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FLUORESCENCE MICROSCOPY OF HEALING GINGIVAL EPITHELIUM

PRELIMINARY REPORT

by

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The study of the tissues of the body in the "ordinary" microscope with polychromatic light suffers from a number of inherent drawbacks. Thin sections of tissues cannot be differentiated from each other to any great degree unless they are artificially stained and, for this purpose, diachromatic stains have been almost universally used. Even these stains do not always give the desired degree of differentiation and to overcome this problem other methods of histological investigation have been developed.

In a study of the healing of the gingival tissues after experimental gingivectomy in dogs (*Persson*, manuscr. in prep.), a number of questions were raised which could not be answered by the usual methods of microscopical investigation. Different tissues, by virtue of their differing chemical composition, can usually be differentiated satisfactorily from each other by the use of diachromatic stains but the differentiation of cells of different ages from the same tissue is a more difficult problem. Young epithelial cells at the edges of healing wounds are usually rather large with a large weakly staining nucleus and poorly staining cytoplasm, while older cells are smaller with a small strongly staining nucleus and strongly staining cytoplasm. These differences are sufficiently marked to differentiate very young from very old cells with diachromatic stains but intermediate stages are not usually distinguishable with these stains. In examining the epithelium covering a healing gingival lesion it is of

considerable interest to know the relative ages of the different cells and this is virtually impossible with diachromatic stains. Fluorescence microscopy is a comparatively recent development and, in many cases, gives better differentiation of tissues than is possible with other methods.

In an attempt to study the origin of epithelial cells in healing gingival wounds, material from an investigation into the healing of the oral epithelium after gingivectomy was examined by fluorescence microscopy. The details and results of an investigation of this material in the "ordinary" microscope using polychromatic light have been described in detail in a paper which will shortly be published by one of us. *(Per-Allan Persson).*

FLUORESCENCE MICROSCOPY

Fluorescence has been simply defined by *De Ment* (1945) as "the emission of light, usually visible, from matter under the influence of an exciting agent". The exciting agent used in the fluorescence microscope is ultra-violet light (wave lengths between 3,000 and 4,000 Å) or ultraviolet + blue light (wave lengths up to 4,800 Å). The source of this light is a carbon arc or high-pressure mercury vapour lamp and the visible rays are removed by passing the light beam through a series of filters. In the eyepiece of the microscope there is another filter which removes the ultra-violet light so that the colours seen by the eye are those excited in the specimen by the ultraviolet light (plus blue light if this is used).

Most tissues fluoresce (this is called "primary fluorescence") when irradiated with ultra-violet light but the emission is so weak in many of the soft tissues of the body that it is of little use in microscopy. Staining the tissues with weak watery solutions of certain dyes called fluorchromes gives them a high degree of fluorescence (called "secondary fluorescence") and this is the method which has been used in the present investigation. The staining properties of these fluorchromes are dependent, to a large extent, on the hydrogen ion concentration in the tissue during staining and on the concentration of the dye. The theory and methods of staining with fluorchromes are dealt with fully

in the works of *Strugger* (1949) and *Hals* (1953) and interested readers are referred to these papers. *Bennett* (1953) tested a number of double fluorchromes in investigating the tissues of the dental pulp and the staining technique and many of the fluorchromes described by him have been used in the present investigation.

The large number of fluorchromes available and their variable effects at different pH values make the number of possible staining combinations very great. In determining the best combination of fluorchromes to differentiate between two particular tissues it is advisable to first determine the Iso-Electric-Point (I.E.P.) of each of the tissues in question. This can readily be done by staining with a single fluorchrome at different pH values and the fluorchrome acridine orange is generally the most satisfactory for this purpose.

The determination of the I.E.P. of a tissue in this way depends on the fact that the tissues of the body are amphoteric and have different staining characteristics on different sides of their I.E.P. They stain with acid dyes at pH values below their I.E.P. and with basic dyes at pH values above this point. Acridine orange is a basic fluorchrome and its colour varies with its concentration. In weak solutions (1: 10,000) acridine orange is green and progressively stronger solutions are yellow then orange and finally red at a concentration of 1: 100. Only imbibition of the acridine orange takes place when tissues are stained at pH values below their I.E.P., and they thus appear green.

At pH values above this point, the cations of the acridine orange are strongly bound to the tissues which then appear orange or red in colour because of the higher concentration. This colour change is quite dramatic and determination of the I.E.P. by this method is relatively simple.

The application of one or more fluorchromes at a pH between the I.E.P.s of the tissues in question will usually give satisfactory differentiation and, with this as a basis, other fluorchromes or combinations of fluorchromes can be tried.

In the present investigation, the following fluorchromes were tested at a pH range of from 2 to 10: — acridine orange, atebrine, auramine, berberine sulphate, congo red, coriphosphine, neutral red, magdala red, primulin, thiazine red, thioflavine S and trypa-

flavine. Aqueous solutions of these stains at a dilution of 1:500 were mixed with an equal quantity of the appropriate buffer solution and applied to the sections for a period of one minute. Full details of the staining technique can be obtained from the paper by *Bennett* (1953).

When these fluorchromes were applied singly, acridine orange was found to give the best overall differentiation between epithelium, connective tissue, dentine and cementum. The finer details of the tissues were, however, not well shown. Thiazine red and atebrine also gave fair differentiation. A number of combinations of fluorchromes were also tested and the best results were obtained with congo red + coriphosphine + primulin at pH 5 and congo red + primulin + acridine orange at pH 5 which were found to give excellent differentiation between the different layers of epithelium. At pH 5, congo red stains the inter-cellular substance between the epithelial cells a dark brown colour and this contrast well with the yellow, green, or light brown colour which the cytoplasm takes from the other stains. These combinations of fluorchromes were particularly useful in differentiating young and old epithelial cells from each other.

PREVIOUS INVESTIGATIONS

The healing of epithelial tissues has been the subject of very many investigations. These have mostly dealt with the healing of skin wounds but the general principles of healing appear to be the same for all epithelia. *Florey* (1945) summarized the modern views on this matter and, discussing the growth of new epithelium over healing skin wounds, wrote: "Cells from the lower layers of the skin edge or corresponding cells from other epithelia can slide across the surface . . ." and later, "mitosis takes place in a limited area which is not, as might be expected, at the growing edge but some little way behind it". In an investigation of healing gingival epithelium, *Orban* and *Archer* (1945) observed the presence of mitosis in cells of the deeper layers of the adjacent epithelium two days after production of the lesion.

The work of *G. Cameron* (1935) and *Menkin* (1941) suggests

that products of inflamed tissue may be responsible for the extremely rapid growth and proliferation of cells which are found in wound healing. Recent work by *Jansen et al.* (1955) has shown that proliferation of oral epithelium is greater after extensive than after simple wounds. As *R. C. Cameron* (1952) has written "Epithelial regeneration is perhaps the most active in the body". He described how epithelial cells manifest an inherent tendency to cover over surfaces and considered that "Epithelial regeneration is partly the result of migration of cells from the margins of the wound, partly due to mitotic division of marginal cells. Such cells manifest an inherent tendency to cover over surfaces, and migration can be impressively rapid when it gets started".

While the skin is able to effect only a relatively simple repair, the cells of reforming mucosae are able to differentiate to a remarkable extent. (*Florey,* 1954). Florey found that restoration of mucosal structure was often complete.

MATERIAL

The material used in this study was taken from dogs at varying intervals of time after experimental gingivectomy. The results of the investigation of this material in the "ordinary light" microscope have been described in a paper which will be published shortly by one of us (Per-Allan Persson) and details of the material and technique can be obtained from that paper.

After removal from the animals, the teeth and surrounding tissues were fixed in 10 % formalin, decalcified in a solution of formic acid and sodium formate and then embedded in paraffin wax. Sections 5 microns in thickness were cut and fluorchromed as described above.

RESULTS AND DISCUSSION

Determination of the Iso-Electric-Points

The results of the determination of the I.E.P. of the different gingival tissues of the dog by treating paraffin sections with the fluorchrome acridine orange are given in the following table.

Determination of the I. E. P. of the gingival tissues of the dog using the fluorchrome acridine orange

	Tissue	Cytoplasm pH	Nucleus pH
Epithelium	Stratum corneum	4—5	—
	Stratum lucidum	5—6	—
	Stratum granulosum	3—4	4—5
	Stratum germinativum	4—5	5—6
	Connective tissue fibres	8—10	

Changes in the Epithelium

In studying the growth of epithelium over a gingival lesion produced by experimental gingivectomy of previously uninfected gingivae in dogs, it was found that healing took place in four stages. These stages were quite distinct from each other but were, of course, continuous with each stage merging into the succeeding one. For the sake of simplicity, they will be described separately.

Stage One

Two days after operation, epithelial cells could be seen in the edge of the blood clot covering the lesion. These cells appeared to have originated from the deeper layers of the epithelium at the edge of the lesion and to have oozed out into the blood clot. Mitosis was observed in only a few cases at this stage and it appears likely that the cells in this first stage are not produced as a direct result of the injury but are cells which would normally have replaced the surface layers of the adjacent epithelium. The number of cells available without increased cell division is obviously very limited and this stage is soon succeeded by stage two with its more active proliferation. This first stage corresponds to the first stage of healing described by *Florey* (1954).

Stage Two

The second stage is first observed two to four days after operation when cell division can be seen in the deeper layers of epithelium some little distance from the edge of the lesion. Rapid proliferation of these deeper layers takes place to form an epithelial process which extends down into the connective tissue like an accentuated rete peg (Figs. 1 a—b, 4, and 9). Serial sections have shown that similar fingerlike processes are produced all round the periphery of the wound. The "peg" formation varies in form from a long finger-like process to a short broad spade-like one, and it has been observed in every specimen removed six or more days after production of the lesion. This "peg" process appears to act as a factory by producing epithelial cells to cover the healing surface of the lesion. The fine differentiation in cell age possible with fluorescence microscopy shows this very well. It seems likely that the "peg" formation is peculiar to oral epithelium and possibly some other closely related types. Preliminary investigation of the healing process in other parts of the oral epithelium has shown that "peg" formation is often present but is much less marked than in the gingival region.

The centre and side of the "peg" next to the lesion appear to be almost entirely composed of young cells. The contrast between the dark-brownish young cells and the lighter brown or yellow older ones was very striking when congo-red + primulin + acridine orange were used at pH 5. In most sections a layer of columnar cells, similar in appearance to the stratum germinativum of normal squamous epithelium, was seen around the periphery of the "peg".

In a few cases, however, young dark-coloured cells occupied one side of the "peg" completely and no columnar basal layer was observed. A sharp boundary was always present between the margins of the "peg" and the connective tissue and a basement membrane appeared to be present. It seems likely that the stimulus for the production of this "peg" may be provided by inflammatory products as the "peg" always appeared at the edge of the area of inflammation. Large numbers of inflammatory cells were present on the side of the "peg" containing most young cells, but very few inflammatory cells were seen on the other side of the "peg" farthest away from the lesion.

The young dark-brown stained cells could be seen passing out of the upper part of the "peg" and through the deeper layers of the epithelium towards the healing edge of the lesion. This can be seen from Figs. 3, 4 and 9 and was strikingly shown with the fluorchromes used. As the cells reach the edge of the old keratinised epithelium they appear to "bubble up" onto the surface. Most of them pass out onto the surface of the healing lesion but some are also seen to slide back along the surface of the old keratinised epithelium. (Figs. 2, 4 and 9). A single or double layer of young epithelial cells lying on the surface of the old epithelium for some distance from the lesion was observed in almost every specimen.

These young cells also appear to have an effect on the edge of the keratinised surface of the old epithelium. The layers of cornified epithelium seem to be opened up at the edge of the lesion and brown stained cells are seen lying between the separated layers (Figs. 4 and 5). It seems likely that this process is one of softening and rounding off of the edge of the lesion. The brown cells lying between the cornified layers are possibly young epithelial cells which have forced their way in between the softened layers but it seems more likely that they are incompletely cornified cells which were originally present in the old epithelium and which have imbibed fluids and become swollen.

In examining the epithelial cells overlying the granulation tissue in the early stages, it is seen that the epithelial cells on the surface are *younger* than those underneath. (Fig. 6). From this it seems reasonable to assume that division of the epithelial cells covering the lesion does not occur in the early stages and that the cells originate from the "peg" formation and slide out over the surface of the cells already there.

The production of epithelial cells by the "peg" appears to be the main source of cells for the healing lesion up to about the ninth or tenth day after production of the lesion.

Stage Three

Cell division and proliferation of the deeper layers of the epithelium on the surface of the lesion can usually be seen after the ninth day and these cells begin to form epithelial processes which

extend into the connective tissue. Young epithelial cells are now produced in the deeper layers of the new epithelium and the "peg" is no longer required and can be seen to become reduced in size. The proliferation of the deeper layers takes place very rapidly and fine epithelial processes grow down into the superficial layers of the granulation tissue (Inner side of Fig. 7 facing the tooth). Organisation of the granulation tissue is well advanced at this stage but lymphocytes and plasma cells are still present in some numbers in the superficial layers. Areas of the lesion nearer the tooth which were not covered by epithelial cells from the "peg" in stage two are now covered by young cells from the deeper layers of the new-formed epithelium. These young cells pass through the deeper layers of the epithelium to the healing edge in the same way as they did from the "peg". Proliferation of this new epithelium proceeds until the surface of the lesion is completely covered. The processes which grow down into the granulation tissue are very fine, often consisting of only one column of cells which are usually cuboidal in shape.

Stage Four

A further stage characterized by excessive proliferation of epithelium on the side of the lesion nearest the tooth was seen in some cases. The fine processes found in stage three had given place to large thick epithelial processes extending into the connective tissue (Fig. 8). Degeneration of the cells in the central areas of these processes was often observed. As this excessive proliferation was never observed on the outer side of the healing lesion, it seems likely that it was caused by the conditions in the bur cut in the tooth. It verged on the pathological and it seems unlikely that it would occur in normal uncomplicated healing of the gingivae.

Epithelium in contact with tooth substance

In the present material, a groove had been cut in the tooth at the level of the gingivectomy incision at the time of operation. Re-attachment of connective tissue with deposition of cementum were observed in only one case while, in all others, epithelium had proliferated down into the groove. In sections stained with

diachromatic stains it appeared that the layers of this epithelium next to the tooth substance might be keratinised. This was only observed in specimens taken a longer time (57 days) after operation. Examination of this material in the fluorescence microscope showed that these cells lying next to the tooth substance were not keratinised. (Fig. 7). Some type of cell degeneration had taken place but the strong secondary fluorescence found with keratinised epithelium was entirely absent.

ACKNOWLEDGEMENTS

The authors are deeply appreciative of the help and advice so generously given by Dr. Gösta Gustafson, Head of the Department of Histopathology at the Royal Dental School, Malmö. Our thanks are also extended to Miss Birgitta Lindenstam for her most efficient technical and secreterial assistance. One of us (W.D.M.) is greatly indebted to the Nuffield Foundation for the award of a dental fellowship.

SUMMARY

Fluorescence microscopy has been used in an investigation of the healing gingival epithelium after experimental gingivectomy in dogs. A number of fluorchromes have been tested to find a combination giving good differentiation between the various gingival tissues and between epithelial cells of different ages. Congo red + primulin + acridine orange at pH 5 proved to be satisfactory in this respect.

As a result of this investigation, the healing of the gingival epithelium has been described in four stages:

(1) Stage one is seen during the first two or three days after operation when cells from the deeper layers of the epithelial edge of the lesion slide out into the blood clot.

(2) Stage two commences about four days after operation. Proliferation of the deeper layers of the epithelium some little distance from the edge of the lesion commences and rapidly forms a large "peg" process which extends down into the connective tissue. Serial sections have shown that similar processes are produced all round the periphery of the wound. The "peg" formation acts as a "factory", producing large numbers of young epithelial cells which stream out from the "pegs" to the healing edge. Here they "bubble up" to the surface, some pass back over the old epithelium, but most

slide over the granulating surface to cover the lesion with layers of epithelial cells.

(3) The third stage is seen after nine days when fine epithelial processes are produced by proliferation of the deeper layers of the new epithelium. These processes extend down into the superficial layers of granulation tissue.

(4) In some cases, a fourth stage is observed in the epithelium adjacent to the tooth. Extensive thick downgrowths of epithelium, often with degeneration of the cells in the centre, are seen.

It has also been shown that, where epithelium grows down next to the tooth, these cells adjacent to the hard substance of the tooth do not become keratinised but undergo some form of degeneration.

RÉSUMÉ

MICROSCOPIE EN FLUORESCENCE DE LA CICATRISATION DE L'ÉPITHÉLIUM GINGIVAL

La microscopie en fluorescence a été appliquée à un travail de recherche sur la cicatrisation de l'épithélium gingival après gingivectomie expérimentale chez le chien. La microscopie en fluorescence, grâce à ses plus grandes possibilités de différenciation cellulaire a été utilisée pour compléter un travail antérieur, effectué à l'aide du microscope optique ordinaire. Un certain nombre de fluorochromes a été essayé pour trouver une combinaison donnant une bonne différenciation entre les divers tissus gingivaux et les cellules épithéliales d'âges différents. Le rouge Congo + primuline + l'orange acridine à pH 5 se sont montrés satisfaisants à cet égard.

En conclusion de cette étude, la cicatrisation de l'épithélium gingival a été divisée en 4 étapes:

1) La première étape se voit durant les 2 ou 3 premiers jours après l'opération, quand les cellules venues des couches profondes de la paroi épithéliale de la lésion glissent à l'intérieur du caillot sanguin.

2) La deuxième étape: commence environ 4 jours après l'opération. La prolifération des couches profondes de l'épithélium commence à quelque distance du bord de la lésion et rapidement

forme une grande extension qui pénetre dans le tissu conjonctif. Des sections en série ont montré que des extensions semblables se produisent tout autour de la périphérie de la cicatrice. L'extension se comporte comme une "usine", produisant un grand nombre de cellules épithéliales qui déferlent hors des extensions vers le bord se cicatrisant. Là elles "bouillonnent" vers la surface, quelques unes revinnent par dessus le vieil épithélium, mais la plupart d'entre elles glissent sur la surface de granulation pour recouvrir la lésion de couches de cellules épithéliales.

3) La troisième étape se voit après 9 jours quand de fins prolongements épithéliaux sont formés à partir des couches profondes du nouvel épithélium. Ces prolongements s'étendent en profondeur dans les couches superficielles du tissu de granulation.

4) Dans certains cas, une 4ème étape succède à la précédente, quand la croissance en profondeur de l'épithélium dans le tissu de granulation devient exagérée. Dans de tels cas, on assiste à la dégénérescence de quelques cellules centrales de ces proliférations.

Il a été démontré également que, là où l'épithélium croit en profondeur au voisinage de la dent, les cellules adjacentes au tissu dentaire dur ne se kératinisent pas, mais subissent une sorte de dégénérescence.

ZUSAMMENFASSUNG

MIKROSKOPIE BEI FLUORESZIERENDEM LICHT VON IN HEILUNG BEGRIFFENEM GINGIVALEPITHEL

Die Mikroskopie bei fluoreszierendem Licht ist angewandt worden bei einer Untersuchung über die Heilung des Gingivaepithels nach experimenteller Gingivektomie bei Hunden. Wegen ihrer grösseren Möglichkeiten bei der Zelldifferenzierung wurde die Mikroskopie bei fluoreszierendem Licht angewandt, um vorangegangene Untersuchungen im Mikroskop bei gewöhnlichem Licht zu vervollständigen. Es wurden eine Anzahl von Fluorchromen untersucht, um eine Kombination zu finden, die eine gute Differenzierung zwischen den verschiedenen Gingivageweben wie Epithelzellen der verschiedenen Entwicklungsstufen ergeben sollte. Kongorot + Primulin + Acridinorange von pH 5 erwiesen sich in dieser Beziehung als befriedigend.

Die Heilung des Gingivaepithels — das Resultat dieser Untersuchung — wird in vier Stadien eingeteilt:

1. Das erste Stadium sind die ersten 2 oder 3 Tage nach der Operation, wenn die Zellen aus den tieferen Schichten des epithelialen Randes der Wunde in das Blutkoagulum eindringen.

2. Das zweite Stadium beginnt ungefähr 4 Tage nach der Operation. Die Proliferation der tieferen Schichten des Epithels setzt ein, etwas vom Rand der Wunde entfernt und bildet sehr schnell einen "Einwuchs"-Prozess, der sich in das Bindegewebe nach unten ausbreitet. Serienschnitte haben gezeigt, dass ähnliche Einwuchs-Prozesse rings um die Peripherie der Wunde entstehen. Dieses Einwachsen des Epithels stellt eine Neubildungsstelle dar, indem eine grosse Anzahl junger Epithelzellen gebildet werden, die von hier zu dem heilenden Rand der Wunde ausströmen. Dort kommen sie wie "Blasen" an die Oberfläche. Einige gehen auch den Weg über das alte Epithel zurück, aber die meisten gleiten über die granulierte Oberfläche, um die Wunde mit einer Lage von Epithelzellen zu bedecken.

3. Das dritte Stadium erkennt man nach 9 Tagen, wenn feine epitheliale Ausläufer von den tieferen Schichten des neuen Epithels gebildet werden. Diese Ausläufer breiten sich nach unten in die oberflächlicheren Schichten des Granulationsgewebes aus.

4. Das vierte Stadium wird in einigen Fällen in dem an den Zahn angrenzenden Epithel beobachtet. In solchen Fällen kann man ein sehr ausgebreitetes Tiefenwachstum des Epithels erkennen, das oft mit einer Degeneration im Zentrum der Zellen einhergeht.

Es ist auch gezeigt worden, dass dort, wo das Epithel sehr nahe am Zahn nach unten wächst, Zellen, die an die Hartsubstanzen des Zahnes angrenzen, nicht keratinisiert werden, sondern einer Form der Degeneration unterliegen.

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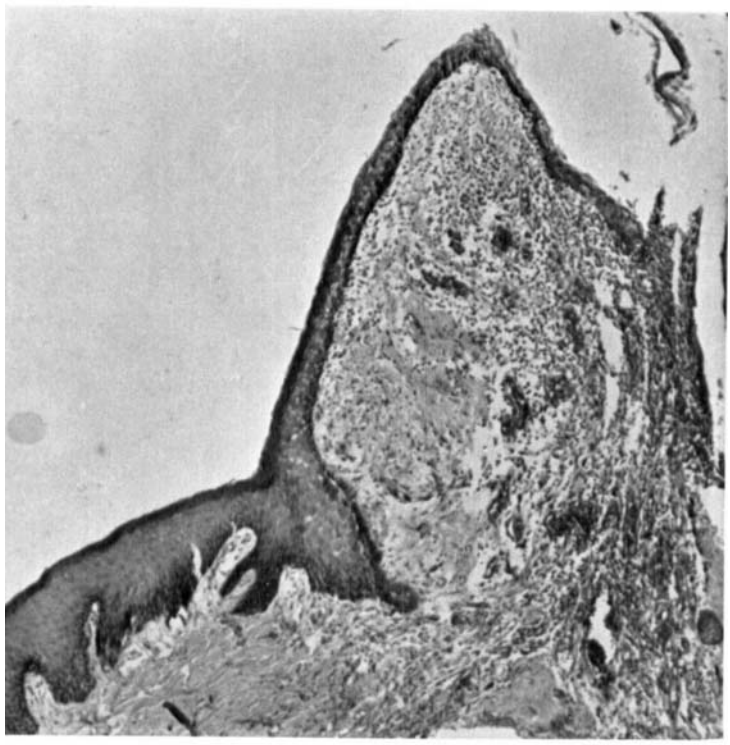

Fig. 1a. Magnification approx. × 50. General view taken in "ordinary light" 7 days after operation and showing the "peg" formation some little distance from the edge of the lesion.

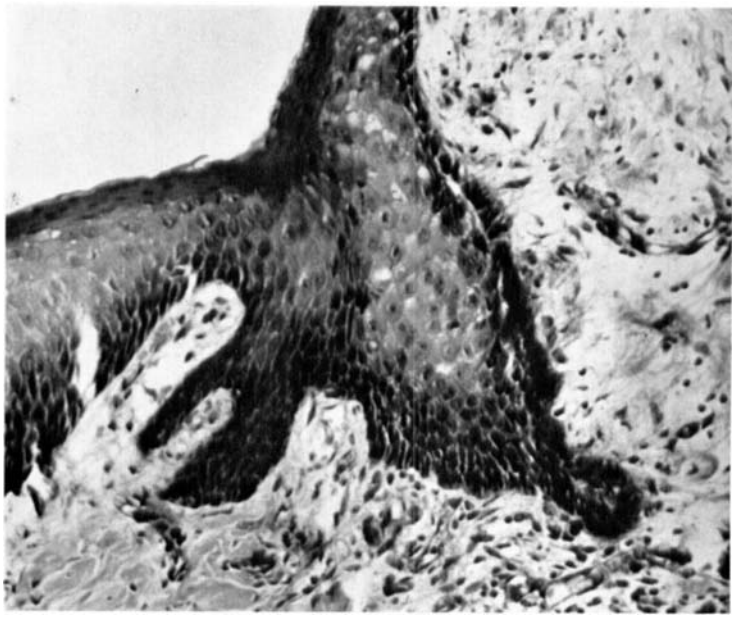

Fig. 1b. A higher magnification (approx. × 120) of the "peg" seen in fig. 1a.

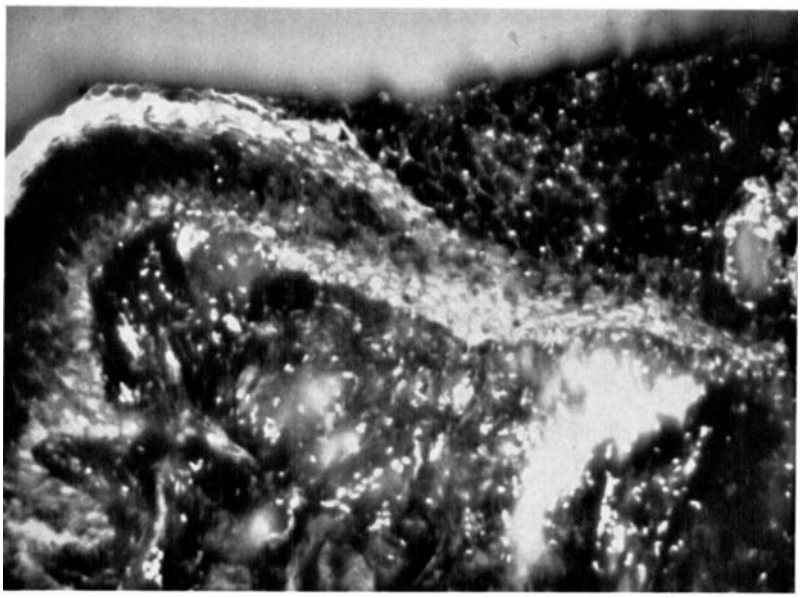

Fig. 2. Magnification approx. × 150. The edge of a fluorochromed specimen 4 days after operation photographed in ultra-violet plus blue light. Dark-stained young cells can be seen to the right on the surface of lesion.

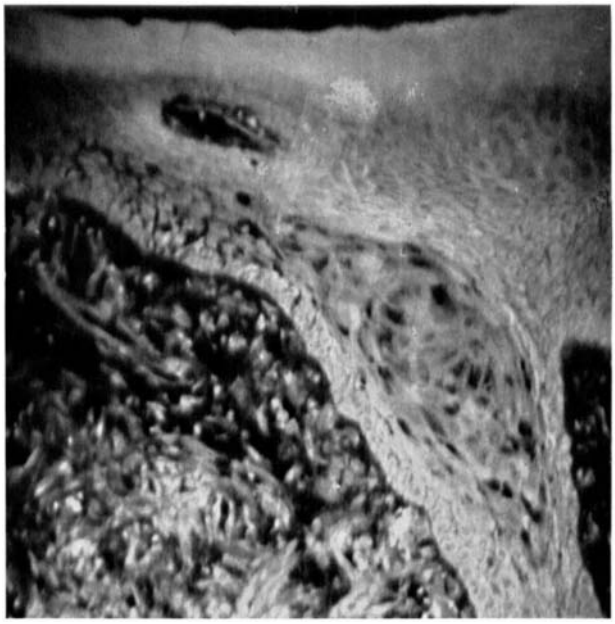

Fig. 3. Magnification approx. × 150. A fluorochromed specimen 9 days after operation photographed in ultra-violet light. The cells in the upper central part of the "peg" can be seen to be rather large, dark and loosely packed and a "stream" of these cells can be followed through the deeper layers of the epithelium to the healing edge of the lesion which lies to the left.

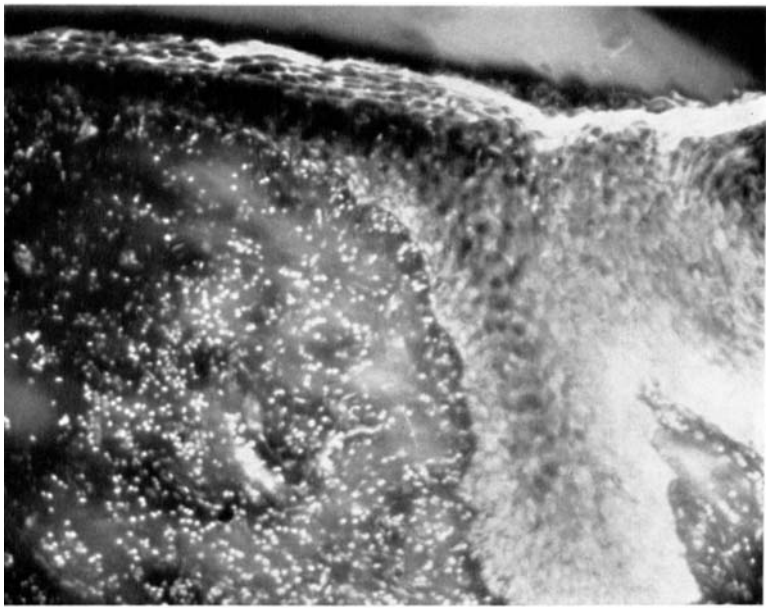

Fig. 4. Magnification approx. × 150. A fluorochromed specimen 8 days after operation photographed in ultra-violet plus blue light. Dark-stained cells can be seen lying between the layers of the keratinised epithelium to the left.

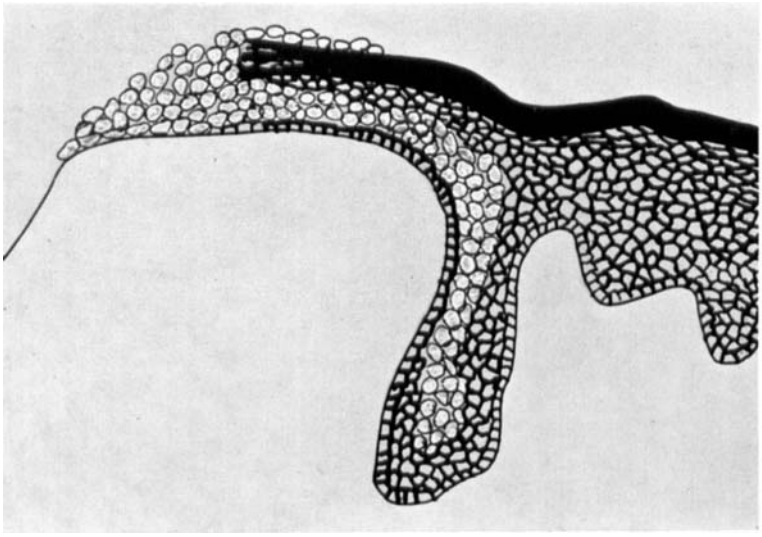

Fig. 5. A diagrammatic representation of the epithelium at the healing edge of the lesion showing the young cells passing out of the "peg" to the surface of the lesion.

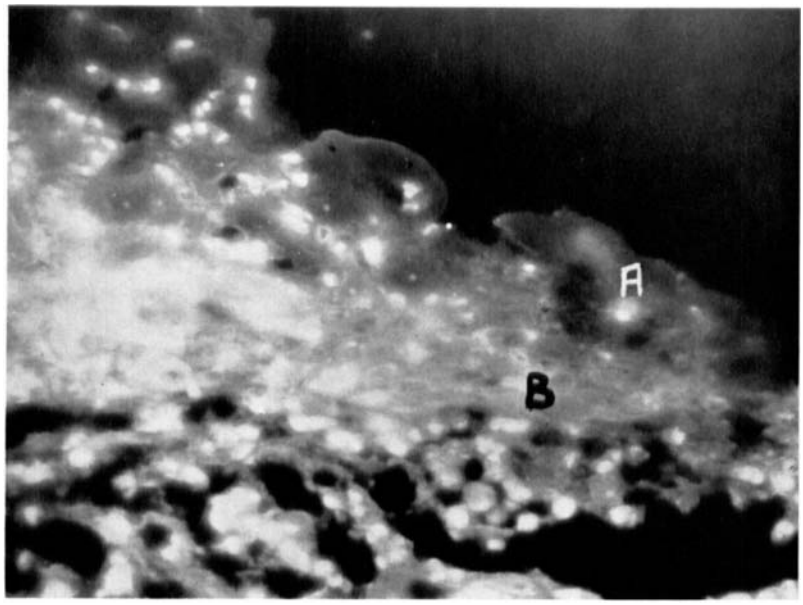

Fig. 6. Magnification approx. × 400. Epithelial cells on the granulating surface of the lesion 7 days after operation. The cells nearest the surface (A) appear quite dark while the deeper-lying cells (B) are somewhat lighter.

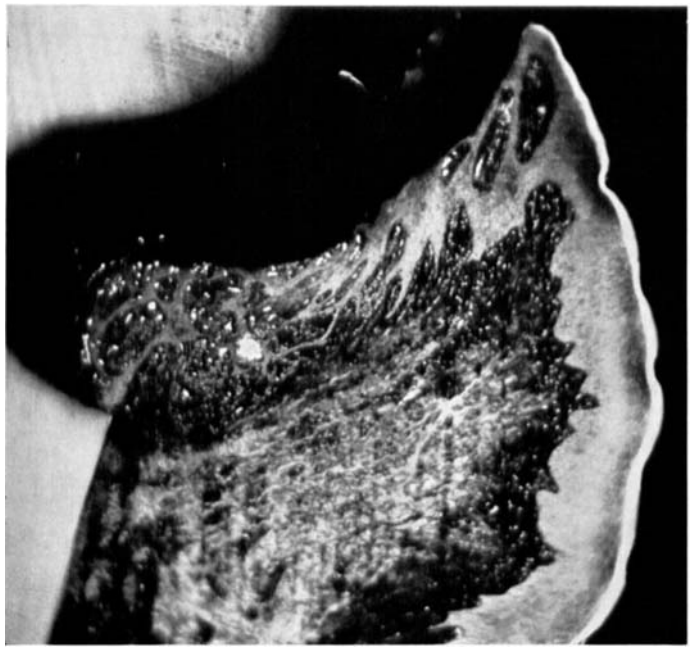

Fig. 7. Magnification approx. × 60. Photomicrograph taken in ultra-violet light of a fluorochromed specimen 57 days after operation. The soft tissues have been pulled away from the tooth during preparation of the section and it can be seen that the layers of epithelium which lay next to the tooth have an entirely different appearance from the outer layers of keratinised epithelium.

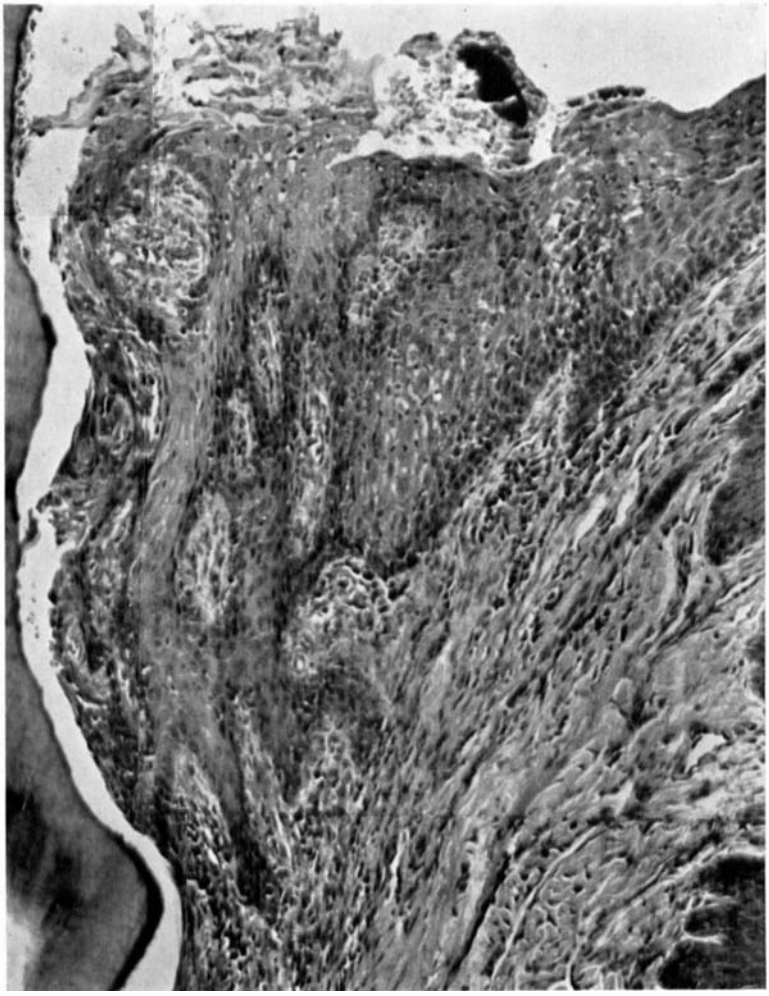

Fig. 8. Magnification approx. × 170. Photomicrograph taken in "ordinary light" showing the extreme proliferation of the epithelium found in stage 4. The cells in the centre of the thick processes can be seen to be degenerated.

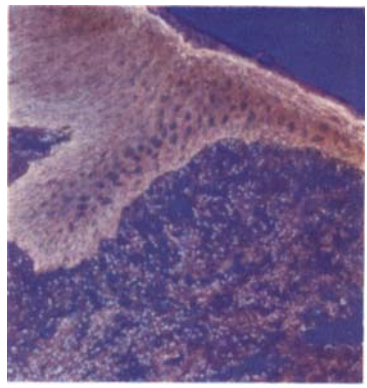

Fig. 9. Magnification approx. × 150. A fluorochromed specimen 8 days after operation photographed in ultra-violet light. Dark-stained young cells can be seen in the "peg" and passing out to the healing edge of the lesion which lies to the right.