THE PROTEIN COMPOSITION OF GLIAL AND NERVE FIBERS

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1. Introduction

In a communication in this Journal it was shown that the electrophoretic band pattern of rat optic nerves did not change markedly after long-standing Wallerian degeneration, at a time when axons had been replaced by neuroglia (fibrous gliosis), thus suggesting that glial and nerve fibers are similar in polypeptide composition [1]. Recent comparative studies of GFA and neurofilament proteins [2], and the isolation of a GFA-like protein from peripheral nerves where glial fibers are not present [3], also point in the same direction, i.e. the similarity of the protein subunit of glial and nerve filaments. GFA is a glial protein as indicated by the fact that it represents the major fraction in severely gliosed tissues and that fibrous neuroglia is selectively stained in immunofluorescence test performed with GFA antisera [4]. In the present report we compare some biochemical and immunological properties of CNS fractions enriched in glial and nerve fibers, using a well established method for the isolation of the axons [5] and a new procedure to obtain preparations enriched in glial fibers. Glial and nerve fibers contain 7–10 nm filaments as a major structural constituent and we thus believe that our studies may contribute to the understanding of this class of cytofilaments intermediate in size between microtubules and actin microfilaments. Microtubules and microfilaments have been extensively studied from the biochemical standpoint, while the intermediate filaments so far have escaped such a detailed biochemical analysis.

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2. Materials and methods

All procedures were carried out at 4°C. Bovine brains were obtained as soon as possible after slaughter at a local slaughterhouse, immediately packed in ice and processed within 1 h. Axonal preparations were obtained with the procedure of Shelanski et al. [5], or with a modification of this procedure [2], with comparable results. No attempt was made to isolate neurofilaments from the axonal preparation since the 2 fractions do not differ significantly in protein and lipid composition [6]. In our attempts to isolate a fraction enriched in glial fibers, a number of standard procedures for the cellular and subcellular fractionation of brain tissue were tried. The enrichment in glial fibers was assessed by the immunodiffusion titer and by immunofluorescence with GFA protein antisera. The amount of nuclear and myelin contamination was determined in cryostat sections stained with toluidine blue. Myelin displays a red metachromasia of brief duration when stained with toluidine blue. Methods using trypsinization to dissociate the tissue [7] could not be used, because of the marked reduction in immunodiffusion titer. The best results were obtained with a modification of the procedure of Fewster et al. [8], aimed at the isolation of glial cells from white matter. After dissection 70–100 g of white matter were finely minced, sieved through nylon meshes of decreasing pore size (760, 452 and 333 μm) and then teased through a piece of nylon

cloth of 130 μm pore size into a medium of 20% (w/v) 'Ficoll' (Pharmacia, N.J.) and Hank's balanced salt solution [9]. The density of 20% 'Ficoll' medium is comparable with 0.7 M sucrose. The tissue suspensions were adjusted to 360 ml by addition of 20% 'Ficoll' and centrifuged at 6000 rev/min for 20 min (SS-34 rotor in a Sorvall RC-2B centrifuge). The compacted floating pads were removed and an axonal fraction was isolated from this material as described before and with comparable results. As indicated by the immunodiffusion titer the pellet obtained by centrifugation in 20% 'Ficoll' was remarkably enriched in GFA protein and there was only minimal myelin contamination. Various attempts at removing the nuclei from this fraction, either by differential centrifugation at higher 'Ficoll' concentration (30–43%), or by density gradient centrifugation in sucrose (0.85–1.4 M) resulted in a decrease of the immunodiffusion titer, and the 20% 'Ficoll' pellet was used for most chemical and immunological studies. Ouchterlony's double immunodiffusion was carried out as described before [10]. The samples were extracted with 0.05 M sodium phosphate buffer, pH 8.0, or with 4 M urea in the same buffer. GFA protein antisera were prepared in rabbits with antigen isolated from multiple sclerosis plaques [11] or from normal human spinal cord (Dahl and Bignami, in preparation). As judged by the appearance of the precipitation lines, antisera raised against protein isolated from normal and pathological tissues were of comparable strength. For morphological studies (immunofluorescence and toluidine blue staining) the fractions were resuspended in a small volume of Hank's balanced salt solution, centrifuged at 8000 rev/min for 15 min and the pellet was cut with a cryostat. The indirect immunofluorescence test was carried out as described before using 1/50 dilutions of the antisera [11]. Non-immune sera and antisera absorbed with GFA protein served as controls. SDS-acrylamide gel electrophoresis was carried out as described by Shapiro et al. [12]. Mol. wt. standards were conalbumin (76 600); human serum albumin (68 000); catalase (58 000); GFA protein (54 000). Protein was determined colorimetrically according to Lowry et al. [13].

Table 1
GFA protein concentration (as shown by the immunodiffusion titer with GFA antisera) in preparations enriched in glial and nerve fibers

	Water-soluble fraction	Urea-soluble fraction (first extract)
White matter	1/2	1/16
Myelin 'wash' pellet[a]	1/16	1/32
Axonal preparation	1/4[b]	1/32
Glial preparation	1/64[c]	1/128
Purified Myelin		Negative

GFA protein was extracted in 0.05 M sodium phosphate buffer, pH 8.0, with and without 4 M urea (w/v, 1:4). The protein content in all preparations was determined according to Lowry et al. [13].

[a] Pellet obtained by centrifugation of the floating pads of myelinated axons resuspended in 0.85 M sucrose, in order to ensure more complete removal of any trapped material [5]. Depletion of the immunodiffusion titer in the axonal preparation could not be obtained by repeating the 'washing' several times.

[b] The low titer in this fraction was probably due to loss of water-soluble GFA protein during incubation in the hypotonic medium used to swell the myelin and thus free the axons from their sheaths [5].

[c] The high titer in the water-soluble fraction was probably due to the lengthy sieving procedure. The amount of GFA protein extracted with buffer from bovine white matter is related directly to the time interval between slaughter and freezing of the brain [14].

3. Results

The immunodiffusion titer with GFA antisera of axonal and glial preparations (20% 'Ficoll' pellet) is shown in table 1. Compared with the starting material (cerebral white matter) both fractions were enriched in GFA protein, but the glial preparation more markedly so. In addition, a larger fraction of the protein was water-soluble in the glial pellet.

Axonal fractions showed a mesh of thin immunofluorescent fibers enclosing non-fluorescent material when stained with GFA antisera (fig.1A). In the glial fraction the immunofluorescent fibers were thicker and more densely packed (fig.1B).

The electrophoretic pattern of the axonal fraction on SDS-acrylamide gels is shown in fig.2A. The dominant band (band c) co-migrated with bovine GFA protein, 54 000 mol. wt. [14]. Additional bands were seen in the high mol. wt. range, 103 000 with the available standards (band a) and immediately below the conalbumin standard (band b, 74 000 ± 1000). The electrophoretic pattern of the glial pellet was similar in some respect to the axonal fraction but also presented a number of differential features. Both preparations shared the major band at 54 000, which co-migrated in mixing experiments. The high mol. wt. bands (about 103 000 and at 74 000) were less intensely stained in the glial fraction. In addition, a series of closely spaced bands migrating ahead of the 54 000 mol. wt. species and ranging down to

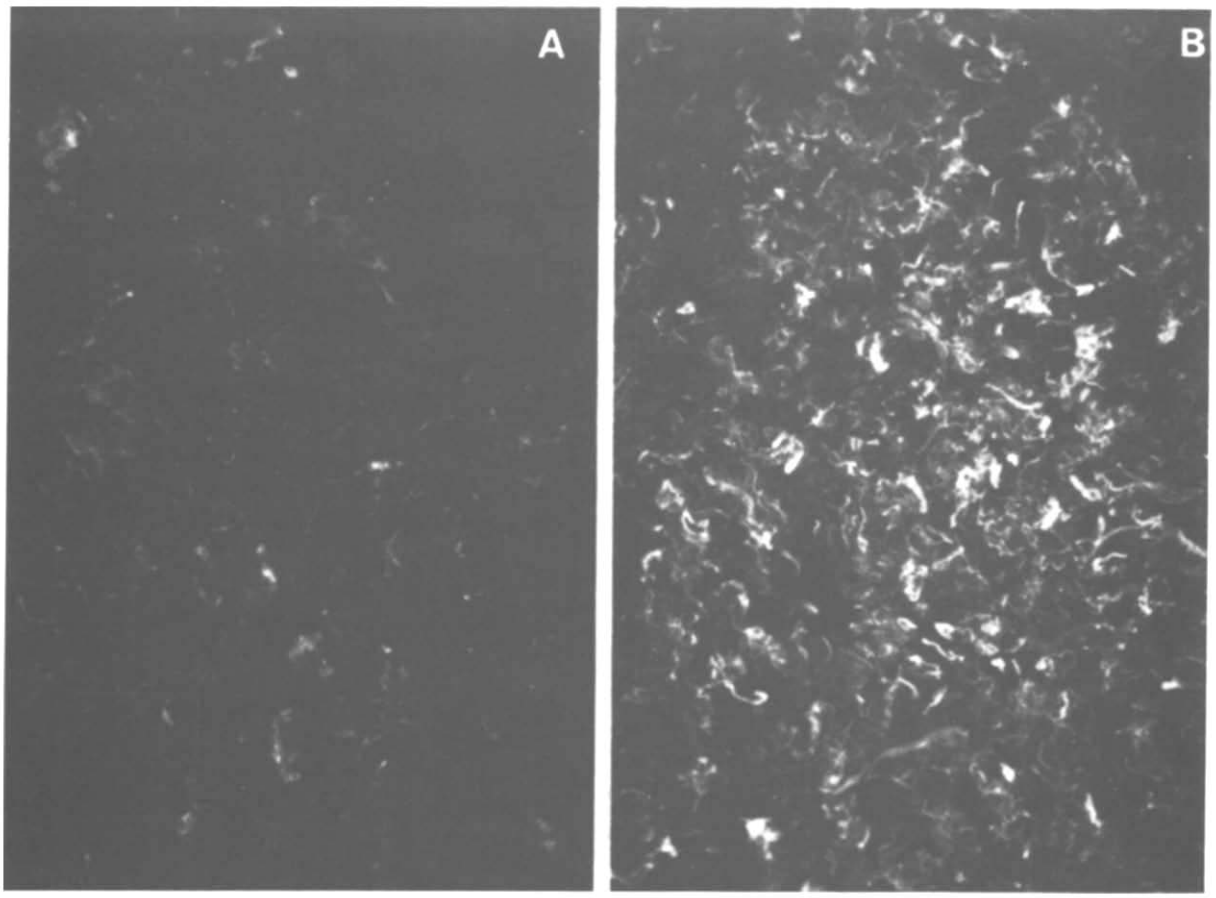

Fig.1. Immunofluorescent staining with GFA antiserum of glial fibers in the axonal and glial preparations. In the axonal preparation (A), thin immunofluorescent fibers form a mesh surrounding nonfluorescent structures, presumably the axons. In the glial preparation (B), immunofluorescent fibers are thicker and more densely packed. GFA antiserum was used in 1/50 dilutions. Non-specific fluorescence was not seen in control sections nonimmune serum at the same dilutions. × 400.

47 000 or to 40 500 depending on the preparation were more prominent in the glial than in the axonal fraction. The mol. wt. of these bands was determined by co-migrating experiments with degraded human GFA protein separating into 7 evenly spaced bands between 54 000 and 40 500 (see fig.1 in [15]).

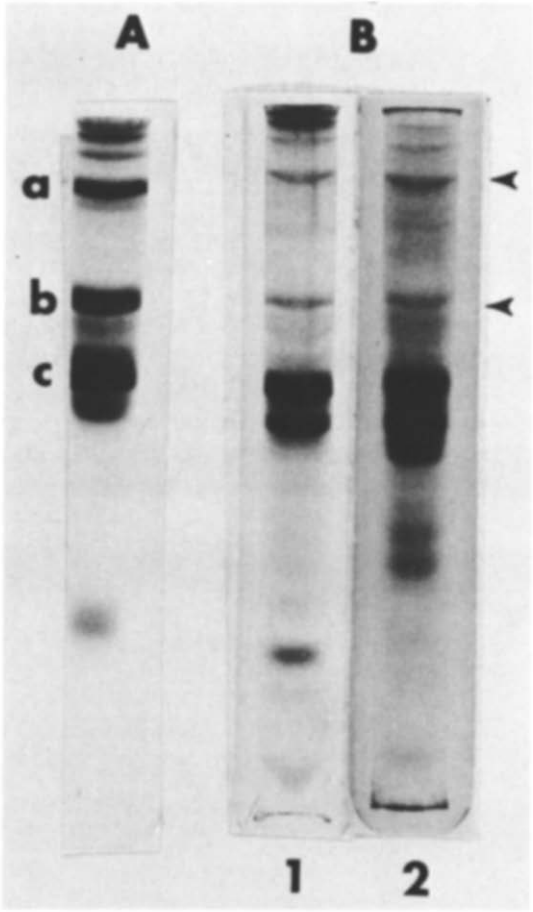

Fig.2. SDS gel electrophoresis at 7.5% acrylamide concentration of axonal and glial preparations. The pellets were extracted in 4 M urea–0.05 M sodium phosphate buffer, pH 8.0. (A) Axonal preparation. The major band (band c) co-migrates with bovine GFA protein, 54 000 mol. wt. [14]. Additional bands are seen at about 103 000 (band a) and at 74 000 ± 1000 (band b). (B) Glial preparation. The major band co-migrates with band c in the axonal preparation. The bands with the electrophoretic mobility of band (a) and (b) in the axonal preparation are weakly stained (arrowheads). Note the multiple bands below the 54 000 mol. wt. species, suggestive of GFA protein degradation [14,15]. The mol. wt. of these bands ranges down to 47 000 (gel 1) or to 40 500 (gel 2), depending on the preparation.

4. Discussion

Electron microscopic studies of the axonal preparation have demonstrated the presence of 2 types of fibers, i.e. nerve fibers and fibers with tight bundles of filaments which were tentatively interpreted as glial [5]. Our findings also indicate that the so called axonal preparation is heterogeneous and contains a mixture of glial and nerve fibers. This is not surprising considering the fine structure of the white matter and the isolation procedure. In this procedure [5] the myelin surrounding the axons is supposed to function as a life vest to float the axons away from the other tissue constituents. The axons are then stripped from the myelin in hypotonic buffer and separated from the myelin by differential centrifugation. In the absence of specific intercellular adhesions between axons and myelin, we believe that the fine glial fibers surrounding the myelin are floated as well as the axons, and that glial and nerve fibers are sedimented together as they become loose from the myelin.

The size of astroglial fibers in the white matter varies considerably, from a thin mesh surrounding the myelinated axons to thicker fibers often attached to blood vessels. It is possible that these were the ones sedimenting in 20% 'Ficoll' and not entrapped in the floating myelin, a hypothesis supported by the immunofluorescence findings showing thicker fibers in the glial pellet compared with the axonal preparation. The electrophoretic band pattern suggested that nerve fibers were not present in significant numbers in this glial fraction, since the high mol. wt. components characteristic of the axonal preparation were less prominent. It is interesting to note that neurofilaments isolated from invertebrate axons are composed primarily of high mol. wt. species [16,17].

Considering that the axonal preparation is also enriched in glial fibers, it could be argued that the protein similar to GFA in this fraction [2] is entirely of glial origin. Although more work is evidently needed to clarify this issue, we still believe on the basis of the evidence presently available that the major band in the axonal preparation, co-migrating with GFA protein 54 000 mol. wt., is at least in part of axonal origin. The electrophoretic pattern of the glial fraction, characterized by multiple bands ranging down to 40 500 mol. wt. is extremely characteristic of GFA degradation [14,15]. Such bands were less

prominent in the axonal preparations, suggesting that the major protein in nerve fibers was less susceptible than GFA to in situ proteolysis. Finally, we have been able to isolate a protein similar to GFA, but lacking immunological activity against GFA antisera, from rabbit sciatic nerves [3]. Peripheral nerves do not contain glial fibers while large myelinated axons are rich in neurofilaments both in the central and peripheral nervous system. Considering the difficulty in separating glial from nerve fibers, and the strong antigenicity of GFA protein [10], we believe that further progress will depend on the isolation of axonal proteins from peripheral nerve.

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