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Lewy bodies in parkinsonism share components with intraneuronal protein bodies of normal brains

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Summary. Histochemical characteristics of the Lewy bodies, in catecholamine neurons of 10 Parkinsonian patients, were compared to those of the spherical protein bodies, the basic protein-rich markers of catecholamine neurons in man. Special methods for proteins and lipids showed that the core of the Lewy bodies, in the neurons of the locus coeruleus and the substantia nigra, contains basic proteins and lipids normally found in the protein bodies. Acid fuchsin and the lipid-soluble fluorescent dye rhodamine B stained the entire core of the Lewy body in the parkinsonian brains and the entire sphere of the protein body in the control brains. Bromsulfophthalein, another acidic dye, which selectively binds to the enzyme gluthathione-S-transferase, had affinity only for a ring-like lamina at the outer layer of the core of the Lewy body and for the outer rim of the protein body. These results demonstrate that Lewy bodies and protein bodies contain similar macromolecular components, that is lipids and two different types of proteins, which also show similar stratification in the two structures. On the other hand, the presence in several neurons of the Parkinsonian patients, of aggregates representing transitional forms between protein bodies and Lewy bodies, indicates that abnormalities of protein bodies precede, and are somehow linked to Lewy body production.

Keywords: Human brain, catecholamine neurons, acidophilic protein bodies, Parkinsonism, Lewy bodies, rhodamine B, bromsulfophthalein.

Introduction

One of the major and unique morphological abnormalities found in the nervous system of individuals with Parkinson's disease is the Lewy body. These eosinophilic inclusions are situated in the perikarya and dendritic processes of nigral as well as other monoaminergic neurons. They have been the subject of intense

investigations as to their origin and composition and considerable speculation exists as to their role in the pathogenesis of Parkinson's disease.

Numerous studies have probed the nature and ultrastructure of Lewy bodies (Duffy and Tennyson, 1965; den Hartog Jager, 1969; Issidorides et al., 1978; Kosaka, 1978; Goldman et al., 1983; Nakashima and Ikuta, 1984; Hirsch et al., 1985; Kahn et al., 1985; Pappola, 1986; Forno et al., 1986; Galloway et al., 1988; Kuzuhara et al., 1988; Bancher et al., 1989) and have demonstrated the presence of basic proteins, phospholipids, tyrosine hydroxylase, neurofilament epitopes and ubiquitin. All these components are differentially partitioned between the electron dense core, its surrounding laminae and the peripheral radiating fibers. Despite these findings the question of the mode of formation of the Lewy bodies remains unanswered. Data pertaining to the gradual emergence of Lewy bodies from existing cellular elements could greatly contribute to the understanding of the pathogenesis of Parkinson's disease.

For the study of cellular abnormalities, which may precede the formation of Lewy bodies, we investigated brains from a group of Parkinsonian patients with overt symptoms of long duration and compared them to matched controls (Issidorides et al., 1978). With the use of phosphotungstic acid haematoxylin (PTAH; Mallory, 1900; Terner, 1966), an acidic dye selective for mitochondrial basic proteins, we found that the substantia nigra zona compacta neurons of the controls displayed abundant spherical acidophilic inclusions in their perikarya and dendrites. In the Parkinsonian brains these inclusions were noticeably reduced or missing. The absence of the spherical inclusions, rich in histone-like basic proteins (Issidorides et al., 1978), was often the only cellular abnormality noticed in otherwise intact neurons of the zona compacta of these specimens. Interestingly, the cores of the Lewy bodies, when present, gave the same PTAH-positive reaction as did the inclusions – termed protein bodies – of the normal neurons. The findings suggested a sharing of macromolecular components between protein bodies and Lewy bodies and raised the question whether the decline of the former was in some way linked to the emergence of the latter in Parkinsonism. The present study is an effort to answer this question and to test the putative metabolic association of protein bodies and Lewy bodies.

Materials and methods

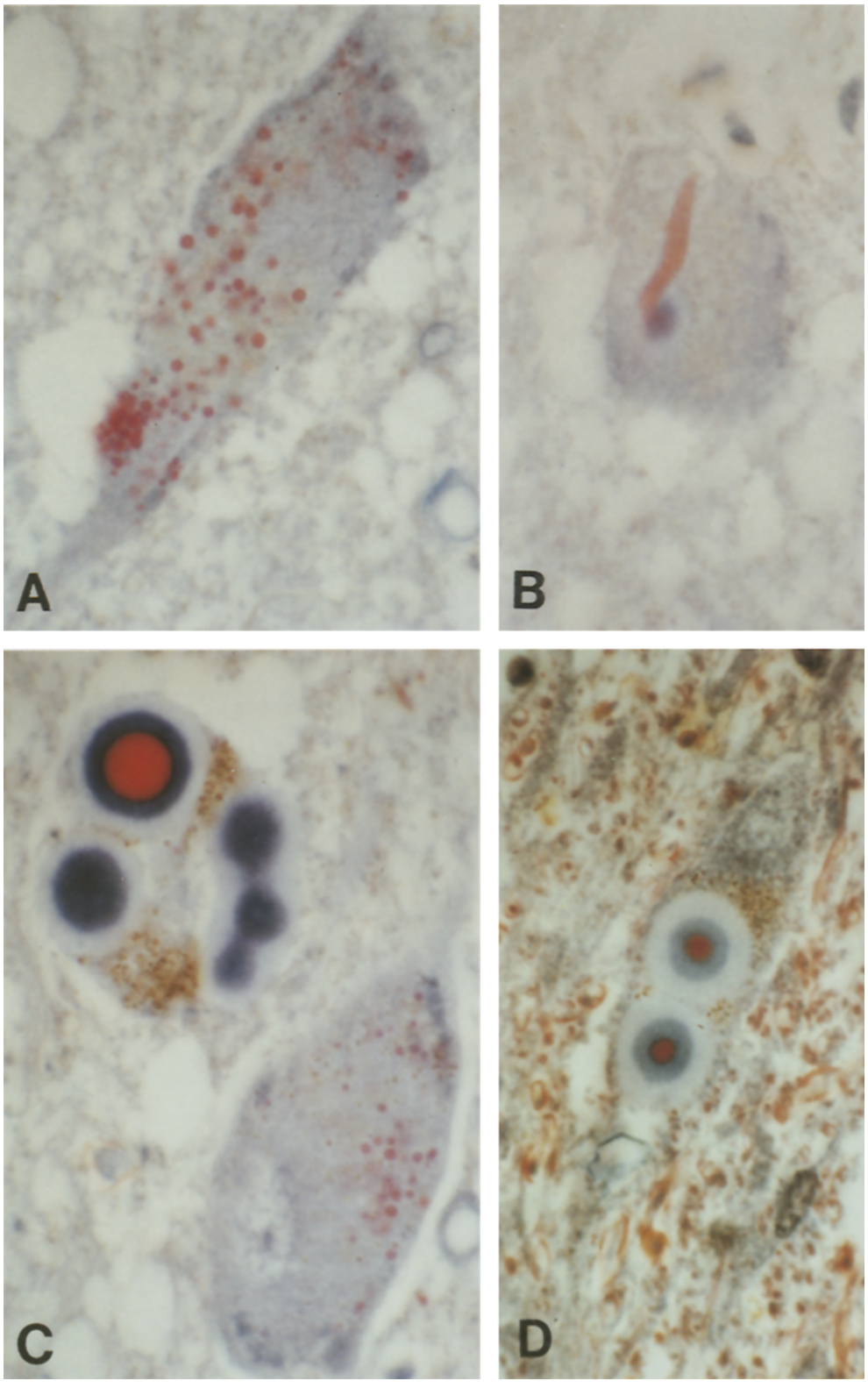
Brains obtained at autopsy and fixed in 10% formalin were provided by the Brain Bank of the Clinical Research Center for Parkinsonism in the Department of Neurology of the Mount Sinai School of Medicine, New York City. The clinical sample included 10 patients with idiopathic Parkinson's disease (ages 57 to 81 years) and five normal controls, free of neurological or psychiatric illness (ages 59 to 76 years). Segments of the pons and midbrain, containing the locus coerulus and the substantia nigra respectively, were dissected out, embedded in paraffin and sectioned at 5 μm. Paired sections of control and parkinsonian tissue were stained simultaneously with each of the methods described below. Mallory's trichrome (Mallory, 1900) was used for the demonstration of acidophilic proteins having different affinities for acid fuchsin, aniline blue and orange G. Liisberg's rhodamine B procedure (Liisberg, 1968) was used for the localization of lipids (Boerner, 1952) of the

class involved in cornification (Clausen and Dabelsteen, 1969). These preparations were studied under fluorescence using epi-illumination and a Leitz filter block N 2.1 for rhodamine B fluorescence. The histochemical application of bromsulfophthalein (BSP), according to Silverman and Glick (1966), was used for the localization of proteins, with which BSP binds stoichiometrically and forms an insoluble blue-purple complex (Nayyar and Glick, 1954). Before staining with BSP, melanin was removed from some of the sections with Gomori's oxidation (Gomori, 1939), which involved treatment with acidified potassium permanganate ($KMnO_4$) for 3 minutes and bleaching with oxalic acid for 1 minute.

Results

Mallory's trichrome method is well suited to the study of protein bodies since the latter, being strongly fuchsinophilic, stand out as bright red globules of various sizes in a pale blue cytoplasm (Fig. 1 A, C). In all of the control brains studied, protein bodies were present in neuromelanin-containing neurons of the substantia nigra and the locus coeruleus, as well as in lipofuscin-containing serotonin neurons of the raphe nuclei. They were scattered in the perikarya and often clustered at the base of a main dendrite (Fig. 1 A). In the parkinsonian brains the catecholamine neurons were depleted of protein bodies, had few melanin granules, and contained Lewy bodies at various stages of formation. Figure 1B shows a neuron of the locus coeruleus which displayed an immature Lewy body, i.e. a small aniline blue-positive mass surrounded by a pale halo. No melanin granules or distinct protein bodies were evident in this neuron. However, a brightly fuchsinophilic tubular structure was connected to the middle of the blue mass (Fig. 1 B). Detailed observation under oil immersion revealed that this structure consisted of closely packed, pressed together and/or fused protein bodies. At the other end of the spectrum, neurons were observed containing up to five Lewy bodies and a fair amount of brown melanin granules in the perikaryon (Fig. 1 C). The largest Lewy body in this neuron displayed a bright red fuchsinophilic core, sharply demarcated from the well circumscribed aniline blue-positive layer, that separated the core from the peripheral clear halo. The smaller diameters and the blue centers of the remaining four Lewy bodies were suggesting that these were either immature or cut tangentially through their blue layers. The second neuron in Fig. 1 C appeared fairly intact except for the absence of melanin and the reduced number of protein bodies. This partly "lesioned" state is representative of a great many neuronal perikarya which were encountered in the locus coeruleus of the parkinsonian brains. In the substantia nigra, twin Lewy bodies were found crowded in the cytoplasm of smaller neurons, in the midst of melanin granules (Fig. 1 D). They displayed the same sharp demarcation between fuchsinophilic center, aniline blue-positive layer and clear halo. In all brain samples examined, stained with Mallory's trichrome procedure, protein bodies and the inner core of the Lewy bodies, invariably, showed identical reactivity.

After the application of Liisberg's rhodamine B method, the protein bodies in the catecholamine neurons of the control brains were selectively fluorochromed and emitted a bright yellow fluorescence, while melanin remained


A
B
C
D

invisible (Fig. 2 A). Liisberg's procedure includes toluidine blue, a basic dye, by means of which a typical Nissl image of the brain tissue is obtained in bright field (unpublished observations). It was, thus, possible to evaluate the degree of melanization and to identifiy the anatomical site of the neurons studied under fluorescence. The massive loss of protein bodies from the catecholamine neurons of the parkinsonian brains was quite easy to detect with this double observations procedure, even when the cell was filled with melanin. Most neurons contained only a small irreglular cluster of fine protein bodies at the base of a main dendrite (Fig. 2 B), while their cell body contained none at all. Occasionally, unusual fluorescing formations were observed. In some cases, these formations consisted of large protein bodies aggregated (fused?) in regular triplet rosettes, which were lined up in a row (Fig. 2 C). In other cases, fluorescing tubular structures, similar to the fuchsinophilic formation of Fig. 1 B, were present in neurons lacking distinct protein bodies. Typical Lewy bodies, sectioned equatorially, displayed a brightly fluorescing central core surrounded by a dull layer (Fig. 2 D). In all specimens, the fluorescence of the core was as bright as that of the normal protein bodies in the control neurons (Fig. 2 A) and that of the fused protein-body-formations in the parkinsonian neurons (Fig. 2 C). Lewy bodies of small diameters did not fluoresce. They were probably cut tangentially through their dull layers.

The localization of the blue staining reaction with BSP in our material was most unusual. This particular acidic dye had affinity for only the outer rim of the protein bodies (Fig. 3 A, arrows) leaving their central part completely unstained. In the Lewy bodies the staining reaction was similarly localized to a ring-like lamina congruent with the outer edge of the core (Fig. 3 B–E). Although Lewy bodies varied in size in the different sites, they presented a uniform stratification after BSP staining in all specimens examined; namely, a pale core encircled by the ring-like BSP-positive lamina, surrounded by a wide, sharply demarcated pale layer and limited by a clear halo (Fig. 3 D). When the neuromelanin was bleached before staining the slide with BSP, it was observed that the "clear" halo at the periphery of the Lewy body was traversed by radially

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Fig. 1. Photomicrographs of neurons of the human brain stem at the levels of the locus coeruleus (**A–C**) and the substantia nigra (**D**). Mallory's trichrome method, × 1,400. (**A**) raphe pontis neuron in control brain showing spherical fuchsinophilic protein bodies scattered in the cytoplasm and clustered at the base of a dendrite. (**B**) demelanized neuron in the locus coerulus of parkinsonian patient, showing early stage in the formation of a Lewy body: a small blue mass with a clear halo is attached to a tubular fuchsinophilic structure. (**C**) locus coeruleus neuron with multiple Lewy bodies showing distinct concentric layers. Largest Lewy body, cut through its equatorial plane, displays red fuchsionophilic core, aniline blue layer, and compact clear halo displacing the melanin granules to its periphery. Note that the second neuron in (C) is completely demelanized, but appears intact and contains protein bodies. (**D**) area compacta neuron in the substantia nigra displaying identical twin Lewy bodies with red cores, blue layers, and unreactive peripheral halos, sharply demarcated from each other

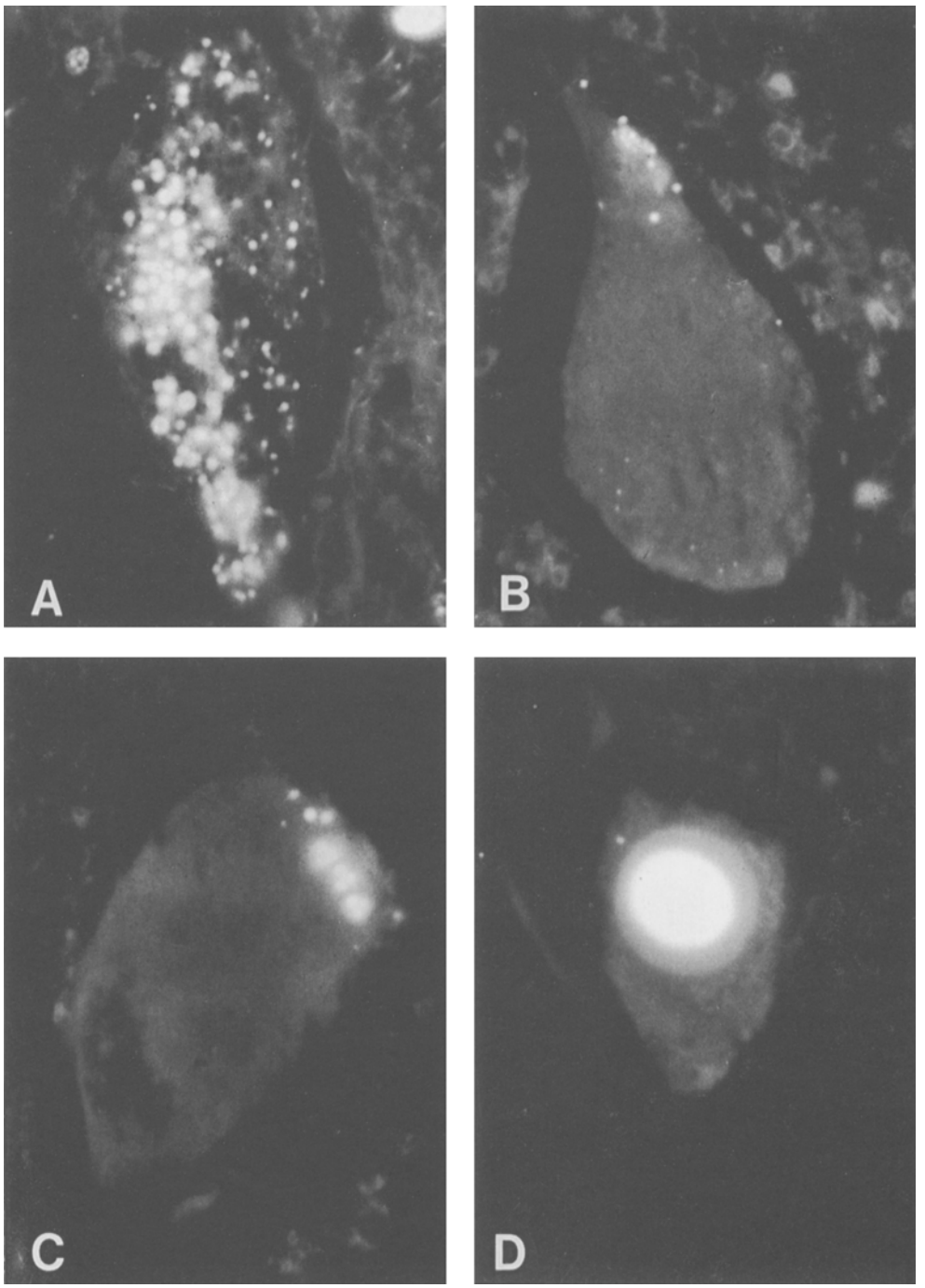

Fig. 2. Fluorescence photomicrographs of catecholamine neurons in the locus coeruleus of control (**A**) and parkinsonian (**B–D**) brains. Toluidine blue – rhodamine B stain, × 1,600. (**A**) Normal concentration of large and small protein bodies in locus coeruleus neuron of control brain. (**B**) depletion of protein bodies from the perikaryon with few small ones remaining at the base of a dendrite. (**C**) depletion of protein bodies from the perikaryon with few large ones fused into triplet rosette groupings. (**D**) large Lewy body with brightyl fluorescing core in shrunken neuron of locus coeruleus

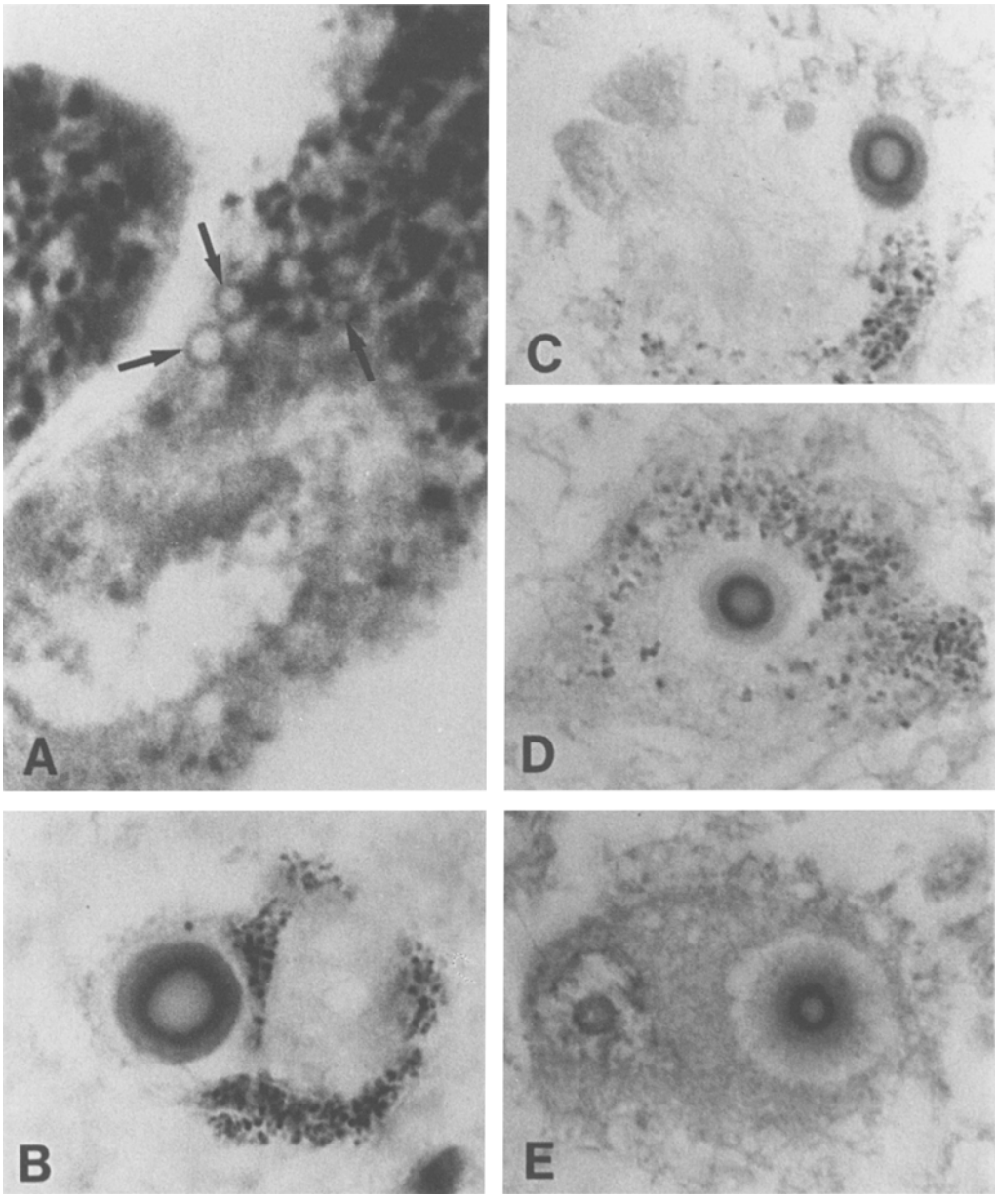

Fig. 3. Photomicrographs of catecholamine neurons in the locus coeruleus of control (**A**) and parkinsonian (**B–E**) brains. Bromsulfophthalein method. Magnifications: (A) = ×2,730, (B–E) = ×1,600. (**A**) protein bodies display a BSP-positive outer rim (arrows) and a clear unstained core. (**B–D**) Lewy bodies display a ring-like BSP-positive lamina between clear central core and pale layer. Note wide unreactive halos around all three Lewy bodies (**B, C, D**). (**E**) neuron treated with acidified $KMnO_4$ before staining with bromsulfophthalein. Note emergence of radially arranged fibers traversing formerly unreactive halo of the Lewy body, and bleaching of melanin granules

oriented pale fibers which emerged from the pale layer and merged with fibrous networks faintly visible now in the cytoplasm (Fig. 3 E).

The results from the application of the BSP staining procedure not only illustrate further the sharing of histochemical components by protein bodies and the core of Lewy bodies, but indicate that the stratification of these protein components follows the same pattern in the two structures: a positive reaction at the rim of the protein body is reflected by the positive reaction at the outer ring-like lamina of the core of the Lewy body.

Discussion

The results obtained indicate that a close relationship exists between protein bodies and the formation of Lewy bodies in idiopathic Parkinsonism. This indication is based upon the identical histochemical reactions of the two structures with a series of stains and, most importantly, upon the existence in some neurons of intracytoplasmic structures that represent transitional forms between protein bodies and Lewy bodies. With the three methods used, i.e. Mallory's (1900) trichrome, Liisberg's (1969) rhodamine B, and Silverman and Glick's (1966) bromsulfophthalein, which addressed the demonstration of different macromolecular components, positive reactions are obtained in the protein body and in the core of the Lewy body. With Mallory's trichrome the sharply demarcated fuchsinophilia of the core justifies the term "acidophilic structure" introduced by Lewy (1912) and replicates the fuchsinophilia of the protein bodies. This fuchsinophilia is due to the presence of histone-like protein structures as suggested in an earlier study (Issidorides et al., 1978). The presence of histone-like proteins in the Lewy body core was confirmed by the positive reaction with mercuric bromphenol blue (Ikeda et al., 1978; Kosaka, 1978; Kosaka et al., 1976) and with the Sakaguchi reaction (Kosaka, 1978; Kosaka et al., 1976) which is the chemical test for arginine. These data correlate well with our results which showed that protein bodies give a positive reaction with mercuric bromphenol blue, which demonstrates histones, as well as a strong fluorescence with phenanthrenequinone (Panayotacopoulou and Issidorides, 1984) which is another chemical test for arginine (Magun and Kelly, 1969). The layer of the Lewy body, sharply demarcated with aniline blue after Mallory's trichrome, has no visible representation in the protein bodies. This layer together with the outer clear halo of the Lewy bodies in locus coeruleus and substantia nigra represent distinct structural macromolecular entities that correlate well with the lamine of alternating densities shown in phase constrast micrographs of Lewy bodies by Duffy and Tennyson (1965).

Rhodamine B, a slightly basic fluorochrome dye with high lipid solubility, has been used to demonstrate lipids by fluorescence microscopy (Boerner, 1952). It has also been reported to be a specific stain for cornification sites in both normal and pathological tissues; its staining affinity is thought to be due to lipids associated specifically with cornified tissues (Clausen and Dabelsteen,

1969). Extended studies have shown that sphingolipids and free sterols constitute the lipid matrix that enmeshes keratin fibers in the stratum corneum (Elias et al., 1977). Since both sphingolipids and sterols can be fluorochromed by rhodamine B in vitro (Boerner, 1952), it is likely that they are the substances responsible for the rhodamine B fluorescence in cornified tissue, in Lewy bodies and in protein bodies. Actually den Hartog Jager (1969) had demonstrated with Baker's acid hematein that the core of the Lewy body contains sphingomyelin, and we have shown that protein bodies also give a positive reaction with Baker's acid hematein (Panayotacopoulou et al., 1989). Moreover, the proteins involved with the lipids of cornification, i.e. keratins and filaggrin, are rich in arginine (Bock and Weiss, 1956; Dale et al., 1981) as are the basic proteins of the protein bodies and the Lewy bodies that were discussed above.

Although cornification and cytokeratins are characteristics of epithelial cells, the coexistence of neurofilament proteins and keratins has been demonstrated in PC-12 cells (Franke et al., 1986), a phaeochromocytoma cell line of neural crest origin commonly used for the study of neuronal differentiation. Moreover cytokeratins have been demonstrated in pyramidal neurons of the hippocampus of adult mouse brain (Masters et al., 1985) and cells of the central nervous system of the adult hamster (Franko et al., 1987).

The staining pattern with bromsulfophthalein, which binds strongly to proteins of the glutathione-S-transferase family (Habig et al., 1974) is of interest in view of the following: a) the outer rim of the protein bodies, which are known to originate in mitochondria (Sekiya et al., 1982; Issidorides et al., 1987), is derived from the outer double membrane of the parent mitochondrion (Issidorides and Pappas, 1988), b) glutathione-S-transferase constitutes 5% of the total proteins recovered in the outer mitochondrial membrane fraction (Morgenstern et al., 1984), c) this enzyme catalyzes the conjugation of glutathione with various xenobiotics. Glutathione-S-transferase is present in the brain (Das et al., 1981). It has been demonstrated in the substantia nigra of both control and Parkonsonian subjects, and could be involved in the removal of free radicals formed from the metabolism of dopamine, or MPTP and other chemical analogues (Perry and Yong, 1986). Glutathione-S-transferase is effectively inhibited by compounds such as serotonin, dopamine, L-dopa and glucocorticoids (Abramovitz et al., 1988); such a high-capacity intracellular binding-protein has the potential to affect the action of these compounds.

In the healthy human brain the protein bodies are almost exclusively found in the perikarya and dendrites of monoamine neurons of the brain stem (Issidorides et al., 1978; Issidorides and Panayotacopoulou, 1978; Panayotacopoulou and Issidorides, 1982), as well as of the monoamine cell groups of the hypothalamus and the forebrain (Issidorides et al., 1985). In Parkinson's disease Lewy bodies are also found in the perikarya and dendrites of the monoamine neurons, in the majority of cases (Ohama and Ikuta, 1976; Halliday et al., 1990). An apparent exception is the presence of Lewy bodies in the cholinergic neurons of the nucleus basalis of Meynert (Lewy, 1912). However, recent studies have

shown that some tyrosine hydroxylase immunoreactive neurons are present in the basal forebrain of the ferret (Henderson, 1987) and monkey (Kohler et al., 1983), as well as in the human cerebral cortex (Gaspar et al., 1987). Furthermore, spherical protein bodies have also been observed within neurons of the nucleus basalis (Issidorides et al., 1985). In other neurological disorders, such as diffuse Lewy body disease, Lewy bodies have been described in non-catecholamine neurons, but their ultrastructural and histochemical characterisitcs differ from the Lewy bodies of the brain stem, in that they often lack a clear halo and they do not contain a central core (Yagishita et al., 1980).

Thus, protein bodies and Lewy bodies in Parkinsonism have a similar distribution within the aminergic systems of the human brain, and also share common chemical components such as basic proteins and rhodaminophilic sphingolipids, as shown in the present study. These findings support our view that a chemical link exists between protein bodies and the formation of Lewy bodies. They also support that Lewy bodies incorporate substances already present in specific inclusion of the healthy neurons, i.e. the protein bodies.

Incorporation of normal cell contituents into Lewy bodies is in agreement with immunohistochemical findings demonstrating the presence of antigenic sites in common with neurofilaments (Goldman et al., 1983; Kahn et al., 1985; Forno et al., 1986; Gallaway et al., 1988), of tyrosine hydroxylase (Nakashima and Ikuta, 1984), of antigens of normal substantial nigra neurons (Hirsch et al., 1985) and of ubiquitin (Kazuhara et al., 1988). It is significant to point out that most of the above immunohistochemical approaches reveal sites *peripheral* to the central core, which in these cases remains unstained. The core of many Lewy bodies, however, is strongly reactive with a monoclonal antibody to paired helical filaments which has been shown to recognize ubiquitin (Bancher et al., 1989).

These results demonstrate that Lewy bodies and protein bodies contain similar macromolecular components, i.e. lipids and two different types of basic proteins which also show similar stratification in the two structures. On the other hand, the presence, in several neurons of the parkinsonian patients, of aggregates representing transitional forms between protein bodies and Lewy bodies, indicates that abnormalities of protein bodies precede, and are somehow linked to, Lewy body production. Taken together, our findings suggest that the disruption of normal cellular metabolism of catecholamine neurons in Parkinsonism, which leads to depletion of monoamines (Ehringer and Hornykiewicz, 1960) and to the decrease or disappearance of protein bodies (Issidorides et al., 1978), does so by arresting their production, or by arresting the *assembly* of basic proteins and lipids into the spherical organelles, and diverting their macromolecules into aberrant formations and, finally, into Lewy body production.

It has often been stated that the chemical identification of the components of the Lewy body may hold the key to understanding the cause of Parkinson's disease (Marsden et al., 1982; Forno, 1986). Our present findings represent a

step in this direction by identifying constituents of normal cellular inclusions from which the Lewy body may possibly originate.

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