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An Assessment of the Immunofluorescence Technique as a Method for Demonstrating the Histological Localization of Tetrahydrocannabinol in Mammalian Tissues

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ABSTRACT: The use of the indirect immunofluorescence technique as a method for demonstrating the histological localization of tetrahydrocannabinol (Δ-THC) has been examined. The experimental protocol was designed in order that optimal staining conditions with respect to temperature, the length of time of incubations and washes, and the dilution of the antisera should be defined. No marked differences were detected between frozen sections of liver from normal and Δ-THC-injected mice. Results from radiotracer experiments using human liver suggest that the success of the method is dependent upon the solubility characteristics of the antigen-antibody complex.

KEYWORDS: pathology and biology, marihuana, histology, antigen-antibody reactions, radioactive isotopes, tetrahydrocannibinol, indirect immunofluorescence technique

The use of histochemical techniques for the detection of sites of drug localization in tissues has great potential in forensic pathology. Such information would be particularly instructive when the drug does not produce any change in tissue morphology. The few methods that are currently available rely mainly on fluorescence procedures. For example, drugs such as chlorpromazine [*1*], and certain carcinogens such as aflatoxin B, *N*-2-fluorenylacetamide, and benzo[*a*]pyrene [*2-4*] have been localized in tissue sections by virtue of their autofluorescence characteristics. However, these techniques are of limited use as few drugs of interest to the forensic sciences fluoresce in the visible region of the spectrum.

Immunocytochemical methods are less restrictive and immunofluorescence procedures have been applied successfully to the detection of phenobarbital [*5*], methadone [*6*], and morphine [*7*]. In this report an assessment is made of the suitability of immunofluorescence for demonstrating the histological localization of tetrahydrocannabinol (Δ-THC), the active constituent of *Cannabis.* Our experiences indicate that the success of the method depends on the solubility characteristics of the antigen-antibody complex.

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Materials and Method

Immunological Reagents

Sheep antisera to Δ-THC were donated by Professor V. Marks, Department of Biochemistry, University of Surrey, U.K. and Dr. C. E. Cooke, Research Triangle Institute, North Carolina, U.S.A. Goat antisera to Δ-THC was purchased from Miles Laboratories, Slough, U.K. Fluorescein isothiocyanate-conjugated antispecies immunoglobulins were obtained from DAKO Immunoglobulins and Miles Laboratories.

Autoradiographic Materials

Δ'(G-3H)Tetrahydrocannabinol, specific activity 19.7 mCi/mg, was obtained from the Radiochemical Centre, Amersham, U.K. The autoradiographs were produced on ^{3}H-sensitive Ultrofilm® (L. K. B. Products).

Microscopy

Prepared sections were examined using a Leitz Orthoplan incident light fluorescence microscope fitted with an XBO 75-DC burner. A wavelength of 390 to 490 nm was selected for excitation.

Procedures

Whole Body Autoradiography

Forty microcuries of ^{3}H-Δ-THC were administered to mice subcutaneously in the middorsal region. Nonradioactive Δ-THC was used to bring the dose injected to 20 μg drug per gram of body weight. The drug was prepared for injection according to the method of Kennedy and Waddell [*8*] in which Δ-THC is triturated with human serum albumin. Animals were killed after 1.5, 3, and 6 h, frozen in acetone at −70°C and blocked at this temperature in methylcellulose. Twenty-micrometre sections were cut and allowed to freeze-dry for two to three days before being positioned on Ultrofilm. They were exposed for up to three months at −20°C.

Preparation of Tissue Sections

Mouse liver tissue was grossly sectioned into blocks and then flash-frozen in liquid nitrogen. The frozen blocks were fixed onto chucks with Tryco-M-Bed® (AMC, London) and 7-μm sections prepared. The sections were thaw-mounted onto alcohol-cleaned glass slides and stored at −20°C under nitrogen until they were stained.

Radiotracer Experiments

Method of Spiking Liver Tissue with Radioactive Δ-THC—Seven-micrometre-thick frozen sections of human liver removed during the course of routine postmortem investigations were spiked with 1 μg of ^{3}H-Δ-THC by overlaying the section for 3 to 4 h with 50 μL of propylene glycol containing the drug. The excess drug was then drained from the slide and the tissue rinsed with 200 μL of phosphate buffered saline (PBS), pH 7.2. The sections were blotted lightly with filter paper before staining.

Scintillation Counting—The activity of the radioactive tissue sections was measured by scintillation counting using a toluene-based scintillant.

Immunofluorescence Staining Procedure

The tissue sections were processed by the indirect immunofluorescence technique [9] as described in Fig. 1. The procedure was carried out at either 20 or 37°C. Sections were stained with antisera for between 30 min and 3 h in a humidified chamber. Unbound antiserum was removed by washing in PBS, pH 7.2, for between 15 min and 1 h.

The antisera were diluted with PBS containing 4% bovine serum albumin (BSA). Primary antisera were diluted between 1:10 and 1:200 while secondary antisera were used at dilutions between 1:10 and 1:80.

Controls consisted of drug-free tissue stained with anti-Δ-THC and Δ-THC-loaded tissue stained with nonimmune antisera.

Results

Autoradiography

The autoradiographs of ^{3}H-Δ-THC distribution in mice demonstrate that a substantial proportion of the injected drug is lost from the animal by biliary excretion, as witnessed by the heavy labelling of the gall bladder and the intestine (Fig. 2). However, demonstrable quantities of Δ-THC become localized in the liver, the adrenal cortex, and the kidney at least 1.5 h after subcutaneous administration. These tissues are therefore suitable for processing by the immunofluorescence technique. No change in this pattern of distribution is detected in animals killed either 3 or 6 h after injection.

Immunofluorescent Examination of Tissue

The immunofluorescence investigation has been resricted to sections of liver tissue. The protocol of the technique was designed in order that the effect of a variety of conditions on

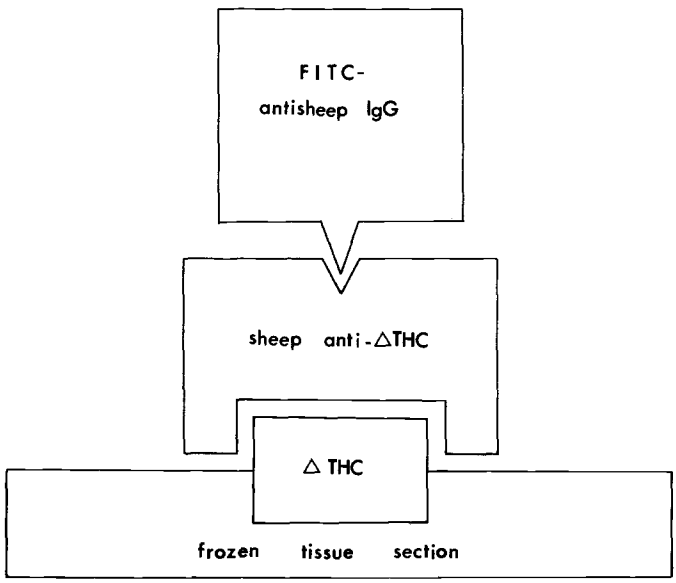
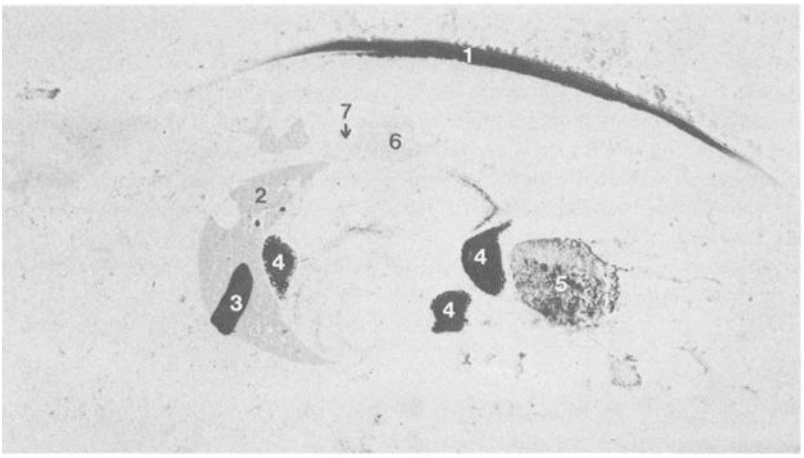


FIG. 1—*The indirect immunofluorescence technique is displayed in which sections containing antigen are first incubated with suitably diluted primary antiserum (Step 1). Excess antibody is removed by washing in phosphate buffered saline (PBS), pH 7.2. A second incubation step is then performed using anti-immunglobulin conjugated to a fluorochrome (Step 2). Excess antibody is again removed by a PBS wash and the sections are then mounted in buffered glycerol pH 8.6.*



1—site of injection
2—liver
3—gall bladder
4—intestine
5—bladder
6—kidney
7—adrenal gland

FIG. 2—*The autoradiographic distribution of Δ-THC in mice is shown 3 h after administration of the drug by subcutaneous injection.*

the demonstration of specific staining could be examined. These included the length of time and the temperature of the incubations with antisera together with the length of time spent in PBS washes. The optimal dilutions of primary and secondary antisera were assessed using chessboard systems of staining [*10–12*]. Three preparations of primary antisera were used, each of which was raised against an antigen (Δ-THC-BSA) that differed only in the position of the hapten-ligand linkage.

Sections of human liver spiked with Δ-THC and sections of liver from mice that had been injected with Δ-THC were stained by the above technique. Animals were killed 3 h after subcutaneous administration of either 20 μg Δ-THC/g body weight or the vehicle used for the injection. No marked differences were detected between control and treatment tissues.

Radioisotope Experiments

The use of radioisotopes enabled an assessment to be made of the degree of leaching of the drug from the tissue during immunofluorescence processing. Frozen sections of human liver spiked in vitro with a known amount of ^{3}H-Δ-THC were removed for counting at each stage of the immunofluorescence procedure. The results are shown in Fig. 3. Although approximately 70% of the drug remained bound to the tissue at the end of the procedure, large losses of activity occurred immediately after incubation with the primary (19% loss) and secondary (7% loss) antisera. Between 1 and 4% of the drug was lost on subsequent PBS washes. Subjection of spiked sections to postfixation in acetone removed a further 67% of the drug from the tissue (Fig. 4).

Discussion

The technique of immunofluorescence has great potential for the detection of drugs in postmortem tissues. The principal advantage over autofluorescence procedures is that there

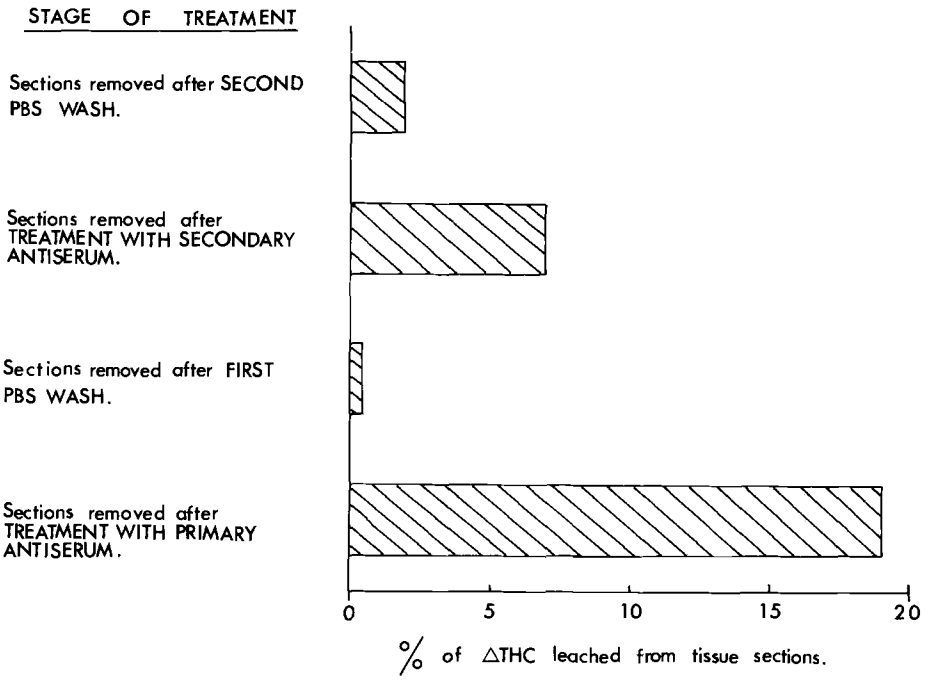


FIG. 3—*Sections of human liver spiked with 1 μg of radioactive Δ-THC were processed by the indirect immunofluorescence technique. Five sections were removed for scintillation counting at each stage of the procedure. The figure shows the amount of Δ-THC leached from the tissue sections at each stage of the procedure.*

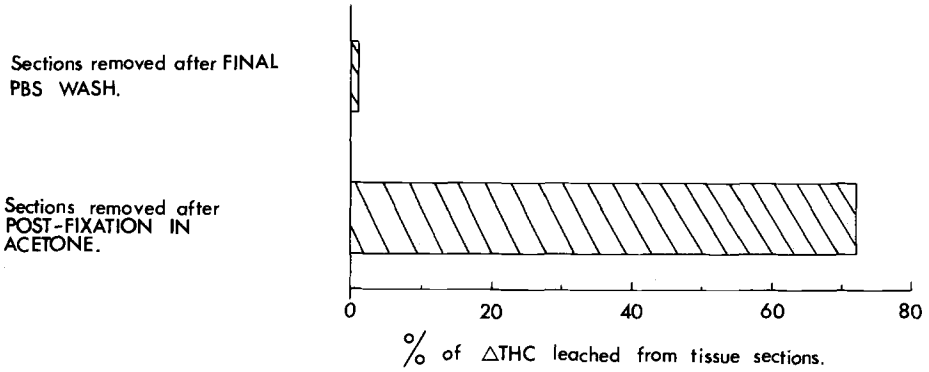


FIG. 4—*Five sections of human liver spiked with 1 μg of radioactive Δ-THC and stained by the indirect immunofluorescence technique were postfixed for 5 min in acetone and washed in PBS before mounting. The figure shows that acetone leaches 67% of the drug from the tissue.*

is no requirement that the drug fluoresce in the visible region of the spectrum. Consequently, provided that suitable antisera are available, the histological localization of a wide range of interesting drugs can be investigated. A number of antisera to drugs are now available commercially and the method has been applied successfully to the histological localization of phenobarbital [5], methadone [6], and morphine [7].

The active constituent of *Cannabis*, Δ-THC, is an extremely lipophilic substance and as such is highly suitable for processing by aqueous histological techniques. The radiotracer ex-

periments have shown that at least 70% of the drug remains in the tissue sections at the end of processing. The importance of limiting the exposure of sections containing Δ-THC to organic solvents in illustrated by the leaching of 67% of the drug by postfixation of stained tissue in acetone.

The experimental protocol was designed in order that optimal staining conditions with respect to temperature, the length of time of incubations and washes, and the dilution of the antisera should be defined. Nonspecific staining was reduced to a minimum by careful selection of a secondary antiserum that interacts weakly with nonspecifically bound constituents of the primary antiserum.

In common with many other drugs Δ-THC is a small molecule (molecular weight, 314.5) and as such it probably has only one antigenic site. It is possible that the position of attachment of the drug to its receptor may mask part of this antigenic site. Should this occur the drug would be inaccessible for binding with its antibody. To overcome this problem, three different primary antisera were used in the experiments, each of which was raised against an antigen that differed in the position of the hapten-ligand linkage (antigen = Δ-THC-BSA; linkage positions in the Δ-THC molecule: C_1, C_5H_{11}, C_{11}). None of these enabled the detection of the drug.

The inability to detect Δ-THC in the liver may be caused by the predominance of certain metabolites of the drug which have little cross-reactivity with the primary antisera. However, it is felt that this is unlikely to be the complete explanation because Δ-THC is not detected by immunofluorescent staining of sections of normal liver spiked in vitro with pure drug.

The information obtained from the radiotracer experiments suggests that Δ-THC forms a water-soluble complex with its antibody as a high proportion of the drug is lost from the sections immediately after incubation with primary antiserum. In addition, further drug is lost after incubation with the secondary antiserum. The failure of the immunofluorescence technique to discriminate between control and treatment tissues may be explained if all of the antibody molecules are used in forming these water-soluble complexes. This hypothesis could be confirmed by examining the leaching of radiolabelled antibody from the tissue.

It is suggested that the formation of such water-soluble complexes should be considered when an experimental protocol is designed for the immunofluorescent detection of drugs. The physical property of lipophilicity and the availability of antisera are insufficient criteria for success.

Acknowledgments

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