this zone. × 8,000. Inset: A higher magnification of one of the junctions and associated thin microfilaments (MF) in the basal web of cells of the intermediate zone of the colony. Intracellular dense plaques similar to those seen in desmosomes are present opposite the points of microfilament attachment. The intercellular dense lines characteristic of this type of junction are absent, however. × 40,000.
ments at the intracellular surfaces of both cell-cell and cell-substrate junctions, which is present at nodal points within the apical and basal webs, is similar to that found in smooth muscle and other nonmuscle cells (17, 33). It has been suggested that this material is analogous to Z disks of skeletal muscle (4, 7, 41). Such material has recently been found to contain the protein α-actinin (34), a protein also located in the intracellular dense material found at the adherens junctions in other vertebrate cells (35). Perhaps the dense material present in the webs is similar to the Z band material of muscle and forms an anchorage for the microfilaments. Treatment of the cells with anti-α-actinin might clarify this point.

During the redifferentiation process, the cell shape changes from squamous to cuboidal; the cell height increases and the horizontal surface area decreases (Table 1). Focal contractions are strongest and most numerous where cells are undergoing the greatest shape changes (Table 1). Few focal contractions are found at the center of the colony where little shape change occurs. This suggests that the focal contractions constitute a mechanism for changing cellular shape during redifferentiation in these cells.

Although there are numerous examples of the role of microfilaments in changing cellular shape during morphogenesis, there have been no previous reports of focal contractions similar to those described above. Microfilaments have been shown to be involved in continuous contractile movements such as ascidian tail resorption (10, 11) and cytokinesis (36–39). However, little cinematographic evidence is available for other morphogenetic movements such as occur in the formation of pancreas (46), chick ovalbumin gland (49, 50), and rat salivary gland (45, 47). The thickness of such preparations would make such studies difficult. Chick and amphibian gastrulation, amphibian neurulation, and epiboly in fish have been the subject of numerous cinematographic studies, but the magnification and filming speeds used are generally so low that focal contractions would not be observed. Examination of these phenomena at higher filming speeds and magnifications would determine whether focal contractions are also associated with these morphogenetic events.

It is very difficult to determine how many of the cells in any one focal contraction are actively contracting and how many are "simply along for the ride." Simultaneous cessation of movement of apical protrusions of several cells at the center of each contraction, plus the prolongation of the contractions when they overlap spatially and temporally, suggests that some form of communication is present among cells in the colony. The presence of electrical communication among colony cells has been aptly demonstrated (18, 31), thus suggesting that such communications may be electrical in nature.

Cells in all areas of the pigmented epithelial clones exhibit apical protrusions which normally extend and retract rapidly but disappear in the presence of CCB. CCB-sensitive microfilaments are also present in the protrusions and just beneath the cell membrane, thus suggesting that these microfilaments, either alone or in conjunction with those of the apical web, are involved in producing the protrusions. Similar groups of microfilaments have been observed in pigmented epithelial cells of rats and monkeys where they are thought to be involved in phagocytosis of detached photoreceptor outer segments (3, 53, 54), and possibly in maintaining the integrity of the microvilli which extend from the cells between the receptor cells of the retina in these animals (6, 7). It is possible that movements of the microvilli seen in cloned cells may represent one or both of these functions.

FIGURE 12 A cross section through cells of the stratified zone of a pigmented epithelial clone after 12 h in 10 μg/ml CCB. The apical web surfaces are convex and smooth, and the nuclei (N) are situated at the tops of the cells. The junctional complexes are no longer obvious, nor is the apical web of microfilaments. CB bodies (CB) are present throughout the cells but are more extensive in the apical areas. × 5,000.

FIGURE 13 A section parallel to the surface of a CCB-treated colony through the base of a cell of the stratified zone. The junctions (J) and basal web (BW) are intact. × 4,000.

FIGURE 14 A higher magnification of microfilaments in the basal web of a CCB-treated cell, showing the microfilaments (MF) and their associated dense bodies (DB) which remain after CCB treatment. Microtubules (MT) can be seen paralleling the course of the thin microfilaments. × 26,000.
Scattered groups of microfilaments are also seen in the interdigitations between adjacent cells. They are probably involved in producing the lateral undulations seen in cells in the outer zones of the colony. At present, their significance is unknown.

The author would like to thank Dr. R. A. Cloney for his help and guidance during the majority of this work, and for his critical reading of the manuscript.

This research was supported in part by an Institutional Cancer Grant of the American Cancer Society, and by British Columbia Medical Research Foundation grant 1977-7.

A portion of this work was performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Washington. The work was completed at The University of British Columbia.

Received for publication 17 July 1978, and in revised form 13 December 1978.

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