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Immunoelectron microscopy of Rosenthal fibers

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Summary. Seventeen intracerebral gliomas containing Rosenthal fibers (RF) were studied by an immunoperoxidase method for localization of ubiquitin (UB), glial fibrillary acidic protein (GFAP), desmin and vimentin (VIM). The majority of RF showed an immunohistochemically negative core surrounded by a ring of overlapping reactions for UB, GFAP and VIM. Many RF were entirely negative for UB and intermediate filaments (IF). Immunoelectron microscopic localization of UB and GFAP was performed on seven selected tumors. UB was found in all RF and on IF in the proximity of RF. GFAP reaction was localized on astrocytic IF, including those trapped within RF, and within the granular component of some RF. In contrast to the light microscopic studies, neither GFAP- nor UB-negative RF were found on immunoelectron microscopy. VIM reaction on IF and a few RF was demonstrated in one tumor processed at low temperature into Lowicryl; it was much weaker than that for GFAP. Many cells with RF contained lysosome-like inclusions with material displaying electron density similar to adjacent RF; few of these inclusions were reactive for UB. It is concluded that RF formation is associated with ubiquitination of astrocytic IF. GFAP- and VIM-immunoreactive IF and products of their disintegration contribute to RF material. It is also suggested that the lysosomal system of astrocytes partially degrades RF.

Key words: Rosenthal fibers – Ubiquitin – Immunohistochemistry – Glial fibrillary acidic protein – Vimentin

Rosenthal fibers (RF) are recognized as a characteristic degenerative change of reactive and neoplastic fibrillary astrocytes [2, 2, 13, 16, 25, 43, 47, 48]. On ultrastructural examination they appear as densely osmophilic irregular masses closely associated with glial filaments [8, 14, 16, 39]. The formation of RF has been attributed to the overproduction or faulty degradation of intermediate filaments (IF) [14, 32] or to the influx of foreign proteins into the affected cells [21, 29]. Although their occurrence is invariably accompanied by intense fibrillogenesis [8, 13, 14, 16], the consistent presence of glial fibrillary acidic protein (GFAP) in RF has been demonstrated only on rare occasions [8, 26, 47]. Most often RF display a peripheral rim of GFAP immunoreactivity around a negative core. Biochemical analysies of RF have shown the presence of ubiquitin (UB) [14], a protein associated with the proteolytic non-lysosomal degradation of other proteins, including those of IF [5, 11, 19, 20, 31, 34, 35, 38, 40, 49]. Subsequent studies demonstrated a peripheral rim of UB reaction around the negative core of RF, resembling that for GFAP [14, 31, 32, 35]. It has been suggested, therefore, that the formation of RF in astrocytes may very likely result from a failure of the UB system to clear the cells of cytoskeletal proteins [14, 31, 32, 34, 35].

To assess the role of UB and glial IF in RF formation, we carried out both, immunohistochemical and immunoelectron microscopic studies on the distribution of UB and IF antigens in the glial tumors with Rosenthal fibers.

Methods

Immunohistochemistry

Light microscopic immunohistochemical studies were carried out on 17 tumors that were selected retrospectively, following electron microscopic documentation of RF in the neoplastic cells. The diagnoses of the tumors are as follows: low grade oligoastrocytoma with protoplasmic astrocytes(9), oligodendroglioma(9), gemistocytic astrocytoma(1), pleomorphic xanthoastrocytoma(1), fibrillary astrocytoma(1), glioblastoma multiforme(1), ganglioastrocytoma(2) and subependymoma(1). Tissue was fixed in phosphate-buffered formalin for 24 to 48 and routinely processed for paraffin embedding. Sections (5 μm in thickness) were placed on glass slides, treated with Lepage Bond glue and used for immunohisto-

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chemistry and routine H&E stains. Sections covered with the primary antisera (the source and dilution of antisera are given in parentheses were incubated overnight at 4 °C. This was followed by either the peroxidase-antiperoxidase (PAP) method [44]: i.e., incubation with swine anti-rabbit (Dako, Santa Barbara, Calif., 1:400) and rabbit PAP (Dako, 1:400) or by the avidin-biotin-peroxidase complex (ABC) method with monoclonal antibodies [23], according to suppliers recommendations (Vector, Burlingame, Calif). GFAP was localized with the monoclonal antibodies (AMAC Incorp. Westbrook, Me, 1:75) and polyclonal antiserum (Dako, 1:200). Seven tumors with the most profuse RF formation were also examined for some or all of the following antigens: vimentin (VIM, Organon Teknika Eurodiagnostic, Oss, Holland, 1:1000), desmin (Dako 1:300), serum albumin (1:40), gamma globulins (1:200), IgM (1:200), IgG (1:1000), IgA (1:1000) as well as kappa (1:1000) and lambda (1:1000) chains of gamma globulins (all Dako).

Double immunostains [22], using a combination of PAP and alkaline phosphatase methods were applied for co-localization of GFAP (with the monoclonal antibodies) and UB in seven cases. The nickel intensification method [41] was used on the sections of seven tumors immunostained for UB or GFAP. In two cases with numerous RF positive for UB and/or GFAP, semiserial sections were predigested with 0.1 % trypsin in 0.1 % calcium chloride and 0.05 M Tris-buffered saline (20 and 90 min), 0.4 % pepsin in 0.01 % HCl (20 and 90 min) as well as pre-treated with 0.5 % Triton X-100 and 8 % sodium metaperiodate for 15, 30, 45 and 60 min at room temperature. For easy identification of RF, all the sections were lightly stained with eosin and cresyl violet.

Antisera Against ubiquitin

Antiserum directed against UB was obtained after immunization of New Zealand White Rabbits (2–3 kg) with either unmodified UB (bovine red blood cells, Sigma, St. Louis, Mo.) Or UB cross-linked to hemocyanin with glutaraldehyde. The antigens were then denatured with sodium dodecyl sulfate. The procedure for preparation of the antigens was adapted from Hershko et al. [20]. The immunization brotocol was as follows: 1 mg denatured antigen (UB or UB-hemocyanin) was emulsified with complete Freund's adjuvant (1:1,v/v) and administered to a rabbit by intramuscular injection (hind leg). This was followed by two subcutaneous injections (neck region) in incomplete adjuvant at 1-week intervals. The animals were test bled 7 and 14 days after the last vaccination. The titer was measured as described [6] and checked for specificity by Western blotting [45]. Another antiserum against UB was obtained as previously published [6].

Electron microscopy

For the routine transmission electron microscopy, small samples of tumor were fixed in 2.5 % glutaraldehyde in 0.1 M sodium-cacodylate buffer, pH 7.2, immediately after surgical removal. The tissues were routinely processed for Epon-Araldite embedding, thick sectioning and toluidine blue staining [33]. One to five blocks were selected for thin sectioning and examination in a Philips 301 electron microscope.

For immunoelectron microscopic localization of UB and GFAP, seven tumors with RF were selected: oligoastrocytoma(4), oligodendroglioma(1), glioblastoma multiforme(1), ganglioastrocytoma(1). Thin sections were collected on nickel grids and etched for 60 min on drops of freshly prepared 8 % sodium metaperiodate [1, 17, 42]. The grids were then gently washed with PBS and placed onto drops of 1 % bovine serum albumin (BSA for 30 min). Grids were then placed directly onto drops of the antisera anti-UB containing 0.1 % BSA (1:25 and 1:100) or anti-GFAP (1:10 and 1:25) and incubated for 16 h at 4 °C. The grids were rinsed in PBS and incubated in protein A-gold for 1 h at room temperature followed by rinsing in distilled water, drying and counterstaining with aqueous uranyl acetate and lead citrate solutions.

One case of ganglioastrocytoma with RF was processed at low temperature into Lowicryl K4M embedding medium (Chem. Werke Lowi, Waldkraiburg, FRG) [28]. Thick and thin sections were subsequently treated in the same fashion as Epon-embedded tissues, except for omission of etching with sodium metaperiodate.

Results

On light microscopic examination RF were most commonly found in astrocytes of mixed oligoastrocytomas, subependymoma and in gliosis around oligodendrogliomas. In the remaining cases (glioblastoma multiforme, gemistocytic astrocytoma and pleomorphic xanthoastrocytoma), RF were present in only a few cells and were only apparent after electron microscopic examination. RF appeared as eosinophilic hyaline masses with a rim of positive reaction for UB, VIM and GFAP around an immunoegative core (Fig. 1). A very few RF displayed a homogenous uniformly strong reaction for GFAP. Double immunostaining showed co-localization of the reactions for UB and GFAP around the negative centre of RF. Positive UB immunoreaction was also noted in beaded processes of scattered astrocytes, usually with

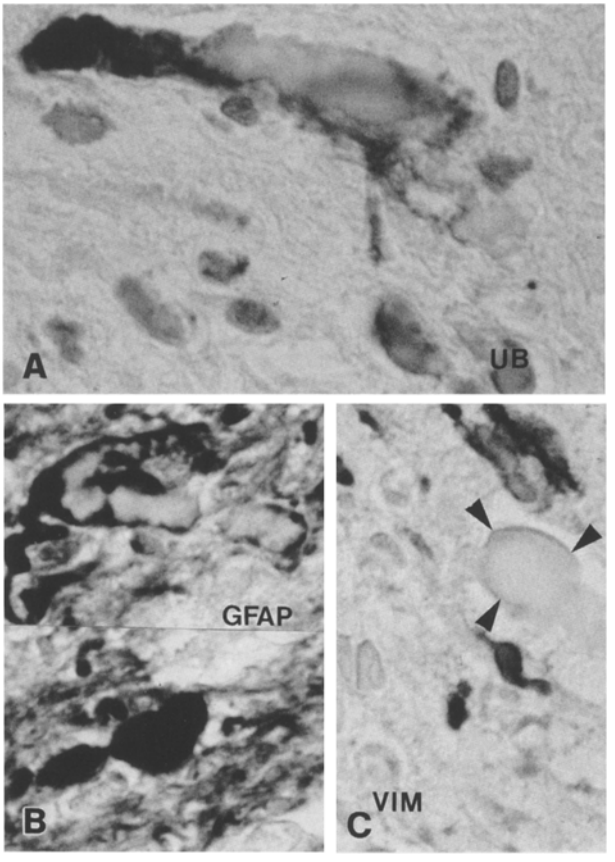

Fig. 1. A Reaction for ubiquitin (UB) at the peripehry of Rosenthal fibers (RF). **B** RF with negative core (*upper*) or diffusely immunoreactive for glial fibrillary acidic protein (GFAP; *lower*). **C** Immunostaining for vimentin (VIM) at periphery of small RF. *Arrowheads* outline large negative RF. **A–C** × 750

RF. The reaction most likely represented either intracytoplasmic collections of small "granular" RF or compacted ubiquitinated filaments. UB was also localized in the cytoplasmic granules of many granular cells in two cases of oligoastrocytoma. Although they were most often only UB positive, double immunostains demonstrated reactions for both, GFAP and UB in some of these granules.

The cytoplasm and processes of many reactive and neoplastic astrocytes showed co-localization of GFAP and VIM. Desmin was found in a significant number of astrocytes only in a case of fibrillary astrocytoma that showed no RF identifiable by light microscopy. Neither predigestion with trypsin or pepsin nor treatment with Triton X-100 or sodium metaperiodate changed the localization or intensity of the reactions for GFAP or UB in astrocytic cytoplasm or RF.

Small intracytoplasmic globules positive for gamma globulins and albumins were present in scattered macrophages and within a few astrocytes, as previously described by others [4].

Immunoelectron microscopy

All RF appeared as conglomerations of a granular or amorphous osmiophilic material of variable electron density, intimately associated with the glial filaments. Antiserum against GFAP decorated IF around RF as well as those that were trapped between the amorphous electron-dense components of RF (Fig. 2). GFAP-immunogold reactivity was also observed on finely granular substance of some RF. Some areas of large RF were composed of electron dense homogenous osmiophilic material without GFAP reactivity or demonstrabel IF.

All the identifiable RF were positive for UB. The majority of UB-immunoreactive RF consisted of a homogenous, relatively lightly osmiophilic material (Fig. 3a). However, some RF also showed a clearly fibrillar substructure and UB immunoreaction on collections of compacted IF (Fig. 3b). The UB reaction was diffusely and evenly distributed on the small RF, while the large ones showed the greatest concentration of gold particles at the periphery (Fig. 3c), in a pattern similar to that seen in PAP preparations (Fig. 1). Only a very few morphologically normal glial filaments displayed UB immunolabelling, they were always in vicinity to RF.

In a case examined for VIM, the reaction was found on glial filaments and within a few RF. The labelling of IF was much less intense and less frequent than with the antibodies against GFAP (Fig. 4).

Astrocytes with RF very often contained cytoplasmic conglomerates of pleomorphic osmophilic material and

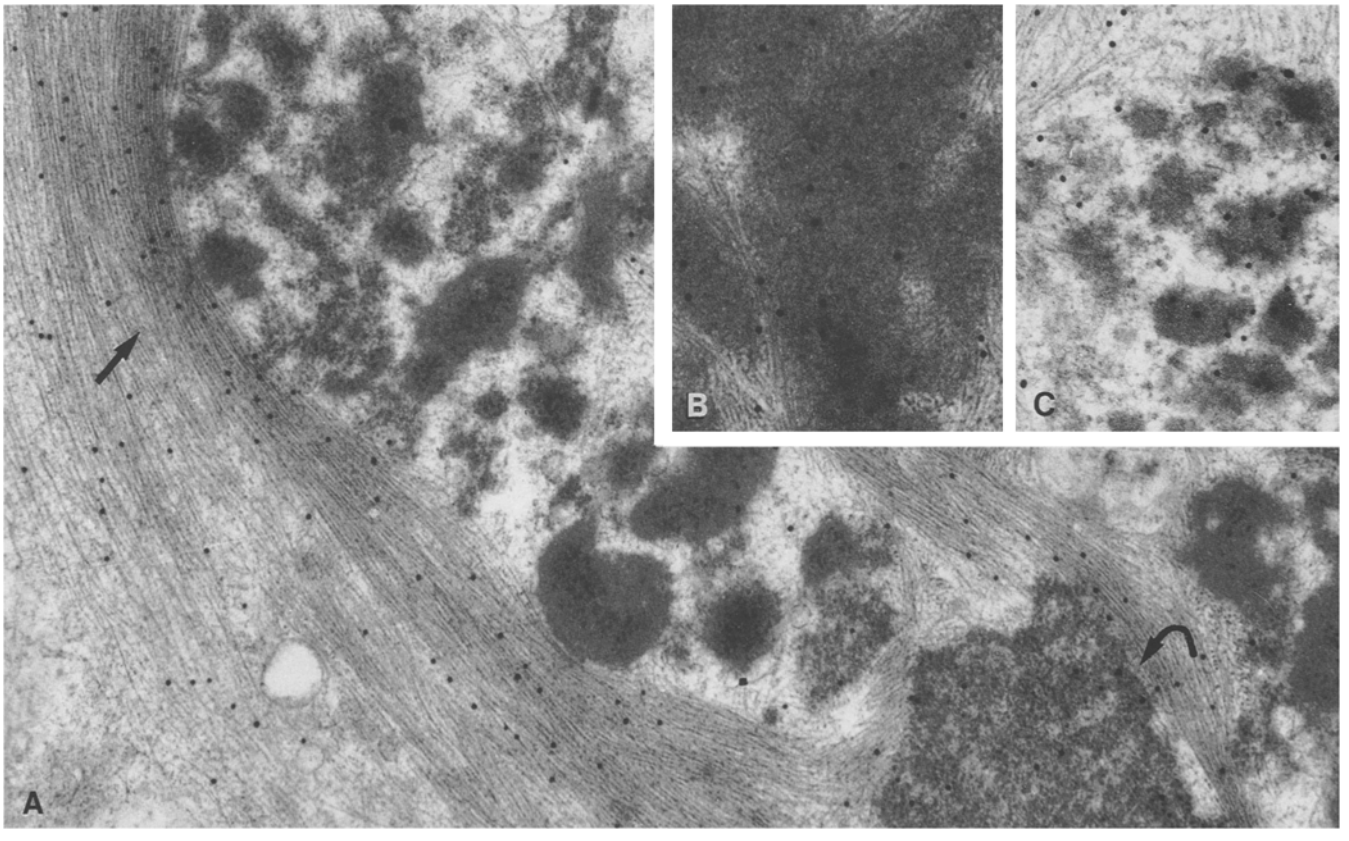

Fig. 2 A–C. Immunogold reaction for GFAP in RF. **A** GFAP-positive intermediate filaments (IF; *arrow*) displaced by the dense, GFAP-negative osmiophilic component of RF. Notice immunogold reaction on lightly granular material of RF (*curved arrow*) not associated with filaments. × 50,600. **B** Higher power of RF, showing entrapped IF decorated with immunogold. × 83,800. **C** Granular material positive for GFAP. × 64,800

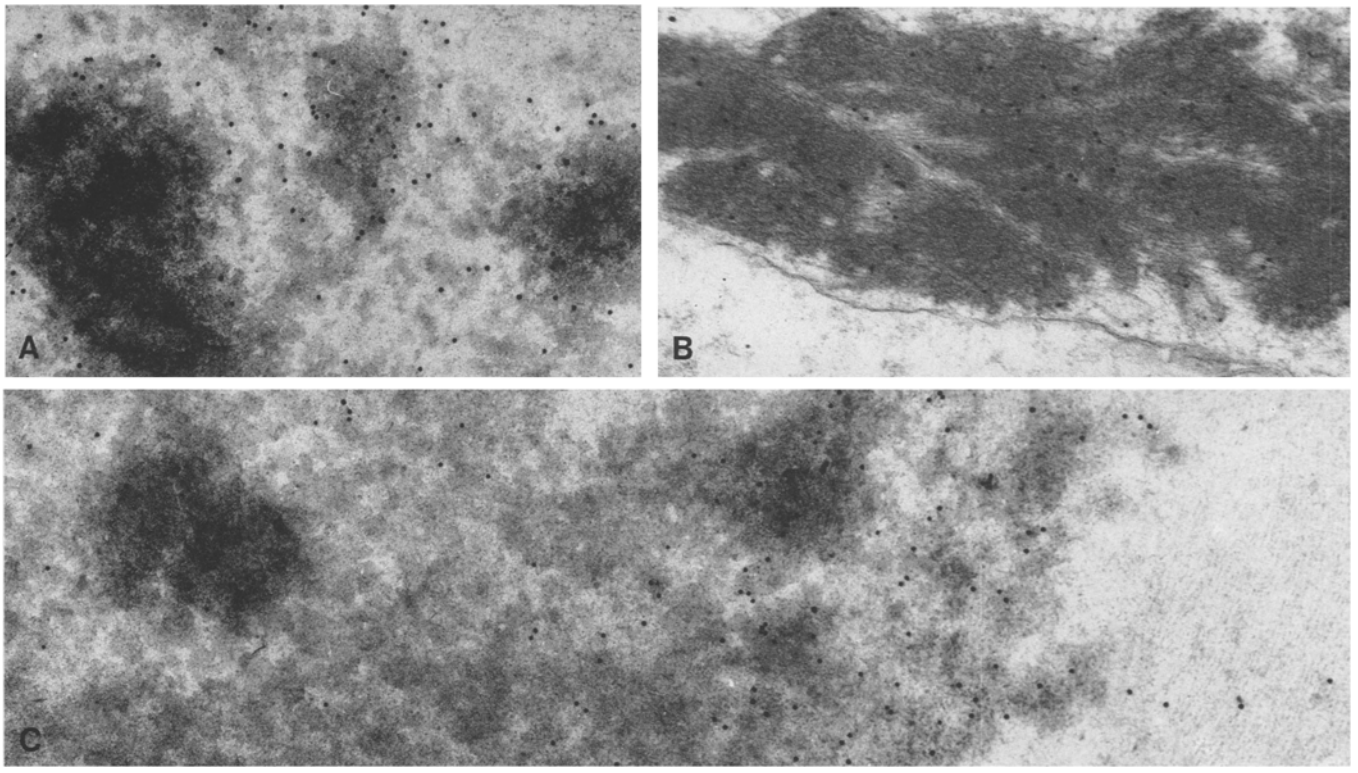

Fig. 3 A–C. Immunogold reaction for UB in RF. **A** Labelling of lightly osmiophilic homogenous deposits not associated with intermediate filaments. × 50,00. **B** Reaction on RF composed of very tightly packed IF. × 38,600. **C** Large RF showing higher concentration of gold particles at the periphery (*right*) than in the center (*left*). Note reaction on few IF at the periphery of RF. × 49,500

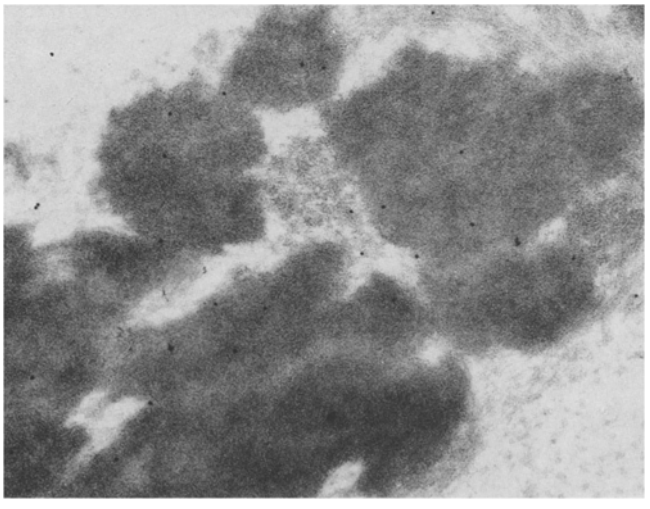

Fig. 4. RF with Immunogold reaction for VIM. × 27,500

scattered lysosomes with a substance morphologically indistinquishable from that of the adjacent RF (Fig. 5). The osmiophilic material in some of these vacuolar inclusions was decorated with gold particles in the immunoreaction for UB. None of the granules were positive for GFAP or VIM, nor displayed IF substructure.

Discussion

The cause and mechanism of RF formation is not well understood. The exclusive presence of RF in the astrocytes in both tissue sections [1, 8, 16, 39, 47, 48] and tissue cultures of astrocytomas [13, 16] underlines the pivotal role of specific products of the astrocyte in the morphogenesis of RF. Negative reactions for plasma constituents in our material speak against the hypothesis of a nonspecific oversaturation of astrocytes with blood proteins [21, 29]. The occurrence of RF solely in the cells with an abundance of IF suggests that RF formation can be attributed to either excessive generation or inefficient degradation of IF [8, 14, 31, 32, 34, 35]. Although the participation of glial filaments to the production of RF has been a controversial issue [7, 25, 43, 47, 48], Western blot analysis of RF demonstrated several different proteins, including UB and components very likely derived from the glial IF, such as VIM and GFAP [14]. Similar to the experience of others [8, 47, 48], scattered GFAP-positive RF were observed in our material. However, much more frequent was the well-recognized restriction of GFAP to the peripheral rim of PAP immunoreactivity around a negative core [3, 7, 14, 25, 32, 43, 48]. This pattern of immunostaining has been attributed to profound changes in the structure and antigenicity of the glial filaments forming RF [14, 25, 32, 34, 43]. Our immunoelectron microscopic findings sug-

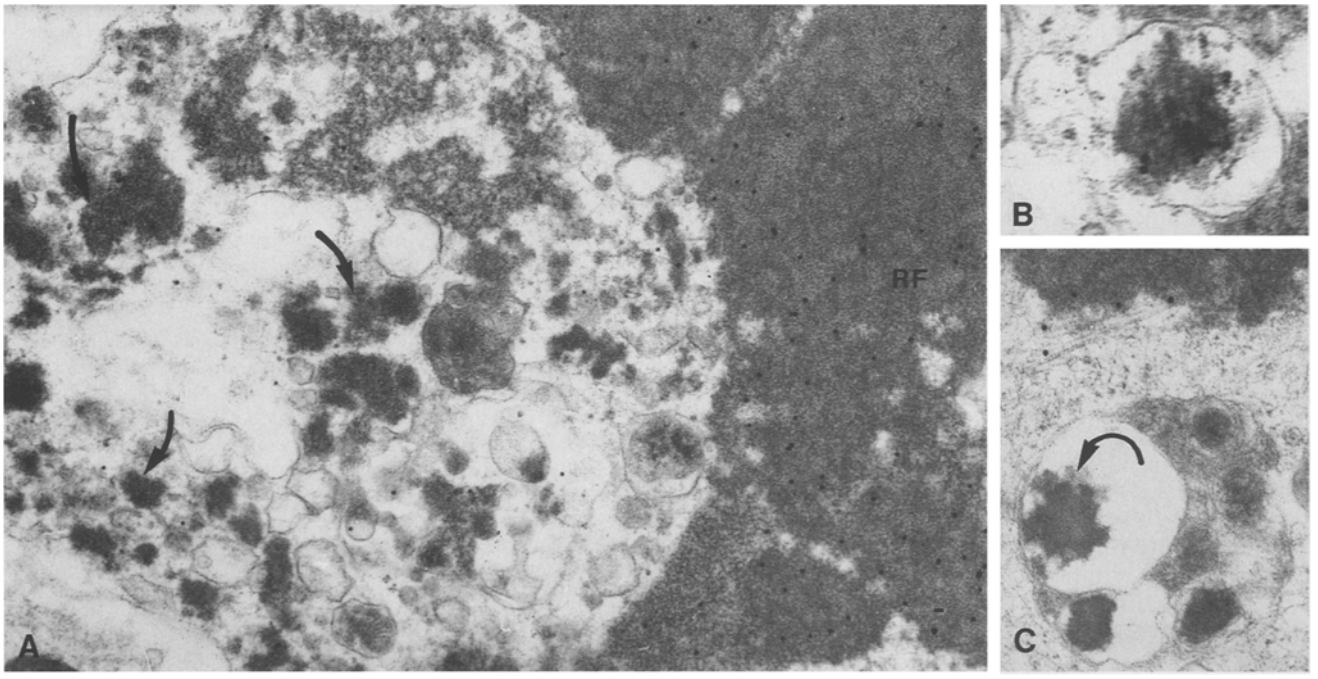

Fig. 5. A Portion of an astrocyte filled with UB-positive large RF (*right*), small granular RF (*arrows*) and with pleomorphic vacuoles. × 37,800. **B** Vacuolar inclusion with UB-positive material indistinquishable from RF. × 49,500. **C** Vacuolar inclusion surrounded by a single membrane and containing electron-dense material (*arrow*) with the same morphological characteristics as that in adjacent RF. Immunogold reaction for GFAP. × 49,500

gest that GFAP reactions of RF were largely due to entrapment of glial IF within RF, and to a lesser degree to the presence of a GFAP-positive granular component of RF. The ultrastructural evidence for changing proportions of intact IF and granular or amorphous material in different RF, may explain their variations in the reported pattern and frequency of GFAP immunoreactivity.

Since VIM constitutes an intrinsic component of a normal astrocyte cytoskeleton [18, 46], biochemical demonstration of its presence and immunohistochemical co-localization of VIM with GFAP in RF is not unexpected. However, reactions for VIM in only a small number of RF in our case, suggests that the contribution of this IF protein to the RF mass is probably less significant than that of GFAP.

The explanation of the role played by glial IF in RF formation is complicated by the discrepancies between the light microscopic and ultrastructural immunohistochemical results. Consistent immunoreactivity of RF for GFAP in the plastic-embedded glutaraldehyde-fixed material [1, 8, 26, 27] is in clear disagreement with light microscopic immunohistochemical studies of paraffin-embedded tissues, usually negative for GFAP in RF [3, 7, 21, 25]. Also in our Laboratory, the pretreatment of paraffin sections in the exact fashion as the plastic-embedded tissues and the application of the detergents or enzymes for PAP and ABC methods, have not increased the frequency or changed the patterns of immunoreactivity of RF for GFAP, VIM or UB. We do not have a satisfactory explanation for this phenomenon.

Immunoelectron microscopy demonstrated consistent presence of UB in all the RF, indicating a crucial role of ubiquitination in their formation. It has been established that one of the major roles of UB is a reversible binding with the abnormal or damaged proteins destined for ATP-dependent proteolysis [11, 19, 20, 40, 49]. Thus, UB probably prevents cell damage by altered proteins [11, 40]. The saturation of the UB system by altered cytoskeletal proteins has been postulated as the cause of the occurrence of RF and other ubiquitinated inclusions in a variety of neurodegenerative disorders [14, 31, 32, 34, 35]. These UB-containing inclusions are not membrane bound, are often filamentous in substructure, and are frequently related to proteins of IF [31, 32, 34, 35, 38]. The fact that UB is rarely bound to normal filaments or other cellular organelles suggests the specificity of the UB system involement in the morphogenesis of these inclusions [37, 38]. This study shows that from the earliest step, RF are ubiquitinated and they appear as amorphous, filamentous or finely granular material continuous with IF. UB immunolabelling of IF in the proximity of RF and the occurrence of RF with filamentous substructure are in keeping with the concept of ubiquitination of IF as one of the initial steps in RF production. Immunoreactivity of the lightly osmiophilic, granular material of RF for GFAP as well as UB, supports the hypothesis of possible conjugation of UB with GFAP and contribution of materials from disintegrated IF to RF [8, 14, 32]. An absence of small UB-negative RF indicates involvement of UB system from the onset of their formation.

However, the occurrence of the UB-negative core in large RF suggests that UB conjugates with RF proteins are perhaps not stable. At some point UB itself is probably cleaved off or becomes degraded.

Our results do not exclude a biochemical heterogeneity of RF. It has been suggested that cross-linkage of ubiquitinated substrate proteins to IF form insoluble complexes bound to the cytoskeleton [9, 10, 40]. It is possible that in addition to the glial filaments, some other cellular proteins (UB-bound or not ubiquitinated) precipitate on astrocytic I F, contributing to RF material. In this alternative process, ubiquitination of IF would occur secondarily, following their alteration by the link with these proteins. Recent studies indicate that RF contain α-B crystallin [30], a protein present in many normal tissues, including astrocytes [15, 24, 50]. A high-affinity of α-B crystallin for nonspecific binding with IF [50] would explain the formation of RF exclusively in the intensely fibrillated astrocytes. However, the most recent studies show accumulation of α-B crystallin in astrocytes without RF production [15], suggesting that this protein alone is not responsible for RF formation.

To our knowledge, the question of the fate of the RF has not been previously addressed. The presence in the cells containing RF of the lysosome-like bodies with material indistinquishable from that of RF, suggests autophagocytic removal of these inclusions from the cytoplasm. A similar mechanism of degradation of altered IF linked with foreign proteins has been documented in experimental conditions [9, 10]. Also Mallory bodies in hepatocytes (that are very similar biochemically and morphologically to RF) are probably degraded with participation of the lysosomal system [12]. The relatively poor development of lysosomes in astrocytes may explain why this pathway of degradation of RF is not sufficient to prevent formation of large RF conglomerates.

In summary, our results suggest that RF arise from the precipitation of proteinaceous, densely osmiophilic ubiquitinated material in fibrillar astrocytes. GFAP and UB are essential components of RF. Ubiquitination of the RF constituents in probably an obligatory pathway for RF production. RF are most likely formed as a result of either insufficient or abnormal ubiquitination of glial IF or the inability of the proteolytic system of affected cells to degrade the UB-tagged cytoskeleton components.

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