STUDY ON THE IMMUNOLOGICAL CROSSREACTIVITY OF NEUROFILAMENT POLYPEPTIDES IN AXONAL PREPARATIONS OF BOVINE BRAIN

Doris DAHL

Harvard Medical School, Department of Neuropathology, and West Roxbury Veterans Administration Medical Center, Spinal Cord Injury Research Laboratory, 1400 VFW Parkview, Boston, MA 02132, USA

Received 28 November 1979

1. Introduction

Following the original observations [1], it is now generally accepted that a triplet of polypeptides at ~200 000, 150 000 and 70 000 mol. wt constitute the mammalian neurofilament [2–4]. The discrepancy between data derived from brain and peripheral nerve filaments has been solved to the effect that a major ~50 000 mol. wt polypeptide in CNS axons prepared by a standard procedure [5] is GFA protein and due to glial fibers copurifying with nerve fibers in this preparation [6]. GFA protein is the subunit of astroglial filaments as indicated by in vitro assembly–disassembly experiments [7]. Peripheral nerves do not contain astroglia.

While the protein composition of the mammalian neurofilament thus appears relatively well established, the relation between the polypeptides in the triplet is still debated. This is an important issue because if they were found similar, their origin from a common precursor should be considered. In effect, preliminary data from one laboratory suggest this to be the case, leading to the hypothesis that the members of the triplet are derived one from another by cellular processing [3]. On the other hand the peptide maps obtained by cyanogen bromide cleavage [8] and by limited proteolysis [9] did not suggest the existance of homologies between the proteins of the triplet. We now present evidence indicating the lack of immunological crossreactivity between the ~50 k and ~70 k mol. wt polypeptides of the triplet and suggesting that the ~200 k and ~150 k mol. wt components are immunologically related.

2. Experimental

2.1. *Preparation of demyelinated axons from bovine brain*

Demyelinated axons were prepared from frozen bovine white matter as in [6].

2.2. *SDS–PAGE*

SDS gel electrophoresis at 7.5% acrylamide done following [10] in 0.1 M sodium phosphate buffer. The sample containing 1% SDS, 1.5% dithiothreitol, 8% sucrose and bromphenol blue were heated for 3 min in a boiling water bath.

2.3. *Elution of non-stained bands from SDS–polyacrylamide gels of axonal preparations*

Elution of non-stained bands for injection into rabbits was done as in [8]. The frozen gels from 23 electrophoretic runs (18 gels/run) were cut and extracted 3 times in distilled water by homogenization and centrifugation at room temperature (200 × g for 10 min). The extracts were combined and freeze-dried. For the absorption of the antisera, non-stained bands from 90 gels were extracted with 9 ml distilled water by homogenization and centrifugation at 200 × g at room temperature. The supernatants were used for absorption of the antisera following dialysis overnight against 3 changes of 0.05 M sodium phosphate buffer (pH 8.0) and centrifugation at 4°C at 12 000 × g. In some experiments the supernatants were concentrated to 3 ml by dialysis against 30% polyethylene glycol (Sigma, mol. wt 6000). All solutions contained 0.1% sodium azide as a preservative.

2.4. *Preparation and absorption of the antisera*

Five rabbits were injected with 4 M urea extracts of the whole axonal preparations. The antigen was boiled with 1% SDS in 0.05 M sodium phosphate buffer and emulsified with an equal volume of complete Freund's adjuvant. The first injection was into the 4 footpads while successive boosters were on several sites in the back. The total amount of protein used for immunization varied from 1.2–22 mg. Eight rabbits were immunized with antigen eluted from the 5 bands identified in fig.1 (2 rabbits/band; bands 1 and 2 were injected together). The antigen from 345 gels was dissolved in 2 ml 0.05 M sodium phosphate buffer (pH 8.0) containing 1% SDS, heated in a boiling water bath for 5 min, emulsified with equal vol. Freund's adjuvant and injected into the footpads. This was repeated the 2 following days subcutaneously in the back. For each band, Freund's complete adjuvant was used for all 3 injections in one rabbit, while in the second rabbit the adjuvant was used only for the first injection. No differences could be detected due to this variation in the immunization procedure. After 3 weeks the rabbits were again injected in the same way and bled 1 week afterwards. From then on each rabbit was bled ~35 ml every 10 days and injected with antigen extracted from 90 gels following each bleeding. Absorption experiments were conducted with diluted antisera as in [6]. The extent of dilution varied with the strength of the antisera. Antisera absorbed with extracts of SDS–polyacrylamide gels served as controls. Some non-specific decrease of the intensity of the staining occurred with concentrated extracts. Immunofluorescent staining of rat cerebellar cortex in acetone-fixed cryostat sections was the main assay to screen the antisera. Weak non-specific staining of axons and stellate astrocytes was sometimes observed in white matter especially at low dilutions with control sera. Anti-neurofilament activity (fig.2) was defined by the intense immunofluorescence of the baskets surrounding the Purkinje cells and by the staining of thin nerve fibers in the inner half of the molecular layer. The baskets are formed by axonal terminals abutting on Purkinje cells. They are characterized by a large content in neurofilaments. No staining was seen in the outer half of the molecular layer. The thin parallel fibers in this location contain mainly neurotubules. Anti-GFA activity (fig.3) was characterized by the staining of Bergmann glial fibers perpendicular to the surface in the molecular layer. The staining was more intense in the outer half of this layer and formed a glial membrane underlying the leptomeninges. The glial fibers scattered in the Purkinje cell layer could be easily differentiated from the thicker baskets demonstrated by the neurofilament antisera. A combination of the neurofilament and GFA patterns was seen with mixed antisera.

3. Results

Fig.1 shows the band pattern of an axonal preparation resolved on SDS–polyacrylamide gel electrophoresis. Two bands were present in the range of the upper component of the neurofilament triplet at ~200 k mol. wt (bands 1,2). These 2 bands were injected together in the rabbits and separately eluted for absorption experiments. Band 3 (~150 k) and band 4 (~70 k) are the other 2 components of the triplet. Band 5 (~50 k) is to a large extent GFA protein [6]. As reported in [8], bands 3–5 were stable and retained their original mobility when re-electrophoresed. Conversely bands 1,2 were often found degraded to smaller components under these conditions.

The results of the immunization and absorption experiments are summarized in table 1. As to the

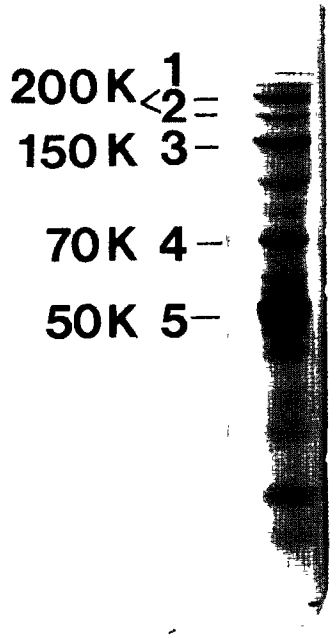

Fig.1. SDS–acrylamide gel electrophoresis of the axonal preparation used for the reported experiments. Bands 1–5 were extracted from the gels for immunization of the rabbits and for absorption of the antisera.

Table 1
Characterization by immunofluorescence of brain filament proteins in axonal preparations of bovine white matter

Antisera[a]	Staining and titer[b]	Residual staining after absorption[a]						
		Axonal prep. (μg/ml)	Human GFA (μg/ml)	Band 1	Band 2	Band 3	Band 4	Band 5
Brain filaments	NF 1:40 GFA 1:40	None (300)	NF (150)	NF GFA	GFA	GFA	NF GFA	NF
Band 1 and 2 ~200 k mol. wt	NF 1:4	None (100)	NF (500)	NF	None	None	NF	NF
Band 3 ~150 k mol. wt	NF 1:20	None (25)	NF (500)	NF	None	None	NF	NF
Band 4 ~70 k mol wt	no staining							
Band 5 ~50 k mol. wt	GFA 1:20	None (300)	None (50)	GFA	GFA	GFA	GFA	None

[a] See fig.1 for identification of bands
[b] Highest dilution of the antiserum allowing bright immunofluorescent staining

Abbreviations: NF, neurofilament staining; GFA, gliofilament staining

absorption experiments, the same results were obtained with concentrated and non-concentrated band extracts, band 2 excepted. Absorption only occurred with concentrated extracts. The 5 rabbits injected with 4 M urea extracts of the axonal preparation produced GFA antisera. Two of these rabbits also produced neurofilament antisera. The immunofluorescent patterns illustrated in fig.2 (neurofilaments) and fig.3 (gliofilaments) were thus combined in the same histological section (fig.4). The gliofilament component was absorbed by human GFA [11] and band 5; the neurofilament component by bands 2,3. The schedule which resulted in the production of mixed antisera was as follows: 8–10 mg protein for the first injection followed by 2 mg boosters in the back every 10 days starting at day 21. The immunological activity was already detectable on day 21 and peaked after 5–6 weeks. Neurofilament antisera comparable in strength to those obtained with the axonal preparation were produced by the rabbits immunized with band 3. The antiserum was absorbed by its own antigen and by band 2. It was not absorbed by bands 1,4,5. Neurofilament antisera were also obtained in rabbits injected with bands 1,2. However, they were weak compared with the other neurofilament antisera as indicated by their immunofluorescence titer. They were absorbed by bands 2,3, but not by bands 1,4,5. The 2 rabbits immunized with band 4 were negative, while those injected with band 5 produced GFA anti-

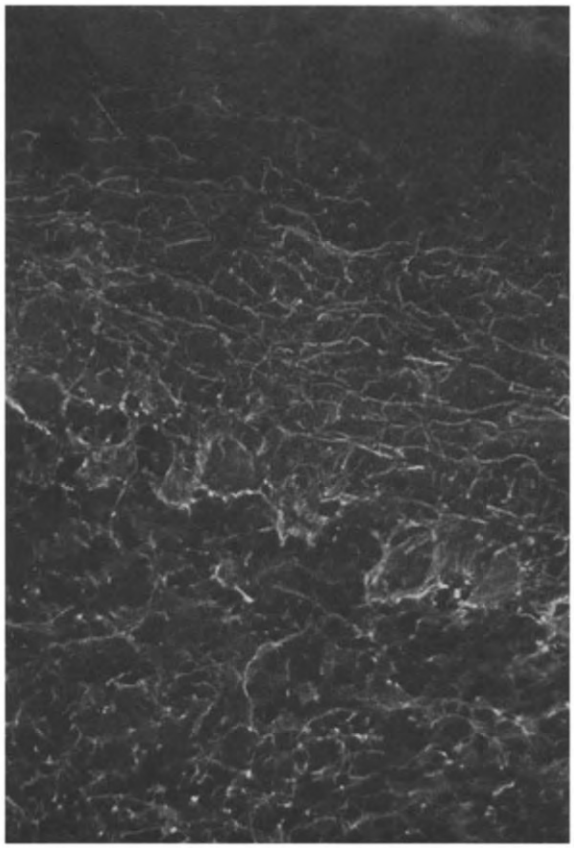

Fig.2. Immunofluorescent staining of the rat cerebellar cortex with neurofilament antisera × 233.

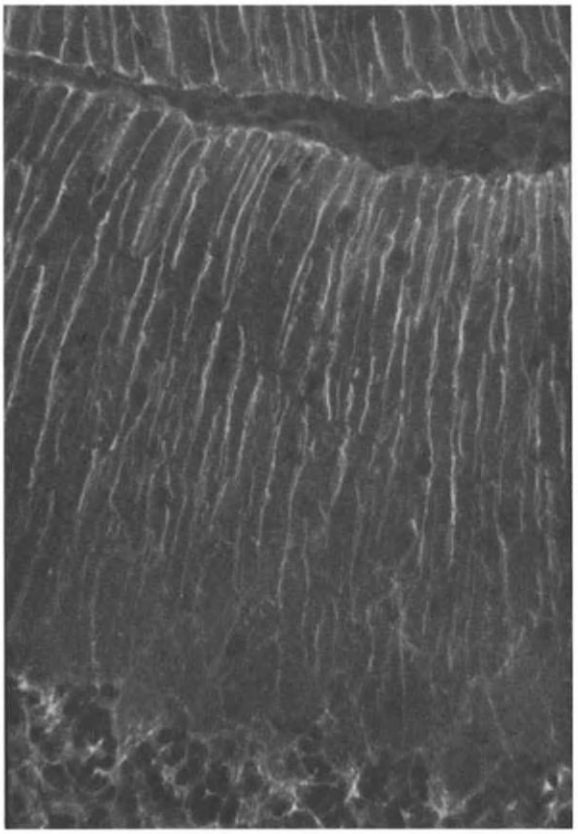

Fig.3. Immunofluorescent staining of the rat cerebellar cortex with GFA antisera × 233.

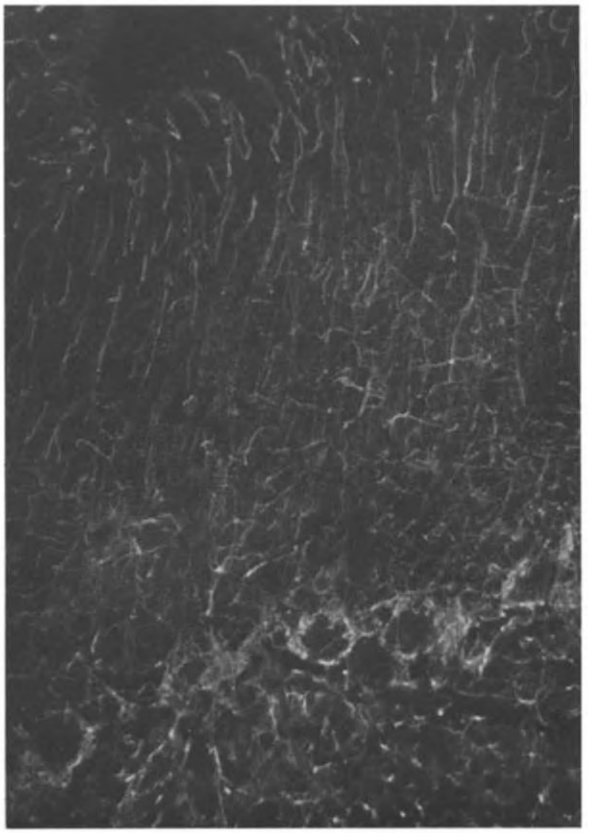

Fig.4. Immunofluorescent staining of the rat cerebellar cortex with mixed antisera (GFA plus neurofilament antisera) × 233.

sera. It should be noted that weak glial staining was also seen with rabbits injected with the other bands of the axonal preparation. This was probably due to trailing of the highly immunogenic GFA protein since the glial activity was absorbed by GFA protein. Neurofilament and GFA antisera were also absorbed by 4 M urea extracts of the axonal preparation. Incubation with trypsin (0.1 mg/ml) at 37°C for 1 h followed by boiling completely destroyed the antigens in the preparation as indicated by absorption experiments. Boiling alone had no effect on the antigenicity. Extraction with 20 vol. chloroform–methanol (2:1, v/v) had no effect on the antigens.

4. Discussion

In cryostat sections of rat cerebellum the neurofilament antisera prepared against the ~150 k mol. wt band of the axonal preparation had the same immunohistological specificity compared with the neurofilament antisera raised with urea-soluble antigen isolated by hydroxyapatite chromatography from chicken brain [12]. The main difference was the strength of the antisera, an important consideration if the antisera are to be used for immunoaffinity chromatography. Chicken neurofilament antisera could be used up to 1:160 dilutions in immunofluorescence tests on tissue sections. With the ~150 k mol. wt antisera dilutions >1:20 resulted in weak immunofluorescent staining. The chicken neurofilament antisera coupled to Sepharose have recently allowed the isolation of the ~70 k mol. wt polypeptide from bovine brain axonal preparations [13]. The other components of the neurofilament triplet were absorbed to the column but could be eluted in 5 M urea at pH 6.5 together with other proteins, while the ~70 k mol. wt polypeptide

was the only protein eluted at pH 2.5.

The data derived from the chicken neurofilament antisera were thus not conclusive with respect to the immunological cross-reactivity of the proteins contributing the neurofilament triplet. They could be interpreted as indicating some degree of crossreactivity [3], differences in immunogenicity between the components of the triplet, or non-specific attachment to the column of the ~200 k and ~150 k mol. wt polypeptides. However, our data indicate that the ~150 k mol. wt component possess antigenic determinants which are not shared by the ~70 k mol. wt member of the triplet. The antisera to the ~150 k mol. wt band were completely absorbed by its own antigen, while absorption with the ~70 k mol. wt band did not affect the intensity of the staining. The antisera were also absorbed by one of the bands in the 200 k mol. wt range. An attractive interpretation of these findings is that the high molecular weight component of the neurofilament triplet generates the 2 other components, with no sharing of antigenic determinants between the ~150 k and ~70 k mol. wt chains. However, the data indicating the immunological relatedness of the ~200 k and ~150 k mol. wt polypeptides in axonal preparations of bovine brain should be interpreted with caution. Trailing of proteins could represent a major problem close to the origin of the gel. The cyanogen bromide peptide maps did not support a structural relationship between the ~200 k and ~150 k mol. wt polypeptides [8]. One possibility is that the differences in sequence in the related peptides were too great for the cyanogen bromide fragments to retain similar mobilities. In addition, it should be noted that protein extracted from the ~200 k mol. wt bands appeared degraded when re-electrophoresed on SDS–polyacrylamide gels. Another point for discussion concerns the production of neurofilament antisera without recourse to the cumbersome procedure of cutting bands for immunization. Strong antisera can be obtained with chicken brain antigen [12]. This paper shows that neurofilament antisera with the same specificity as those directed against the ~150 k component of the triplet can be obtained with 4 M urea extracts of the axonal preparation. These antisera also display GFA activity but this can be absorbed with GFA isolated by hydroxyapatite chromatography from human autopsy material [11] or from bovine spinal cord autolyzed in vitro [14].

Acknowledgements

The technical assistance of Carol Crosby and Sandra Zapatka is gratefully acknowledged. I also wish to thank Dr Amico Bignami for his advice in immunohistology. This work was supported by USPHS grant NS 13034 and by the Veterans Administration.

References

[1] Hoffman, P. N. and Lasek, R. J. (1975) J. Cell Biol. 66, 351–366.
[2] Anderton, B. H., Ayers, M. and Thorpe, R. (1978) FEBS Lett. 96, 159–163.
[3] Liem, R. K. H., Yen, S.-H., Salomon, G. D. and Shelanski, M. L (1978) J. Cell Biol. 79, 637–645.
[4] Schlaepfer, W. W. and Freeman, L. A. (1978) J. Cell Biol. 78, 653–662.
[5] Schook, W. J. and Norton, W. T. (1976) Brain Res. 118, 517–522.
[6] Dahl, D. and Bignami, A. (1979) Biochim. Biophys. Acta 578, 305–316.
[7] Rueger, D. C., Huston, J. S., Dahl, D. and Bignami, A. (1980) J. Mol. Biol. in press.
[8] Dahl, D. (1979) FEBS Lett. 103, 144–147.
[9] Thorpe, R., Delacourte, A., Ayers, M., Bullock, C. and Anderton, B. H. (1979) Biochem. J. 181, 275–284.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[11] Dahl, D. and Bignami, A. (1976) Brain Res. 116, 150–157.
[12] Dahl, D. and Bignami, A. (1977) J. Comp. Neurol. 176, 645–657.
[13] Dahl, D. (1980) Biochim. Biophys. Acta in press.
[14] Bignami, A., Dahl, D. and Rueger, D. C. (1980) in: Advances in Cellular Neurobiology (Fedoroff, S. and Hertz, L. eds) vol. 1, Academic Press, New York, in press.