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PRELIMINARY OBSERVATIONS ON THE EFFECTS IN VIVO AND IN VITRO OF LOW DOSE LASER ON THE EPITHELIA OF THE BLADDER, TRACHEA AND TONGUE OF THE MOUSE

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Abstract

The effects of low dose CW laser were studied by in vivo and in vitro systems. The experimental tissues that were used included bladders, tracheas and tongues as experimental tissues. Buddings (round surface projections) from the transitional epithelium of bladder were frequently observed 3 days after laser treatment in both in vivo and in vitro systems. The trachea and tongue were less affected. In both the in vivo and in vitro systems, some epithelial cells of the trachea showed decreased microvilli and cilia 3 days after treatment whereas the epithelial cells of the tongue revealed no response to laser treatment in both systems. Low dose laser, however, appeared to promote the rate of healing of experimental tongue ulcer: healing was about 1 day earlier in the laser treated than non-treated animals and vessel infiltration and epithelialization were detected earlier in the treated.

KEY WORDS: Low dose, laser, bladder, tongue, trachea, ulcer, culture.

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Introduction

Since the early discoveries of the laser (Maiman, 1960), numerous studies have been conducted on the biological effects of this form of radiation. The majority of these works focused on the retina and described the biological effect of the laser on this tissue (Flocks and Zweng, 1964; Noyori et al., 1964; Jones and McCartney, 1966; Kohtiao et al., 1966; Zweng et al., 1966; Synder, 1967; Campbell et al., 1968; Marshall and Mellerio, 1967, 1968; Fine et al., 1968; Leibowitz and Peacock, 1969; Taleff et al., 1969; Watts, 1969; Ham et al., 1970, 1974; Lerche and Beeger, 1976; Zuclich and Connolly, 1976; Bahte and Rassow, 1977; Gibbons and Allen, 1977; Lerche et al., 1978; Yew and Chan, 1978). The physical property of the laser techniques used in these reports was mostly thermal and often led to scar formation (Landers et al., 1976). Subsequently, the heating effect of lasers has been applied to other systems as well to achieve certain forms of therapeutic and surgical advantages (Kaplan and Sharon, 1976; LaForte et al., 1976; Ben-Bassett et al., 1978; Cochrane et al., 1980; Kana et al., 1981). These studies have used lasers to control blood loss in surgery, help in removal of tumors and to promote the healing and strengthening of intestinal anastomoses.

More recently, the low dose effects of lasers (employing low output CW lasers) have attracted new attention. The low dose laser, although unable to transmit the more intense heat of earlier systems, can on the other hand, stimulate biological synthesis in the retina (Yew et al., 1982a) or inhibit growth in cultures (Yew et al., 1982b). These effects create a new dimension for further investigation of the low dose CW laser effects on biological systems and the results may very well open new avenues of application in medicine. The purpose of the present paper is to determine the effects of low dose laser on different types of epithelial cells both in intact animals (in vivo) and in culture systems (in vitro).

Materials and Methods

In all cases, a He-Ne laser (Spectra

physics) of 16 mW/cm power density was employed and the animals used were albino mice (strain ICR) of 2.5 months old. The animals were reared in the university animal facility and all mice were free of specific pathogens.

Bladder (in vivo systems)

Six animals were used in this experiment. All animals were anesthetized with Nembutal (1 g/kg). A median incision was made above the pubic symphysis in each animal. The bladder was then exposed and a cut was made in the anterior wall of the bladder to expose the interior of the bladder. Three animals served as control and were not treated with the laser. For the other 3 animals, a laser beam was applied through an optic fiber to the exposed internal bladder surfaces for 5 minutes. The bladders were then closed and the incisions sutured. After 3 days, the animals were sacrificed and the bladders processed for scanning electron microscopy.

Bladder (in vitro system)

Three animals were used in the experiment. The animals were sacrificed and the bladders excised. Two pieces of tissue 1 mm^3 in size were obtained from each bladder. Each sample was placed on a wire-mesh raft (luminal surface up) and cultured in an organ culture medium of TC 199 with 10% fetal calf serum in a culture chamber of 95% O_2 and 5% CO_2 (Trowell, 1959). After 1 h of incubation, 3 pieces of the culture were then irradiated by laser for 5 minutes. The other 3 pieces were not irradiated and served as control. All treated and untreated cultures were returned to the incubation for another 3 days and the cultures processed for scanning electron microscopy.

Trachea (in vivo system)

Six animals were used in this experiment. Under 1 g/kg Nembutal anesthesia, a medium incision was made in the neck of each animal and the trachea (level:rings 3 to 5) exposed. A hole (1 mm^2) was drilled through the anterior wall of each trachea of all 6 animals but the laser beam was applied through the hole into the trachea of only 3 animals for 5 minutes. That is, 3 animals served as experimental and 3 as control. The wound was then closed around the hole and the animals were sacrificed after 3 days. That part of the trachea that was exposed was taken out in each animal and processed for scanning electron microscopy.

Trachea (in vitro system)

Three animals were sacrificed. Two small pieces of trachea at rings 3-5 level (1 mm length) were removed from each animal. Each tracheal ring was cut open and with the surface ciliated epithelium facing upward, was cultured on mesh in TC 199 with 10% fetal calf serum as described above. After 1 h in culture, 3 pieces of the culture were irradiated with the laser for 5 minutes whereas the other 3 pieces served as control. All the cultures were returned to incubation for another 3 days. The cultures were then processed for scanning electron microscopy.

For morphometric counting of microvilli and cilia in both in vivo and in vitro systems, the following method was employed. For all the 4 groups of the experiment on trachea, control in vitro, experimental in vitro, control in vivo and experimental in vivo, 3 specimens of each group were used. The surface microvilli and cilia were counted separately in 20 randomly selected cells per specimen from photographs at 15000X. The results were depicted as histograms.

Tongue (in vivo system)

Six animals were used in the experiment. 3 served as control and were not irradiated. For the other 3 animals, a laser beam was directed onto the mid-dorsal surface of each tongue through an optic fiber for 5 minutes while each animal was anesthetized with 1 g/kg Nembutal. After 3 days, the control and treated animals were sacrificed and the tongue excised and processed for scanning electron microscopy.

Tongue (in vitro system)

Three animals were used in the experiment. The animals were sacrificed and their tongues were excised. Two pieces of the tongue 1 mm^3 in sizes were cut out from the mid-dorsal zone of each animal and each specimen was put in organ culture medium, TC 199 with 10% fetal calf serum, and cultured as described in the previous paragraph (for the bladder). After 1 h, 3 cultures were irradiated by laser for 5 minutes whereas the other 3 cultures were not irradiated and served as control. All the treated and control cultures were returned to incubation for another 3 days and then processed for scanning electron microscopy.

Laser Effect on the Healing of Experimental Tongue Ulcer

Tongues of intact animals were employed. Twenty-four animals were anesthetized with 1 g/kg Nembutal. Under anesthesia, an area of 1 mm^2 on the mid-dorsal surface of each tongue was stripped of its surface epithelial layer (epithelium) using a sharp scalpel or with scissors. Slight bleeding followed and the wound was washed with saline. The control (12 animals) were left untreated. The wounds of the other 12 animals were irradiated with the laser beam for 5 minutes and the beam was delivered through an optic fiber while the animal was anesthetized. All control and experimental animals were injected with tetracycline IP (10 mg/kg). The animals (3 controls and 3 experimentals for each time point) were sacrificed 8, 24, 48, 72 h after surgery and the ulcerated specimens processed for scanning electron microscopy.

All samples were fixed in 2.5% glutaraldehyde in cacodylate buffer for 2 h and then washed in cacodylate buffer. After washing and dehydrating through graded series of alcohol, each sample was put into Freon TF and absolute alcohol mixture for 15 minutes and then into 3 changes of Freon TF, 15 minutes each. They were then critical point dried and coated with gold. Observations were made using a 35 CF JSM scanning electron microscope.

In order to evaluate quantitatively the increase in the number of blood vessels (capillaries) in the regions of experimental ulceration, 24 animals were used. Experimental ulcers were made under anesthesia on the mid-dorsal regions of the tongue as stated above. Twelve animals were left as controls while the ulcers of the other 12 (animals) were irradiated with the laser beam through an optic fiber for 5

minutes as in the previous experiment. Half of the control and half of the experimental animals were sacrificed at 8 h after surgery and the other half at 24 h after surgery. The areas with the ulcer were removed, fixed in neutral buffered formalin, dehydrated in graded series of alcohol and cleared in xylene. After clearing, the tissues were embedded in paraffin. Sections were then cut 6 μm through the central regions of the ulcers and either stained with H & E or stained with the DAB method for peroxidase (Pearse, 1972) which made the red cells in capillaries more prominent to facilitate counting of these vessels. From the histological sections, the number of capillaries per high power field (600X, Nikon microscope) in the lamina propria right beneath the lesion and at the edges of the lesions were counted. For each animal, 6 random areas beneath the lesion and 6 random areas at the edge of the lesion were counted. The mean with the standard deviation were computed from all the samples of all the specimens.

Results

Bladder

Scanning electron microscopy revealed that the luminal surface of the transitional epithelium of the bladder of all 3 control mice demonstrated very little specific surface characteristics. The cells approximated hexagonal shapes and had a microvillus apical surface which bulged into the bladder lumen (fig. 1). After low dosage laser irradiation, there were ball-like projections from the surface of these transitional cells (figs. 2a & 2b). The projections ranged in size from 3 to 4 μm and projected from portions of the apical surface of some cells. Unlike the control cells, microvillus projections were absent from the surface. The control cells were devoid of any ball-like projections as compared to the treated samples. In the control cultured transitional epithelium (i.e., those cultured but not irradiated), the apical surface of epithelial cells was wrinkled and had fewer microvilli. When these samples were compared with the in vivo group, the cells from the in vitro group appeared flatter (fig. 3). In the laser-treated cultures, ball-like buddings arose from the surfaces of the transitional cells (fig. 4) like their treated in vivo counterparts. These buds were as large as 7 to 8 μm in diameter and had convoluted surfaces. Apart from these changes, a small number of transitional cells in the treated cultures revealed some surface wrinkling (i.e., shrinkage) (fig. 5).

Trachea

In the pseudostratified epithelium of the control mouse trachea, 2 types of surface projections were observed. From the apical surface of 1 type of cell, long cilia were seen which were about 1.5 μm or longer in length. From the bulging surfaces of other cells, small microvillus-like projections (usually of length 0.2 to 0.3 μm) were noted. Both types of projections were seen along through the entire luminal surface of control samples (fig. 6). Tissues from all the culture groups showed a decrease in the number of cilia and microvillus projections when compared with the tracheas of intact animals (fig. 7). All specimens from the control trachea (3 specimens) and those from intact animals after irradiation (3 specimens) revealed very little morphological difference. However, in the laser treated in vivo, a decrease in the groups of cilia and microvilli were observed when compared with the control in vivo (figs. 8, 9). There was a further decrease in these surface projections in the treated culture (i.e., treated in vitro) when compared with the control culture (i.e., control in vitro) (figs. 10 and 11).

Morphometric measurements on the numbers of cilia and microvilli in the tracheal epithelial cells of the 4 groups (control in vivo, experimental in vivo, control in vitro and experimental in vitro) are depicted in figs. 12-15. There are different frequency distributions in the 4 groups. On the whole, cells in the experimental (laser treated) groups had less cilia and microvilli than the control and the in vivo cells also had more cilia and microvilli than the in vitro. The number of microvilli in the tracheal cells of control in vivo was 187.7 ± 37.8 ($\bar{x} \pm$S.D.) versus 164.5 ± 36.7 in the tracheal cells of laser treated in vivo. The number of cilia in the tracheal cells of control in vivo was 62.3 ± 14.4 versus 52.6 ± 5.3 in the tracheal cells of laser treated in vivo. All differences between control and experimental were highly significant ($p<0.01$, t test). The number of microvilli in the tracheal cells of control in vitro was 122.6 ± 14.1 versus 74.3 ± 17.8 in the tracheal cells of laser treated in vitro. The number of cilia in the tracheal cells of control in vitro was 41.7 ± 7.1 versus 24.5 ± 6.9 in the tracheal cells of laser treated in vitro. All differences between control and experimental were highly significant ($p<0.001$, t test).

Tongue

The surface epithelium lining the tongue of the mouse was lined by stratified squamous epithelium with numerous projecting papillae (fig. 16). Sometimes the projections were cornified. In all the 4 groups (control in vivo), laser treated (in vivo), control culture (in vitro), laser treated culture (in vitro), very little differences could be detected. The papillae of laser treated groups appeared somewhat shorter in size than control (fig. 17).

On the other hand, after experimental ulceration of the tongue, obvious differences in healing were evident between the control and the laser irradiated groups. Figs. 18, 19 showed the lesion 1 h after denudation of the surface epithelium. The lamina propria was exposed. Connective tissue cells and fibers as well as blood cells were apparent. 8 h after making the lesion, both control and laser treated animals still had a lot of debris in the lesion (fig. 20), and more vessels were seen infiltrating into the lesion of the treated group (fig. 21). Twenty four h after, epithelialization of the lesion by squamous cells was apparent but only in the laser treated specimens (fig. 22). By 48 h, debris still covered the ulcer of most control specimens with little epithelialization although a

lot of vessels had migrated into the wound (fig. 23). At this time, the ulcer of the laser treated specimens were covered by epithelium and had healed (fig. 24). By 72 h, healing was also largely completed in the control specimens (fig. 25) but the treated sample demonstrated well healed epithelium (figs. 26, 27).

Morphometrical measurements of paraffin-embedded sections ($\bar{x}\pm$S.D.) indicated that the number of capillaries per high power field (600X) in the lamina propria beneath the lesion was 5.3±1.2 and at the edge of the lesion was 4.3±1.2 for the control at 8 h after surgery. For the laser treated animals 8 h after surgery, the number of capillaries per high power field (600X) in the lamina propria beneath the lesion was 9.8±1.9 and at the edge of the lesion was 6.6±1.1. Employing the t test, the difference between the control and experimental was highly significant ($p<0.01$ for the edge of the lesion and $p<0.001$ for the center of the lesion).

24 h after surgery, the number of capillaries for high power field (600X) in the lamina propria beneath the lesion was 8.1±2.5 and at the edge of the lesion was 6.1±2.1 for the control. For the laser treated animals 24 h after surgery, the number of capillaries per high power field (600X) in the lamina propria beneath the lesion was 11.6±4.2 and at the edge of the lesion was 9.1±3.4. Employing the t test, the difference between the control and experimental was slightly less significant than the 8 h groups ($p<0.1$ for the edge of the lesion and $p<0.2$ for the center of the lesion).

Discussion

Our results indicate that the laser effect on the culture system is very similar to the response in the intact animals for the responses of the epithelia in the bladder and the tongue. The culture system of the trachea was however more sensitive than the trachea of the intact animal. Care must therefore be taken when generalizing results from culture for use in intact animals. Furthermore, no serious deleterious cellular effects as reported in the embryonic lung after CO_2 laser irradiation (Shepanek et al., 1979) was registered in our cases. Of the 3 types of epithelium, the transitional epithelium of the bladder is most sensitive to low dose laser irradiation. After irradiation, budding from the surface is a consistent feature. Such budding represents perhaps a response to injury. For the pseudostratified epithelium of the trachea, loss of cilia and microvilli are features after irradiation. The tongue appears to be most resistant to low dose laser irradiation and does not appear to sustain injury.

One of the most conspicuous advantages of low dose laser in the present study is in the acceleration of healing of the experimental ulcer of the tongue. The quickened rate of healing is perhaps related to the more rapid vascularization induced by the low dose laser. Earlier epithelialization is another factor and may be a consequence of rapid vascularization as early vascularization will bring in sooner the nutrients required for cellular synthesis leading to epithelialization. Kovacs et al. (1974) using the ear chambers of rabbit as experiment, concluded that laser could induce increased vascularization of newly formed tissues. This effect is achieved by 1) activation of the vessels and/or 2) increase phagocytic capacity of the macrophage and/or 3) loosening the fibrin network of the clot (Kovacs et al., 1974). Morphometrical studies in our case also indicated an increase in the number of capillaries in the laser treated group 8 h to 24 h after lesion. This is most obvious at sites beneath the lesion 8 h after. Promotion of skin healing has been documented in the rats after He-Ne laser stimulation (Kana et al., 1981). Kana et al. attributed the phenomenon to the possible increase in collagen synthesis after low dose laser irradiation. In their case, however, no attention was paid to epithelialization.

The low dose laser effect represents a new category of possible application of lasers to medicine. However its effects - both deleterious & advantageous must be clearly understood before it can be put to good use. The present paper endeavours to promote further interest along this line.

Acknowledgements

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Fig. 1. Normal transitional epithelium of bladder in control animal. Note hexagonal shape with minute surface projections. Bar = 1 μm

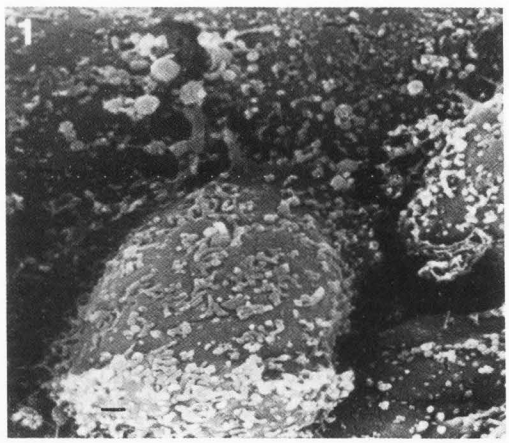
Fig. 2a. Transitional bladder epithelium (in vivo) after laser irradiation. Note ball-like budding from surface (arrow). Bar = 1 μm

Fig. 2b. Transitional bladder epithelium (in vivo) after laser irradiation. Note ball-like budding actually continuous (arrows) with transitional epithelium. Bar = 1 μm

Fig. 3. Control bladder epithelium after 3 days in culture. Note few surface projections but wrinkled on surface. Bar = 1 μm

Fig. 4. Bladder epithelium in culture after laser irradiation. Note ball-like budding with convoluted surface (arrow). Bar = 1 μm

Fig. 5. Bladder epithelium in culture after laser irradiation. Note shrinkage and wrinkling of some cells (arrow). Bar = 1 μm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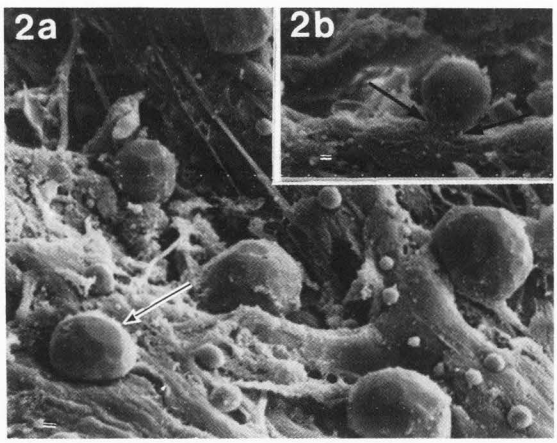
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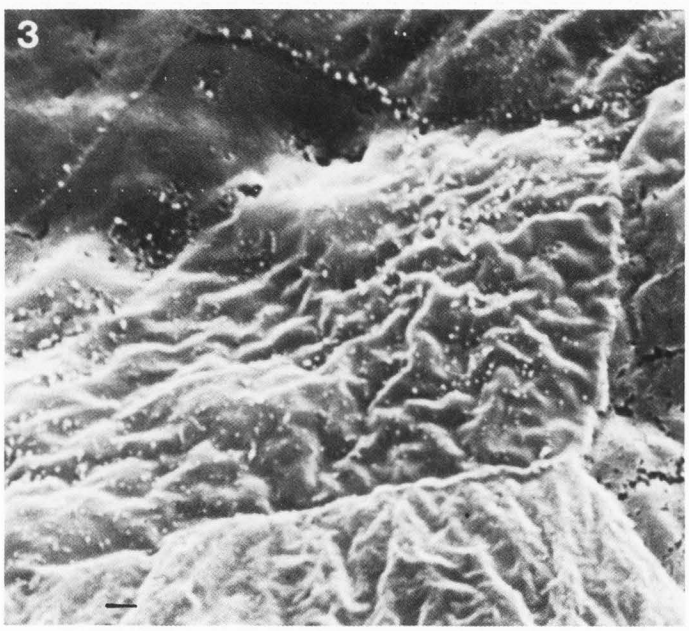
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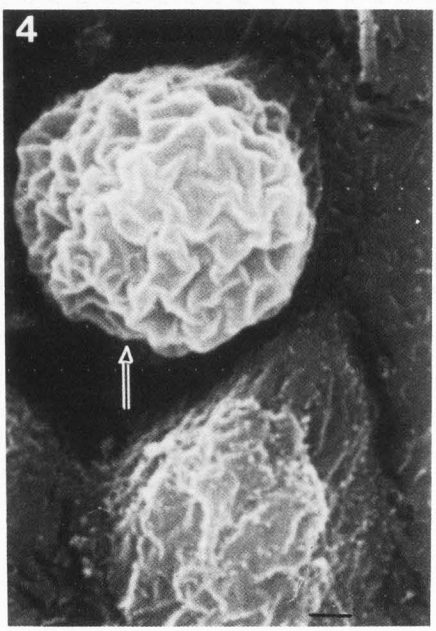
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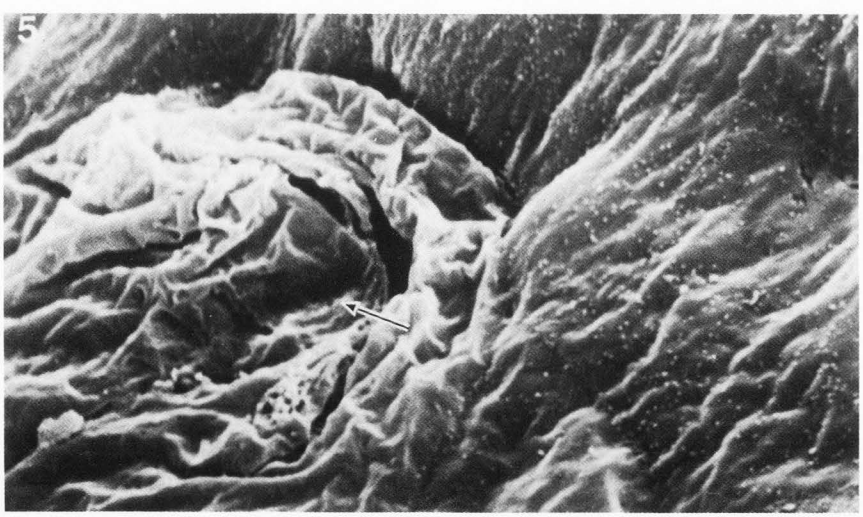
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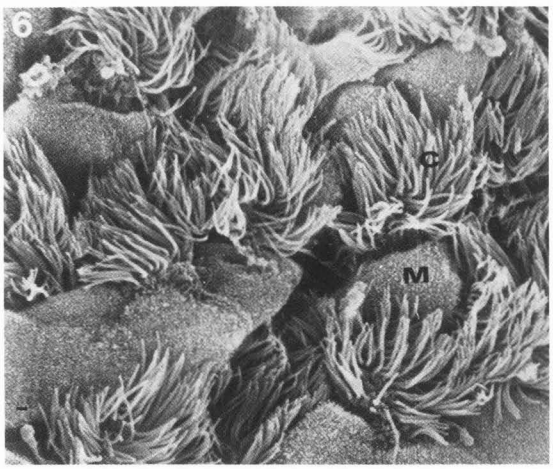
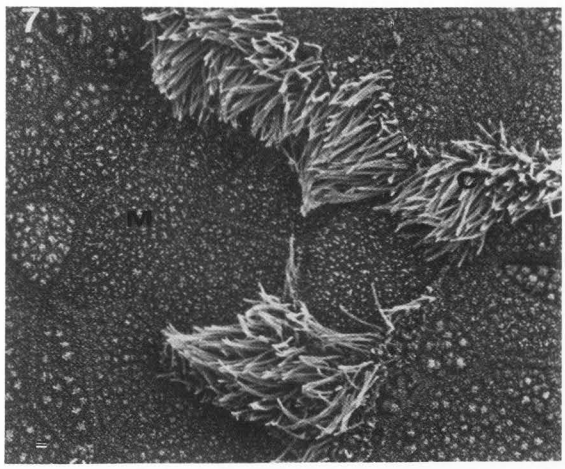
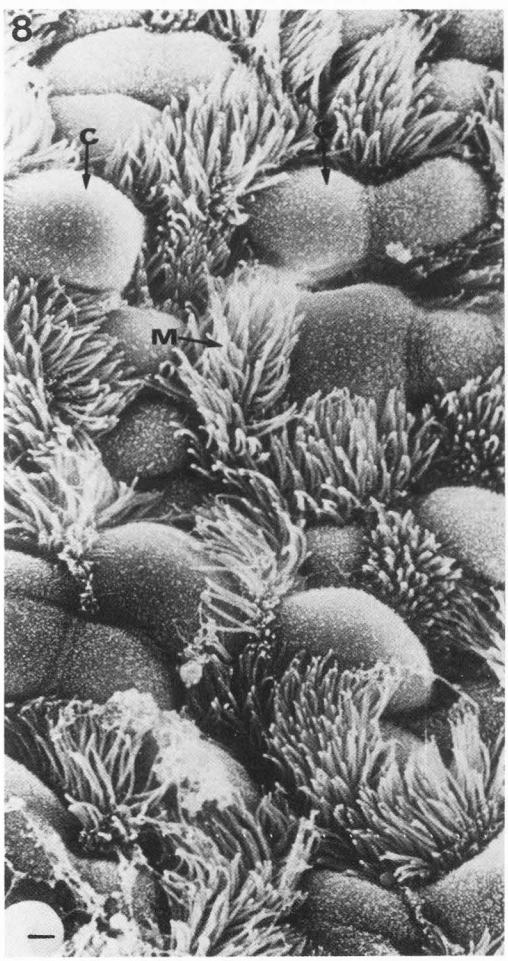






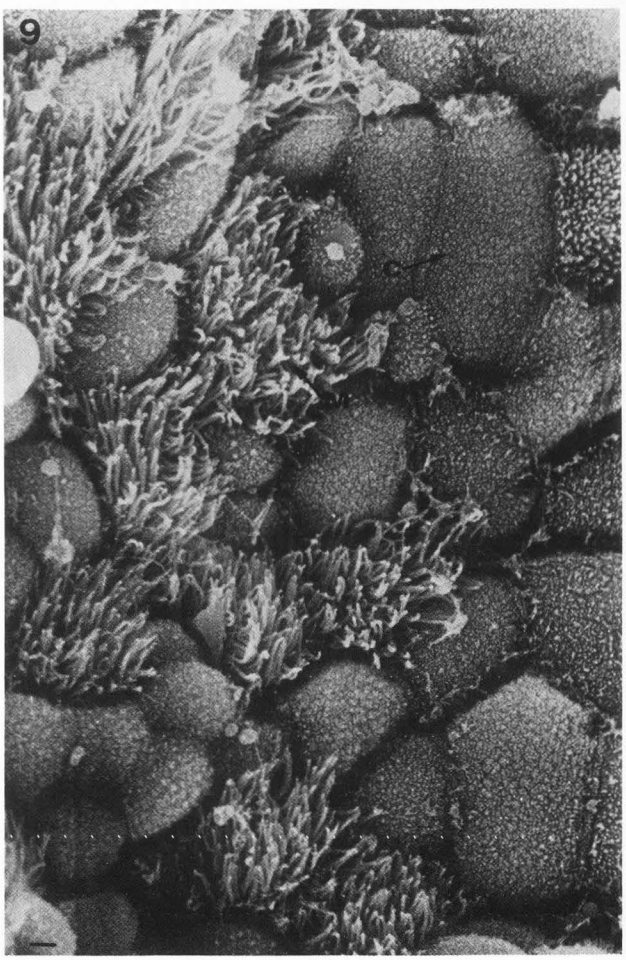
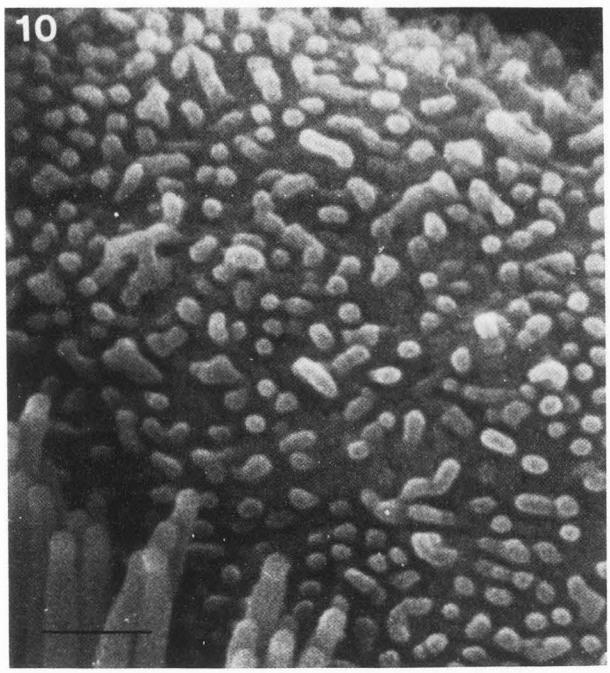
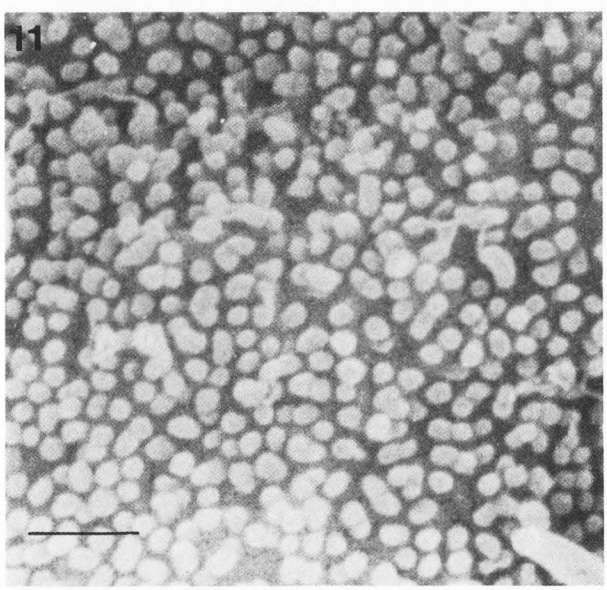


Fig. 6. Normal pseudostratified epithelium of trachea in control animal in vivo. Note cilia (C) and microvilli (M) on surface of cells. Bar = 1 μm

Fig. 7. Pseudostratified epithelium of trachea in culture (in vitro control). Note flattened surface and fewer cilia (C) and microvilli (M) when compared with in vivo control. Bar = 10 μm

Figs. 8, 9. (Fig. 8) Pseudostratified epithelium of trachea in vivo in animal 3 days after laser irradiation. Note less cilia (C) and microvilli (M) in comparison with control in vivo (Fig. 9). Bar = 1 μm





Figs. 10, 11. (Fig. 10) Trachea epithelium in culture after laser irradiation. Note there are fewer microvilli (Fig. 10) than control culture (Fig. 11). Bar = 1 μm

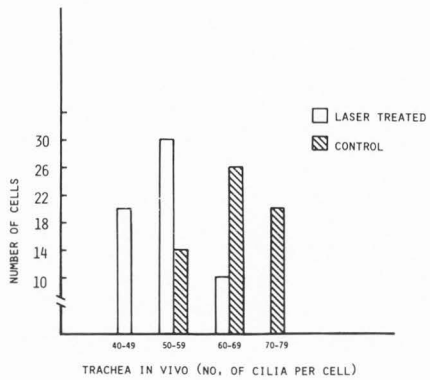


Fig. 12. Histogram denoting the frequency distributions of the number of cilia per epithelial cell of trachea in the control in vivo and laser treated in vivo.

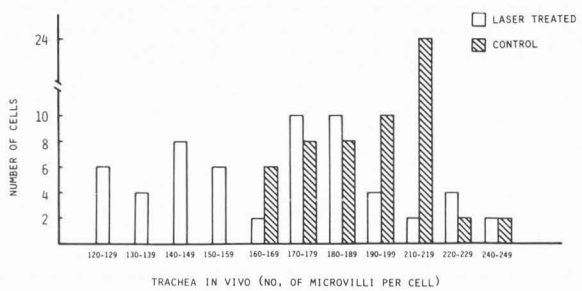


Fig. 13. Histogram denoting the frequency distributions of the number of microvilli per epithelial cell of trachea in the control in vivo and laser treated in vivo.

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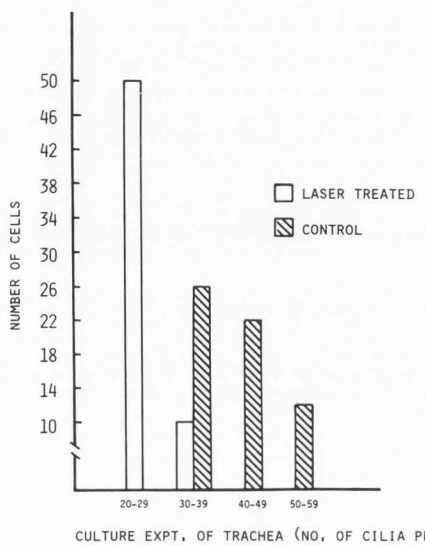

Fig. 14. Histogram denoting the frequency distributions of the number of cilia per epithelial cell of trachea in the control in vitro and laser treated in vitro.

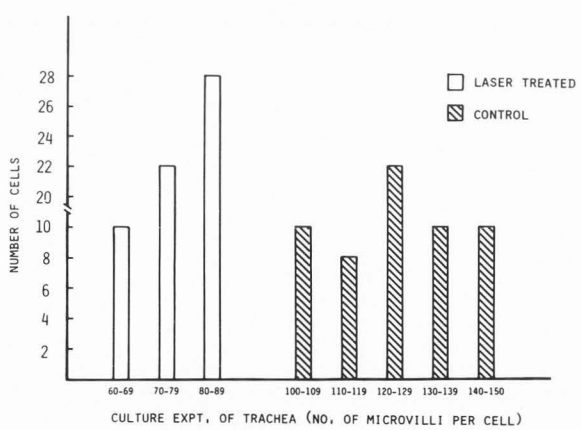

Fig. 15. Histogram denoting the frequency distributions of the number of microvilli per epithelial cell of trachea in the control in vitro and laser treated in vitro.

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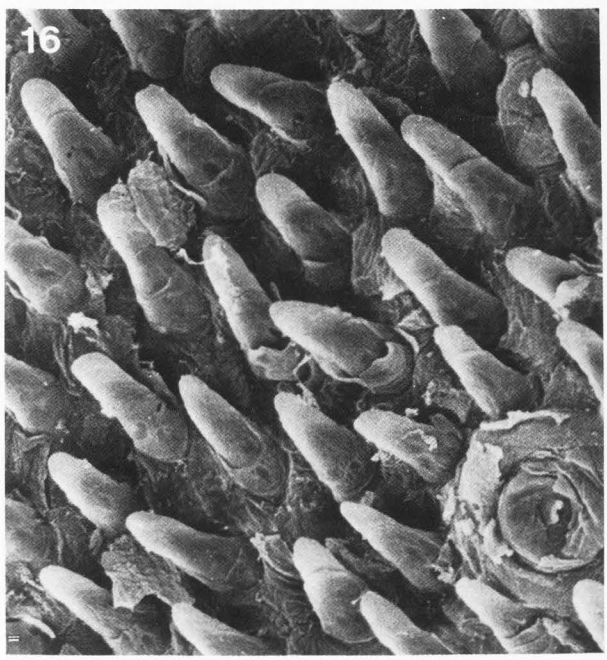

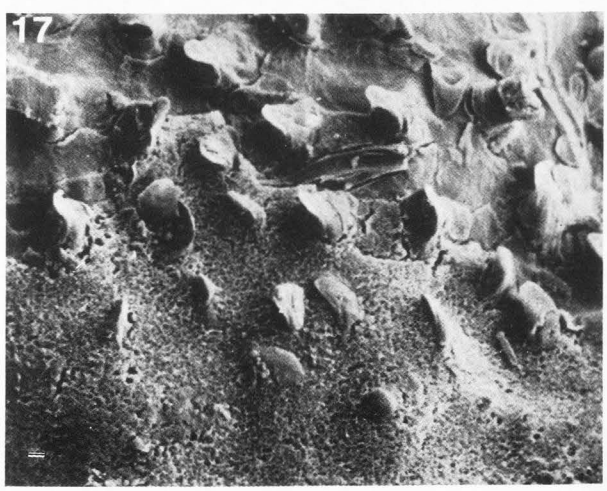

Fig. 16. Normal tongue epithelium in vivo. Note papillae on stratified squamous epithelium. Bar = 10 μm

Fig. 17. Tongue in vivo after laser irradiation appears very similar to control tissue except shorter papillae. Bar = 10 μm

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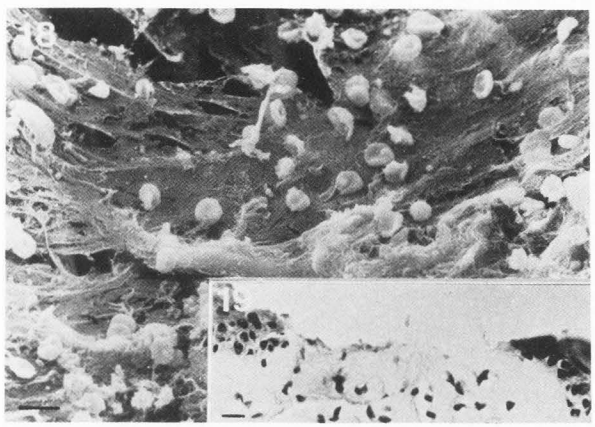
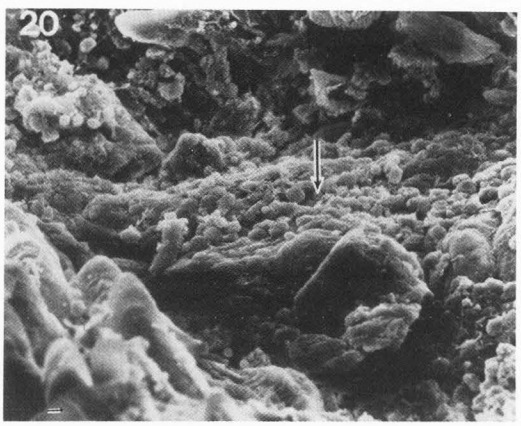
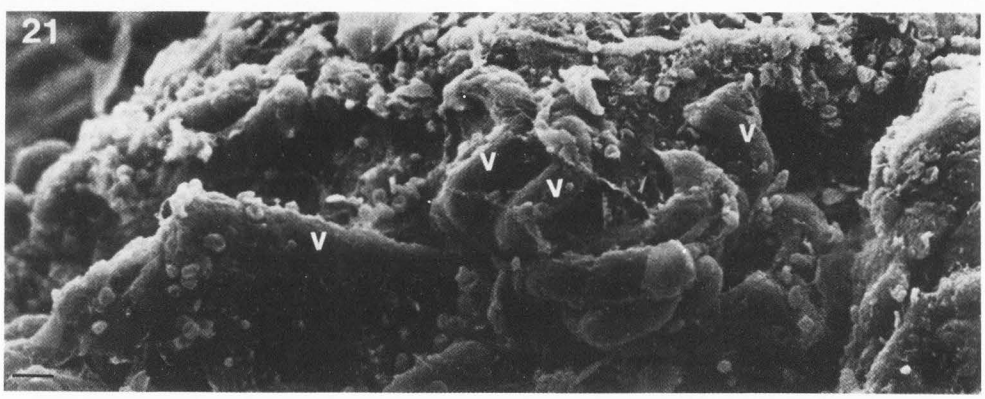
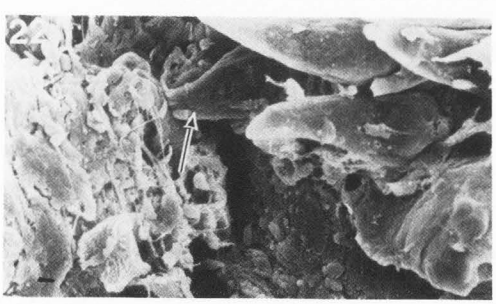








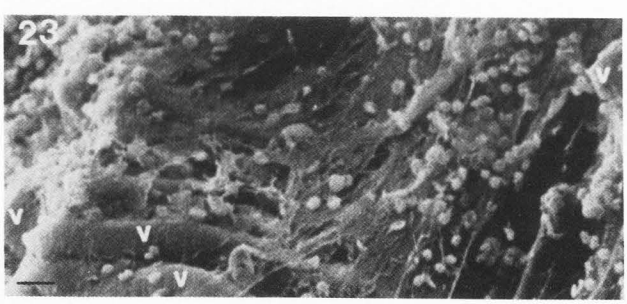


Fig. 18. Tongue denuded of surface epithelium, i.e., ulcer. Note blood cells and connective tissue in scanning electron micrograph. Bar = 10 μm

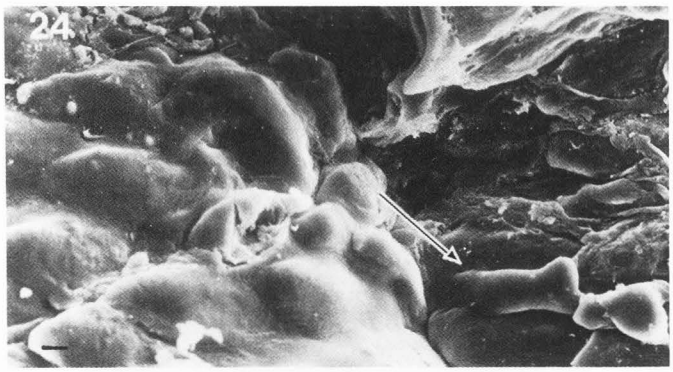
Fig. 19. Light micrograph of ulcer lesion on tongue. Bar = 10 μm

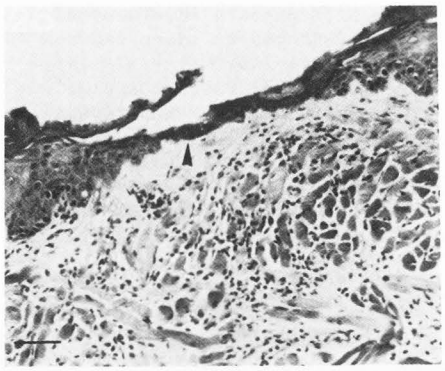
Fig. 20. Tongue ulcer 8 h after surgery. Note excessive amount of debris (arrow). Bar = 10 μm

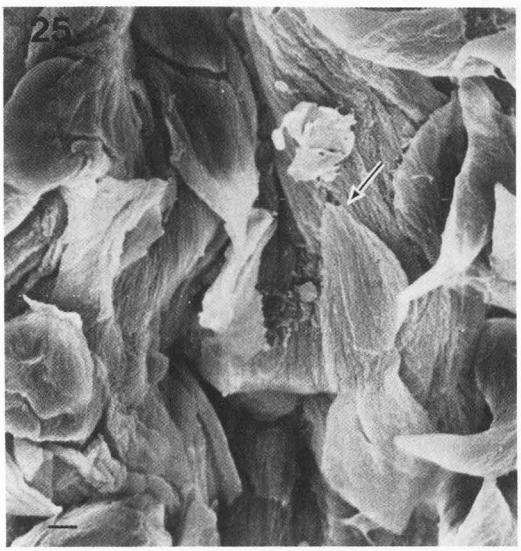
Fig. 21. Infiltration of ulcer by blood vessels (v) (after laser treatment). Bar = 10 μm

Fig. 22. Beginning of epithelialization of laser treated ulcer (arrow) 24 h after surgery. Bar = 10 μm

Fig. 23. Healing of control ulcer with vessels (v) migrating into it 48 h after surgery (without laser treatment). Bar = 10 μm







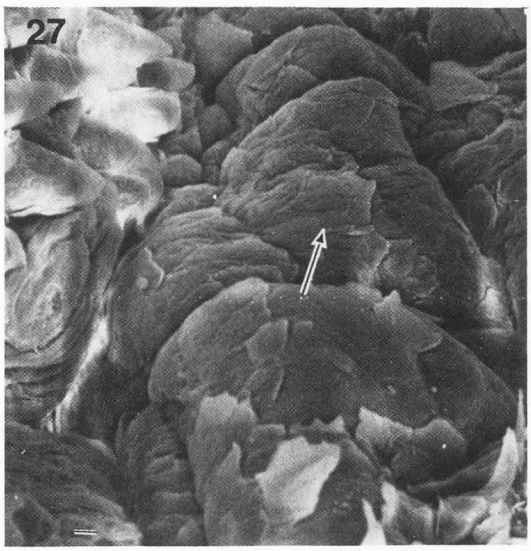


Fig. 24. Epithelialization of laser-treated ulcer is almost completed by 48 hours after surgery (arrow). Bar = 10 μm

Fig. 25. 72 hours later, control ulcer is also covered by thin epithelium (arrow). Bar = 10 μm

Fig. 26. Light micrograph showing well healed ulcer (laser treated) 72 h after surgery (arrow). Bar = 10 μm

Fig. 27. Well healed laser treated ulcer 72 hours after surgery (scanning electron micrograph) (arrow). Bar = 10 μm

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Discussion with Reviewers

A.P. Evan: Have you performed TEM studies on the ball-like projections after laser treatment to determine what cellular changes are taking place to produce these structures?
Authors: No, not as yet.

Reviewer III: The number of cilia per mouse tracheal cell appears lower than that generally reported in the literature for tracheal ciliated cells. With what degree of accuracy can the counts be made, considering the difficulties involved in imaging sufficiently well all the cilia of a cell in any one micrograph?
Authors: The difference may be due to a difference between genetic strains and perhaps also the breeding environment. We feel that the counts were accurate as they were done under high magnifications and the cilia were white contrasting a dark background and were very conspicuous. In spite that the epithelial cells have slight bulging surfaces, a view from the top still shows all the cilia.

Reviewer III: Would you speculate as to how low dose laser treatment may accelerate vascularization of a wound? Does it stimulate endothelial cell division or rate of migration?
Authors: We think it probably stimulates both endothelial cell division as well as the rate of migration. We are hoping to conduct further studies along these lines.