translocation. Hence organelle motility is controlled exogenously and the translocation-defective variants may be useful in characterizing the cellular components involved in this process (*12*). Preliminary studies have shown that the inability of erythrophoroma cells to carry out cyclic AMP–induced carotenoid droplet dispersion may be due to a defective protein kinase (*13*). Similar studies with the melanized variants should provide additional information.

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8. In addition to using standard cloning techniques (*2*), we devised a method to establish melanogenic clones which exploited their colonial growth and high buoyant density. Single colonies of melanin-laden cells were manually isolated from the initial cultures and subcultivated in fish serum–supplemented medium for 8 to 10 weeks. The cells were then harvested with 0.25 percent collagenase in phosphate-buffered saline and resuspended in 60 percent (by volume) Percoll (Pharmacia) in phosphate-buffered saline. Centrifugation at 1000*g* for 10 minutes produced a virtually pure pellet of melanized cells, which were replated in fish serum–supplemented medium.
9. The conventional terminology for normal melanogenic cells distinguishes between melanophores and melanocytes on the basis of their ability or inability, respectively, to translocate their pigment organelles. The terms melanophoroma and melanocytoma make the same distinction for the melanized clones of tumor cells.
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Quantitative Autoradiographic Mapping of Herpes Simplex Virus Encephalitis with a Radiolabeled Antiviral Drug

Abstract. *2′-Fluoro-5-methyl-1-β-D-arabinosyluracil (FMAU) labeled with carbon-14 was used to image herpes simplex virus type 1–infected regions of rat brain by quantitative autoradiography. FMAU is a potent antiviral pyrimidine nucleoside which is selectively phosphorylated by virus-coded thymidine kinase. When the labeled FMAU was administered 6 hours before the rats were killed, the selective uptake and concentration of the drug and its metabolites by infected cells (defined by immunoperoxidase staining of viral antigens) allowed quantitative definition and mapping of HSV-1–infected structures in autoradiograms of brain sections. These results show that quantitative autoradiography can be used to characterize the local metabolism of antiviral drugs by infected cells in vivo. They also suggest that the selective uptake of drugs that exploit viral thymidine kinase for their antiviral effect can, by appropriate labeling, be used in conjunction with clinical neuroimaging techniques to define infected regions of human brain, thereby providing a new approach to the diagnosis of herpes encephalitis in man.*

Herpes simplex virus type 1 (HSV-1) causes severe sporadic encephalitis in man with high mortality and morbidity (*1*). The course of this illness can be altered by treatment with the antiviral drug vidarabine (*2*), and newer drugs with more selective antiviral action may further improve the therapeutic outcome (*3, 4*). However, a critical factor in successful management of herpes encephalitis is early diagnosis. Brain biopsy is at present the only definitive means of early diagnosis, leaving the clinician with a difficult decision that may result in delayed or inappropriate therapy (*5*).

In an effort to find a noninvasive, accurate diagnostic test for HSV-1 encephalitis, we have examined the possibility of exploiting the selective uptake by infected cells of an antiviral drug that serves as substrate for virus-coded thymidine kinase. By using radioactively labeled 2′-fluoro-5-methyl-1-β-D-arabinosyluracil (FMAU), a potent new antiviral agent (*6*), and quantitative autoradiography (*7*), we have now demonstrated the selective uptake of FMAU by HSV-1–infected brain regions in the rat. We predicted that, after penetration into the brain, labeled FMAU would be selectively phosphorylated by viral thymidine kinase expressed in infected cells, and that the metabolic products (nucleotides and perhaps DNA) "trapped" within these cells could be assessed quantitatively in autoradiograms of brain sections in a manner analogous to that of [2-^{14}C]deoxyglucose phosphate (*7*). We here present data showing that infected regions do indeed selectively sequester and concentrate labeled FMAU and that these regions can be mapped autoradiographically.

Focal encephalitis was produced in female CD rats (Charles River Laboratories; 150 to 200 g) by intraocular inoculation of the right eye with 2.6×10^6 plaque-forming units of the F-strain of HSV-1 suspended in a volume of 20 μl (*8*). The intraocular route was chosen because it avoids the need for direct intracerebral injection with attendant trauma and because a reproducible, anatomically stereotyped focal encephalitis ensues (*9*). In some experiments, the rats were immunosuppressed by intraperitoneal injection of cyclophosphamide (250 mg/kg) administered 1 day after viral challenge in order to produce more severe brain infection. The rats were killed on day 5 or 6 after inoculation of the virus. Radioactive FMAU (labeled with ^{14}C at the number 2 position of the pyrimidine ring) with a specific activity of 47.5 μCi/mg (12.3 nCi/nmole) was injected intravenously (5 mg/kg) (*10*). The [^{14}C]FMAU was injected into three rats 24 hours before they were killed, and into the remaining rats 6 hours before they were killed. Samples of arterial blood were obtained at frequent intervals and analyzed for ^{14}C activity by liquid scintillation counting. Arterial blood pressure was monitored continuously and arterial blood gases were sampled intermittently by way of femoral cannulae. Previous studies have shown that in the rat most FMAU is excreted unchanged in the urine and only a minor fraction undergoes metabolic alteration (*11*). The animals were killed by injection of a lethal dose of pentobarbital (42 mg). Their brains were removed rapidly, frozen in Freon (cooled to −40°C in dry ice)

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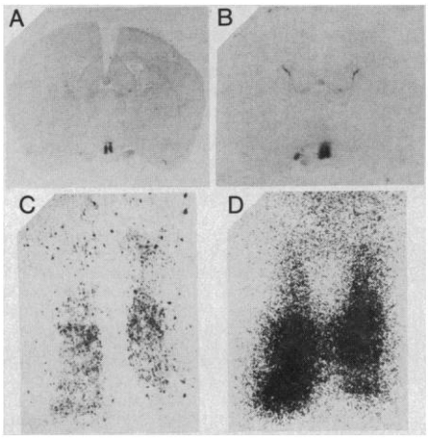

Fig. 1. Correlation of the distribution of PAP-defined viral antigen and ^{14}C activity in brain sections of a cyclophosphamide-treated rat killed 6 days after intraocular inoculation of HSV-1 and 24 hours after [^{14}C]FMAU injection. (A) Low-powered view of PAP-stained section with light hematoxylin counterstain. The peroxidase reaction product stains viral antigens darkly in the infected suprachiasmatic nuclei and adjacent regions of the medial hypothalamus bilaterally; lighter staining of antigens present in the left optic tract and supraoptic nucleus is less well-appreciated at this magnification. (B) Autoradiogram prepared from an adjacent section demonstrates that the regions of increased ^{14}C activity correspond to those of viral antigen with the exception of lateral walls of the lateral ventricles where viral antigen was not detected. (C) Higher magnification of the infected suprachiasmatic nuclei from PAP-stained section shown in (A). (D) Higher magnification of the infected suprachiasmatic nuclei from autoradiographic section shown in (B).

within 2 to 3 minutes of death, and stored at −70°C. Coronal brain sections (20 μm) were prepared for quantitative autoradiography of ^{14}C, with MR-1 (Kodak) film being used according to the methods of Sokoloff *et al.* (*7*). In addition to quadruplicate serial brain sections (taken at 30-section intervals), sections of eye, liver, and skeletal muscle along with a series of 11 ^{14}C-labeled methylmethacrylate standards (Amersham) were included on each film. Tissue sections immediately adjacent to those selected for autoradiography were processed for peroxidase-antiperoxidase (PAP) staining of HSV-1 antigens, as previously described (*12*). Autoradiographic images were digitized by means of a Vidicon-based EyeCom II image-processing system linked to a PDP 11/23 microcomputer. Optical density in defined brain regions of interest was then converted to ^{14}C activity concentration (nanocuries per gram) by using a calibration curve derived from analysis of the ^{14}C standards.

In a group of ten rats that received [^{14}C]FMAU, seven were infected with HSV-1, and three were uninfected control rats. These control rats were subjected to a cortical freeze lesion (*13*) to test the effects of necrosis and breakdown of the blood-brain barrier on drug distribution. In autoradiograms prepared from each HSV-1-infected rat, ^{14}C activity exceeded background within infected regions, and these regions correlated closely with viral antigen staining in each case (Fig. 1). Data from a representative animal studied 6 hours after [^{14}C]FMAU injection are given in Table 1. In this animal, the concentration of [^{14}C]FMAU in the plasma decreased rapidly from 1097 nCi/g at 1 minute to 370 nCi/g at 35 minutes and thereafter more slowly to a final concentration of 187 nCi/g at the time of death. The background distribution of ^{14}C activity in the brain was quite uniform, with only minor variations among uninfected regions. In contrast, within infected structures (including the retina, optic chiasm, suprachiasmatic nuclei of the hypothalamus, left optic tract, left thalamus, central mesencephalon, and pineal gland) activity was consistently increased, ranging from 2 to more than 13 times that of background. The intensity of uptake appeared to vary with the density of infected cells and stage of viral replication within each of these areas. In the choroid plexus, ^{14}C activity was similar to that of blood and liver, and represented the only uninfected region exhibiting activity above that of background in the brain. Results in this animal were typical. In all other infected animals studied, including those treated with cyclophosphamide and the nonimmunosuppressed, a similar correlation was found between [^{14}C]FMAU uptake and regional infection as defined by PAP staining, with the ratio of activity in infected areas to that in noninfected areas being of a similar magnitude. In animals killed 24 hours after isotope injection, isotope uptake was similarly enhanced within infected brain regions compared to uninfected brain, with the exception of the lateral walls of the lateral ventricles where higher activity was noted (Fig. 1B), apparently within the uninfected ependymal lining. In the three animals with cortical freeze lesions, no increase in isotope uptake was detected in or surrounding the area of necrosis, indicating that increased local drug concentration was not merely a result of altered blood-brain barrier permeability.

Our results show that [^{14}C]FMAU is selectively taken up by infected brain cells and that this tracer can be used to map the distribution of HSV-1 infection. Quantitative autoradiography, which permits measurement of labeled drug uptake by a limited number of infected cells, should prove valuable in studies of the metabolism of this and related drugs whose mechanism of action involves phosphorylation by viral thymidine kinase. In this regard, quantitative autoradiography is superior to liquid scintilla-

Table 1. Regional tissue concentrations of ^{14}C in an HSV-1-infected rat that received [^{14}C]FMAU 6 hours before it was killed. The data are from sections of uninfected liver, infected (right) eye, and representative levels of brain showing focal infection.

Tissue and region of interest	Area* (pixels)	Viral antigen staining†	Radioactivity (nCi/g ± S.D.)
Liver	3395	−	188.25 ± 13.19
Eye			
Retina, posterior	45	+	2447.81 ± 172.66
Retina, inferior	49	+	1528.07 ± 140.36
Brain			
Background left cortex	621	−	104.35 ± 4.88
Left ventricular choroid plexus	27	−	188.59 ± 9.18
Optic chiasm, right	15	+	325.12 ± 16.46
Background, left cortex	1221	−	95.29 ± 4.32
Left hypothalamus	33	+	1170.91 ± 66.90
Right hypothalamus	27	+	1138.10 ± 99.57
Left optic tract	9	+	1105.89 ± 77.34
Background, left cortex	441	−	96.78 ± 4.66
Background, right central thalamus	441	−	67.47 ± 3.90
Left dorsomedial thalamus	35	+	1183.53 ± 163.31
Left lateral thalamus	15	+	198.21 ± 21.00
Background, left cortex	775	−	106.57 ± 5.53
Central midbrain	45	+	468.81 ± 23.96
Background, left cortex	1287	−	83.08 ± 7.70
Background, central brainstem	1287	−	110.97 ± 9.98
Pineal gland	63	+	1509.18 ± 97.65

*Area of the defined region of interest assessed in the digitized autoradiogram. †Staining of viral antigens with PAP in the adjacent serial section.

tion counting of organ lysates for the assessment of labeled drug uptake, since with the latter technique high uptake by a small number of cells is likely to be masked by the large volume of background activity associated with uninfected brain (*14*).

Our results enhance the prospect of using radioactively labeled antiviral drugs to detect HSV encephalitis in humans. Further studies are required to define conditions optimizing the autoradiographic contrast between infected and background brain tissue. It may also be possible to use the related antiviral pyrimidine nucleoside 2′-fluoro-5-iodo-1-β-D-arabinosylcytosine (FIAC) (*4*) for diagnostic purposes. Incorporation of a positron- or gamma-emitting isotope of iodine into the FIAC might be exploited by positron emission (*15*) or single photon (*16*) tomography, or perhaps even by clinical gamma scanning, to image brain infection in man.

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Type II Collagen Autoimmunity in Otosclerosis and Meniere's Disease

Abstract. *Antibodies to collagen types I, II, and IV were measured in patients with otosclerosis and patients with Meniere's disease. Levels of antibodies to type II collagen were significantly higher in these patients than in control subjects, while no differences were found among levels of antibodies to collagen type I or type IV. These observations suggest a possible role for type II collagen autoimmunity in the etiology of otosclerosis and Meniere's disease.*

The causes of otosclerosis and Meniere's disease are obscure. Histopathologic changes in otosclerosis include spongification of the ossicles and otic capsule (*1*). Meniere's disease is characterized by auditory and vestibular symptoms attributable to the accumulation of excess fluid in the membranous labyrinth of the ear (endolymphatic hydrops) (*2*). The diseases have not been known to be related.

We recently described autoimmune hearing loss, vestibular dysfunction, and otospongiotic lesions in rats. These animals had characteristics of both human otosclerosis and Meniere's disease (*3*). All the abnormalities appeared to be associated with autoimmunity against collagen—in particular, type II collagen. It was, therefore, of interest to test otosclerosis and Meniere's disease patients for the presence of antibodies to collagen.

Serum was obtained from patients at the Shea Clinic, Memphis, Tennessee. Bovine type II collagen and human collagen types I and IV were isolated and purified (*4*). (Bovine type II collagen was used because of the difficulty in obtaining adequate quantities from human cartilage. It cross-reacts extensively with human type II collagen.) The purity of the collagen preparations was determined by amino acid analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and uronic acid analysis. In each case there was no detectable contamination by noncollagenous proteins, proteoglycans, or other types of collagen. Immunoradiometric assay was performed by a modification of the procedure described by Clague *et al.* (*5*). Collagen was dissolved in phosphate buffer (*p*H 7.6; ionic strength, 0.4*M*) by stirring overnight at 4°C. The concentration was adjusted to 25 μg/ml based on spectrophotometric determination of specific absorption at 230 nm. To each well of a 96-well polystyrene plate was added 100 μl of the collagen solution. Dynatech Removawell strip holders were used so that individual wells could be easily separated for scintillation counting. (Use of a 96-well plate facilitates the handling of samples during subsequent multiple wash steps.)

After overnight incubation of the plate at 4°C, unbound collagen was removed by washing with 0.15*M* NaCl and 1 percent ovalbumin was added to block nonspecific absorption of antibodies. Incubation was resumed for 1 hour; then the plate was washed three times with 0.15*M* NaCl containing 0.05 percent Tween 20. (All buffers used subsequently also contained Tween 20 to prevent nonspecific absorption.) Dilutions of human serum were added to the collagen-coated plate, the plate was incubated at room temperature for 4 hours, and unbound antibodies were removed by three washes with 0.15*M* NaCl containing 0.05 percent Tween 20. Antibodies bound to the collagen were detected by adding ^{125}I-labeled staphylococcal protein A (Amersham) (10,000 count/min per well). After incubation of the plate for 2 hours, excess protein A was removed by three washes and the wells were separated and placed individually in a gamma scintillation

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