In Vivo Localization of Injected Carcinogen by Fluorescence Quartz Rod Microscopy

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SUMMARY

A technic whereby carcinogenic hydrocarbon particles injected into living animals can be identified by fluorescence microscopy of living lung fields is described. The method utilizes high pressure mercury light sources, with appropriate filters at the source and microscope optics for fluorescence examination; a quartz rod as light carrier to the lung field; and special thoracotomy on living rats and rabbits for exposure of the area to be examined. The particles stood out wherever localized as long as 5 months after injection. They could be studied by display on a television camera and photography of the monitor image. Film was too slow for in vivo instant recording. Damage, as revealed by ordinary microscopy, was not very obvious, and further in vivo methodology to detect alterations in the physicochemical nature of areas involved is suggested. Liver, spleen, kidney, brain, muscle, bone marrow, and skin also revealed the particles after post mortem fresh examination.

Fluorescence of carcinogenic hydrocarbons upon proper excitation is well known and has been utilized in chemical identification (3, 4, 7, 8, 15). After application to biologic tissues, these compounds have been studied by their characteristic emission in skin (1, 5, 20, 23, 24), in post mortem examination of fluids, cells, and organs after various forms of administration (5, 6, 16, 21, 22) and in vitro tissue extracts (2, 8, 18, 19, 25). Various reports have dealt with the status of the fluorescence spectrum after in vivo combination with tissue (2, 6, 18–25), but direct in vivo observation on internal organs has heretofore been limited to the fresh post mortem examination.

With the development in this laboratory of a technic whereby standard quartz rod transillumination (10) can be adapted for fluorescence observation on living internal organs (11), it appeared possible to attempt in vivo observation of injected carcinogens, especially in lung. The method of localization proved, after some initial difficulties, to be quite simple and is described herein with early results.

MATERIALS AND METHODS

Illumination.—A rod of quartz with a flat ground end (Fig. 1) carries light from an Osram or Hanovia Xenon-Mercury high pressure type lamp. A filter (Fig. 1A) (Corning #5860 or #5874) is placed at the source to narrow transmission, at 3650 Angstrom Units. Advantages of either filter will appear in the “Results” section. The illuminated hollow tip of the rod drips isotonic fluid and is placed so that its beam is directed at approximately 45 degrees over the tissue surface to be examined. A microscope (stereobinocular or standard monocular body without condenser) is brought over the tissue, and focus is achieved in the white light. Then with the above-mentioned

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filter at the source and with an additional 'stop' filter (B) (Corning #3389) at the ocular, fluorescence from the tissue and carcinogen is viewed.

Such observation of delicate tissue autofluorescence as well as faint carcinogen fluorescence at high power (500–900×) depends upon a strong light source. Ordinary G.E. type A-H4 low pressure mercury vapor lamps have not proved satisfactory; the 1000 watt G.E. type A-H6, although water cooled and difficult to set up, is quite adequate. Hanovia 1000 watt Xenon-Mercury air cooled or the smaller and simpler Osram lamps are preferable.

**Animals.**—Rats and rabbits have been used in this study. Rats were of the Sprague-Dawley strain, approximately 6 weeks old when started on injections. Rabbits of similar age in most of the experiments were white New Zealand. Controls were litter-mates where possible; otherwise animals of similar age and stock were used.

In total, 125 animals were used in the experiments reported here, but deaths from trial injection preparations mentioned below and occasional anesthetic or surgical deaths have reduced the reportable cases to 72: 56 rats and 16 rabbits.

**Preparation of materials and method of injection: dosages.**—Considerable difficulty was encountered in arriving at a carcinogen suspension with the desired qualities—low toxicity and small, relatively uniform particle size. Suspensions containing large particles plugged pulmonary vessels upon intravenous injection. If the particles were too small (below 4 μ) or in very dilute dispersion, they were difficult to see. 3,4-benzpyrene, 20-methylcholantherene, and 1,2,5,6-dibenzanthracene were the chemical compounds used. Gelatin suspensions2 (10 mg/cc) when injected produced heavy distribution throughout lung but proved quite toxic. Five per cent alcohol suspension or simple serum suspensions were the least toxic, the serum in all respects the most successful.

Injections3 were for the most part intravenous, although the intratracheal route was tested to establish its feasibility for this study.

Doses effective for the demonstration of the particles in rats ranged from a total of 0.5–5.0 mg. of dibenzanthracene administered, for the most part, serially over periods of 2 weeks in three to seven injections. However, the carcinogen could be identified sparsely distributed after single injections. The concentration in the solution

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A. Kindly supplied by Mr. Sidney Laskin of the New York Postgraduate Institute of Industrial Medicine.

B. Removal of the carcinogen is not as important as the size of the particles. Rabbits received totals of from 0.6 mg. to 35.0 mg. in divided doses over periods of 2 weeks. Single doses resulted in identifiable but widely scattered particles. Again, the particle size of the dispersion and not the concentration of substance is what is of importance for this method of demonstration of carcinogen.

Observations on the living lung were made at different intervals after the injections. These intervals range from a matter of a few seconds to a few hours, and thereafter to a few days, through 2-, 4-, 6-, and 8-week periods, and then at wider intervals including 6, 8, and 12 months.

**Operative technic.**—*In vito* observations on living lung have been carried out according to the method of Irwin, Burrage, Aimar, and Chesnut (9). Under pentobarbital anesthesia an animal was tracheotomized, and a thoracotomy was performed4 while oxygen was administered through the tracheal tube. The lungs were gently inflated until, with just the right balance of anesthesia and oxygenation, respiratory motion of the diaphragm ceased. An animal may be maintained in this state for many hours and can recover after proper closure.

**Observation of area.**—The quartz rod was then brought to the area on which observations were desired, and the microscope was swung into position (Fig. 1). Observations at high power could be carried out on surface alveolar walls, pulmonary arterioles, and pulmonary venules. By looking inside alveolar sacs, one could occasionally make out the mouths of alveolar ducts and respiratory bronchioles. Magnifications included stereoscopic up to 120X and monocular up to 530X by use of 10X ocular and 53X water-immersion objective.

Single-frame photographic recording was best accomplished with sensitive black and white film (Kodak Tri-X Negative) or, if color was desired, Super Anscochrome. However, there was considerable difficulty in getting a clear picture of a living field, which moved with cardiac motion even after respiratory motion had ceased. The most sensitive motion picture film available to us did not register adequately the fluorescence of the carcinogen, although, by long exposure, good still photographs on the film mentioned above were obtainable from lung fields after death. It was then discovered to be quite feasible to display the living lung field and the carcinogen with an Image Orthicon television camera and to photograph the television monitor. The sensitivity of the Image Orthicon was great enough to pick up the faint fluorescence of the carcinogen,
and this image, having been electronically transduced to a monitor, was easily photographed. Such methodology related to light absorption and fluorescence is discussed in references 12–14.

**RESULTS**

**LUNG**

1. *Normal appearance in white light quartz rod microscopy.*—In white light illumination, examination of the exposed lung by the method outlined above revealed alveoli as bright reflecting surfaces. At 100X and beyond, blood in capillaries could be seen to be coursing over the alveoli. Inter-alveolar septa appeared as dark margins except where blood vessels were present. Wandering white cells were not infrequent. An occasional large blood vessel on the surface could be seen, as well as infrequent surface bronchioles. The mouths of alveolar ducts and respiratory bronchioles could be made out by peering directly through alveoli. The walls of these structures were of an irregular outline with a yellow hue. Only on section of fresh lung can intrapulmonary bronchi be made out. No attempt has been made to include in the figures such details of lung histology. Full description and pictures of normal lung by in vivo microscopy appear in detail in reference 9. The above is included for comparison with the following on fluorescence in vivo.

2. *Normal appearance in fluorescence microscopy.*—With a Corning #5874 filter at the light source, there is a bright purple to red hue thrown over the normal illuminated surface. A Corning #5880 filter at the source permits less total exciting light to fall on the surface but also sends less reflected color of the excitant to the eye. This situation affords a better view of autofluorescence and carcinogen fluorescence. For photography or television display, a #3389 filter (Fig. 1B) produces further contrast by eliminating much of the reflected light from the source while it transmits the greater part of the fluorescence. Autofluorescence from normal alveolar surfaces is a faint blue. Small blood vessels are nonfluorescent, although very large vessels have a bright blue outer wall. Variations in intensity of autofluorescence occur, due to changes at which the angle of light strikes rounded alveolar protuberances and the general curve of lung surface. Surface bronchioles fluoresce a faint blue of greater intensity than do alveolar surfaces. On section of freshly removed inflated lung, bronchi may be seen as a bright blue, identifiable immediately by intensity of fluorescence and size of structure. Extrapulmonary bronchi and trachea have bright blue cartilage rings and darker blue to purple surface tissue. When these tubes are cut open and exposed to fluorescence-exciting illumination, the epithelium is dull green in color. Mucus is a dark yellow.

3. *Appearance of carcinogen.*—A carcinogen-injected animal gives a startlingly different picture, because, in addition to the autofluorescence described above, the lung, even at 8X or 38X magnification, is dotted with bright-colored dots. These are immediately identifiable, since their intensity is much brighter than that of the surrounding area. With the #5874 filter a variety of colors ranging from purple to blue-green to yellow is seen by the eye. This variation in color impression on the retina depends on how much of the exciting light source reaches the eye, but this statement is not to be taken to mean that the actual fluorescence emission varies depending on the excitant. The #5880 filter gives the particles a visual appearance less varied and more generally blue than the colors produced by the #5874. The difficulty in subjective determination of color leads logically to an extention of this work which will be mentioned in the next section.

Figure 2 shows the bright fluorescent spots produced by particles of dibenzanthracene in rabbit lung, photographed in color but printed on black and white at 10X magnification. A #5874 filter was used to narrow the transmission of a 1000-watt Xenon-Mercury Hanovia lamp. Figure 3, of rat lung, shows the presence of a single particle of dibenzanthracene excited by light from an Osram lamp with a #5874 filter at the source and a #3389 filter at the eyepiece of the microscope, 10X magnification. However, this image was photographed from the monitor screen of an Image Orthicon television camera placed at the ocular of the microscope. Figure 2 required 2 minutes’ exposure time on color film, whereas Figure 3 was achieved by taking a ½ second black and white polaroid photograph of the television monitor. Figure 4 shows the same lung field as Figure 3 except that filters were removed, permitting white light to illuminate the field with consequent disappearance of the particle. Measurement comparison of any refractile spot seen in Figure 4 with the position of the actual particle of Figure 3 will reveal the difficulty of truly locating a single particle in the area without the fluorescence examination.

Particle size varied with the suspension method, but the smallest particles lodged, after intravenous injection, in vessels of arteriole and capillary caliber. Recent experimentation has demonstrated particles as small as 4 μ, but the size range is generally 10–500 μ. Two weeks after intratracheal
Injection of 1,2,5,6-dibenzanthracene in gelatin, yellow, green, and purple particles were seen at the surface of peripheral alveoli and, at sacrifice, were seen to be distributed throughout the parenchyma. It is of interest to note that particles were not seen in the trachea or bronchi (intra- or extra-pulmonary) of animals given either intravenous or intratracheal injections. Presumably the particles, sprayed intratracheally into bronchi or trachea and then searched for 2 weeks later, had been washed out by ciliary action. However, particles sprayed on the opened trachea at operation could easily be identified. Intravenous distribution from injection was apparently not close enough to the surface to be seen.

Our observations indicate that morphologically apparent damage in vivo following injection of carcinogenic material is very slight. Particles of dibenzanthracene have been located, lodged in vessels showing intact flow as long as 2½ months after injection. There was possibly some thickening of the vessel wall to be seen, and not infrequently an alveolus in the vicinity of an identified carcinogen particle appeared to be collapsed. This was by no means an invariable finding, and slight trauma in normal control observations could also produce collapse. However, in control non-injected animals such slightly injured areas could be reinflated by gently increasing \( \text{O}_2 \) pressure, but the carcinogen-injured alveoli did not reinflate. Such collapse was noted as early as 9 days after injection.

The longest period of time over which particles have been identified after injection is 5 months. In this case a rat which received six injections totaling 2.5 mg. of dibenzanthracene showed particles of 4–8 \( \mu \) in size located in surface inter-alveolar septa. Animals showed a decreasing number of particles over a period of days—20, 28, 41, 76—after injection, but no obvious fluorescence color changes. Examination has been performed 5, 6, 8, and 10 months after the last injection; and no trace of damage correlated exactly to the areas of carcinogen can be noted in vivo.

**DISCUSSION**

This paper is intended to illustrate a method of localization and study of injected carcinogen, a method that is applicable to living lung. Since the original design of the project was to show that it is possible to carry out such localization of fluorescence, the study has centered, up to now, on this aspect and not on histologic analysis or on tumor production.

To be truly revealing of the dynamics of carcinogenesis, more than such in vivo microscopy is obviously necessary. The method suffers at the moment from the relatively large particle size, and yet it is felt that data of interest may come.

**OTHER ORGANS**

After injection of dibenzanthracene in adequate quantity as indicated in gelatin, on examination of the freshly cut surface of organs by directing the quartz rod at them, using 32× magnification, one could see with ease particles in liver, spleen, bone marrow, kidney, muscle, heart wall, and brain. The autofluorescent pattern of each organ was also readily seen and was quite characteristic—strikingly so in the case of kidney and bone. Particles stood out wherever located as colored dots, with the color variation similar to that of the lung deposits.

In certain animals of an early series, a yellow fluorescent line was noted in the cortex of bone of carcinogen-injected rabbits and rats. This was, at first, thought to be carcinogen associated, and the possibility was stated in a presentation of this work (13). The yellow line has subsequently been proved to be antibiotic administered unsuspected in the drinking water of the injected series and by chance not to the controls. No yellow line has appeared in bone cortex of any animal controlled subsequently; and when oxytetracycline was purposely administered, the yellow line appeared as Milch, Rall, and Tobie4 have demonstrated (17).

**FIG. 1.**—Line drawing of entire apparatus and animal. Light source, preferably Osram or 1000-watt Xenon-Mercury Hano-via, air cooled. Note hollow rod for dripping fluid over lung. Filter A is either Corning #5860 or #5874, Filter B is Corning #8889.

**FIG. 2.**—Print from color photograph (Super Anscochrome) of fluorescence of 10× magnified lung surface field of rabbit examined one month after injection of gelatin suspension of dibenzanthracene. Particles are seen as bright dots. Filter #8874 at light source. Exposure time, 2 min.

**FIG. 3.**—Photograph of television monitor displaying single fluorescent dibenzanthracene particle in rat lung field (10× magnification). Exposure time of polaroid film at the monitor was \( \frac{1}{2} \) sec. #8874 filter at light source and #8889 filter at eye-piece of microscope.

**FIG. 4.**—Same lung field as Figure 3, but filters removed so that field is displayed in white light.
from study of the area adjacent to the particle involved in the tissue-particle interaction. Subjective fluorescence color impressions, it has been pointed out, are unsatisfactory, and therefore this laboratory has turned to actual dispersion of the fluorescence spectral lines by spectroscopy of the microscope image. Such spectroscopy should compare interestingly with data from other laboratories reporting on fluorescence of tissue-carcinogen states (6, 19, 20, 25). Experiments to date indicate that it is quite possible to obtain such spectra from living lung fields, and the results will form part of a later publication. Possibly other physicochemical techniques may be applied to the images in these fields.

It is hoped that the method may be improved and may be applicable to other laboratories with their own particular interests and techniques.

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