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Another Look at Lead Inclusion Bodies

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ONE of the characteristic signs of chronic intoxication with lead, in man and in experimental animals, is the presence of intranuclear inclusion bodies in renal tubular cells. Histologic studies and electron microscopy have revealed general features of the inclusion bodies, such as heterogeneity with respect to various stains, and the presence of microfibrillar structures.[1-11] It has been noted repeatedly that some of the inclusions are acid-fast after application of carbol-fuchsin, and that they contain little or no DNA, as judged by results of the Feulgen reaction (see refs. in Table 1). Sometimes the inclusions are surrounded by contracted chromatin,[8,10] but the weight of evidence has made it appear unlikely that they are derived from nucleoli.[7,10,11] To learn more about the nature of such intranuclear inclusions, we have studied kidneys of rats poisoned with lead subacetate. For this purpose, various methods involving light and electron microscopy were utilized.

The findings now to be reported indicate that the microfibrils and other, amorphous components of typical lead inclusion bodies in cells of proximal convoluted tubules are proteins which appear *not* to be histones. The fine structure of the microfibrils and the possible relation of microfibrils to chromatin will also be considered, as well as the disposition of DNA that often surrounds the inclusion bodies. Furthermore, it will be shown that in chronic intoxication with lead, nonspecific intranuclear inclusions that differ markedly from the typical ones may develop in cells of distal convoluted tubules.

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Materials and Methods

Animals and Treatments

Thirty-six female Sprague-Dawley rats, initially weighing approximately 200 gm., were given a series of intraperitoneal injections of 1% lead subacetate (reagent grade, Matheson Coleman & Bell Co.) in 0.9% NaCl solution. They were given from 9 to 25 injections during periods of 3–9 months and killed at intervals. The kidneys from 22 of these animals were examined by light and electron microscopy. Each rat received 2–4 injections per month, the number depending upon blood counts (degree of anemia, number of RBC with basophilic stippling, number of reticulocytes) and change in weight. Twelve female Sprague-Dawley rats served as controls. They were given intraperitoneal injections of 0.9% NaCl solution. The animals were housed in individual cages and were given Purina Rat Chow and water ad libitum.

Preparation of Tissues

For routine light microscopy, tissues were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. They were then stained in various ways, to be mentioned. Sections for light microscopy were also cut from blocks prepared for electron microscopy as described below; these sections were stained with toluidine blue [12] or with iron hematoxylin,[13] followed by toluidine blue or by basic fuchsin.

For electron microscopy, some blocks were fixed in 2% redistilled glutaraldehyde in Millonig's phosphate buffer [14] for 3 hr., then thoroughly rinsed in the buffer, and afterwards "postfixed" for 1 hr. with 1% OsO_4 in Millonig's phosphate buffer. Other blocks were fixed for 1 hr. with 1% OsO_4 in Millonig's phosphate buffer. All blocks were dehydrated in graded concentrations of ethyl alcohol, followed by propylene oxide; they were embedded in Epon 812 epoxy resin, essentially as described by Luft.[15]

In an attempt to achieve added contrast, phosphotungstic acid (PTA) was added to the 75%, 95%, and 100% ethyl alcohol used for dehydration (2% concentration of PTA).

Thin sections to be studied were stained with 7% aqueous uranyl acetate, with Reynolds' lead citrate, or with both stains in sequence (uranyl stain first). As a routine, unstained sections from each block were also examined.

The methods given by Monneron and Bernhard [16] for enzyme digestion of sections embedded in Epon were also applied, using RNase (50 Kunitz units/mg.), DNase (3780 dornase units/gm.), and pronase (45,000 PUK units/gm.; Calbiochem, Los Angeles).

The indium stain of Watson and Aldridge [17,18] was also used. For this purpose, tissues were fixed in 10% acrolein. The blocking reactions and the reaction with indium were carried out as recommended by Watson and Aldridge.[17] Blocks were embedded in Vestopal, also described by Watson and Aldridge.[17]

Ultrathin sections, prepared for electron microscopy, were mounted on carbon-coated grids and examined with a Siemens Elmiskop I electron microscope, operated at 80 kv., or with an RCA EMU-3B electron microscope, operated at 50 kv.

Results

Light Microscopy

The observations by light microscopy on material fixed in 10% formalin and embedded in paraffin are essentially in agreement with those

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previously reported by others. The findings are given in Table 1. Figures 1–6 show typical results. Several features may be noted. Thus, after application of the Feulgen stain,[19] about 50% of inclusion bodies were surrounded by Feulgen-positive material, which was present either as a narrow, peripheral red rim, or in clumps (Fig. 3 and 4). In a few instances, the Feulgen-positive material at the periphery of an inclusion body was connected by a Feulgen-positive spur or bridge with Feulgen-positive material of another inclusion body. Attachments to nucleoli were not found. We never encountered Feulgen-positive material in the center or bulk of an inclusion body, but only at the periphery. Application of fast green after extraction of DNA with hot trichloroacetic acid[20] left the inclusion bodies faintly rose-colored (Fig. 5), except for an occasional thin rim that was green, as was the unextracted residue of chromatin. Thus, only the rims of inclusion bodies may have contained histones. By the mercuric bromphenol blue method for proteins,[21] the inclusions were stained dark purple-blue, whereas nucleoli were blue or light blue (Fig. 6).

Electron Microscopy

As shown in Fig. 7–10, profiles of sectioned inclusions vary in size and shape, but most of them are roughly circular or elliptical. They contain fibrils which are embedded in a somewhat granular matrix. In most instances, an outer zone, composed largely of a loose mesh of fibrils, and a central, more compact core may be distinguished. The outer zone varies greatly in thickness; at its periphery one usually sees protruding fibrils averaging 120 Å in thickness. There is considerably less "amorphous" matrix in the outer zone than in the core. Some of the granules may be artifacts introduced by the lead and/or uranyl stains. The differentiation of two zones, periphery and core, was a feature of most of the inclusion bodies. It did not depend on the method of fixation. It was well marked after primary fixation with OsO_4 (Fig. 11), or with glutaraldehyde (Fig. 7–10), and it was present in unstained sections as well as in those stained with lead citrate (Fig. 12), or with lead citrate as well as with uranyl acetate (Fig. 7–11). After the latter (double staining) procedure, the cores appeared very dense whenever there was much amorphous background (Fig. 7, 8, 10, and 11). But some of the smallest inclusions (early ones?) did not have distinct cores and outer zones.

In general organization and fine structure, the inclusion bodies described are quite unlike chromatin and nucleoli. Nucleoli were always separate and distinct from inclusion bodies. This is shown in Fig. 9 and

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Table 1. Staining of Intranuclear Inclusions in Renal Tubular Cells

	Authors (by Ref. No.) reporting on human material						Authors (by Ref. No.) reporting on material from rats						
	2, 3	5	6	11	1	9	2, 3	4	8*	10	9	11	Pres. rep.
H&E	eos.	NR†	NR	eos.	eos.	eos.(?)	eos.	eos.	eos.	pale gray-green to red	eos.	eos.	eos.
Acid-fast Ziehl-Neelsen	+	±	NR	+ or −	NR	+ ur. sed.‡	+	+	+	NR	+	+	+ or −
Feulgen	NR	±	±	+ or −	−	NR	NR	NR	−	−	NR	+ or −	+ or − at periph.; − at ctr.
Feulgen after TCA extraction	NR	±	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Direct Schiff	NR	−	±	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
PAS	NR	NR	−	NR	NR	NR	NR	NR	NR	NR	NR	NR	−
Masson	orange	NR	NR	red	NR	NR	NR	NR	NR	red	NR	red	NR
UV fluorescence	NR	NR	NR	NR	NR	NR	NR	NR	+	+	NR	NR	NR
Methyl green pyronine	NR	NR	pink	NR	NR	NR	NR	NR	NR	NR	NR	NR	green to pink
Toluidine blue	NR	NR	blue	NR	NR	NR	NR	NR	NR	bluish green	NR	NR	bluish gray
Light green	NR	NR	NR	NR	NR	NR	NR	NR	NR	green	NR	NR	NR
Basic fuchsin	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	+
Fast green	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	−§
Bromphenol blue	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	dark purple

None of the reports included data on staining with phloxine.
* Rats reported on were both wild and laboratory animals.
† Data not reported.
‡ Urinary sediment.
§ Negative except for rim, which is often positive.

10. The fine structure of nucleoli that were situated in nuclei with inclusion bodies appeared normal (Fig. 9, 10, and 13).

Preparations treated with indium gave a clear differentiation of the nucleolonema, nucleolar particles, and perinucleolar chromatin from material in inclusion bodies (Fig. 13, 14, and 18). Furthermore, as is shown in Fig. 13, 14, and 18, the inclusion bodies appeared unstained by indium or, at most, stained very faintly, whereas nuclear chromatin, nucleolar RNA (and associated DNA), and ribosomes in the cytoplasm, were heavily stained.

The thickness of fibrils in inclusion bodies, as seen in profile, ranges between 100 Å and 130 Å. Close scrutiny suggests the presence of structural detail within the fibrils (filaments?) (Fig. 15–17), particularly in material impregnated with PTA (Fig. 15 and 16). After staining with both lead citrate and uranyl acetate, the density of fibrils to electrons was considerably increased. This appeared to be due mainly to the lead stain. The fibrils did not, however, have a uniform thickness. Possibly, relatively thick fibrils are composed of more substructures then are thinner ones. Variation in thickness of fibrils was observed after all staining procedures, as well as in unstained material that had been fixed in glutaradehyde. Impregnation with PTA made the fibrils more dense to electrons than the surrounding chromatin or nuclear membrane (Fig. 15). This effect appears to be similar to the enhancement of contrast produced by PTA in fibrous actin, myosin, and collagen. After fixation with glutaraldehyde, followed by treatment with OsO_4, the fibrils had essentially the same appearance as after primary fixation with OsO_4. The lead citrate stain enhanced contrast of fibrils markedly (Fig. 12), whereas uranyl did so only slightly.

Fibrils at the periphery of inclusion bodies were sometimes connected with, or attached to, compact chromatin (Fig. 18). Judging from the appearance of these connections and from their relative frequency, they may not have been fortuitous. They were observed in material fixed with glutaraldehyde and postfixed with OsO_4, in material fixed with OsO_4, and in material fixed with acrolein and exposed to the indium stain.

Sections from tissue fixed with glutaraldehyde, postfixed in OsO_4, and embedded in Epon, were processed for treatment with enzymes according to the methods of Monneron and Bernhard.[16] Application of pronase for short periods gave the most impressive results. After 10 min. of exposure to 0.5% pronase, at pH 7.4 (in water, adjusted with dil. NaOH), the outer zones of inclusion bodies had been almost completely digested while the cores had been attacked less severely (Fig. 20 and 21). By contrast, chromatin, ribosomes, nucleoli, and cytoplasmic

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structures appeared intact. Thus, there was a differential effect. Exposure of sections to 0.5% RNase in water at pH 6.8 for 1 hr. had no discernible effect on the inclusions, but it did not affect cytoplasmic ribosomes either. Treatment with 0.2% DNase at pH 6.4 (in water, adjusted with diluted NaOH) for 1 hr. resulted in some reduction of contrast in the chromatin, but had no visible effect on inclusion bodies.

That the tissue was embedded in Epon undoubtedly hindered the effectiveness of enzymatic digestion. Only the results obtained with pronase were definite enough to warrant further consideration.

Nonspecific Inclusions

Another kind of inclusion body may be found in lead poisoning, and may at the level of light microscopy, be mistaken for the specific intranuclear inclusion bodies just described. As shown in Fig. 21, the fine structure of such inclusions differs from that of the characteristic inclusions of lead intoxication. The former inclusions are not specific since similar bodies have been found in other cells under various conditions, possibly related to fixation.[22] The nonspecific inclusion bodies contain a mixture of structural components, among which myelin figures are most prominent (Fig. 21). In other instances, we have observed similar inclusions that appeared to be derived from components of the cytoplasm. Thus, a very deep invagination of the nuclear envelope by cytoplasmic structures (e.g., mitochondria, lysosomes, and myelin figures) may develop in injured cells, and if planes of sectioning pass through the invagination, the resulting configuration may simulate nuclei with inclusions, but nuclear membranes would surround the enveloped material. However, in Fig. 21, distinct nuclear membranes around the inclusions cannot be seen, and therefore the latter may really be intranuclear, their origin being unknown.

Discussion

The observations presented above indicate that the fibrillar material in lead inclusions is a protein and that, most likely, this protein is not a histone. These inferences are based on the following findings, considered together: The inclusions give negative reactions with fast green after extraction with trichloroacetic acid, but they stain intensely with mercuric bromphenol blue, and are eosinophilic; the fibrils in the inclusions are stained positively by PTA, and are digested preferentially by pronase.

It has been suggested by Landing and Nakai [6] that the acid-fastness of the inclusions may be due to sulfhydryl groups. To us it appears more

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likely that the acid-fastness is a physical phenomenon, as in the case of tubercle bacilli, particularly since the inclusions were not all equally acid-fast.

As judged from the results of the Feulgen stain, at least the cores of inclusion bodies contain no DNA. Moreover, staining with indium strikingly differentiates the fibrils from chromatin, ribosomes, and nucleolar components. Hence it is unlikely that the fibrils contain either DNA or RNA.

The fine structural basis for the Feulgen-positive outer rim of many of the inclusion bodies is not clear. It seems most likely that such Feulgen-positive material represents chromatin that surrounds inclusion bodies, though it might be partly depolymerized DNA, located between the peripheral fibrils. But the negative results obtained after staining with indium are not in favor of the latter possibility. However, on purely morphologic grounds, the fibrils in the inclusions appear to have some relation to chromatin.

Though the occasional projection of fibrils into condensed chromatin might lead one to suppose that fibrils originate from chromatin, the evidence is insufficient to support such an inference. One of the major questions to be answered first is whether these fibrils are a degradation product of pre-existing, normal nuclear components, or whether they are an abnormal protein, synthesized *de novo*. Perhaps they are derived from soluble (normal or abnormal) nonhistone protein molecules by a process of polymerization. If so, high resolution electron microscopy of suitably prepared material should reveal subunits in the fibrils. Several observations (Fig. 16–18) suggest that the fibrils may be composed of smaller filaments, and the fine structural detail so far visualized is compatible with the existence of multiple helical structures as basic constituents of the fibrils.

The loose fibrillar mesh at the peripheries of inclusion bodies may consist of fibrils that are younger than those situated in cores, since it is reasonable to suppose that enlargement of an inclusion body would occur at its periphery.

One may ask whether the essential structural units of the fibrillar protein are synthesized in nucleoli. If so, our findings provide no clues since they have not demonstrated connections between nucleoli and inclusion bodies. Precursors of fibrils (monomers or subunits), however, may be released from nucleoli to be assembled (polymerized) elsewhere. That the inclusion bodies do not result from nucleolar activity was suggested by Müller and Stöcker,[23] who found that neither ^{3}H-cytidine nor ^{14}C-l-phenylalanine were incorporated into inclusion bodies

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in time periods during which these compounds were incorporated into nucleoli situated within the same nuclei as the inclusions. Two other possibilities are that the inclusions, and specifically the fibrils, result from degradation and restructuring of pre-existing intranuclear components, or that they are assemblies of cytoplasmic precursors. Since there is no plausible conceptual framework for relating the intranuclear inclusions to cytoplasmic protein synthesis, we favor the hypothesis that the inclusions result from degradation and restructuring of a pre-existing intranuclear protein.

The fibrillar component in the inclusions and the general features of core and periphery have been described by others;[7-11] but detailed studies of the fine structure of the inclusions, of effects of fixatives and contrasting procedures, and of enzymatic digestion, have not been published previously. Wachstein,[2,3] Landing and Nakai,[6] and Müller and Ramin [10] proposed that the inclusions are composed mainly of protein. Landing and Nakai,[6] Müller and Ramin,[10] and Beaver [7] considered the inclusions to be distinct from nucleoli. We agree with these conclusions.

It is apparent that precise chemical data on the constitution of the inclusion bodies are needed as a basis for investigations of their genesis. To make such chemical analyses feasible, methods for the preparation of fibrils in highly purified state must be developed. We would suggest that the significant features in the genesis of the inclusions are likely to be changes in regulatory mechanisms that are relevant beyond the problem of lead intoxication.

Summary

Intranuclear inclusions characteristic for lead poisoning were investigated by light and electron microscopy, using various preparative techniques. The inclusions were produced in cells of renal tubules of female Sprague-Dawley rats by repeated intraperitoneal injections of lead subacetate. The characteristic inclusions in cells of proximal convoluted tubules were always distinct from nucleoli; the latter appeared normal. Feulgen staining and electron microscopy revealed that some inclusion bodies were surrounded by Feulgen-positive material, but did not contain such material. After treatment with trichloroacetic acid, the inclusions were not stained by fast green, and from this result it was inferred that they do not contain histones. They were, however, stained heavily by mercuric bromphenol blue and by basic fuchsin. They were distinctly more sensitive to attack by the proteolytic enzyme pronase than were chromatin or nucleoli, as demonstrated in thin sections by electron

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microscopy. The most common type of intranuclear inclusion had a compact core and a circumferential fringe. The latter consisted largely of a loose mesh of fibrils ("microfibrils") that varied considerably in thickness (100–130 Å). Close scrutiny has suggested that the microfibrils are composed of an undetermined number of filamentous structures, perhaps in helical configuration. The smallest filaments seen measured about 40 Å in thickness. The cores of inclusion bodies contained a compact, relatively amorphous, sometimes granular matrix, as well as fibrils. Less frequently, relatively small inclusion bodies were encountered which, in electron micrographs, displayed only fibrils.

The fibrils were well preserved after fixation with glutaraldehyde, osmium tetroxide, or acrolein. They were only moderately osmiophilic. Treatment with alcoholic phosphotungstic acid during dehydration markedly enhanced the contrast density of the fibrils. In thin sections, stained only with lead citrate, the fibrils had considerably greater contrast density than chromatin, nucleolar components, and the nuclear membrane. They also differed from chromatin, nucleolar components and ribosomes, by their lack of affinity for indium.

Some fibrils at peripheries of inclusion bodies extended into condensed chromatin, but it has remained unclear whether or not they were actually connected with chromatin.

In cells of distal convoluted tubules some nuclei contained another, nonspecific type of inclusion. The latter appeared as membranous whorls (myelin figures) with some amorphous osmiophilic material. These inclusions were readily differentiated by electron microscopy, but not by light microscopy.

We conclude that the fibrils, and much of the amorphous material in the lead inclusion bodies, are composed of protein other than histone, that the fibrils do not contain nucleic acids, and that they are composed of smaller, filamentous structures. The fibrils may have been derived from a protein that was originally associated with chromatin. They do not appear to have any direct relation to nucleoli or nucleolar products.

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Illustrations followed
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