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DETECTING PLANT SILICA FIBRES IN ANIMAL TISSUE BY CONFOCAL FLUORESCENCE MICROSCOPY

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Abstract—Silica fibres from the inflorescence bracts of the grass *Phalaris canariensis* L. cause dermatitis, and have been implicated in the aetiology of oesophageal cancer in northeastern Iran. Here we describe a method for labelling these fibres so that they can be located in mammalian tissue. Fluorescein was covalently linked to isolated, purified fibres with the silane coupling agent 3-aminopropyl triethoxysilane. The labelled hairs were then rubbed into the backs of mice. These were later killed and their skin fixed, stained and sliced at a thickness of 250 μm. A confocal laser scanning microscope gave brilliant images of the fibres at any depth up to 100 μm or more beneath the surface of the slice. Fibres penetrated deeply into the dermis. Several cubic millimetres of tissue could be surveyed in 1 h. The number of fibres present was approximately 2 mm^{-3} initially, falling to 0.1 mm^{-3} after 7 days.

INTRODUCTION

THE grass *Phalaris canariensis* L. is farmed commercially in many parts of the world including the United Kingdom. Its seeds are enclosed in lemmae which bear fine silica fibres, known anatomically as inflorescence macrohairs, which can cause skin irritation and dermatitis among people who handle them. Dermatitis is prevented in seed warehouses by protective clothing and vacuum extraction systems, modelled on the systems used in woodworking factories.

However, hairs from *P. canariensis* have been found to contaminate the diet in a region of northeastern Iran where there is a high incidence of oesophageal cancer (O'NEILL *et al.*, 1980). Similar plant silica hairs (in this case derived from millet) have been found in the diet of a part of China where the same disease has a similar incidence (O'NEILL *et al.*, 1982). In addition, there are several other parts of the world where exposure to other silica fibres derived from plants could be related to cancer incidence, for example, sugar cane farming in Florida (BOENIGER *et al.*, 1988). Purified *P. canariensis* silica fibres have been shown to promote skin cancer in laboratory mice (BHATT *et al.*, 1984). There is, therefore, a real possibility that plant silica fibres might present a long-term health hazard.

The mechanisms of mineral fibre carcinogenesis are not understood (MOSSMAN *et al.*, 1990; SELIKOFF, 1990). The chemical constitution of the fibre does not seem to be important, and carcinogenic mineral fibres can be formed from a wide range of compounds, including tremolite, crocidolite, chrysotile, erionite and glass in human epidemiology. An even wider range has been found to be carcinogenic in animal

experiments. It seems that carcinogenicity depends only on the durability, size and shape of the fibres (STANTON, 1974; STANTON *et al.*, 1977). Unfortunately, it is still not clear precisely what shapes of fibres are important, nor exactly how they might affect growth and differentiation: probably there is more than one mode of action. One possibility, that long fibres induce proliferation by altering cell shape, remains uninvestigated (MAROUDAS *et al.*, 1973).

In the past 15 years a considerable body of information has been obtained concerning the inflorescence macrohairs of *P. canariensis*, and more is known about them than about any other silicified plant structure. Besides the studies mentioned above, much work has been carried out on their structure and development (HODSON *et al.*, 1984; HODSON and BELL, 1986; SANGSTER *et al.*, 1983a,b; SANGSTER and HODSON, 1986), and their chemistry is also well understood (PERRY *et al.*, 1984a,b, 1987). They are larger and more uniform than any other mineral fibre which might be associated with cancer, and although only 0.5 μm wide at the point, their shafts are thick, with a mean maximum diameter of 15 μm, and they are 200 μm long (BHATT *et al.*, 1984). Similar lengths do occur in other kinds of fibre, but only as a small component of a wide size distribution. Glass fibre has an approximate median diameter of 1 μm and a wide range of lengths; the asbestos minerals have still smaller diameters and still greater ranges of lengths (O'NEILL *et al.*, 1986).

The hairs of *P. canariensis* are difficult to see under the microscope in spite of their size, because of their transparency and the similarity of their refractive index to that of the mounting medium. In addition, like the other fibres, their hardness means they are torn out as sections are cut. In consequence, we still do not know what proportion of these fibres enters the body either in animal experiments or in human exposures, nor whether they migrate to other sites or remain in their original position, nor how long they can persist, nor which cells they could interact with, nor how these interactions might lead to disease.

This paper describes an investigation into the possibility of labelling these fibres for light microscopy. The fibres of *P. canariensis* can be covalently linked to fluorescein, and when such fibres are embedded in skin they are easily visible in fluorescence or confocal microscopes. The brightness of the label allows relatively large volumes of tissue to be surveyed. Confocal microscopy allows three-dimensional imaging of the fibres and the cells surrounding them. This technique therefore offers a new approach to the study of the interactions of plant silica fibres with the cells of animal tissues.

MATERIALS AND METHODS

Silica fibre isolation

The sample of purified *P. canariensis* inflorescence hairs used had previously been used for carcinogenesis experiments. This material had originally been collected from the dust extraction plant in the seed cleaning installation of Darling Downs Grain Exporters Pty Ltd, Mount Tyson, Queensland 4356, Australia (the choice of supplier was dictated by the season; a northern hemisphere source was Manor Farm Granaries, Brington, Huntingdon, Cambridgeshire PE18 0PY, U.K.).

The purification process has been reported before. It involved repeated sedimentation, followed by digestion for 72 h in boiling 20% nitric acid to dissolve organic material. The final product was a white flocculent dust consisting of essentially pure

silica fibre with a mean length of 150 μm (range 40–300), and a mean diameter of 14 μm (range 7–30). The fibres were sharply pointed at one end; the radius of this point was 0.25 μm (BHATT *et al.*, 1984).

Silica fibre labelling

Silica was labelled with fluorescein by the method described by VAN BLAADEREN and VRIJ (1992). It makes use of a silane coupling agent, 3-aminopropyl triethoxysilane (APS), to link fluorescein isothiocyanate (FITC) with exposed silica groups on the fibres. A new surface coating of pure silica is then deposited from tetraethoxysilane (TES).

The procedure was as follows: 25.9 mg of dry FITC (Sigma Chemical Co. isomer 1, molecular weight 389.4) was added to 6.985 ml of dry ethanol mixed with 0.1837 g of APS (Janssen) in a dry nitrogen gas atmosphere. This solution was stirred overnight in a sealed container, the product of this being called FITC–APS. Then 4.2 ml of FITC–APS was added to 2.81 g of the isolated fibre suspended in 130 ml of ethanol–ammonia (a mixture of 1 volume of 25% ammonia with 10 volumes of ethanol), and the mixture stirred at 1000 rpm for 2 h, and then the fibres were allowed to settle to the bottom of the vessel. The supernatant was removed with a vacuum line, and the sediment then washed four times by suspending it in 100 ml volumes of ethanol. This was followed by three washes in ethanol–ammonia.

The washed and labelled fibres were then suspended in 130 ml of ethanol–ammonia and stirred with 28 μl of TES for 2 h. The effectiveness of the coating was tested by immersing a few fibres in carbon disulphide and observing them under an u.v. lamp. Carbon disulphide quenches the emission from fluorescein if it comes into molecular contact with it. This test was repeated after three further additions of 28 μl of TES and one addition of 560 μl. At the end of this process, quenching was considerably reduced.

The same method was used to label with rhodamine isothiocyanate (Sigma).

Implantation of labelled silica in skin

Young adult male mice of the Schneider strain were treated with silica fibre on their dorsal surfaces. First, the hair was clipped from an area about 15 × 30 mm centred over the vertebral column between the pectoral and pelvic girdles. One day later, silica fibres were applied to this prepared surface by rubbing with a (doubly) gloved thumb. The thumb was dipped into a quantity of fibres and the fibres which adhered to the glove rubbed over the skin with 10 strokes, both backward and forward. This dipping and rubbing process was itself repeated 10 times, to make a total of 100 strokes in all. Moderate pressure was used. The mice showed no obvious signs of discomfort or inflammation in the treated area of skin, either immediately or later.

Four hours after this treatment some of the mice were killed by carbon dioxide asphyxiation. The dorsal surface was then treated with a depilatory; two cosmetic depilatories were tried and both performed well with no significant differences. The depilatory cream was allowed to remain for 3 min, and then the hair was washed off under running water. An area of skin measuring 12 × 25 mm was then dissected from the dorsal surface. This was clipped onto a small slip of card to keep it flat. The flattened skin samples were immersed in 4% formalin in buffered saline, and allowed to remain there for at least 2 h. Some of the mice were allowed to survive for 7 days; they were then treated in the same way.

Sectioning

Thick sections, perhaps better called slices, were cut from these fixed skin samples. Immediate observations could be made by cutting frozen tissue; it was not possible to cut frozen sections any thicker than 30 μm, but many of the fibres survived this process. Wax-embedded sections could be cut more thickly, up to about 250 μm, which ensures that fibres outside the cutting plane are not disturbed. Mouse skin is thinner than ordinary tissue samples and the times in the reagents, and in the embedding medium were short. The slices were cut on a base sledge microtome. Because of their unusual thickness, they would not stick together in a ribbon as would ordinary sections, so they were picked up individually with a glass 'hockey stick' and handled gently, and care was taken to avoid disturbing the fibres in the slices by pressure or bending stresses.

Staining and mounting

Slices to be stained were first dewaxed and washed in successive dilutions of alcohol, and then in distilled water. They were then immersed in acid fuschin (1:1000 in water) for 1 min. This was followed by three washes in water and 1 min in TOTO (benzothiazolium-4-quinolinium dimer, Molecular Probes Inc., Oregon, U.S.A.) at a dilution of 1:10 000 in water. After three further washes in water, the slices were rinsed in 70% ethanol, dehydrated in absolute ethanol and cleared in xylene. They were finally transferred to slides and mounted in Fluorolite (supplied by Raymond A. Lamb).

Labelling after implantation

To test the possibility that silica might be labelled *in situ*, slices of mouse skin which had been treated with unlabelled silica fibres were used. The slices were mounted on slides, de-waxed and washed free of xylene with 100% ethanol as before. They were then flooded with FITC-APS for 2 min. After three further washes in ethanol, they were returned to water for staining with acid fuschin. They were then dehydrated again and mounted in Fluorolite as before.

Microscopy

A confocal laser scanning microscope was used to make fluorescence images. The equipment consisted of a Nikon Optiphot epifluorescence microscope fitted with a Nikon planapochromat $\times 60$ NA1.4 immersion objective, a Zeiss Plan-Neofluar $\times 25$ NA0.8 immersion objective and a Nikon $\times 10$ dry objective (Nikon Instruments Division, Shropshire, U.K.). An MRC 600 Bio-Rad laser scanning confocal attachment was mounted on this microscope (Bio-Rad Microscience Ltd, Hertfordshire, U.K.), and used in conjunction with the Krypton–Argon Mixed Gas laser emitting at 488, 568 and 647 nm. The 488 and 568 bands were selected from the beam with the 'dual exciter' setting on the Bio-Rad attachment, and the K1 and K2 filter blocks were used to collect the two fluorescence emissions. The output of the laser was attenuated with the number 2 setting of the neutral density filter to prevent any degradation of resolution. Single scans at the normal rate with the pinhole set to 20% of maximum (3 mm on the scale) and the gain set to 60 gave acceptable images. Some increase in quality was achieved by averaging five scans with the Kalman command, and this was done for the images shown here. When fibres did not lie exactly in the horizontal plane, a Z-series of up to 100 images at intervals of 1 μm was collected and

projected using the 'maximum' option of the Bio-Rad software. Measurements were then made by changing the angle of projection to a plane including the fibre axis. This was done with the 'ThruView' three-dimensional imaging program from Bio-Rad. The effectiveness of this program was confirmed by comparison with the 'VoxelView' programme mounted on a Silicon Graphics Indigo computer (Fairfield Imaging, Forest Row, Sussex RH18 5EZ, U.K.). Both programs gave similar results.

Safety considerations

Silica fibres can cause dermatitis after repeated exposure and have been shown to promote cancer in mice. Only skin contact and ingestion have been associated with disease; they seem to be too heavy to offer any respiratory hazard. Gloves were always worn, and the work done in an enclosed space. All the surfaces which had been exposed to the fibre were wiped with damp tissues which were bagged for incineration after work had finished.

RESULTS

Labelling

This labelling procedure produced brilliantly fluorescent fibres. They remained bright after several minutes' exposure to the laser. Three-dimensional image arrays could be collected without any fading, even with the highest power objective. Labelling was not limited to the surface of the fibre, and confocal optical sections showed fluorescence everywhere except in the axial void. Fibres labelled with rhodamine were also brilliant, but fluorescein was used in preference in the subsequent work.

Yield

Only 25% of the starting weight of material was recovered. The loss could have been due to fragmentation, since small particles are removed by the washing process. Fragmentation might have been caused by the rate of stirring chosen; in future it might be best to set this rate at the minimum necessary to keep the fibres in suspension.

Counterstains

Various counterstains were investigated in a search for good images of the cells surrounding the fibres. Haematoxylin and eosin in combination gave a good confocal image in thin sections but absorbed too much light for imaging in slices. Acid fuchsin gave acceptable images of the cytoplasm in slices, and good contrast with fluorescein. Nuclear stains were less satisfactory. The nucleic acid dye TOTO was tenacious and brightly fluorescent, but emits in the fluorescein channel. Unfortunately, the gain in this channel had to be kept low because of the brilliance of the labelled silica. Nevertheless, the combination of acid fuschin and TOTO made it possible to distinguish the main features of the anatomy of the skin. The monochrome reproductions shown here are less informative than the colour originals.

Imaging fibres in tissue slices

It was easy to find fibres in tissue slices. Figure 1 shows a typical example of a slice cut on the day of treatment. Many of the fibres were lying on the tissue surface, some

had penetrated a short distance, and relatively few had penetrated deeply into the dermis. Much of the silica was in the form of broken debris, indicating that the fibres had fragmented. It is not clear what proportion of this fragmentation occurred during labelling and how much when the fibres were brought into contact with the skin. However, some fibres survived. Figure 2 shows an intact fibre which is 213 μm long and up to 8 μm wide, which is well within the range of sizes in the original sample.

The fluorescein emission showed a clear distinction from the emission of the cornified layer, germinal layer, the dermis and the underlying muscle. However, cell outlines and nuclei were often indistinct. We could not determine whether any cells became attached to the fibres, or spread along them, or suffered any other sort of alteration.

Persistence of fibres in skin

There were fewer intact fibres in skin when it was sectioned 7 days after treatment. A search of 12 slices only yielded one such fibre (Fig. 3). Repeated searches on other occasions indicated that about 10 slices had to be searched to detect a fibre. This finding makes it possible to estimate the numbers of fibres present in the tissue. The slices were 15 mm wide, 250 μm thick, and the band surveyed extended 100 μm from the cornified layer down into the dermis. Their volume was therefore 0.375 mm^3. A calculation based on the number of slices which had to be searched to detect a fibre shows that there were approximately 2 fibres mm^{-3} immediately after exposure, falling to 0.1 mm^{-3} 7 days later.

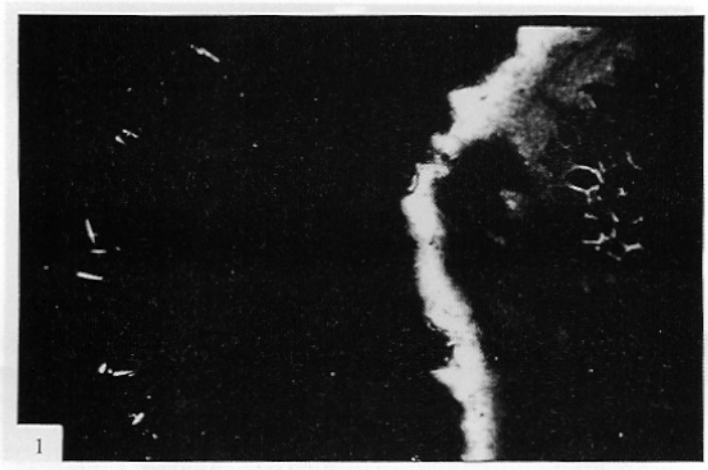
Figure 3 is a single confocal image. Confocal images are optical sections, and at high magnification they only show the whole fibre if it lies exactly parallel to the stage of the microscope. Like most fibres, this one is tilted, and so only the tip shows in this image. A projection of the same fibre is shown in Fig. 4. Twenty sections were made to encompass the whole fibre, and the maximum projection option of the Bio-Rad software was then used to record the brightest pixel at each point in the series. This allowed the whole fibre to be visualized. The fibre is 35 μm long in the XY plane; it was the longest fibre found after 7 days.

Small fluorescein-stained particles (up to 5 μm in diameter) were common after 7 days. These were found at any depth below the surface of the skin, though more commonly in the upper levels of the dermis. They may have resulted from the break-up of fibres; further work would be needed to determine how their total volume is related to the volume of fibres initially present, or how they could have become dispersed to adopt the rather uniform distribution seen at this time. However, it seems clear that

FIG. 1. Transverse slice of skin fixed on the day of treatment. Confocal images in which the green emission (excited at 488 nm) is on the left side and the simultaneously collected red emission (excited at 568 nm) on the right. The location of the fibres in relation to the skin surface has been determined by merging to form a colour image. This image is not reproduced here, but shows that some of the silica fibres lie on the surface of the skin and others penetrate through the epidermis. Magnification $\times$ 300.

FIG. 2. Single channel image at higher magnification, showing a silica fibre of unusual length. It is 213 μm and up to 8 μm wide. Magnification $\times$ 590.

FIG. 3. Merged image of the same field, with acid fuschin revealing epidermis, dermal musculature and hair follicles.



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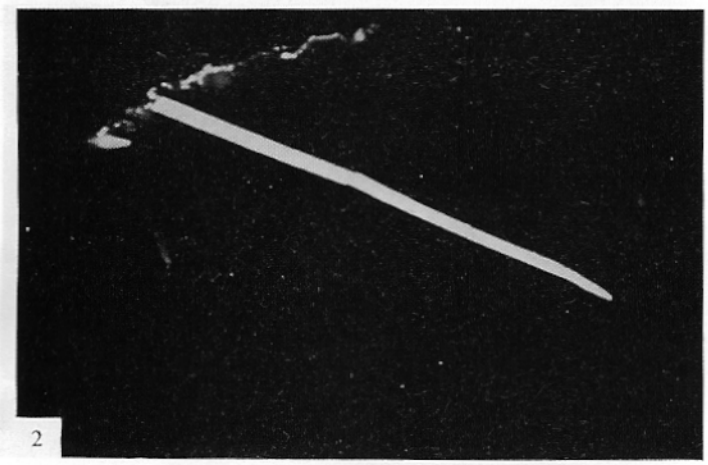

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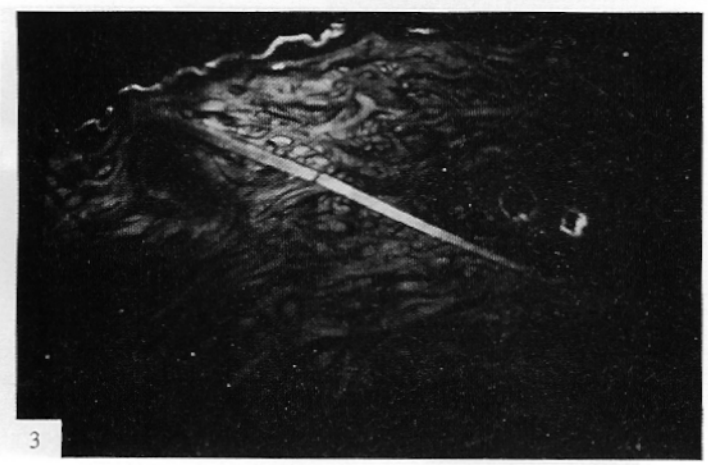

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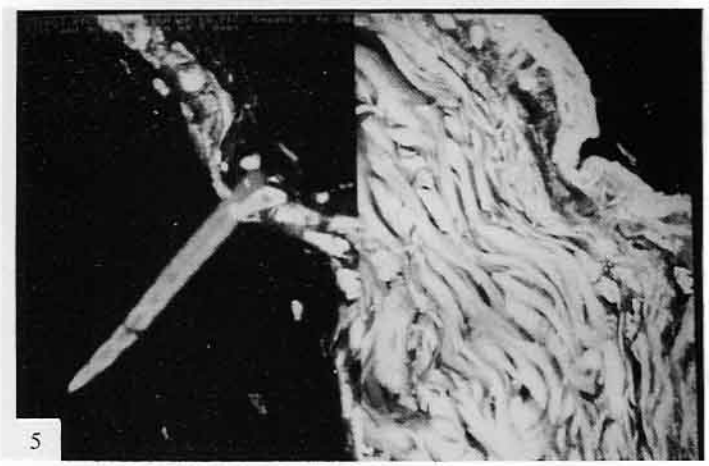

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6

some of the loss of fibres from the skin which occurred during the first few days must have been due to breakage after implantation.

Labelling in situ

Labelling of silica *in situ* was also found to be possible, but was hampered by background staining. Fluorescein–APS could be applied to skin slices in the same way as to isolated silica, and reacted strongly with unlabelled fibres embedded in them. It reacted to a lesser extent with cells and connective tissue, but this tissue fluorescence was much greater in absolute terms because of the much greater mass of skin present. Only fibres which lay near the surface of the slice could be imaged after *in situ* labelling (Fig. 5).

DISCUSSION

These observations show that plant silica fibres can be labelled with fluorochromes in such a way that they can be detected in tissue slices. Slices are so much thicker than sections that considerably increased volumes of tissue can be surveyed. Even very small numbers of fibres can be counted.

In addition, fibres in tissue slices remain in undisturbed contact with the cells surrounding them. It will therefore be possible to use a confocal microscope to study the local effects of fibres on growth and differentiation. Such a study can be expected to lead to a fuller understanding of the action of silica fibres on the organization of animal skin.

It might also be possible to detect silica in human *post mortem* or surgical specimens in this way. It was found that fluorescein–APS can be linked to silica in tissue slices, but that background staining of the tissue causes enough glare to restrict imaging to fibres near the surface of the slice. We do not understand the mechanism of the bonding between fluorescein–APS and protein, but if it is a chemical reaction with free isothiocyanate groups it could be avoided quite simply by ensuring that they are all taken up by APS.

It has been shown here that either fluorescein or rhodamine can be coupled to silica. It therefore seems likely that any fluorescent isothiocyanate can be used to label silica with APS. Choice, then, will be simply dictated by the excitation wavelengths available

FIG. 4. Similar merged image taken from skin fixed 7 days after treatment, at higher magnification. Here the × 60 NA 1.4 lens was used, and its short depth of focus means that only part of the fibre is visible in this single confocal image. Magnification × 2430.

FIG. 5. Projected image of the same field shown in Fig. 3. Here 20 optical sections at 1 μm increments along the *Z*-axis have been integrated to gain depth of focus. The yellow signal is on the left, and shows nuclei in the germinal layer of the epidermis as well as silica; the red signal is on the right, and shows dermal musculature, epidermal cytoplasm and cornified layer. The whole length of the fibre can be seen; there is a fracture near the tip. It is 50 μm long, and the diameter at the midpoint is 3 μm. Magnification × 1440.

FIG. 6. Images of a fibre stained *in situ*. Unstained silica fibre was rubbed on the skin, which was then fixed and sliced. Slices were reacted with fluorescein–APS. Here the two channels both display yellow emission, but at different gains. The skin is less bright than the fibre, but the amount of fluorescein is nevertheless enough to obscure any fuschin signal, and so the details of the tissue are obscured. This fibre lies about 25 μm below the surface of the slice. It is 35 μm long. Magnification × 860.

from the laser and the counterstains desired. It will be possible to use any of the new labelling molecules which are now being developed for antibodies and DNA probes.

The success of this coupling method for silica suggests that other types of mineral fibre might be labelled in the same way. It seems likely that both man-made glass fibres and the various types of asbestos might expose silicate groups on their surfaces which would be available for reaction with APS. Searches for these smaller objects must take more time because they disappear so rapidly as the microscope is defocused, but imaging (as distinct from searching) should not be a problem since self-luminous objects can be detected even when their size is below the resolution limit of the light microscope. The possibility that glass and asbestos fibres could be labelled with APS for imaging by confocal microscopy in animal tissues deserves investigation.

As yet no effects of the fibres on the anatomy of the skin have been detected. In spite of the promise of this approach, not even the cells in contact with them have been identified. It is possible that the brightness of the label has obscured adjacent cells, since there is some spill-over of the fluorescein signal into the rhodamine channel which could obscure a small region surrounding the fibre. Even if the region obscured by spill-over is very small it might be important because cells attached to long linear substrates become very small in transverse section. The nucleus is compressed to about 3 μm, and the remainder of the cell may be less than 1 μm in diameter (O'NEILL *et al.*, 1990). If spill-over is the problem, a simple reduction in labelling will reveal the cells in contact with the fibres. However, it is plain that the choice of counterstains, basic fuschin and TOTO, is only provisional. They give imperfect images of the cells making up the dermis and epidermis.

Confocal and conventional microscopy require different kinds of stain. A confocal microscope can penetrate thick specimens only when the stain is sparsely distributed. When the whole mass of the specimen is stained, too much light is absorbed, and the strong uniform staining which is optimal for thin microtome sections is just the opposite of what is required. We need to select stains specific for single components of the tissue. One possible candidate is rhodamine-labelled phalloidin, which has been found strikingly effective for displaying the three-dimensional anatomy of skin keratinocytes in multilayered cultures (KUBLER *et al.*, 1991). Other specific probes might also be effective. In addition, the specific antibodies also used by Kubler *et al.* fulfil all requirements apart from the small molecular size preferred for reasonably rapid diffusion into skin slices. The question as to whether probes or antibodies could be made to stain slices has not yet been investigated.

Another way of improving the staining would be to introduce small quantities locally by microinjection. A small quantity of stain would offer less obstruction to the beam, and would be just as effective as bulk staining if it was positioned at the site of a fibre. Apparatus which would help to do this by keeping the needle in a constant position in relation to the point of focus of the microscope is now commercially available (Zeiss, Welwyn Garden City, Hertfordshire AL7 1LU, U.K.).

The quality of the images might also be improved with a different mounting agent. The mounting agent we used was developed for thin sections and might not be optimal for tissue slices, where transparency is a primary requirement. Mountants also influence the emission of the fluorochromes under laser irradiation, and can give remarkably different results in confocal laser scanning microscopy (BECKER *et al.*, 1991).

It is plain that many of the silica fibres studied here did not persist in the skin for more than a few days. Perhaps 95% of them were lost within a week, either to the exterior or to other sites in the animal's body. It is not clear whether this rate of loss would be continued, or whether the remaining fibres are persistent. Earlier experiments on the cancer-promoting activity of these fibres do not give any information on persistence. They indicated that these fibres promoted cancer as effectively as croton oil, but only when applied repeatedly. Single applications were not attempted (BHATT *et al.*, 1984).

It seems possible that persistence might be dependent upon mechanical strength, and that the fibre preparation used might have been specially fragile. When these fibres were first purified, acid was used to free them of organic material. *Phalaris canariensis* fibres only contain 40% of silica, and the remainder is made up of polysaccharide, with some protein (PERRY *et al.*, 1987). This material was removed in order to ease the task of explaining their cancer-promoting activity. However, removing the polysaccharide might also have weakened their structure. Native, unextracted, fibres might be more persistent.

These images were made with a krypton laser. The argon-ion laser was just as good for imaging silica when it was labelled with fluorescein, but not so good for imaging cells. The krypton laser's 564 nm band excited rhodamine more efficiently, and gave better separation between the fluorescein and the rhodamine channels. This made it less likely that the brilliance of a labelled fibre could obscure adjacent cells. A reasonable balance between the brightness of fibres and cells is of course still desirable, and the labelling and staining methods we have reported here need some improvement to take full advantage of this property. In addition, the 564 nm emission caused silica fibres labelled with rhodamine to give images as bright as fluorescein, so that there is a free choice of counterstains excited at either 488 nm (fluorescein, TOTO), 564 nm (rhodamine, fuschin) or 647 nm (cyanine). It remains to be seen whether either of these properties are critically important.

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