American Journal of Pathology, Vol. 143, No. 5, November 1993
Copyright © American Society for Investigative Pathology

Short Communication

In Situ Hybridization of HIV-1 RNA in Retinal Vascular Wall

Isabelle Reux,* Anne-Marie Fillet,† Jean-Guy Fournier,‡ Henri Agut,† Marc Gentilini,§ Phuc LeHoang,‖ and Jean-Jacques Hauw*

From the Laboratoire de Neuropathologie Raymond Escourolle, I.N.S.E.R.M. U.360, Association Claude Bernard, Paris; Laboratoire de Bactériologie-Virologie,† Association Claude Bernard, CERVI, Service des Maladies Infectieuses et Parasitaires,§ Service d'Ophtalmologie,‖ Groupe hospitalier La Pitié-Salpétrière, Paris, France and I.N.S.E.R.M. U.43,‡ Hôpital Saint-Vincent de Paul, Paris, France*

In situ *hybridization of human immunodeficiency virus-1 (HIV-1) has been performed on eight eyes from eight distinct acquired immune deficiency syndrome patients (three cases had a normal fundus examination and five presented with cytomegalovirus retinitis). The eyes were removed at autopsy and frozen immediately. Contiguous 10-μ cryostat sections were obtained and tested with a HIV probe labeled by nick-translation with [^{35}S]-ATP. HIV-1 RNA was detected in the retina of two acquired immune deficiency syndrome patients. The first positive case presented with typical ophthalmological and histopathological cytomegalovirus retinitis, the second one was not related to cytomegalovirus, according to clinical or histopathological classical criterias. HIV-1 was localized in retinal vascular walls. This shows that there is an active replication of HIV in retina of some acquired immune deficiency syndrome patients. (Am J Pathol 1993, 143:1275–1279)*

Ophthalmic lesions occur in up to 70% of acquired immune deficiency syndrome (AIDS) patients.[1–3] They range from asymptomatic cotton-wool spots to blinding retinal necrosis. These ocular disorders are either the result of direct infection by human immunodeficiency virus (HIV) or may involve interactions with other infectious opportunistic agents such as cytomegalovirus (CMV). To elucidate the role of HIV-1 in AIDS retinopathy, we performed immunocytochemistry and *in situ* hybridization to examine affected eyes for the presence of HIV-1.

Materials and Methods

Cases

Eight cases were submitted to ophthalmic examination performed 60 days or less before death. Two HIV-1-seronegative cases were studied as controls. The eyes were removed at autopsy, frozen in isopentane cooled by liquid nitrogen, and stored at −80 C. They were cut into two parts with an electrical metal saw. Then, contiguous 10-μ cryostat sections were obtained.

Histological Study

Tissues were fixed in 4% formaldehyde for 10 minutes, then hematoxylin-eosin staining was performed.

Immunocytochemistry

After acetone fixation for 10 minutes, sections were incubated for 20 minutes in Tris buffer saline (TBS). HIV-p24 mouse monoclonal antibodies (M857 Dako) at 1/10 dilution were applied for 45 minutes. Slides were gently rinsed in TBS and then incubated for 45 minutes with rabbit anti-mouse immunoglobulins

Supported by grants from the NIH, the Agence National de recherche sur le SIDA, and the Fédération Française des Aveugles.

Accepted for publication August 4, 1993.

Address reprint requests to Dr. I. Reux, Laboratoire de Neuropathologie Raymond Escourolle, Groupe Hospitalier La Pitié Salpétrière, 83, boulevard de l'Hôpital, 75013 Paris, France.

(Z259, Dako) at 1/25 dilution, rinsed again in TBS, and incubated in alkaline phosphatase-mouse anti-alkaline phosphatase complex (APAAP D 651, Dako) at 1/50 dilution for 45 minutes. Alkaline phosphatase substrate-chromogen solution (Naphtol AS-BI phosphate, 50 mg; dimethylformamide, 0.6 ml; 0.05 mol/L Tris buffer, pH 8.7, 100 ml; 1 mol/L levamisole 100 µl; 4% sodium nitrite, 0.5 ml; 5% new fuschsin in 2 mol/L HCl, 0.2 ml) was incubated for 20 minutes. Slides were washed in TBS and then tap water. They were counterstained with hematoxylin and mounted aqueous mounting medium. Every incubation was made at room temperature in a moist chamber. Negative controls included the study of eye sections from an HIV-I-seronegative patient, the use of respiratory syncytial virus antibody as an irrelevant monoclonal antibody, and immunoperoxydase staining in the absence of primary antibody. Positive controls were HIV-1-infected sections from brain tissue.

In Situ *Hybridization*

As a probe, we used pBT1 plasmid containing a 9-kb fragment of the HIV-1 genome inserted in the plasmid pUC 18.[4,5] The HIV probe was labeled by nick-translation with [^{35}S]ATP (Amersham, Les Vlis, France) to a specific activity of 8.10^7 cpm/µg. The plasmid without insert was labeled in a similar manner and used as a control for nonspecific hybridization. Frozen sections collected on clean microscope slides were fixed in 4% formaldehyde for 10 minutes and stored at −20 C until use. Before hybridization, the sections were incubated with 0.2 mol/L HCl for 10 minutes, then with 10 µg/ml proteinase K at 37 C for 15 minutes. Hybridization was carried out overnight under a sealed siliconized coverslip at 37 C with 0.1 µg/ml DNA probe in a solution containing 50% freshly deionized formamide, 10% dextran sulfate, 600 mmol/L NaCl, 10 mmol/L Tris HCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% denhart, 1 mg/ml *Escherichia coli* and yeast RNA, 500 µg/ml herring and salmon sperm DNA, and 10 mmol/L dithiotreitol. The solution was denatured (3 minutes at 100 C, immediately cooled in ice) before adding dithiotreitol. After hybridization, the slides were washed in 50% formamide 4× standard saline citrate (SSC) for 10 minutes, twice in 50% formamide 2× SSC for 30 minutes, once in 2× SSC for 30 minutes, in 0.1× SSC at 55 to 60 C for 10 minutes and in 0.1× SSC at 37 C for 15 minutes. The tissues were dehydrated in increasing concentrations of ethanol and coated with Amersham LM-1 emulsion. Autoradiographic exposure was for 8, 19, and 25 days at 4 C and was developed in Kodak D19 and stained with Mayer's hemalun. As negative controls, two HIV-1-seronegative patient's retinas were hybridized with the HIV probe. As positive controls, HIV probe was tested in HIV-1-infected lymphocytes and HIV-1 p24 antigen-positive brain.

Results

Cases

Individual findings for the eight patients are summarized in Table 1. The average age at death was 35.8

Table 1. *Systemic and Ocular Disorders in Eight cases of AIDS*

Case no.	Age (years)	Risk	Systemic disorders	Ocular disorders	Zidovudine	Anti-CMV treatment
1	29	1	Progressive multifocal leukoencephalopathy	None	No	None
2	32	H	Mucocutaneous candidiasis, meningoencephalitis, cryptosporidiosis	None	Yes	None
3	32	Unknown	Meningoencephalitis, frontal and cerebellar tumor	None	Yes	None
4	34	H	Cerebral toxoplasmosis, pneumocystis carinii pneumonia, CMV sclerosing cholangitis, salmonella sepsis	Bilateral CMV retinitis	Yes	Foscarnet
5	47	H	Mucocutaneous candidiasis, cytolytic hepatitis, pneumonia	Bilateral CMV retinitis	Yes	Foscarnet
6	33	H	Mucocutaneous candidiasis, pneumocystis carinii and CMV pneumonitis	Bilateral CMV retinitis	No	Ganciclovir
7	44	H	Cerebral toxoplasmosis, mucocutaneous candidiasis, CMV pneumonia, peripheral neuropathy	Bilateral CMV retinitis	Yes	None
8	35	H	Cutaneous Kaposi's sarcoma, pulmonar tuberculosis, mucocutaneous HSV and candidiasis, colitis	OD: CWS, OS: CMV retinitis	Yes	Foscarnet

H = homosexual, 1 = sexual intercourse with an HIV-1-seropositive drug addict woman, CMV = cytomegalovirus, HSV = herpes simplex virus, CWS = cotton-wool spots.

years. Six of the patients were male homosexual and the other two were an heterosexual patient who had sexual intercourse with an HIV-1-seropositive drug addict woman and another patient with an unknown risk factor. Three patients had normal fundus examination, four had bilateral and one unilateral CMV retinitis. Six of them received zidovudine. CMV retinitis was treated in three cases by foscarnet, in one case by ganciclovir, and one case was not treated. The average delay between the last ophthalmological examination and death was 38.9 days (9 to 60). Two HIV-1-seronegative cases were studied as controls: a 78-year-old man who died from vertebrobasilar stroke and a 25-year-old woman who died from viral meningoencephalitis. The brain used as control for HIV-1 RNA hybridization was from a 30-year-old homosexual man who presented cutaneous and gastric Kaposi's sarcoma and bilateral CMV retinitis. He had a normal cerebral tomodensitometry 1 month before death and died from bilateral bronchopneumonia.

Histological Study (Table 2)

In patients with normal fundus, histological examination was normal in two cases (no. 1 and no. 2). However, it showed typical cytomegalic cells in case no. 3. There was typical CMV retinitis (Figure 1) concordant with ophthalmological results in the other five cases.

Immunocytochemistry and in Situ *Hybridization (Table 2)*

No HIV-1 p24 antigens were detected by immunohistochemical methods. HIV-1 RNA was detected in two cases (Table 2). The morphology of sclera, choroid, and retina was well preserved and permitted the topographic analysis of results. In one case (no. 4), there was a typical CMV retinitis on clinical and histological grounds. In the other case (no. 2), ophthalmic and histological examination were normal. Silver grains were seen principally within vascular walls (Figure 2). They were also seen in a few structures of the ganglionic cell layer (Figure 3), which were unidentified by classic morphological techniques. The clusters of silver grains were rare and were not confined to retinitis areas. The specificity of the hybridization reaction was confirmed by the presence of the background signal alone when slides from the same case were hybridized with the labeled control plasmid and when control tissues from HIV-1-seronegative patients were hybridized with the HIV-1 probe. In addition, HIV signal were demonstrated in HIV-infected lymphocytes and HIV-infected brain.

Discussion

Retinopathy is a major complication of AIDS. The precise role of HIV and its interactions with other viral agents, such as CMV, have not been determined. HIV-1 has been detected in retina by immunocytological techniques,[6-9] in retina cultures,[6,8-10] and by polymerase chain reaction.[7] This did not allow precise localization. Only Pomerantz, et al[6] in 1987, reported the precise localization of the glycoprotein 120 and of the p-24 HIV-1 antigens in the cytoplasm of the capillary endothelial cells as well as in neuroretinal cells in the two studied cases. We report here the *in situ* hybridization of HIV-1 RNA in the retina of two AIDS patients. The first positive case (no. 4) presented typical ophthalmological and histopathological CMV retinitis; the retina of the second case (no. 2) was not infected by CMV, according to clinical or histopathological classical criterias. HIV-1 was localized in retinal vascular walls, confirming Pomerantz et al's results.[6] However, we could not determine which cells (endothelial cells, pericytes, microglia, or even Müller cells and astrocytes) were infected because microglia[11] and macroglia (Müller cells more than astrocytes)[12] have

Table 2. *Ocular Microscopic Findings and Results of Immunohistology and in Situ Hybridization for the Detection of HIV-1*

Case no.	Histological study microscopic findings	Immunochemistry HIV-1 p24 antigen	*In situ* hybridization HIV-1 RNA
1	Normal	–	–
2	Normal	–	+
3	Large cells in retina with viral inclusions, CMV retinitis	–	–
4	Large cells in retina with viral inclusions, CMV retinitis	–	+
5	Large cells in retina with viral inclusions, CMV retinitis	–	–
6	Retinal necrosis, cytomegalic cells in neurosensorial retina and pigment epithelium, CMV retinitis	–	–
7	Large cells in retina with viral inclusions, CMV retinitis	–	–
8	Large cells in retina with viral inclusions, CMV retinitis	–	–

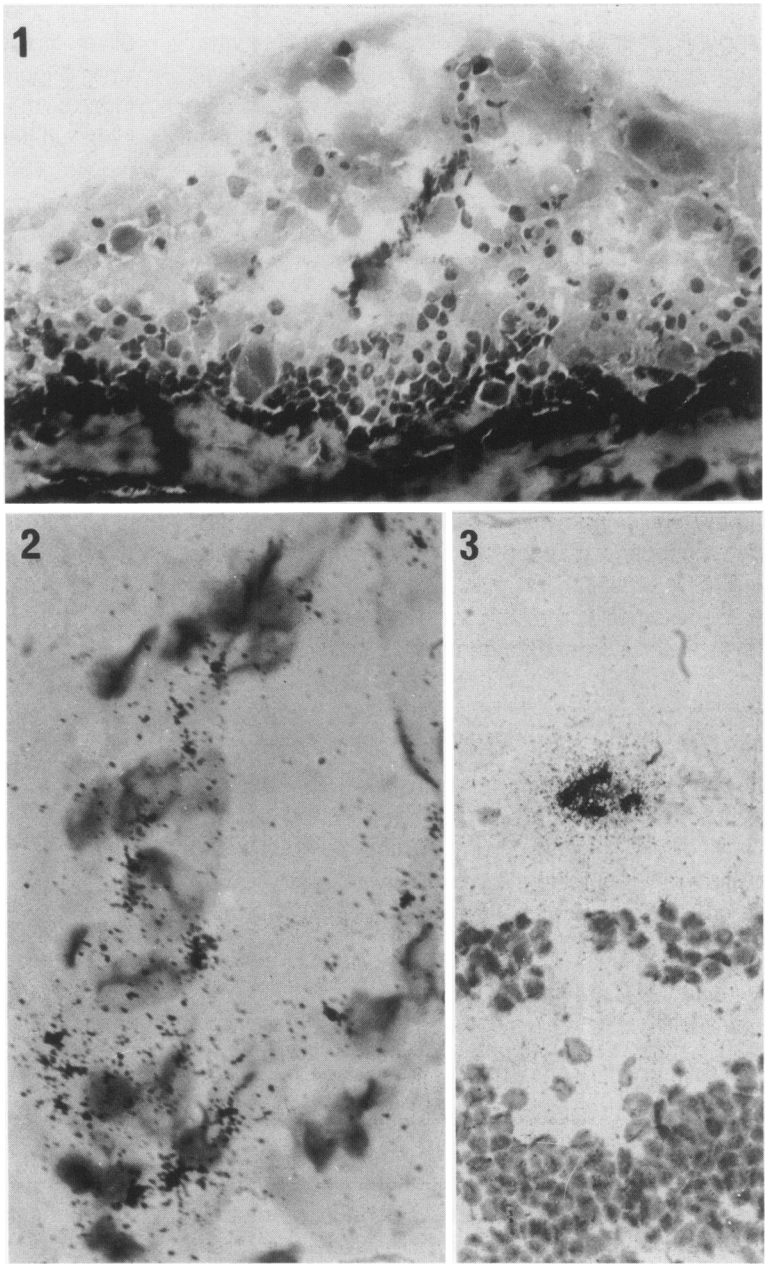

Figure 1. *Cytomegalic cells with intracytoplasmic inclusions are seen in the retinitis area (H&E; original magnification: 25×).*
Figure 2. *Formaldehyde-fixed frozen sections of retina from patient no. 4, hybridized with ^{35}S-radiolabeled HIV-1 DNA probe. Some cells of vascular wall of the retina contain viral RNA (original magnification: 40×).*
Figure 3. *Formaldehyde-fixed frozen sections of retina from patient no. 4 hybridized with ^{35}S-radiolabeled HIV-1 DNA probe. The morphology of the cells within the ganglionic cell layer is concording with a vascular wall (original magnification: 25×).*

been shown to be components of the perivascular walls. On central nervous system, Vazeux et al[13] performed immunocytological double labeling, which suggested that microglial/macrophages cells were infected by HIV. Every as yet identified structure that was labeled was vessel. The few unidentified structures that were seen in the ganglionic cell layer may be also small vessels. *In situ* hybridization was more sensitive than immunocytochemistry, inasmuch as we have detected RNA HIV-1 in two of eight cases and none HIV-1 p24 antigen. Kennedy et al[14] detected CMV genome by *in situ* hybridization but not HIV-1 in three of five patients whose retina was formaldehyde-fixed and paraffin-embedded. The little percentage of HIV-1 detection may have revealed latent or weak productive infection. Both cases positive for HIV-1 received zidovudine, which inhibits viral replication at a late stage and therefore is not expected to clear cells of latent virus. Surprisingly, no HIV-1 RNA was detected in the two patients who did not receive zidovudine. HIV-1 has been hybridized in vascular walls within a brain used as control confirming data of previous immunocytological studies.[15,16] HIV-1 infection causes

retinal microvascular abnormalities clinically characterized by cotton-wool spots, intraretinal hemorrhages, ischemic areas, and maculopathy.[2]

Acknowledgments

We thank Mrs. O. Freytag for technical assistance.

References

1. Palestine AG, Rodrigues MM, Macher AM, Chan CC, Lane HC, Fauci AS, Masur H, Longo D, Reichert CM, Steis R, Rook AH, Nussenblatt RB: Ophthalmic involvement in acquired immunodeficiency syndrome. Ophthalmology 1984, 91:1092–1099
2. Pepose JS, Holland GN, Nestor MS, Cochran AJ, Foos RY: Acquired immune deficiency syndrome, pathogenic mechanisms of ocular disease. Ophthalmology 1985, 92:472–484
3. Jabs DA, Green WR, Fox R, Polk BF, Bartlett JG: Ocular manifestations of acquired immune deficiency syndrome. Ophthalmology 1989, 94:1092–1099
4. Fournier JG, Prevot S, Audouin J, Le Tourneau A, Lebon P, Diebold J: Fine analysis of HIV-1-RNA detection by in situ hybridization. Modern Pathology of AIDS and Other Retroviral Infections. Edited by Racz P, Haase AT, Gluckman JC. Basel, Karger, 1990, pp 62–68
5. Hantaï D, Fournier JG, Vazeux R, Collin H, Baudrimont M, Fardeau M: Skeletal muscle involvement in human immunodeficiency virus infection. Acta Neuropathol 1991, 81:496–502
6. Pomerantz RJ, Kuritzkes DR, de la Monte SM, Rota TR, Baker AS, Albert D, Bor DH, Feldman EL, Schooley RT, Hirsch MS: Infection of the retina by immunodeficiency virus type I. N Engl J Med 1987, 317: 1643–1647
7. Qavi HB, Green MT, SeGall GK, Font RL: Demonstration of HIV-1 and HHV-6 in AIDS-associated retinitis. Curr Eye Res 1989, 8:379–387
8. Cantrill HL, Henry K, Jackson B, Erice A, Ussery FM, Balfour HH: Recovery of human immunodeficiency virus from ocular tissues in patients with acquired immune deficiency syndrome. Ophthalmology 1988, 95: 1458–1462
9. Schmitt-Gräff A, Neuen-Jacob E, Rettig B, Sundmacher R: Evidence for cytomegalovirus and human immunodeficiency virus infection of the retina in AIDS. Virchows Arch [A] 1990, 416:249–253
10. Srinivasan A, Kalyanaraman S, Dutt K, Butler D, Kaplan HJ: Isolation of HIV-1 from vitrous humor. Am J Ophthalmol 1989, 108:197–198
11. Lassmann H, Zimprich F, Vass K, Hickey WF: Microglial cells are a component of the perivascular glia limitans. J Neurosci Res, 1991, 28:236–243
12. Holländer H, Makarov F, Dreher Z, van Driel D, Chanling T, Stone J: Structure of the macroglia of the retina: sharing and division of labour between astrocytes and müller cells. J Comp Neurol 1991, 313:587–603
13. Vazeux R, Brousse N, Jarry A, Hénin D, Marche C, Vedrenne C, Mikol J, Wolff M, Michon C, Rozenbaum W, Bureau JF, Montagnier L, Brahic M: AIDS subacute encephalitis. Identification of HIV-infected cells. Am J Pathol 1987, 126:403–410
14. Kennedy PGE, Newsome DA, Hess J, Narayan O, Suresch DL, Green WR, Gallo RC, Polk BF: Cytomegalovirus but not human T lymphotropic virus type III/lymphadenopathy associated virus detected by in situ hybridisation in retinal lesions in patients with the acquired immune deficiency syndrome. Br Med J 1986, 293:162–164
15. Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MBA: Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. Proc Natl Acad Sci USA 1986, 83:7089–7093
16. Rhodes RH, Ward JM, Cowan RP, Moore PT: Immunohistological localization of human immunodeficiency viral antigens in formalin fixed spinal cords with AIDS myelopathy. Clin Neuropathol 1989, 8:22–27