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Review
Viral Etiology of Parkinson's Disease: Focus on Influenza A Virus

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Some clinical reports and epidemiological data suggest that a virus may play a role in the etiology of Parkinson's disease (PD). Following intracerebral injection of a neurovirululent strain of influenza A virus into mice, the virus was found to be particularly localized in neurons of the substantia nigra and hippocampus. Although efforts to detect virus particles in the brains, or antibodies in the serum or CSF of patients with PD have been generally unsuccessful, recent immunohistochemical work has revealed the presence of complement proteins and the interferon-induced MxA in association with Lewy bodies and swollen neuronal processes. Although a viral etiology for PD is not now widely accepted, we proposed such an hypothesis. Neurovirulent influenza A virus is a candidate, but some other viruses or complex infection of these viruses may be responsible for the formation of Lewy bodies and the later death of nigral neurons. Copyright © 1996 Elsevier Science Ltd.

Parkinson's disease   Influenza A virus   Complement protein   Factor Xa

INTRODUCTION

In 1990, Hudson and Rice [1] proposed a viral hypothesis with respect to the ALS–parkinsonism–dementia complex of Guam. They suggested that, "during the acute phase of infection, the influenza or similar virus may be carried in the circulation and can gain access to the nervous system. A virus within a neuron at any site within the nervous system may be either a whole or a defective virus. After a latent interval these may mutate to a form that may suppress macromolecular synthesis and cause degeneration of the neuron... the mutant gene and/or its product may then be transferred to adjacent neurons producing a continuous loss of interconnected neurons... the influenza A virus is a prime candidate because of its historical relationship with encephalitis lethalgica."

Acute, often transitory parkinsonism has been reported during viral encephalitis. The causative viruses include Japanese encephalitis B [2], coxackie B2 [3], Western equine encephalitis [4], influenza A [5] and herpes simplex [6]. Antigens of influenza A virus have been detected in the brains of persons with post-encephalitic parkinsonism [7]. Magnetic resonance imaging (MRI) indicates predominant involvement of the substantia nigra in patients developing

parkinsonism following Japanese encephalitis [2]. Bojinov [8], Shen *et al.* [9] and Lin *et al.* [10] all reported post-infectious parkinsonism with nigral pathology, but they were not able to identify a particular virus. The case report by Lin *et al.* [10] is particularly interesting. A positron emission tomography (PET) scan gave results similar to those in Parkinson's disease (PD), that is a decreased striatal [18F]-fluorodopa uptake, particularly in the putamen, and a relative excess of D2 receptors. These data indicate that viruses may be pathogens capable of producing selective nigral pathology. One may speculate that certain asymptomatic virus infections might manifest as PD several years later by the processes described by Hudson and Rice [1].

Encephalitis lethalgica is one example of a chronic disease where a form of parkinsonism results from a possible viral infection. Eighty percent of the patients afflicted with encephalitis lethalgica had chronic progressive parkinsonism. However, the pathology differs from that of PD in many respects. Similarities with Guamanian Parkinson–dementia complex and with progressive supranuclear palsy have been suggested [11]. The co-occurrence of encephalitis lethalgica and the 1918–1919 influenza epidemic led to speculation of a causal association between the two epidemics. The swine influenza virus (H1N1) was implicated as the most probable etiological agent for the influenza pandemic. Immunofluorescent staining

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showed antigens of the influenza A viral strains WSN and NWS in the brains of six cases of encephalitis lethalgica [7]. These antigens were seen in the nuclei of nerve cells in the hypothalamus and midbrain. Other than the influenza A virus, only coronaviruses in the MHV and HCV-OC43 antigenic group are suspected of producing chronic damage to the dopamine-producing neurons of the substantia nigra (SN) in humans [18].

Most epidemiological studies have failed to identify an intimate relationship between obvious exposure to a virus and PD. However, two reports have strongly supported an infection with influenza as the cause of PD. An increased risk of developing idiopathic Parkinson's disease in individuals born during the 1918–1919 influenza pandemic has been reported [12]. A case–control study evaluating risk factors for PD suggested that the frequency of severe flu-like illness was higher in the PD group than in controls [13]. For several reasons, we have recently directed our work towards testing the hypothesis that the influenza A virus is important in the etiology of PD, and the relevant data will be the focus of this review.

NEUROVIRULENT INFLUENZA A VIRUS

The most common viral epidemics are certainly those due to influenza. Significant epidemics due to influenza A virus occur every 2–3 years. However, only a few cases of encephalitis occur in these epidemics, and the exact frequency of CNS complications from influenza A is hard to document. Although there is one report [19] of the isolation of influenza A, such success has not been widely experienced. Frankova et al. [20] were able to isolate influenza A virus from brains of humans dying with influenza. They showed that ependymal cells were immuno-positive for the virus antigen.

Influenza A viruses are RNA viruses with extra-ordinary latent, infective and mutational properties, and are defined as those strains from any species which possess the group-specific cross-reacting internal ribonucleoprotein and matrix protein antigens. They have two principal surface antigens, haemagglu-tinin (HA) and a neuraminidase (N), which vary antigenically from strain to strain. The HA glyco-protein performs two crucial functions in the early phase of a virual infection. Haemagglutinin is respon-sible both for the binding of the virus to cell surface receptors and for mediating liberation of the viral genome into the cytoplasm through membrane fusion. The variations can involve major (shift) or minor (drift) alterations and HA and N vary independently. Consequently each strain designation carries an indi-cation of its major antigenic make-up as well as the year of isolation.

The human influenza A viruses expressing neuro-virulence are the early H1N1 strains A/NWS/33

(NWS) and A/WSN/33 (WSN). The NWS and WSN strains have been maintained in many laboratories and have retained their unique pathogenic properties [21]. Neurovirulence has also been reported for recombi-nant strains between WSN and A/Aichi/2/68 (H3N2) [22,23]. The mechanism of the neurovirulence has been studied using mice. Infection of mice with these viruses by intracerebral inoculation caused a meningo-encephalitic condition [24]. Using various recombinants between the WSN and NWS strains, Nakajima and Sugiura [23], and Sugiura and Ueda [22] clearly demonstrated that the WSN neuraminidase is the principal factor determining neurovirulence. The role of WSN neuraminidase has been considered to be the facilitation of the cleavage of the HA polypeptide. On the other hand, matrix protein (M) and probably nonstructural protein (NS) proteins play a role as helpers or accessory virulence factors, enabling effi-cient virus replication in the brain.

ENTRY, DISSEMINATION AND PENETRATION INTO THE CNS

Potential entry routes into the organism are mucosal membranes of the upper respiratory tract. In general, primary replication must occur in these target cells, and then the virus must reach the CNS by either the blood stream (hematogenous spread) or via nerves (neural spread). Following hematogenous spread, neuroinvasive agents penetrate the CNS through the choroid plexus or through endothelial cells. Reinacher et al. [24] studied in detail the spread of neurovirulent influenza A virus in mice following intranasal inocu-lation. They showed that viruses invade the brain by the hematogenous route (viremia) and/or via centri-petal transport along cell processes from the nasal mucosa to the CNS (olfactory and trigeminal routes).

TROPISM OF INFLUENZA A VIRUS

The essential component of the receptor for influ-enza A viruses is sialic acid. Influenza A viruses specifically recognize N-acetylneuraminic acid. It is generally believed that the spread of a virus and tissue tropism depend largely on a proper match between the cleavability of the viral glycoprotein by an endo-peptidase and the availability of the protease in the host [25]. Therefore, tropism of influenza A virus could be determined by the proteases of the host cells as well as the presence of the receptor (sialic acid), leading to a localized infection despite the widespread occurrence of the sialic acid. The blood-clotting factor Xa [25] and tryptase Clara [26] are two enzymes which have been identified as influenza virus-activating proteases. How-ever, there is no clear evidence of such activating proteases in the human respiratory tract.

Our recent immunohistochemical study using an antibody to human factor Xa (FXa) revealed that the 54
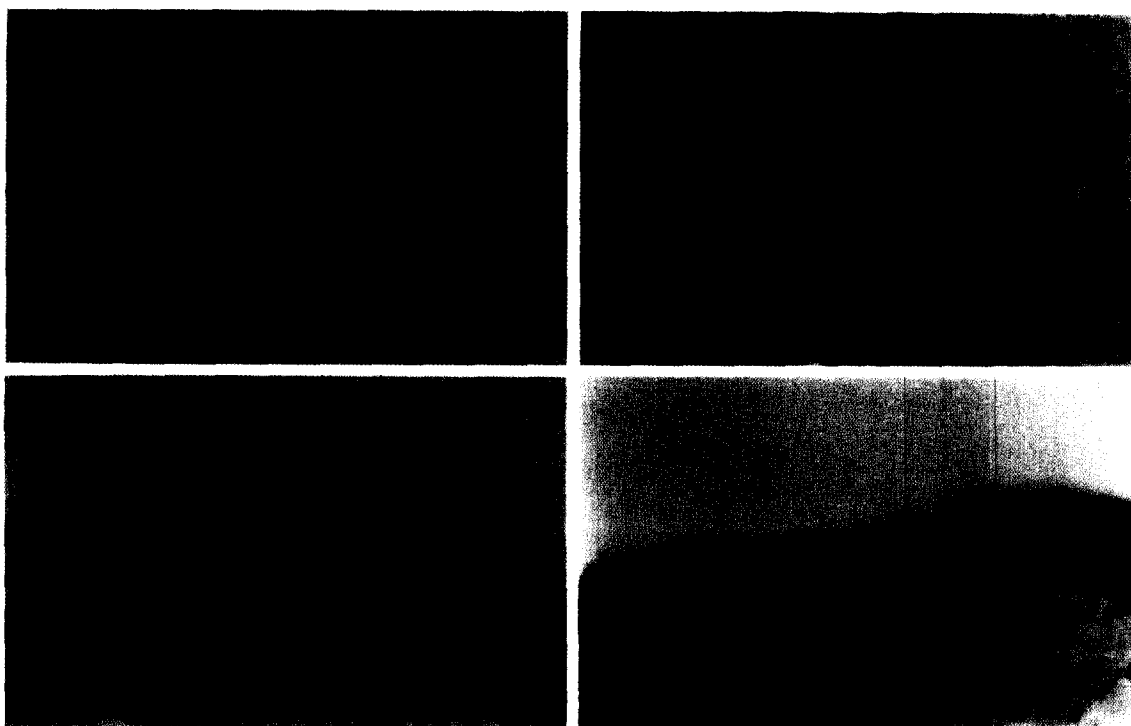
FIGURE 1. Immunostaining with antibodies to FXa in the brain (A,B), bronchus (C) and nose (D). A: FXa immunolabeling was seen in the neurons of the oculomotor nucleus. B: In the substantia nigra, positive staining (arrows) was seen in the neuronal cytoplasm. C: Intense FXa staining was seen in the bronchial epithelia. D: Factor Xa staining in the middle concha showed positive immunolabeling in the epithelium as well as in macrophages (arrow).

kDa form of FXa can be found in the nose, bronchus, duodenum and brain (Fig. 1)[27]. The FXa immunoreactivity in the respiratory tract was exclusively localized to the nose and bronchus. The FXa localization on the apical surface of those tissues is of particular interest in view of the fact that some para- and orthomyxoviruses targeting these tissues require FXa-like endoproteinases for their ability to replicate and spread, and bud from the apical surface [25]. Furthermore, we observed positive immunolabelling in some brainstem neurons, such as in the SN, oculomotor nucleus, locus ceruleus and pontine nuclei. White matter microglial cells were also positive for FXa. Positive immunolabelling in some catecholamine neurons is particularly interesting with respect to influenza A infection.

THE POSSIBILITY OF INFLUENZA A VIRUS PERSISTENCE

Among the various cell types in the CNS, viruses frequently persist in neurons, suggesting that these cells can evade immune surveillance. A recent study suggested that a general mechanism of cell death in hosts infected with influenza A virus is apoptosis [28]. Influenza is an acute viral infection which has never been thought to develop into a chronic state. However, the possibility can not be excluded that the virus might persist in the organism for a long time, without cytopathic effects or apoptosis. The persistence of influenza A virus (WSN strain) in cell cultures has

been described [29, 30]. Tentsov et al. [31] reported that virus-specific sequences were found for a long period of time (83 days) in children born to mothers who contacted influenza during pregnancy. As in the culture study of Frielle et al. [29], M protein could not be detected, but viral nucleocapsides persisted in such children. The production of defective interfering influenza viruses has been said to increase the probability of endosymbiotic infection, and the presence of a temperature-sensitive mutant appears critical to the maintenance of a persistent infection [32].

EXPERIMENTAL MODELS OF INFLUENZA VIRUS ENCEPHALITIS [33]

Experiments on the direct inoculation of the neurotropic NWS strain of influenza virus into mouse brain have already been undertaken, but a clear invasion into neuronal cells has not been shown [24,34,35]. Intranuclear neuronal inclusions were, however, described by Miyoshi et al. [34]. Recently, we also studied the clinical symptoms and the localization of neurovirulent influenza A virus in mice after intracerebral injection. We used four different strains of influenza A virus [A/WSN/33 (H1N1, WSN); A/Aichi/2/68 (H3N2,Aichi) and two recombinants, R96; and R404BP. The detailed genotype of each virus has been described in our previous paper [33]]. The gene derivations of these strains are presented in our recent paper. Each mouse received an
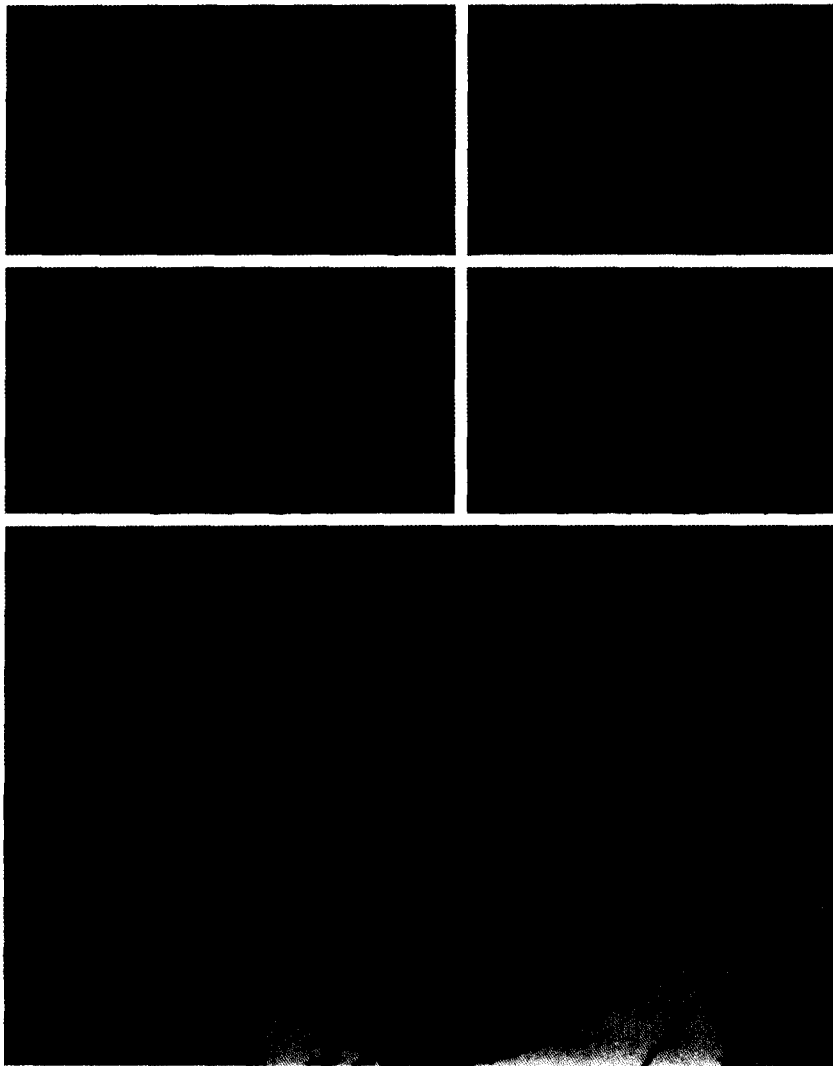
FIGURE 2. Immunohistochemistry using the anti-WSN antibody in mice from the R404BP group on day 3 (A, B) and day 7 (C, D, E). A: Low-power photomicrograph demonstrating WSN-immunonoreactivity in the bilateral medial parts of the SN and VTA. B: High-power photomicrograph of A shows that the positive immunoreactivity is localized in some neurons, neurites and the neuropil surrounding them. C: Low magnification photomicrograph indicating more prominent staining in the SN than at day 3, now extending to the SNC laterally and SNR ventrolaterally. Additional labeling is seen in the central grey (CG). D: Higher magnification of C shows that a large number of neurons with neurites extending dorsolaterally into the SNC are strongly positive. E: Higher magnification of D. The definite and progressive involvement of the substantia nigra showed a selective and reproducible invasion of influenza A virus into the parenchymal tissues of the mouse brain.

intracerebral inoculation through a double-tapered needle of 30 ml of phosphate buffered saline containing 510 plaque forming units (PFU) of the virus to be tested. Immunohistochemistry with anti-WSN antibody (rabbit polyclonal, 1:10,000) was done. The virus strains with the WSN gene segment coding for neuraminidase (NA) induced meningo-encephalitis in the mice. The mice inoculated with the R96 strain, which has only the NA gene from the WSN strain, had mild symptoms and weakly positive immunoreactivity to the anti-WSN antibody in meningeal regions. Both the WSN and R404BP strains, which contain the WSN gene segments coding for both NA and matrix protein (M), were clearly neurovirulent both clinically and pathologically. On day 3 following inoculation with either of these strains, WSN-immunoreactivity was seen in meningeal and ependymal areas, neurons

of the circumventricular regions, the cerebral and cerebellar cortices, the SN zona compacta (SNC) and the ventral tegmental area (Fig. 2A, B). On day 7, meningeal reactions as well as neuronal staining was still seen, and advanced immunoreactivity was evident in the SNC (Fig. 2C, D, E) and hippocampus. Double immunostaining demonstrated that the WSN antigen was only seen in neurons and not in microglia or reactive astrocytes. Immunostaining for a lectin, maackia amurensis agglutinin that recognizes the Neu5Ac a2,3 Gal sequence which serves as a binding site for influenza A virus on target cell membranes, showed that positive immunoreactivity was localized to the ventral SN and hippocampus.

On day 14, we performed a virus plaque assay from whole brains of the surviving mice and found WSN strain virus with a titer of 1.2×10^4 pfu/ml, suggesting

an adaptation to the mouse brain during this period.

These results suggest that nigral (and hippocampal) neurons may be especially vulnerable to attack by neurovirulent influenza A virus.

THE SEARCH FOR DIRECT EVIDENCE OF THE INFLUENZA A VIRUS IN PD

Gamboa [7] did not find immunolabelling for the neurovirulent influenza A virus antigens NWS and WSN in five patients with PD. Marttila et al. [14], using the complement-fixation technique, showed no significant difference in serum levels of the antibody to influenza A in a comparison of 444 PD patients and a similar number of healthy controls. Elizan et al. [15] also found no significant increase of antibody level in serum or CSF of PD patients when compared with controls. Schwartz and Elizan, using electron microscopy, immunofluorescence, and tissue culture techniques, searched for viral particles and virus specific products, including NWS, in human autopsied brains [16]. However, they could not detect any virus. Additional studies with sensitive nucleic acid hybridization methods have not revealed any evidence of the presence of influenza A viral RNA in PD brain tissue [17]. In our laboratory, we have attempted to detect viral products using the polymerase chain reaction (PCR), but so far we have been unsuccessful.

Thus, there is little direct evidence as yet for the persistance of influenza A virus in PD brains. However, further PCR experiments using different primers are necessary, because of the possibility that a virus within a neuron may be a defective one.

POSSIBLE IMMUNE SYSTEM PROTEINS IN HUMAN BRAIN WHICH MAY REFLECT INFLUENZA A INFECTION

There are several types of natural immunity to viruses and virus-infected cells. Certain viruses and virus-infected cells directly activate the complement system which may in turn lyse the viruses or virus-infected cells. In addition, antibodies may cross-react with viral glycoproteins or other structures leading to viral inactivation or interference with virus maturation. Interferon (IFN) and interferon-induced protein also induce an antiviral state in host cells. If these proteins appear in pathological regions of PD brain tissues, a viral hypothesis should be listed as a prime candidate for the cause of PD.

Complement proteins [36]

In an immunohistochemical study of the SNs from 11 PD cases and 5 controls, we found that that Lewy bodies (LBs) in PD are recognized by antibodies to complement proteins (Fig. 3) [37]. In PD, but not in controls, intra- and extraneuronal LBs and dendritic spheroid bodies were stained by anti-human C3d,

C4d, C7 and C9 antibodies. However, we were not able to detect proteins of the alternative complement pathway such as fraction Bb of factor B or properidin. Activation of the complement system is important in amplifying the proteolytic cascade, opsonizing tissue for phagocytosis and destroying invaders. The association of complement and its inhibitors with Alzheimer's disease lesions, and the presence of complement activated oligodendroglia [38] in some neurodegenerative disorders have been well described as characteristics of the central nervous system pathology. The complement cascade can be activated not only by immunoglobulins, but also by other factors such as trypsin-like enzymes, myelin or a virus. Our results on the association of complement proteins with LBs and dendritic spheroid bodies, the pathological characteristics of the nigral damage in PD [37], suggest that classical complement activation may contribute to the nigral pathology. A virus may be one of the candidates for the activation of complement.

Interferon and interferon-induced protein mxa [39,40]

The recent nomenclature of interferons (IFNs) is based primarily on sequencing. It designates leukocyte IFNs as α-IFN and ω-IFN, fibroblast IFN as β-IFN, and immune IFN as γ-IFN. Infection by various viruses activates the α-IFN and β-IFN genes. It has been suggested that the production of α-IFN in various tissues may be stimulated by factors other than viral infection and may have a role in normal physiology [41,42]. In line with this suggestion, traces of α-IFN have been demonstrated in the CSF of normal individuals [43], and constitutive expression of α-IFN has been shown in normal human brains as well as in the brains of patients with multiple sclerosis [44]. Akiyama et al. [45] and Yamada et al. [39] reported α-IFN immunoreactivity in neurons and microglia (Fig. 4A) in human brain. Punctate staining of neurons was diffusely seen in cortical areas. This may have reflected non-specific binding of a-IFN to gangliosides in neuronal membranes [46,47].

A wide variety of cell types have small numbers of high affinity receptors for α/β-IFNs [47]. Various α-IFN subspecies, as well as β-IFN and ω-IFN, but not γ-IFN, can compete with each other for the same binding sites on human cells. After binding to the cell, IFN molecules are transported into the nucleus within a few minutes by facilitated receptor-mediated endocytosis [47]. Immunohistochemically, we recently showed that an α-IFN receptor (α-IFNR) is localized to brain microglial cells (Fig. 4B) as well as to macrophages in infarct areas [40]. Soluble forms of the α-IFNR protein (p40) were seen in plasma, consistent with a previous study [48]. Microglial localization of both α-IFNR and α-IFN suggest that α-IFN may be coupled to receptors in postmortem human brain tissue. So far, however, we have not determined whether microglial cells produce α-IFN in

FIGURE 3. Immunohistochemistry with the antibody to human C4d. A: In the substantia nigra of a PD case, a positively stained Lewy body can be seen (peripheral staining). B: Immunoelectron microscopy showed that the reaction products surround the core.

an autocrine fashion or not. In the SN tissues in PD patients, we could not find any structures other than microglia which stained with the antibodies to α-IFN or its receptor. In the experimental mice with WSN intracerebral inoculations, IFN-positive microglia appeared in the areas of influenza A invasion.

MxA (molecular mass of about 78 kDa) is an interferon-induced protein which is encoded by a gene located on the distal part of the long arm of human chromosome 21 [49]. In contrast to mouse Mx1 protein, which accumulates in the nuclei of IFN-treated cells, tMxA accumulates in the cytoplasm [50]. MxA plays a role in defense against vesicular stomatitis virus, influenza virus and measles virus at a transcriptional [51], post-transcriptional [52] and translational level [53], respectively. The Mx proteins found in several species all have sequence elements

typical of a GTPase in the amino-terminal portion of the molecule [54, 55]. The GTP binding core domain has been thought to be essential for anti-influenza A activity in the murine Mx1 protein [56].

We found a clear involvement of MxA in LBs and swollen neuronal processes in PD (Fig. 5). The large punctate staining for MxA seen by immunoelectron microscopy [57] might suggest that the MxA protein in LBs forms aggregates, as reported for Mx1 [55].

Immunohistochemical and histochemical studies have revealed various components of LBs. These have been tentatively divided into four groups [58]: (1) postulated structural elements of the LBs fibrils such as neurofilaments; (2) proteins implicated in the cellular response to fibrils, such as ubiquitin; (3) enzymes of the phosphorylation/dephosphorylation system, such as Ca^{2+}/calmodulin-dependent kinase-II;
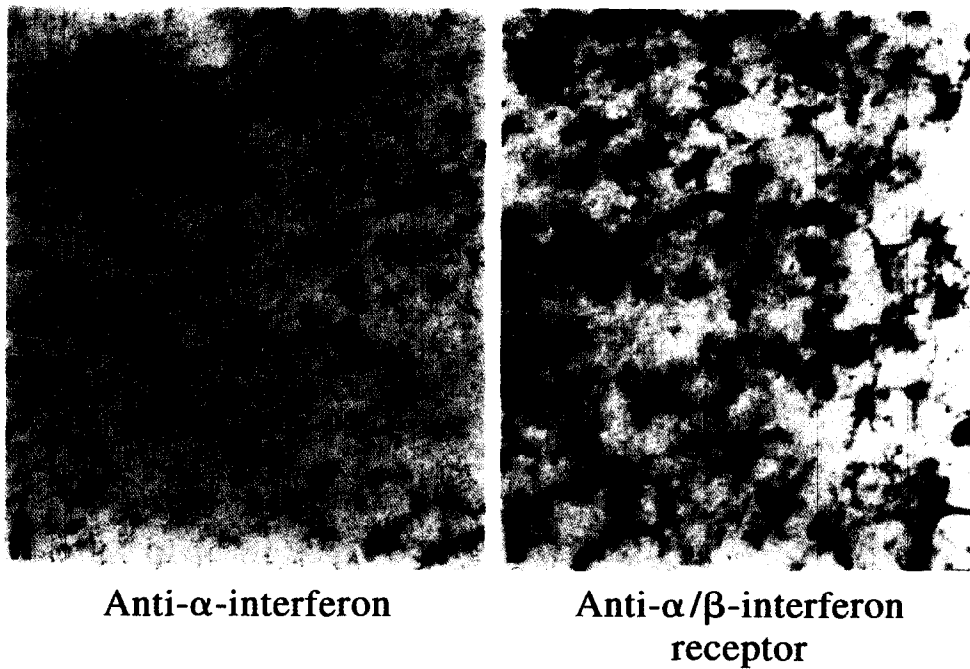
Anti-α-interferon

Anti-α/β-interferon receptor

FIGURE 4. Immunostaining by antibodies to α-IFN and an α/β-IFN receptor in PD brain tissues. A: Staining for α-IFN in the parietal white matter shows aggregates of punctate immunoproducts (arrow heads). B: Staining for α/β-IFN receptor in parietal white matter shows intense immunopositive cells with microglial morphology, as well as capillary staining. The substantia nigra of PD cases did not show any staining other than that of microglia.
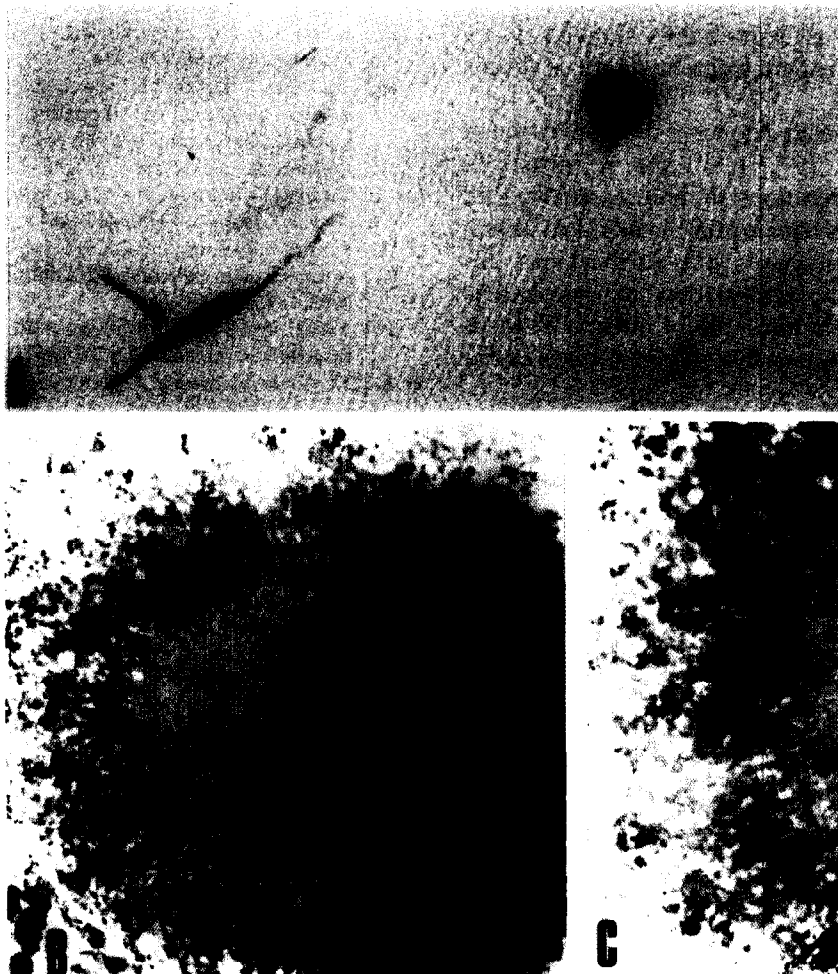

FIGURE 5. Immunostaining by an antibody to MxA in PD substantia nigra. A: The staining for MxA shows intracellular LBs and a swollen neuronal process (arrow). B: Immuno-electron-microscopy showed that irregularly shaped reaction products are dispersed in the peripheral fiber structures of a LB. C: Higher magnification of B.

and (4) cytosolic proteins, such as tyrosine hydroxylase. Pollanen et al. [58] proposed a model of LB pathogenesis involving self-assembly and aggregation of proteins, post-translational modifications and proteolysis. They emphasized the contribution of neurofilaments to the fibril formation of LBs. Although the mechanism for LBs fibrillogenesis remains unclear, the present study suggests that induction and aggregation of MxA protein may play some role in LB formation.

The co-localization of complement proteins and MxA may support a viral mechanism for the formation of LBs, although its says nothing as to the nature of the virus. Another possibility, in view of the appearance of tyrosine hydroxylase in LBs, is that the accumulation of MxA in LBs may indicate that it has some physiological function in neurons.

A HYPOTHESIS

This review of recent literature, and our own studies, indicate that: (1) nigral neurons are positive to factor Xa, a determining protease for influenza A virus tropism; (2) LBs, swollen neuronal processes and dendritic spheroid bodies, which are specific pathological markers in PD, contain complement and MxA proteins; and (3) animal experiments showed that a major target of neurovirulent influenza A virus strains are the nigral neurons.

Several viruses cause an extensive reorganisation of cytoskeletal elements [59]. Influenza A virus, for example, produces alterations in actin assembly and distribution, as well as concurrent biochemical alterations in intermediate filaments [60]. Furthermore, this virus induces the phosphorylation of cytoskeletal proteins [61]. It may therefore be of interest to focus future attention on the possible role of viruses in the formation of LBs.

These data, as well as some epidemiological evidence, strongly suggest the possibility of a viral etiology for PD. Further research to detect viral products in PD patients is very much needed. The detection of abnormal cellular products induced by influenza A infection, which may lead to LB formation and later death of nigral neurons, should also be investigated. Further studies to establish an experimental model following this hypothesis are now in progress in our laboratory.

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