Axonal Damage Revealed by Accumulation of β-APP in HIV-positive Individuals without AIDS

SHU F. AN, MD, PhD, BRUNO GIOMETTO, MD, MIKE GROVES, MSc, ROBERT F. MILLER, MBBS, FRCP, ANDREW A. J. BECKETT, FIBMS, FRANÇOISE GRAY, MD, PhD, BRUNO TAVOLATO, MD, AND FRANCESCO SCARAVILLI, MD, PhD

Abstract. The presence of neuropsychological disturbances in HIV-positive, pre-symptomatic individuals is a controversial issue. Neuroimaging studies have not shown brain atrophy or hypointensity in the white matter, whereas proton magnetic resonance spectroscopy has revealed some abnormality of cerebral biochemical composition. Using an antibody to β-amyloid precursor protein (β-APP), we previously demonstrated frequent and widespread axonal changes in the brains of AIDS patients. In this study, we extended the use of β-APP to asymptomatic patients in order to establish a possible morphological correlation with neuropsychological disorders. Brain samples from 29 patients were examined. Results showed bundles of β-APP-positive axons in 8/29 cases (27%). The changes, seen in both superficial and deep white matter, were either focal or diffuse, could not be visualized by silver or ubiquitin stains, and did not coexist with any change in distribution or morphology of astrocytes and microglial cells. We conclude that in HIV-positive asymptomatic individuals, axonal changes: (a) may be related to the state of immune activation with consequent presence of toxic substances, including cytokines, observed in these patients; (b) may represent mild changes that could undergo repair, unless other pathological events, such as the supervening of the AIDS stage and the specific encephalitis, make them permanent.

Key Words: Axonal changes; β-APP; HIV-positive asymptomatic patients; Immune activation; Neuropsychological disorder; Polymerase chain reaction (PCR); Reversible.

INTRODUCTION

Up to 30% of patients in the late stage of HIV infection may suffer from HIV-associated cognitive/motor disorder, which, in 10% of the cases, is the presenting feature of HIV infection (1). The pathological counterpart of this clinical syndrome is a leukoencephalopathy that can be accompanied by nerve cell loss (2). With regard to the early asymptomatic phases of HIV infection, it is known that HIV is already present both in CSF (3) and in the brain substance (4). Evidence for a subclinical decline in cognitive performances in HIV-1–infected patients, prior to the appearance of clinically significant immunodeficiency, is still a controversial issue: whereas some neuropsychological studies have demonstrated deficits (5–7), others found this group of patients asymptomatic (8, 9). Although neuroimaging with magnetic resonance imaging (MRI) has not shown atrophy or white matter T2 signal hyperintensity in association with symptomatic late stage HIV infection, proton magnetic resonance spectroscopy has demonstrated abnormalities of cerebral biochemical in asymptomatic HIV-1–infected individuals (10). The neuropathological correlate of this may be the white matter pallor (11) and the state of immune activation as indicated by the expression of major histocompatibility complex (MHC) class II antigens, leading to production and release of cytokines (4) and discrete nerve cell loss (12).

It is obvious from the above studies that if neuropsychological symptoms are present during the asymptomatic stages of the infection, these are so minimal as to defy in many instances the most sophisticated tests and tools. Moreover, the initial nerve cell loss found in some of the patients in this group (12), while showing that HIV has already triggered a process that will eventually cause irreparable damage to the central nervous system (CNS), is probably not severe enough to produce any symptoms.

There is growing evidence suggesting that HIV-associated cognitive/motor dementia may not be the homogeneous entity previously suggested (13) and that some pathogenetic mechanisms still evade our comprehension. Likewise, it is possible that the clinical and pathological abnormalities, so far described in HIV-1–positive asymptomatic patients, may not encompass the whole spectrum of the process. In particular, as most studies have interpreted the clinical abnormalities in relation to neuronal loss and myelin pathology, no attention has been paid to the condition of the axons and their possible role in the pathogenesis of the disorder. Indeed, they have been considered for a long time to be unaffected in HIV encephalitis (HIVE) until the terminal stages.

Silver impregnation is the classical method to study axons; however, with this technique changes can be detected only 12 to 24 hours (h) after damage has taken place (14). Moreover, ubiquitin does not reveal abnormal axons consistently (15).
More recently, by using an antibody to β-amyloid precursor protein (β-APP), which reveals early and subtle axonal changes, we investigated the possibility of the presence of axonal injury in patients with HIV and the relationship that this injury might have with neuropsychological abnormalities (16). Accumulation of β-APP was observed in all HIV brains studied and in a small number of AIDS cases without obvious brain abnormalities. The patterns of immunostaining varied from globular structures to bundles of parallel formations. These results showed, first, that axonal injury is a common neuropathological feature in AIDS, and, secondly, that it can be located in close proximity to the gyri, a region usually spared by HIV (2). It was suggested that, of the two types of changes, the one that appears as bundles of parallel formations could represent reversible axonal damage, and it was speculated that it might explain the fluctuation of neuropsychological symptoms often seen in individuals with AIDS.

In this study, we apply β-APP antibody to the brains of HIV-positive, asymptomatic patients in an attempt to contribute, from a morphological point of view, to a better understanding of the neuropsychological disorders in this group of individuals.

MATERIALS AND METHODS

Patients

Forty-eight anterior cerebral frontal lobe samples were examined. They included 29 from HIV-1–positive, asymptomatic patients. Their deaths were caused by drug overdose or accident, and in a number of patients the HIV status was not known until an autopsy was performed. No physical examination or biochemical tests are recorded for any of the patients. Twenty-one of the brains are from the series of one of the authors (FG), 6 are from the Edinburgh MRC AIDS Brain Bank (Dr. J. Bell), and 2 are cases of the Institute of Neurology. In addition, 14 negative control brains (9 HIV-1–negative brains of drug addicts and 5 normal brains) and 5 positive controls consisting of HIV were used.

Neuropathology

Routine neuropathological examination included histological and histochemical methods. Biotinylated lectin (RCA120, Vector Labs, UK) has been used as a microglia/macrophage marker. Incubation with lectin was followed by application of avidin-peroxidase complex and the visualization was achieved by chromogen. The Bielschowsky silver staining method was used to visualize normal and abnormal axons.

Immunohistochemistry

Sections were deparaffinized by washing 3 times with xylene, then were treated with 0.3% hydrogen peroxide in methanol (to block endogenous peroxidase), and were washed in tap water and phosphate-buffered saline. Sections were then heated for 5 minutes (min) twice in a microwave oven at high power in 0.1 M sodium citrate buffer, pH 6.0, for GFAP (Dako Ltd, UK), p24 (DuPont Diagnostics, UK), and β-APP (Boehringer Mannheim, UK) staining. For A4 (Dako Ltd, UK), 90% formic acid pretreatment for 20 min was necessary before heating in a microwave oven, while for detection of ubiquitin (Dako Ltd, UK), trypsin digestion was applied to retrieve antigens. After blocking nonspecific immunoglobulin binding with 5% normal swine serum for 10 min at room temperature (RT) in Tris-buffered saline (TBS), the sections were incubated with anti-p24, anti-β-APP, and anti-ubiquitin antibodies for 1 h at RT. After further rinses in TBS and incubation with biotinylated secondary antibodies for 30 min at RT and reaction for 30 min with an avidin-peroxidase complex at RT, the reaction was finally developed with 3,3’-diaminobenzidine, and sections were counterstained with Meyer’s hematoxylin.

Polymerase Chain Reaction (PCR)

Nested PCR was used to detect HIV sequences in paraffin-embedded brain tissues of all 48 cases studied. The area of the paraffin section including meninges was removed with a scalpel from the specimen to be used for PCR; the blade was replaced for each case examined. The technique for DNA preparation from formalin-fixed, paraffin-embedded tissue has been described in previous reports (17). The primers used in this study were designed to amplify a sequence of HIV pol gene. Of the reaction mixture, 50 µl contained 0.3 mM of each primer, 0.2 mM of dATP, dCTP, dGTP and dTTP, and 2.5 U Biotaq DNA polymerase in PCR buffer (16 mM [NH₄]2SO₄, 67 mM TRIS-HCl [pH 8.8], 3 mM MgCl₂, 0.01% Tween-20). Thirty-two cycles of the first round of PCR were performed with 1 µg extracted DNA, each cycle consisting of thermal denaturation at 94°C for 1 min, and annealing and extension at 63°C for 2 min. A 144-bp fragment was amplified at the first round of PCR by pol 1 (3181-3203, 5’-CAG GAA AAT TAG CGA GAA TGA GG) and pol 2 (3324-3302, 5’-CCC ATG TTT CTT TGT GTA GT). The second round of PCR was amplified by pol 3 (3228-3247, 5’-CAA TTA ACA GAG GCA GTG CA) with 5’ digoxigenin (Dig) -11-DUTP end-labeled and pol 4. In this round of PCR, 28 to 30 cycles were performed with 2.5 µl of PCR product obtained from first round amplification of 50 µl of reaction product. Thus, a 97-bp Dig-labeled PCR product was obtained after the second-round of amplification. The PCR product was analyzed by electrophoresis and Southern transfer followed by chemiluminescence (Boehringer Mannheim, UK) detection. As the sensitivity of our PCR method was in the range of 5 to 10 copies, the possibility of blood alone reaching the threshold of detection was ruled out, as the amount of blood included in the sample could not contain more than 1 to 2 copies (see 18).

Human β-globin gene was amplified in all HIV-1 PCR-negative and -positive individuals to confirm that the quality and quantity of extracted human DNA was suitable for the amplification. Amplified β-globin products were visualized by ethidium-bromide staining of agarose gel electrophoresis.

RESULTS

Neuropathology

Neuropathological findings in the brains of HIV-1–positive, asymptomatic patients have been previously reported (4), and included astrogliosis (Fig. 1a) in 28, hyperplasia of microglia (Fig. 1b) in 23, and chronic lymphocytic meningitis in 9. On the other hand, myelin
Fig. 1. Photomicrographs of the white matter of an HIV-1–positive, asymptomatic individual stained with anti-GFAP antibody (a), lectin RCA-120 (b), and anti-β-APP antibodies (c & d). (a) illustrates the moderate astrogliosis (×520); in (b) a few microglial cells show slightly increased numbers of cytoplasmic processes (×480); (c) shows a number of β-APP bundles (×120) that in (d) appear to consist of axons, some of which are beaded (×800).

pallor was minimal in 3 cases and absent in the others. One brain was completely normal.

Presence of multinucleated giant cells, a typical morphological feature of HIVE, as well as astrogliosis, hyperplasia of microglia, and myelin pallor was seen in all 5 cases with AIDS. Bielschowsky staining did not show obvious swollen axons in any of the HIV-1–positive asymptomatic individuals, in any of the HIV-negative controls, and in 3 of the AIDS cases. In the other 2 cases with AIDS, isolated swollen axons were seen (Table).

### PCR

HIV-1 DNA was detected by PCR in 14 out of 29 asymptomatic HIV-infected cases without AIDS. All 5 cases with AIDS were also HIV-1 DNA positive. No HIV-1 DNA was detected in 14 HIV-negative controls.

### Immunohistochemistry

p24 was negative in all the HIV-positive, asymptomatic patients and the controls. Four out of 5 cases with HIVE were p24 positive.

No β-APP immunostaining was seen in HIV-negative controls. Accumulation of β-APP was found in 8 out of 29 asymptomatic HIV-positive patients, in whom it appeared as bundles of parallel structures (Fig. 1c) identifiable as axons (Fig. 1d). Of these 8 cases, 4 were HIV-1 DNA positive and 4 were negative on PCR. The changes were focal in 3 cases and diffuse in the others, and in only a few instances did they colocalize with vessels. No cell body took up stain, and staining was never seen in the gray matter. Regarding the localizations of the immunostaining within the white matter, it could be found in both gyral and central white matter regions, whereas the U fibers were only marginally involved in one case. On the other hand, in the 5 cases with HIVE, globular structures were also seen in 2 cases, and in the other 3, bundles of parallel formations were predominant.

No ubiquitin immunostaining was seen in any of the asymptomatic HIV-positive cases. One case with HIVE showed deposition of ubiquitin-reactive material on globular structures, corresponding topographically and morphologically to β-APP–positive globular formations. A4 staining was negative.

Serial sections stained with GFAP and β-APP antibodies and with RCA-120 were examined. The presence of bundles of β-APP–positive axons did not correspond to an alteration of either the number or the morphology of either astrocytes or microglial cells.

### DISCUSSION

Beta-amyloid precursor protein (β-APP) represents a normal constituent of nerve cells. It accumulates in damaged axons (19–23) and, after head injury, it can identify subtle and early (up to 2 h after the event) changes (24–26).

In a previous study of brains of patients with AIDS (16), evidence of β-APP was found to vary from globular structures, usually seen in the deep white matter, to bundles of parallel formations, identifiable as abnormal axons; the latter were found only in the peripheral regions of the white matter, including that of the gyri. Giometto et al (16) concluded that globular structures indicated chronic lesions, whereas the bundles correlated with acute changes. In the present investigation we have been able to detect axonal abnormalities in the brains of 8 out of 29 (27%) HIV-1–positive, asymptomatic individuals. In these cases, only bundles of positive axons are seen that are localized in both gyral and central white matter. Previous neuropathological studies in patients with AIDS have mentioned axonal changes only in a single patient (27).

With regard to the brains of asymptomatic HIV-positive patients, we instead observed that, whereas discrete nerve cell loss is present in only a small number of cases (12) (2/36, both showing β-APP-positive staining), abnormal axons are present in 27% of the cases and are mostly (5/8) diffuse. We therefore came to the conclusion that axonal changes are more obvious than nerve cell loss.
Data to support neurotoxicity in HIV infection are numerous in the literature; however, they all refer to the effect of HIV, or parts of it, on nerve cells in vitro. As for pathological changes of axons, damage to the nerve fibers, albeit to their terminals, has been reported in AIDS by Pittaluga et al (28). Moreover, axonal degeneration and a decrease in axonal population of the optic nerve have been demonstrated by Tenhula et al (29) in a study of 12 AIDS patients: they observed a 40% decrease in the mean axonal population with the mean axonal diameters not differing markedly from control cases. They conclude that these changes may not only be secondary to retinal damage, but also may be the expression of an AIDS-related primary optic neuropathy.

With regard to the pathological events taking place during the early stages of HIV infection, Buzy et al (30) reported that half of 28 CSF samples are neurotoxic on mouse hippocampal cultures or rat retinal ganglion cells.

Our results are therefore the first demonstration in man that axonal changes do occur in brains of asymptomatic HIV-positive patients and support the possibility that these patients may present with neuropsychological abnormalities. They correlate with the report by Price et al (13) of functionally significant abnormalities consisting of cognitive impairment and general motor slowing during this stage in up to 15% of the patients. Furthermore, Wilkinson et al (10) showed a significant difference in the N-acetyl/Choline ratio between asymptomatic (Centers for Disease Control [CDC] Group II/III) HIV-positive and seronegative individuals, confirming previous reports (31, 32).

Following the description of axonal changes in asymptomatic patients, two questions arise. The first is regarding their pathogenesis. A number of possible mechanisms have been put forward to explain the neuropathological changes in AIDS, and these have been reviewed by Lipton (33). They include the effect of gp120 (34), the damaging effect of which can be delayed (34–36), and the condition of immune activation (37) with release of a number of substances that may be toxic to nerve and glial cells (38).

With regard to gp120, Brenneman and coworkers described dystrophic neurites following intraventricular injection of this glycoprotein in young rats (39–41), and Pittaluga et al (28) have shown that HIV-1 gp120 potentiates the NMDA-evoked noradrenaline release. A similar excitotoxic mechanism is associated with post-traumatic axonal damage (42). In the animal model, as well as in man, there is evidence that axonal damage could eventually result in axonal swelling and disruption. In addition, trauma results in an increase of various cytokines (43–45) that are also reinforcing the excitotoxic neurodegenerative effect (46). Therefore, as brains of both AIDS patients (37, 47, 48) and HIV–positive individuals (4) express cytokines, it is not surprising that axonal abnormalities were found in AIDS by Giometto et al (16) and that similar changes were described in brains of HIV-positive, asymptomatic individuals in the present study.

The second question concerns whether the axonal changes described above are permanent or, at least to some extent, reversible. The latter possibility would postulate a damage of moderate severity that could be repaired and would also postulate that the conditions producing it would not be permanent. As for the first condition, while it cannot be tackled directly on the basis of the material available in this study, were the pathogenesis of HIV-related changes and postaxonal injury to be similar, some clues might be provided by the results of experimental trauma: following a trauma of mild severity, some axons show reactive changes, without evidence of disruption of the axolemma (49). Furthermore, whereas in rats a mild trauma capable of eliciting behavioral changes may fail to produce obvious changes (50), it leads to delayed brain lesions when combined with transitory ischemia delivered within a short period from the injury (51, 52). In pathogenetic terms, it means that a mild traumatic injury may not elicit a level of neuroexcitation sufficient to reach the threshold necessary for the production of irreversible changes (53); these, on the other hand, could be obtained after a second insult (ischemia) is added within a short period from the initial trauma. Similarly, in HIV–positive, asymptomatic individuals it is conceivable that minor damage to the axons could either fail to appear or to persist, provided that the initial insult is temporary and minor and is not potentiated by a second insult. Regarding the conditions that could lead to the repair of an axonal injury in HIV–positive individuals, it has been reported that metabolic changes visible on magnetic resonance spectroscopy in AIDS patients have disappeared after retroviral treatment (54, 55). Although both the lack of available clinical data on our patients and the events leading to their demise prevented any information about their immunological status (in particular the CD4) to be obtained, the possibility exists for a virus-related insult to persist only temporarily during this stage, as it is likely that immunological defenses are still able to counter the virus; consequently, expression of cytokines may cease, as has been observed for TNF-α, which persists for only 12 to 24 h after mild trauma (44). During the phases of non-production of toxic substances, the axonal flow could be re-established and the accumulation of β-APP could disappear. It is therefore possible that in the brains of HIV–positive patients, axons could go through a phase of functional damage, highlighted by accumulation of β-APP, but this would not necessarily be followed by fragmentation and degeneration.
REFERENCES

10. Wilkinson ID, Miller RF, Miszkiel KA, et al. Cerebral proton magnetic resonance spectroscopy in asymptomatic HIV infection. AIDS 1997;11:289–95


52. Jenkins LW, Moszynski K, Lyeth BG, et al. Increased vulnerability of the mildly traumatized rat brain to cerebral ischemia. The use of controlled secondary ischemia as a research tool to identify common or different mechanisms contributing to mechanical and ischemic brain injury. Brain Res 1989;477:211–24


Received June 18, 1997
Revision received August 5, 1997
Accepted August 5, 1997