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Filterable Forms of *Nocardia*: An Infectious Focus in the Parkinsonian Midbrains

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1. Introduction

Nocardia is a strictly aerobic gram-positive, partially acid-fast mycelial bacterium that superficially resembles the fungus (Beaman & Beaman, 1994). The bacterium causes L-dopa-responsive movement disorder accompanied by neuronal inclusions resembling Lewy bodies, suggesting a possible link between *Nocardia* and Parkinson's disease (PD) (Kohbata & Beaman, 1991). PD is a slowly progressive, acute monophasic neurological disorder with a lengthy prodromal phase before the onset and with longer duration of disease (Fearnley & Lees, 1991; Hawkes, 2008). It is characterized pathologically by neuronal loss, reactive gliosis, and Lewy bodies in the remaining neurons in the midbrain substantia nigra (Greenfield & Bosanquet, 1953). Spread of Lewy bodies occurs from the brainstem through midbrain and basal forebrain to the cerebral cortex (Braak et al., 2003). Furthermore, the transfer of the nigral lesion to fetal grafts in PD patients with fetal nigral transplantation happens (Braak & Tredici, 2008; Li, et al., 2008). The transfer experiment of the nigral lesion to experimental animals did not attain satisfactory results by using antibiotic-containing homogenizing buffer (Bethlem & Jager, 1960). PD is probably caused by an environmental agent rather than a hereditary factor. The environmental agent may be parasitic to midbrain nigral tissues and bacterial in nature. Heredity might play a role in predisposing certain individuals to PD cause.

Antibody to *Nocardia* is found in the serum of PD patients (Kohbata & Shimokawa, 1993). The 125 human brain specimen (including PD, Dementia with Lewy bodies, and other patients) is not positive for filamentous gram-positive nocardiae by Gram staining (Lu et al., 2005), though *Nocardia*-like beaded cells were present in the midbrain nigral lesions of two patients suffered from encephalitis with a parkinsonian syndrome (Bojinov, 1971 ; Kohbata & Beaman, 1991). On the other hand, an accumulation of acid-fast lipochrome bodies, morphologically identical to filterable forms of *Nocardia* (i.e., filterable nocardiae), within the neuroglia of midbrain nigral lesion is dense in the early stage of PD (Kohbata et al., 1998).

Filterable nocardiae are gram-negative, acid-fast, and PAS-positive granular cells. They are, in vitro & in vivo, morphologically characterized by gelatinous masses containing yellow-fluoresced granules under ultraviolet light when stained with acridine orange and grow to be cylindrical tubules such as that of mycelial bacterium in the presence of erythrocyte lysates. An experimental infection causes a late-onset movement disorder after a long incubation period. Filterable nocardiae, degeneratively generated from *Nocardia*, have a high predilection for erythrocytes and spread through neuroglia to neurons in the mouse brain as intracellular parasites (Kohbata et al., 2009). We attempted to investigate their isolation from the nigral tissues of patients with PD and to detect their presence in the midbrain.

2. Subjects and methods

2.1 Subjects

Six PD patients (aged 65 to 82 years; median age, 72 years) and four patients without neurological disorder (aged 60 to 70 years; median age, 65 years), serving as age-matched controls, were selected from the archives of the Department of Pathology, Chubu National

Subject	Age (yr)	Gender[a]	Duration[b] (yr)	Cause of death	Hoehn & Yahr[c]	Reference
Parkinson's disease						
PD1	65	m	2	Pneumonia	II	Kohbata et al., 1998
PD2	68	m	9	Suicidal attack	III	Kohbata et al., 1998
PD3	75	m	5	Pneumonia	V	This study
PD4	82	f	10	Leukemia	V	This study
PD5	68	f	19	Pneumonia	V	Kohbata et al., 1998
PD6	73	m	26	Choking	V	This study
Control						
C1	60	m	-	Pulmonary cancer	-	Kohbata et al., 1998
C2	61	m	-	Pulmonary cancer	-	Kohbata et al., 1998
C3	70	m	-	Pulmonary cancer	-	Kohbata et al., 1998
C4	70	m	-	Sepsis	-	This study

Table 1. Clinical characteristics in PD and control patients examined.
a, Gender; f = female, m = male.
b, Duration indicates the time from clinical diagnosis of PD to death. c, Hoehn & Yahr 1967.

Hospital (Aichi, Japan), Nagano Red-Cross Hospital (Nagano, Japan), Shinshu University (Nagano, Japan), and Fuji National Hospital (Shizuoka, Japan). Details concerning PD and control patients were shown in the Table. Two suspected PD patients (sPD; female, 78 years old, sPD2 male; 75 years old), in addition to that of PD patients 1 and 4 as shown in Table, were picked up to obtain several frozen midbrain samples of the nigral tissue. Informed consent was obtained from the patients and their family members. The privacy rights of all subjects were always observed. PD was diagnosed by neurologists on the basis of the results of neurological examinations (Kohbata et al., 1998). Paraffin-embedded tissues, serially sectioned into 5-µm sections, and then placed on glass slides according to an atlas (Haines, 1987). The sections at the level of the caudal substantia nigra were examined. The sections were stained with hematoxylin and eosin (H&E). All procedures were performed in compliance with relevant laws and institutional guidelines. The protocol was approved by the appropriate institutional committees.

2.2 Culturing of brain samples

Frozen midbrain samples, ca. one cubic cm in volume, were dissected into small pieces and inoculated into 50 ml of brain heart infusion supplemented with 0.4% (w/v) yeast extract (BYE). After one-day incubation at 37 ºC with shaking (130 strokes per minute), the culture was centrifuged for 10 min at 5000 rpm. Supernatant was filtrated through a 0.45 µm and then through a 0.22 µm filter. Filtrate samples were transferred into equal volumes of BYE broth medium and incubated at 37 ºC with shaking for two days. One hundred microliters of filtrate samples was inoculated into 50 ml of BYE broth medium with or without 1% (v/v) erythrocyte lysates to be incubated. Erythrocyte lysates were prepared as described previously (Kohbata et al., 2009). Culture samples at various incubation periods were applied to glass slides with a loop and fixed by heat. Samples were histochemically and immunohistochemically examined as described in 2.5. Light microscopy.

2.3 Detection of DNA in the culture samples

DNA was prepared as described previously (Kohbata, 1998; Kohbata et al., 2009). Briefly, five milliliters of extraction buffer (100 mM Tris-HCl, pH 9.0, 40 mM EDTA), 1 ml of 10% SDS, 3 ml of benzyl chloride were added to 10 ml of the culture samples or to 1 wet gram of filamentous gram-positive nocardiae within sterile tubes. For collection of filamentous gram-positive nocardiae, one milliliter of stock culture was transferred into 50 ml of BHI broth and incubated at 37 ºC with rotational agitation (150 rpm) as described previously (Kohbata & Beaman, 1991). At 16 h after inoculation, the bacterial pellets were harvested by centrifugation for 15 min at 3000 rpm. The bacterial pellets were washed three times with sterile DW. The tube was vortexed and incubated at 50 ºC for 30 min with shaking, ensure the two phases remained thoroughly mixed. Three milliliter of 3 M of sodium acetate, pH 5.0, was then added, and the tube was kept on ice for 15 min. After centrifugation at 5000 rpm at 4 ºC for 30 min, the supernatant was collected, and DNA was precipitated with isopropanol. The samples were applied to the wells of a 0.8% gel run in TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 8.0). Bands were visualized by staining with ethidium bromide (1 µg/ml), destained with distilled water, and photographed. The procedure employed was as follows: A) PCR amplification. B) Restrictive digestion analysis of genome DNA. C) Subcloning of DNA. Amplification of gene fragments and polymerase chain reaction-mediated synthesis were performed as described previously (Kohbata et al., 2009). Genome

DNA was incubated for 1 hr at 37 ºC in the presence of 5 U of *Eco*R1 or *Hin*d III (Nippon gene, Tokyo, Japan) according to the manufacture's instructions. The fragments produced were separated on a 0.8% gel run in TAE buffer. Several fragments were produced by complete *Hind* III digestion. Eight fragments, each composed of nearly 1000 bases, were selected and inserted into a pUC118 (Takara, Tokyo, Japan) plasmid vector system. Purified plasmids were sequenced as described previously (Kohbata et al., 2009). Sequence analysis was performed through queries of GenBank using the Basic Local Alignment Search Tool (BLAST).

2.4 Light microscopy

Smear samples of broth cultures were Gram-stained. Via a PAS, smear samples or midbrain sections were stained. For immunostaining, smear samples or midbrain sections were stained with antiserum, the specificity of which has been already confirmed (Kohbata et al., 2009). Processing was performed according to conventional peroxidase-antiperoxidase complex or avidin-biotin complex protocols. 3, 3'-diaminobenzine was used as the chromogen. Midbrain sections of PD patients 1, 2, and 5 were stained with 0.1% acridine orange (AO, Polyscience Inc., PA., USA) in McIlvaine's buffer for 4 min. Several sections were stained with 10 μg/ml of 4, 6-diamidino-2-phenylindole (DAPI, Polyscience, PA, USA) and observed with an epifluorescence microscope (Fluorophoto VFR, Nikon, Japan) as described previously (Kohbata, 1998; Kohbata et al., 2009).

3. Results

3.1 Morphological and immunological features of filterable isolates

Several clusters composed of numerous gram-negative granules were observed in one-day cultures of PD patient 1 culture filtrates (Fig. 1A). In two-day cultures, the same granular cells were also seen. Many immunoreactive brilliant granules were present in the clusters (Fig. 1B). PAS-stained clusters were observed (Fig. 1C). Large globules composed of small red PAS-positive granules were seen in the smears from the one-day culture supplemented with erythrocyte lysates (Fig. 1D). Several clusters of gram-negative granules were also seen in one-day culture of PD patient 4 culture filtrates (Fig. 1E). In two-day cultures, similar gram-negative granules were observed. Numerous brilliant immunoreactive granules were present in brown clusters (Fig. 1F). Large globules composed of gram-negative granules were seen when supplemented with erythrocyte lysates. PAS-positive clusters were not seen. In one-day cultures from patient sPD1, many clusters of numerous gram-negative granules were observed (Fig. 1G). They were not reactive to the antiserum (Fig. 1H). Any gram-negative or gram-positive granules were not observed in one-day and two-day cultures of sPD patient 2 filtrates.

3.2 DNA genomic features of filterable isolates

Genomic DNA was detected in broth cultures PD4 (3 & 4 on Fig. 2A), but not in broth cultures PD1 (1 & 2 on Fig. 2A). PCR-mediated synthesis failed to amplify the 16S rDNA of filterable organisms. Genomic DNA digested with *Eco*R1 or *Hind* III revealed a profile (4 & 5 on Fig. 2B) different from that of *Nocardia*. Eight fragment sequences were analyzed. The 4295-base genome was AT-rich, with a G+C content of 41%. G+C content in each fragment varied from 39 to 48%. Most fragments contained several A/T homopolymers. One of these fragments revealed that the genome was AT-rich with a G+C content of 40%; it contained six
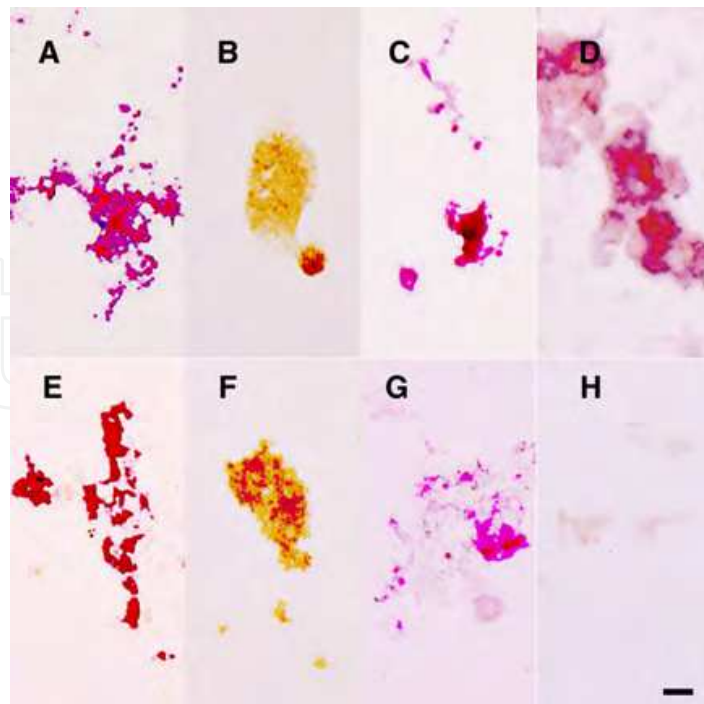
Fig. 1. Light micrographs of broth cultures. Gram-stained (A), immunostained (B), and PAS-stained (C) smears of BYE broth cultures at one day after inoculation of PD1 culture filtrates. PAS-stained smears of BYE broth medium inoculated with PD1 filtrates when supplemented with erythrocyte lysates (D). Gram-stained (E) and immunostained (F) smears of BYE broth cultures at one day after inoculation of PD4 culture filtrates. Gram-stained (G) and immunostained (H) smears of BYE broth cultures at one day after inoculation of suspected PD1 culture filtrates. Panel A through H are the same magnification (bar = 10 μm).

tetramers and three pentamers of adenine or thymine within its 679-base sequence. BLAST analysis indicated that each fragment sequence bore no similarity to genes found in any other sequenced organisms.

3.3 Eosinophilic and immunoreactive inclusion-bearing midbrain neurons of the VL pars compacta and central gray area of PD patient 1

Neurons harbored eosinophilic inclusions such as a peripheral halo with eosinophilic body (Fig. 3A), with small eosinophilic body (Fig. 3B), with eosinophilic body containing a dense center (Fig. 3C), and with double eosinophilic dense bodies (Fig. 3D). Each of next immunostained sections revealed reactive inclusion bodies (Fig. 3E), reactive granular inclusions (Fig. 3F), inclusion bodies with reactive peripheries (Fig. 3G), and tubule-like bodies with reactive peripheries (Fig. 3H). Inclusion bodies within midbrain neurons were negative on several sections stained with PAS.

3.4 Neurons harboring immunoreactive, PAS-positive inclusions and degenerative neurons in the pars compacta of PD patient 2

Immunoreactive and PAS-positive inclusions occupied one half of the neuronal cytoplasm (Figs. 4A & 4B). Immunoreactive corpora were composed of a deformed portion (arrowheads on Fig. 4A). Red inclusions within shrunken pigmented neurons and partially
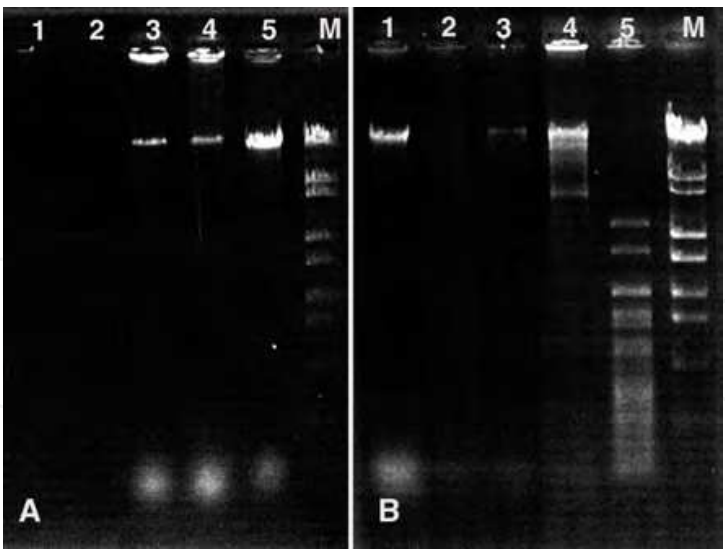
Fig. 2. Genome DNAs in the broth cultures. Detection of genomic DNA (A). 1; 1-day culture of PD1. 2; 2-day culture of PD1. 3; 1-day culture of PD4. 4; 2-day culture of PD4. 5; genomic DNA of *Nocardia*. M; marker 6 (Wako, Tokyo, Japan). Restrictive digestion analysis using *Eco*R1 and *Hind* III (B). 1; genomic DNA of *Nocardia*. 2; digested genomic DNA of *Nocardia* with *Eco*R1. 3; digested genomic DNA of *Nocardia* with *Hind* III. 4; digested genomic DNA of filterable organisms collected from patient PD4 with *Eco*R1. 5; digested genomic DNA of filterable organisms collected from patient PD4 with *Hind* III. M; marker 6. The preparation method is described in the text.
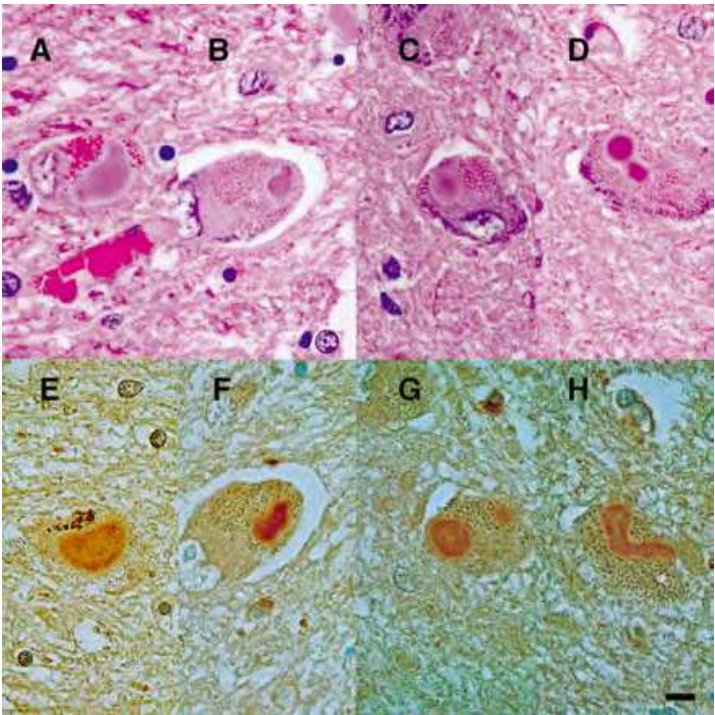
Fig. 3. Light micrograph of the VL pars compacta and the central gray area neurons of patient PD1. H&E-stained sections of the VL pars compacta (A) and of the central gray area (B, C, and D). Immunostained sections of the VL pars compacta (E) and of the central gray area (F, G, and H). Magnification of panels a through h is the same (bar = 10 μm).

red-colored ring-like inclusions within pigmented neurons were observed (Figs. 4C & 4D). The cytoplasm was filled with numerous brilliant brown granules. The same granules were deposited in neuroglia (Fig. 4E). Numerous brown granules were localized within the neuronal cytoplasm (Fig. 4F). One half of the cytoplasm was occupied by granule-containing gelatinous masses composed of a deformed portion (arrowheads on Fig. 4G).
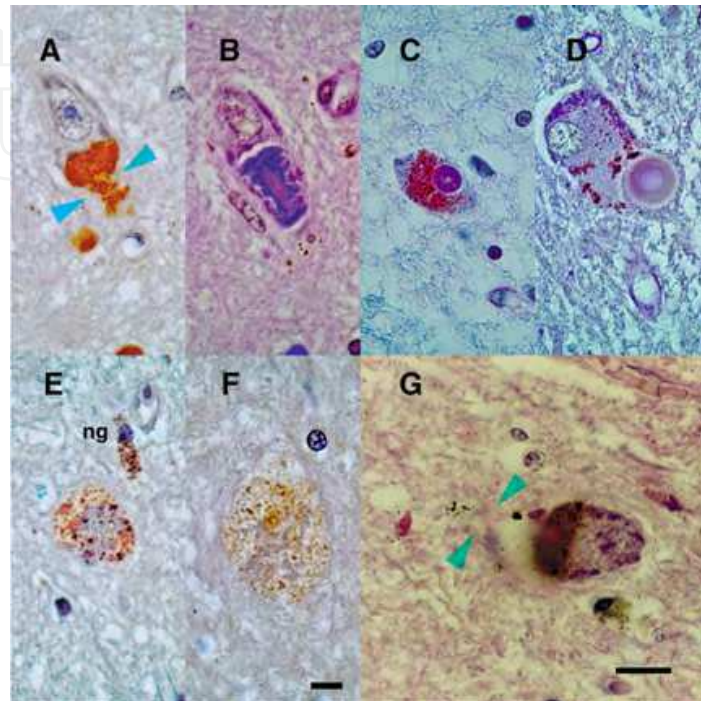

Fig. 4. Light micrographs of the VM and VL pars compacta neurons in PD patient 2. Immunostained (A) and PAS-stained next (B) sections of the VM pars compacta. PAS-stained sections (C) of the VM pars compacta and that (D) of the VL pars compacta. Immunostained sections (E and F) of the VL pars compacta. Magnification of panels a through f is the same (bar = 10 µm). H&E-stained section (G) of the VL pars compacta. Bar = 10 µm. ng; neuroglia.

3.5 Immunoreactive and PAS-positive corpora in the VM pars compacta of the substantia nigra of PD patients

The blue spots, probably identical to astroglia, were predominantly present in the VM pars compacta of PD patient 1 (Fig. 5A). In the same location, many PAS-positive spots were present among the blue spots (Fig. 5B). In higher magnification of the nigral region shown by an arrowhead on Fig. 5A or Fig. 5B, faintly immunoreactive (Fig. 5C) and PAS-positive (Fig. 5D) corpora were seen among the blue spots of which size was similar to that of corpora. An examination of other tissue section reveals that several immunoreactive (Fig. 5E) and many PAS-positive (Fig. 5F) spots were present. In higher magnification of the nigral region shown by an arrowhead on Fig. 5E or Fig. 5F, immunoreactive granule-containing (Fig. 5G) and PAS-positive corpora (Fig. 5H) were seen. Many immunoreactive (Fig. 6A & 6E) and PAS-variable (Fig. 6B) spots were seen in PD patient 2. In higher magnification of the nigral region shown by an arrowhead on Fig. 6A, 6E, 6B or 6F, immunoreactive (Fig. 6C & 6G) or PAS-positive (Fig. 6D) granule-containing corpora were observed. Faintly PAS-positive corpora were present (Fig. 6H). Several corpora were likely
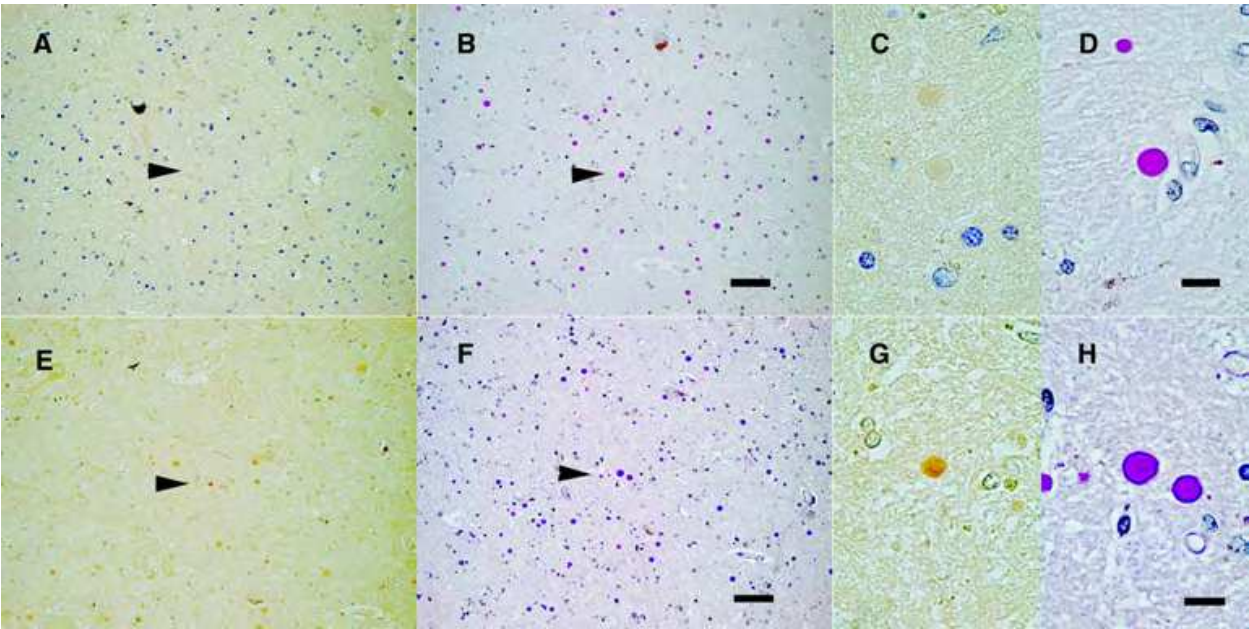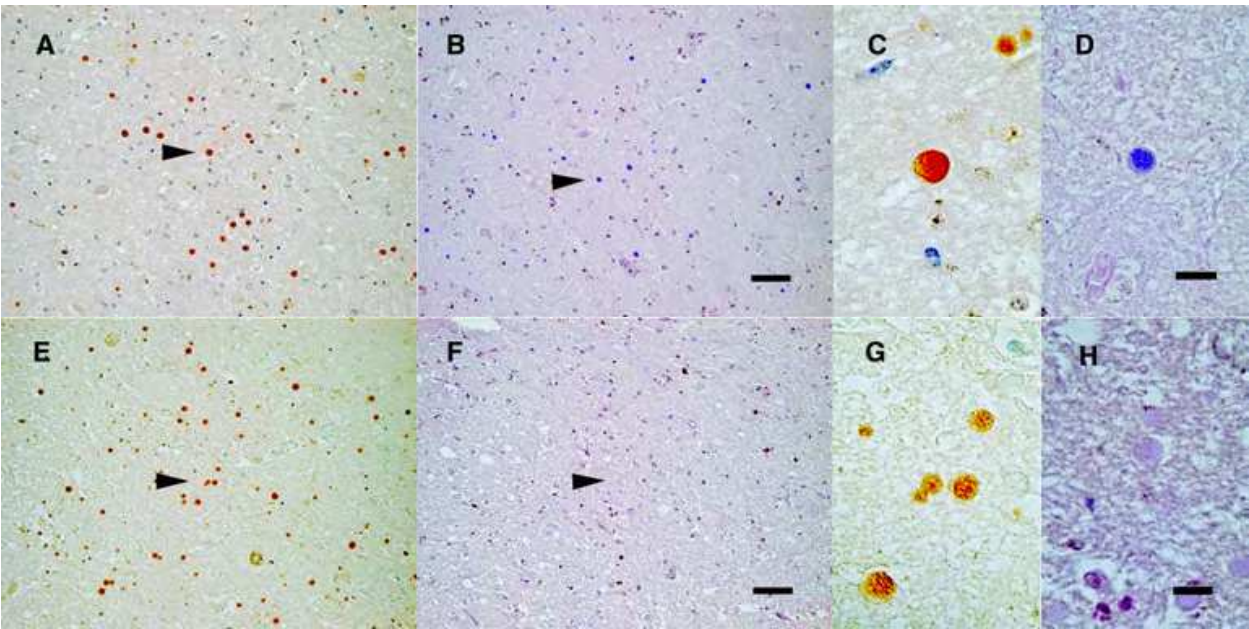
Fig. 5. Light micrographs of the VM pars compacta of the substantia nigra in PD patient 1. Immunostained sections (A, C, E, & G). PAS-stained sections (B, D, F, & H).   Panels A, B, E, and F are the same magnification (bar = 50 µm). Panels C, D, G, and H are the same magnification (bar = 10 µm). The section composed of panels A through D is 120 µm-distant from other section of panels E through H in paraffin sections.

Fig. 6. Light micrographs of the VM pars compacta of the substantia nigra in PD patient 2. Immunostained sections (A, C, E & G). PAS-stained sections (B, D, F, & H). Panel A, B, E, and F are the same magnification (Bar = 50 µm). Panel C, D, G, and H are the same magnification (bar = 10 µm). The section composed of panels A through D is 285 m-distant from other section of panels E through H in paraffin sections.
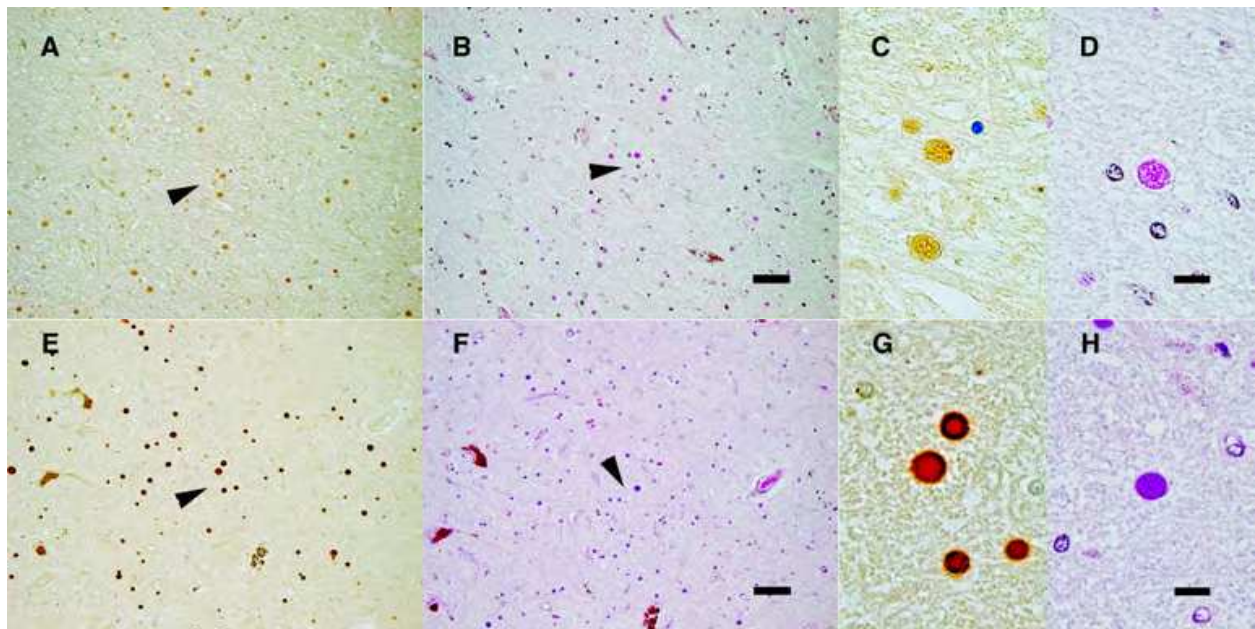
Fig. 7. Light micrographs of the VM pars compacta of the substantia nigra in PD patients 4 and 5. Immunostained sections of PD patients 4 (A & C) and 5 (E & G). PAS-stained sections of PD4 (B & D) and PD 5 (F & H). Panels A, B, E, and F are the same magnification (Bar = 50 µm). Panels C, D, G, and H are the same magnification (bar = 10 µm).

composed of immunoreactive granules. Many immunoreactive (Fig. 7A) and PAS-positive (Fig. 7B) spots were seen in PD patient 4. In higher magnification of the nigral region shown by an arrowhead on Figs. 7A or 7B, immunoreactive and PAS-positive granule-containing corpora were observed (Figs. 7 C & 7D). The same corpora were present in the VM compacta of PD patient 3. Many immunoreactive (Fig. 7E) and PAS-positive (Fig. 7F) spots were seen. In higher magnification of the nigral region shown by arrowhead on Fig, 7E or Fig. 7F, immunoreactive and PAS-positive corpora were observed (Figs. 7G & 7H). The same corpora were present in PD patient 6. A few immunoreactive spots were seen (Fig. 8A), but PAS-positive spots were not evident (Fig. 8B). In higher magnification of the nigral region shown by an arrowhead on Fig. 8A or Fig. 8B, immunoreactive (Fig. 8C) or pink-colored (Fig. 8D) corpora were present in control patient 1. In control patient 2, several immunoreactive (Fig. 8E) and PAS-positive (Fig. 8F) spots were seen. In higher magnification of the nigral region shown by an arrowhead on Fig. 8E or Fig. 8F, immunoreactive granule-containing (Fig. 8G) and pink-colored (Fig. 8H) corpora were observed. The same corpora were seen sparsely present in the substantia nigra of control patients 3 and 4.

3.6 PAS-positive corpora and gelatinous mass harboring acid-fast or AO-positive granules in the midbrain of PD patient 1

Many PAS-positive granule-bearing neuroglia (arrows on Fig. 9A), clusters of PAS-positive granules (Fig. 9B), PAS-positive large corpora (Fig. 9C), and acid-fast granule-containing gelatinous masses (Fig. 9D) were present in the VL pars compacta. When stained with AO, many fluorescent granules variable in size were seen within corpora (Fig. 9E). Near an autofluorescent granule-bearing neuron (an arrow in Fig. 9F), gelatinous masses were not autofluorescent.   When stained with AO they fluoresced brilliantly nearby lipofuscin-bearing neuron (an arrow in Fig. 9G) and were found to be composed of fluorescent granules of similar size at higher magnification (Fig. 9H).
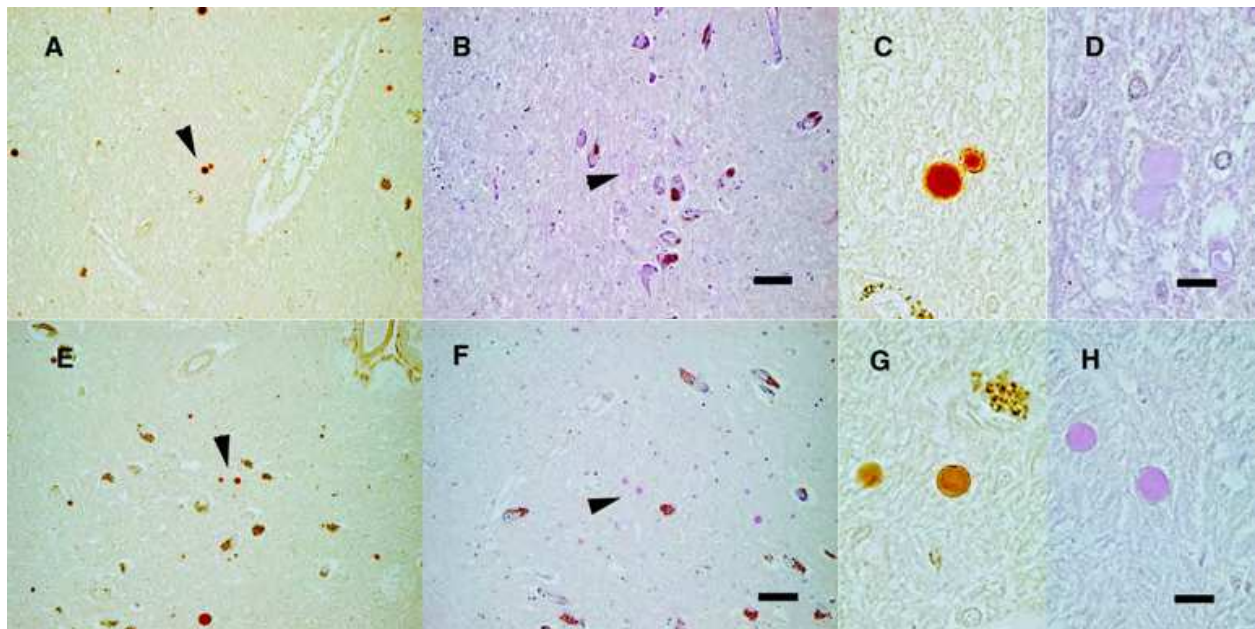
Fig. 8. Light micrographs of the VM pars compacta of the substantia nigra in control patients C1 and C2. Immunostained sections of control patients C1 (A & C) and C2 (E & G). PAS-stained sections of C1 (B & D) and C2 (F & H). Panels A, B, E, and F are the same magnification (Bar = 50 µm). Panels C, D, G, and H are the same magnification (bar = 10 µm).

3.7 Neurons connected to AO-positive gelatinous masses and degenerative neurons in the pars compacta of PD patient 1

The slide sections following that shown in Fig. 9G revealed gelatinous masses connected to the same lipofuscin-bearing neurons (arrowheads on Figs. 10A & 10B). Brilliantly yellow-fluorescent neuron-like bodies were present nearby several melanin-pigmented neurons (Fig. 10C). In H&E-stained sections, they appeared as invisible ghost cells (an arrow on Fig. 10D). Under higher magnification of the VL pars compacta shown by an arrow on Fig. 10D, they were observed as gelatinous masses containing many granules (Fig. 10E).

3.8 AO- and DAPI-positive granule-containing gelatinous masses and immunoreactive corpora connected with neurites in PD patients 2, 3, and 5

AO- and DAPI-positive granules were localized within gelatinous masses in the same section of PD patient 2 (Figs. 11A and 11B). In PD patient 5, gelatinous masses containing AO- and DAPI-positive granules were also observed. Immunoreactive corpora (Fig. 11C) were observed to be connected with neurites containing PAS-positive granules on the next section (an arrowhead on Fig. 11D). Immunoreactive corpora were composed of a deformed portion (an arrowhead on Fig. 11E) where PAS-positive granule-bearing neurites connected on the next section (an arrowhead on Fig. 11F).

4. Discussion

4.1 Isolation study of the nigral tissue samples

Gram-negative, immunoreactive, and PAS-positive granular cells were observed in broth cultures of PD patient 1 filtrates. Gram-negative and immunoreactive granular cells were
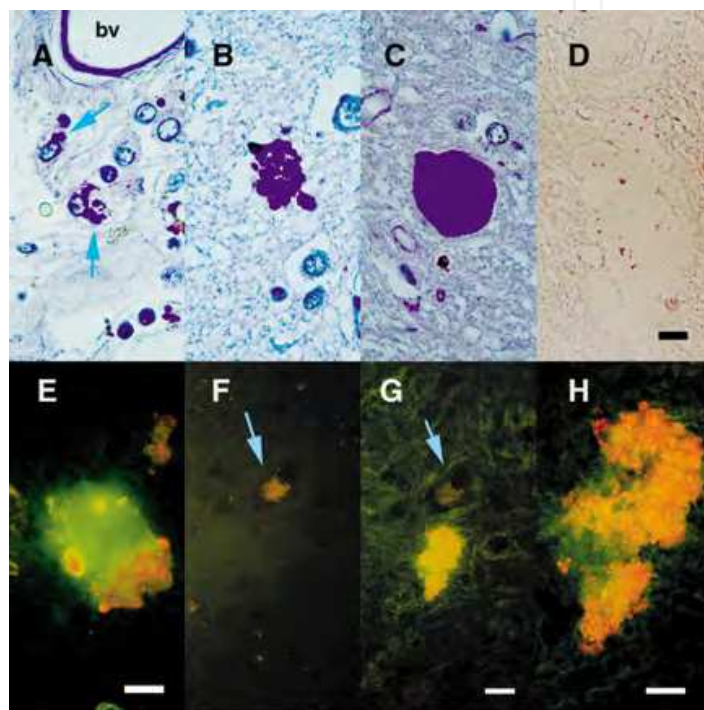
Fig. 9. Light micrographs of the VL and VM pars compacta and the central gray area of PD patient 1. PAS-stained sections (A, B, and C) and acid fast-stained section (D) of the VL pars compacta. Panels A through D are the same magnification (Bar = 10 µm). Acridine orange-stained sections from the central gray area under ultraviolet light (E). Bar = 10 µm. Unstained (F) and acridine orange-stained (G) sections from the VM pars compacta under ultraviolet light. Panels F and G are the same magnification (bar = 25 µm). Acridine orange-stained section from the VM pars compacta under ultraviolet light (H). Bar = 10 µm. bv; blood vessel.
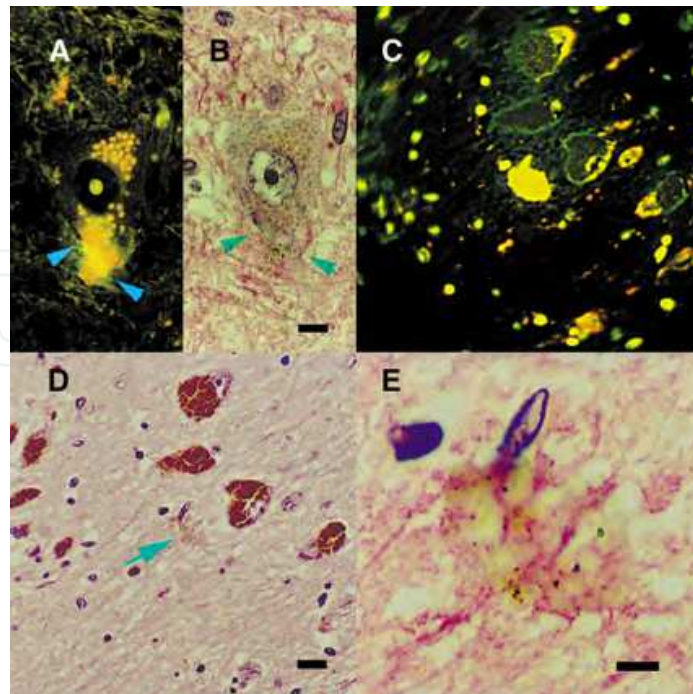
Fig. 10. Light micrographs of the different VM and VL pars compacta of PD patient 1. Acridine orange-stained (A) under ultraviolet light and H&E-stained (B) sections from the VM pars compacta. Panels A & B are the same magnification (Bar = 10 μm). Acridine orange-stained section under ultraviolet light (C) and H&E-stained (D) section from the pars compacta. Panels C and D are the same magnification (bar = 25 μm). H&E-stained section (E) from the pars compacta. Bar = 10 μm.
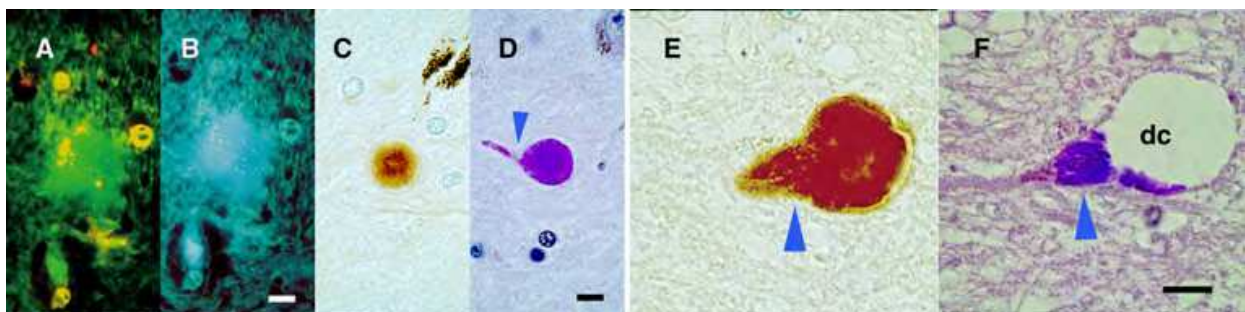

Fig. 11. Micrographs of the VM and VL pars compacta of PD patients 2, 3, and 5. Acridine orange-stained (A) and DAPI-stained (B) sections of the VM pars compacta of PD2 under ultraviolet light. Panels A and B are the same magnification (bar = 10 μm). Immunostained (C) and PAS-stained (D) sections of the VM pars compacta in PD3. Panels C and D are the same magnification (bar = 10 μm). Immunostained (E) and PAS-stained (F) sections of the VL pars compacta in PD5. Panels E and F are the same magnification (bar = 10 μm). dc; detached corpora.

also observed in that of PD 4 filtrates. Both of the growth were enhanced by erythrocyte lysates supplemented. The two filterable organisms shared morphological, immunological, and physiological features with filterable nocardiae. One may postulate **first** that filterable nocardiae are likely born inside the cyst of *Nocardia* under aerated conditions and released into an environmental wind to colonize a preferable unknown site of infection (Kohbata, 1998; Kohbata et al., 2009). Filterable nocardiae may cause air-borne infection, localize within an as-yet unknown primary niche, and occupy erythrocytes as their preferred niche. Via erythrocyte, filterable nocardiae might infect other hosts horizontally or vertically. **Second**, intraerythrocytic pathogen may seed astroglia. After an invasion of astroglia, filterable nocardiae might produce cysts and preferentially multiply in the midbrain nigral tissues of PD patients, by lacking a cylindrical tubule formation. The primer, amplifying the 16S rDNA of *Nocardia*, was not effective in the detection of 16S rDNA of filterable organisms. The first trial aiming to detect of 16S rRNA, specific to gram-positive filamentous nocardiae (i.e., so-called *Nocardia*), in Lewy body-containing brain specimens did not attain satisfactory outcomes (Chapman et al., 2003; Lu et al., 2005). Filterable nocardiae might happen to be undergoing degeneration inside a new pathogenic niche.

4.2 Distribution and localization in control or PD patients

Immunoreactive and PAS-positive corpora were present in the substantia nigra of control patients. The observed corpora's morphological features are identical to those of corpora amylacea (i.e., astroglial PAS-positive inclusions). A literature reveals that corpora amylacea become microscopically detectable in the central nervous system in the first decade of life. After the age of 50, corpora amylacea increase numerous in the central nervous system (Cavanagh, 1999). Seropositivity for *Nocardia* is frequent in healthy aged as well as young subjects (Hubble et al., 1995, Kohbata & Shimokawa, 1993). Corpora amylacea are likely to be filterable nocardiae in origin. Filterable nocardiae form PAS-positive cysts at the tips of cylindrical tubules in the presence of erythrocyte lysates. Many corpora, morphologically & immunologically identical to filterable nocardiae cysts, were densely present in the substantia nigra of PD patients when compared with that of control patients. Several corpora were present in the VL pars compacta of PD patients. Gelatinous masses containing granules were connected with midbrain neurons (Figs. 10A & 10B) and present in perikarya (Fig. 4G). Immunoreactive and PAS-positive granules were present in neurites (Figs. 11C, 11D, 11E, & 11F) or perikarya (Figs. 4A & 4B). Filterable nocardiae may multiply inside astroglia, where they might invade neurons through astroglial-neuronal synapses. Immunoreactive granules were deposited in neuronal cytoplasm (Figs. 4E & 4F) as well as in various stages of Lewy body (Figs. 3E, 3F, 3G, & 3H). The gelatinous masses containing granules occupied ghost neurons (Fig. 10E). Filterable nocardiae may be involved not only in the neuronal loss but also in the Lewy body formation. Spread of Lewy bodies from the brainstem through midbrain and basal forebrain to the cerebral cortex might result from an invasion by filterable nocardiae through blood stream.

4.3 Host response to their presence

A comparison with the VL pars compacta of age-matched controls reveals that numerous melanin-pigmented neurons were absent from PD patients. The corpora in the VM pars compacta of PD patient 1 were faintly to weakly immunoreactive when compared with not only that of other PD patients but also that of control patients. In the early stage of PD, many

dense clusters of acid-fast lipochrome bodies were observed. Many doughnut-like acid-fast pathogens are present on the surface or the inside of the mouse midbrain one week following the onset of movement disorder (Kohbata et al., 2009). Filterable nocardiae likely invade the midbrain substantia nigra, probably the VL pars compacta, on a massive scale from the disease onset to the early stage. Microglia, only protecting neuroglia against infection, may become activated to gather at and rapidly attack the site of infection (Verkhratsky & Butt, 2007). Faintly immunoreactive spots (Figs. 5A & 5E) may be subject to the host immune response. Filterable nocardiae's invasion and the host immune response might result in a severe loss of the VL pars compacta dopaminergic neurons, which could ensue and lead to the onset of disease. The substantia nigra of PD patients is likely to be under activated microglia in comparison to that of control patients (McGeer et al., 1998; Mirza et al., 2000). Filterable organisms, isolated from early stage PD patient 1, were PAS-positive and immunoreactive. The corpora were intensely PAS-positive and faintly immunoreactive. From the broth cultures of PAS-negative filterable organisms PD 4, sufficient amounts of genomic DNAs were obtained. Filterable organisms PD 1, under stressed conditions, were PAS-positive. The PAS-positive thick layers may inhibit yields of the genomic DNAs by using the extraction buffer.

4.4 Host intracellular digestive system

Immunoreactive and PAS-positive corpora, of which size was similar in the VM pars compacta, were different in their shape and size in the VL pars compacta. An electron microscopic study of AC-positive corpora, shown in Fig. 9E and measured as ca. 30 µm in diameter, revealed several wall-free prokaryotes measured as ca. 0.5 µm in diameter (Kohbata et al., 2005). Filterable nocardiae may penetrate perivascular astroglia, multiply to be clusters of granules with 1.0 µm in diameter and form large corpora of which diameter was 25 µm (Fig. 9C). The neuronal cytoplasm was occupied with numerous immunoreactive granules (Figs. 4E and 4F), granule-containing (Fig. 4G), and AC-positive gelatinous masses (Fig. 10E). Filterable nocardiae are not likely to be trapped into heterophagic vacuoles fused with lysosomes to be degraded, suggestive of intracellular digestive system not functional. Mutations in the glucocerebrosidase genes encoding lysosomal enzymes emerge as strong genetic determinants predisposing people to PD (Aharon-Peretz et al., 2004). Filterable organisms may be not degraded by lysosomal enzymes in astroglia as well as neurons. Corpora amylacea might be an infectious focus in the PD midbrains. Intraneuronal PAS-positive inclusions (i.e., Lafora bodies), resemble or identical with corpora amylacea, are pathologically hallmark of Lafora disease (Minassian, 2001). The digestive system of astroglia as well as neurons may be not functional by the genetic lesions including impaired autophagy and mutations in the lysosomal protein glucohydrolase (Aguado et al., 2010; Minassian el al., 2000). Lafora bodies are distributed throughout the central nervous system (Schwarz & Yanoff, 1965). Intraneuronal PAS-positive inclusions, shown in Figs. 4B & 4C, partially resemble Lafora bodies (Namba, 1968). Filterable nocardiae could potentially invade the substantia nigra, dentate nucleus, thalamus, globus pallidus, and the 3rd & 4th layers of the cerebral cortex.

4.5 Adaptation of filterable nocardiae

An intraerythrocytic pathogen may seed astroglia to form corpora amylacea through the blood-brain barrier. Ever since the first decade of life, filterable nocardiae might infect the central nervous system of normal subjects, but rapidly to be eliminated. PAS-positive

granules were present within perivascular neuroglia (arrowheads on Fig. 9A). Gelatinous masses containing many acid-fast granules were present in the VL pars compacta (Fig. 9D). Acid-fast lipochrome bodies are abundantly present around blood vessels in the early stages of infection, but not in later (Kohbata et al., 1998). PAS-positive filterable nocardiae, present during all stages of PD, are likely to adapt to survive within midbrain astroglia as well as neurons. Dust-like DAPI-positive granules were present within gelatinous masses (Fig. 11B). The filterable nocardiae, as intracellular parasites, were likely to adapt and survive as PAS-, AO-, DAPI-positive, or immunoreactive small granules inside both astroglia and neurons in PD patients. DAPI staining is used for the detection of AT-rich endosymbiotic bacteria (Sun et al., 2009). *Nocardia*, closely related to *Mycobacterium*, belong to the high-G+C subdivision of gram-positive eubacteria (Woese, 1987). The leprosy-causing pathogen *Mycobacterium leprae* remains uncultivatable on artificial medium and shows a high predilection for neuroglia of the peripheral nervous system. *M. leprae* has undergone major deletions yielding a smaller genome size and lower G+C contents (Cole et al., 2001). Eight fragment sequences of filterable organisms were analyzed to be AT-rich with lower G+C contents. Most fragment contained several A/T homopolymers. The shift toward high A+T content that is common in host-restricted symbiotic bacteria leads to increased occurrence of A/T homopolymers (Moran et al., 2009). An intracellular lifestyle inside erythrocytes, astroglia, or neurons may lead to the genomic DNA degeneration by the retention of the morphological, immunological, and physiological features under host-restricted conditions. It was possible to isolate filterable organisms present in the nigral lesions for investigation of their morphological, immunological, physiological, and genomic DNA features. This will facilitate challenge and isolation studies of filterable organisms which may be a cause of PD.

5. Conclusion

An isolation study of filterable nocardiae from several frozen nigral tissue samples of PD patients was performed. A preferential site of their growth in the midbrain of six PD patients was histochemically and immunohistochemically assessed. Filterable organisms isolated from two PD patients shared the same morphological, antigenic, and physiological features with filterable nocardiae. PCR-mediated identification was not successful. The partial genomic DNAs were AT-rich with a G+C content of 41 %, containing several A/T homopolymers. Neuronal vacuoles, ghost, and shrunken nigral neurons occupied by immunoreactive, PAS-positive granules, or granule-containing gelatinous masses were present in patients with early stages of PD. Immunoreactive and PAS-positive corpora were densely distributed throughout the VM pars compacta of the substantia nigra in six PD patients. Gelatinous masses or immunoreactive corpora likely connected with midbrain neurons were observed. The results suggest that filterable organisms isolated from the nigral tissue samples likely originate from the degeneration of filterable nocardiae. Filterable nocardiae may multiply within astroglia, through which they might invade midbrain neurons, and could play a significant role in both neuronal loss and Lewy body formation.

6. Acknowledgements

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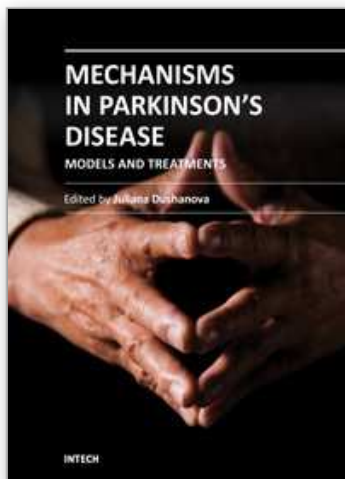
Mechanisms in Parkinson's Disease - Models and Treatments

Edited by Dr. Juliana Dushanova

Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

How to reference

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Shunro Kohbata, Ryoichi Hayashi, Tomokazu Tamura and Chitoshi Kadoya (2012). Filterable Forms of Nocardia: An Infectious Focus in the Parkinsonian Midbrains, Mechanisms in Parkinson's Disease - Models and Treatments, Dr. Juliana Dushanova (Ed.), ISBN: 978-953-307-876-2, InTech, Available from: http://www.intechopen.com/books/mechanisms-in-parkinson-s-disease-models-and-treatments/filterable-forms-of-nocardia-an-infectious-focus-in-the-parkinsonian-midbrains

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