# DEMONSTRATION OF A PROTEIN IMMUNOCHEMICALLY RELATED TO GLIAL FIBRILLARY ACIDIC PROTEIN IN HUMAN FIBROBLASTS IN CULTURE

Elisabeth BOCK[☆], Søren RASMUSSEN[☆], Morten MØLLER* and Peter EBBESEN[+]

[☆]*The Protein Laboratory, University of Copenhagen, 34 Sigurdsgade, DK-2200 Copenhagen N,* **Anatomy Dept. B, University of Copenhagen, 1, Universitetsparken, DK-2100 Copenhagen Ø* and [+]*Institute of Medical Microbiology, Tumorvirology Dept., 22, Juliane Mariesvej, DK-2100 Copenhagen Ø, Denmark*

Received 13 September 1977

## 1. Introduction

The glial filament, 8–10 nm in diameter, is morphologically similar to the neurofilament and the intermediate filament found in a variety of cells and tissues [1–7]. A soluble protein has been isolated from gliotic areas from brain [8]. Antisera prepared against this protein give apparently specific glial staining by indirect immunofluorescence methods [9], and the protein has therefore been termed the glial fibrillary acidic protein (GFA). It has been proposed that this protein was the subunit of the glial filament. Recent comparative studies of GFA and neurofilament proteins [10], and the isolation of a GFA-like protein from peripheral nerves, where glial fibres are not present [11] indicated similarity of the protein subunit of glial and nerve filaments. This finding strongly suggests the possibility that cytofilament in other non-neural cells might also be composed of similar protein subunits. The characteristics of the neurofilament protein and GFA differ very much from those of actin and of tubulin and, therefore, encourages the consideration of intermediate filaments as a separate class of organelles on a biochemical as well as a morphological basis. In this study we report the demonstration of a protein immunochemically related to GFA present in human fibroblasts.

## 2. Materials and methods

### 2.1. *Purification of GFA*

GFA was purified from normal human brain obtained at autopsy approximately 8 h post mortem using hydroxylapatite chromatography as described by Dahl and Bignami [12]. The purified protein was tested by means of sodium dodecylsulphate polyacrylamide gel electrophoresis, using a gradient gel (PAA 4/30, Pharmacia Fine Chemicals). The GFA preparation consisted of 4 polypeptides in the range from 55 000–40 000 daltons.

### 2.2. *Fibroblast cultures*

Whole skin biopsies were obtained from healthy adults. One mm pieces were seeded in plastic flasks in Modified Eagles Medium with 10% fetal calf serum and kept at 37°C, 5% $CO_2$. After 6 weeks the bottom was covered with fibroblasts whereas other cell types had died off. Secondary and tertiary cultures were established after loosening of the cells with trypsin. For extraction of antigens secondary and tertiary cultures derived from three different individuals were used. The nutrient medium was removed and the cultures washed twice in 10 ml phosphate buffered saline (PBS, pH 7.4, 37°C), and loosened by a rubber policeman. The antigens were extracted with 0.05 M sodium phosphate buffer, pH 8.0 after a first extraction of the cell suspension with 0.01 M sodium phosphate/0.01 M $MgCl_2$ buffer, pH 6.2 (extract A). The suspension was centrifuged at 40 000 × *g* for 30 min. The supernatant was studied by crossed immunoelectrophoresis and used for absorption of anti-GFA activity. In some experiments the cell suspension was extracted by the phosphate buffer, pH 8.0, directly and used for crossed immunoelectrophoresis (extract B).

2.3. *Antiserum against GFA*

Human brain GFA isolated from multiple sclerosis plaques was kindly supplied by Dr L. F. Eng, USA, and antiserum was raised in rabbits. The antiserum reacted with only one antigen when tested against extracts of human brain. This antigen was identified as GFA using purified GFA [13]. The specificity of the antiserum was shown to be identical to a GFA antiserum kindly supplied by Drs Dahl and Bignami, USA [13].

2.4. *Immunohistochemical staining*

Secondary and tertiary cultures were washed in PBS and fixed at 4°C for 30 min in 1% formaldehyde, freshly prepared from paraformaldehyde. They were thereafter sequentially incubated in 30 min steps in the following reagents: (1) anti-GFA rabbit antiserum (diluted 1:150 or 1:300); (2) peroxidase labelled swine anti-rabbit IgG (diluted 1:20). The dilutions were performed in phosphate buffered saline. Each step was followed by washing in phosphate-buffered saline for 3 × 5 min. By a 5–15 min reaction with a mixture containing 0.05% 3,3′-diaminobenzidinetetrahydrochloride and 0.005% hydrogen peroxyde in 0.05 M Tris–HCl buffer (pH 7.6), the peroxidase bound to the cells formed the brown insoluble reaction product. Control of the specificity of the staining was performed by omitting either anti-GFA rabbit antiserum or peroxidase labelled swine anti-rabbit IgG. Furthermore the specific anti-GFA was replaced by preimmune rabbit serum or anti-GFA antiserum absorbed with purified GFA. In order to avoid soluble antigen–antibody complexes in the absorbed antiserum the optimal ratio between GFA and anti-GFA was determined by Sewell titration [14].

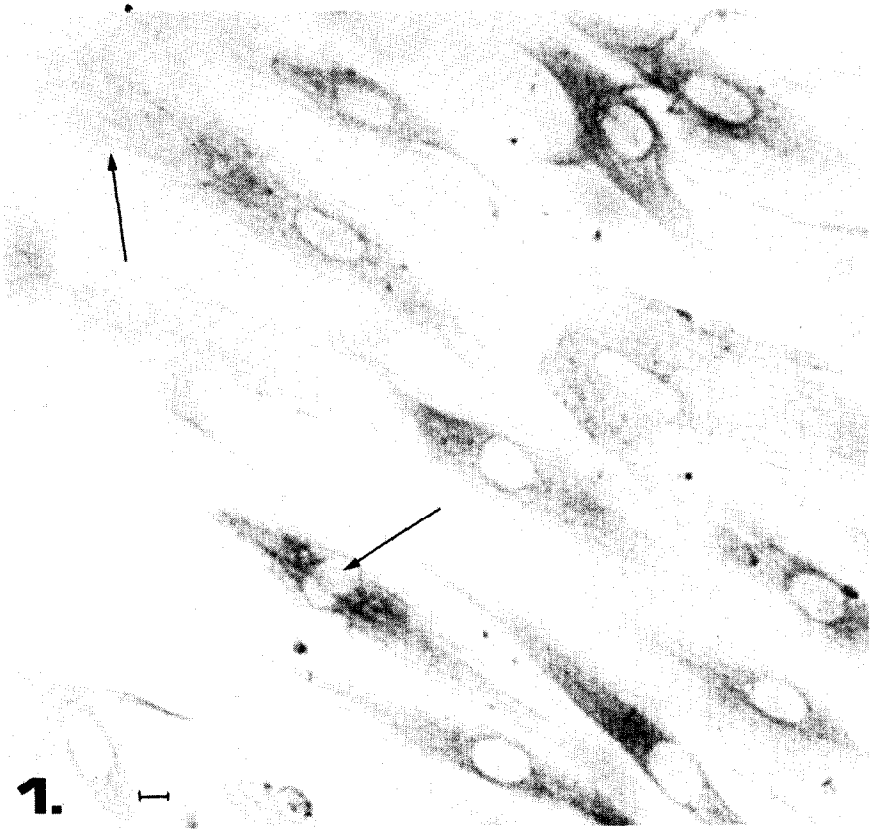


Fig.1. Immunohistochemical demonstration of GFA-positive filaments (arrows) in cultured fibroblasts. The indirect peroxidase-labelled immunoglobulin technique has been used for the demonstration. The bar represents 10 μm.

2.5. *Immunodiffusion test*

Extracts of fibroblasts were analysed by Ouchterlony's double diffusion test [15] in agarose gel for 24–48 h.

2.6. *Crossed immunoelectrophoresis*

This was performed as described by Weeke [16]. The antigens were separated by agarose gel electrophoresis (1st dimension electrophoresis). After having turned the electric field 90° the electrophoresis was continued into an antibody containing gel (2nd dimension electrophoresis). The antigen–antibody complexes precipitate in arches delimiting an area proportional to the amount of antigen and inversely proportional to the concentration of antibodies. GFA antiserum absorbed with fibroblast culture extract was tested by means of crossed immunoelectrophoresis with intermediate gel [17]. This is a modified crossed immunoelectrophoresis in which an intermediate gel containing the antiserum under investigation is placed between the 1st and the 2nd dimension gel. During the 2nd dimension electrophoresis GFA migrate through the intermediate gel and thereafter through the 2nd dimension gel. Depending upon the titre of the absorbed antiserum in the intermediate gel GFA will be completely, partially or not retarded by immunoprecipitation in the intermediate gel, indicating no absorption, partial absorption or complete absorption of the antiserum.

## 3. Results

By phase-contrast microscopy the cultivated fibroblasts were seen as bipolar cells with long extending processes. Using GFA antiserum all the cells in the cultures were stained by the immunohistochemical procedure, fig.1. The peroxidase reaction product was confined to a cytoplasmatic filamentous network. No staining of the nucleus was seen. When the fibroblasts were treated with preimmune control sera, no staining was observed, fig.2. Furthermore, the specific staining of the cells was completely abolished when antiserum absorbed with the purified human brain GFA protein was used in the immunohistochemical reaction. However, extracts of fibroblast cultures (extracts A and B)

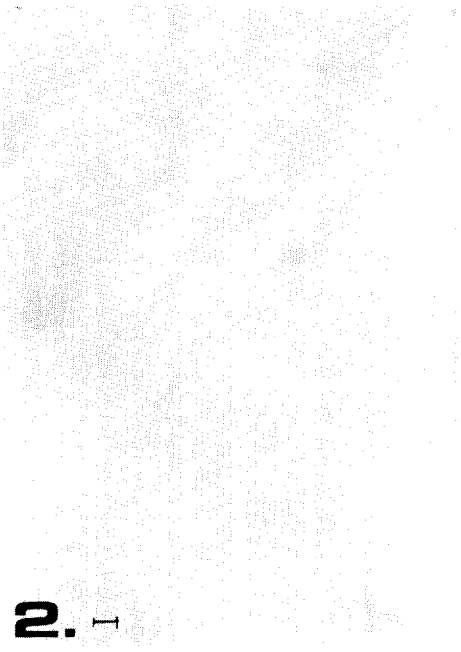


Fig.2. Control picture from cultured fibroblasts. Rabbit serum has been used in the immunohistochemical reaction instead of the specific GFA antiserum. The bar represents 10 μm.

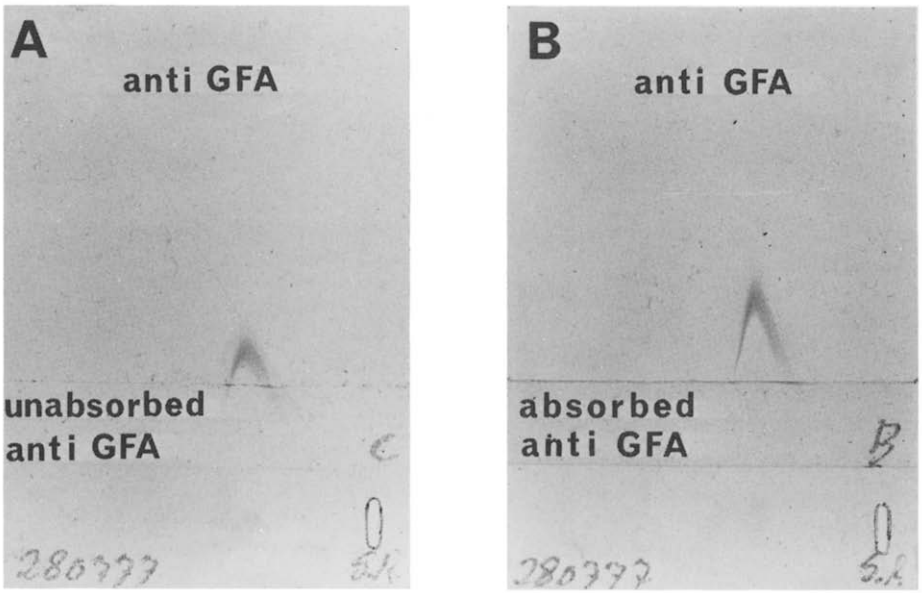


Fig.3. Crossed immunoelectrophoresis with intermediate gel of 0.33 μg purified human GFA. The reference antiserum in the upper gel was anti GFA (0.533 μl/cm²). The intermediate gel in fig.3A contained 200 μl consisting of 2 μl anti-GFA and 198 μl 20 mM Tris–barbital buffer pH 8.6. The intermediate gel in fig.3B contained 200 μl consisting of 2 μl anti-GFA and 198 μl fibroblast extract A corresponding to 102 μg protein. A pronounced decrease of anti GFA titre was seen after absorption with fibroblast extract. For explanation see Materials and methods.

were unable to form immunoprecipitate either by Ouchterlony's double diffusion test or crossed immunoelectrophoresis. When GFA antiserum was absorbed with fibroblast (extract A) a decrease in GFA antiserum titre was demonstrated by means of crossed immunoelectrophoresis with intermediate gel, see fig.3. This indicated that although the fibroblast extract did not contain an antigen which was able to form insoluble antigen/antibody precipitates with GFA antiserum, the fibroblast extract contained an antigen which was able to absorb some GFA-antibody activity. The general effect of the fibroblast extract on rabbit immunoglobulins was tested by addition of fibroblast extract A to a rabbit antiserum against human transferrin. No changes in antibody titre could be demonstrated in this control experiment.

## 4. Discussion

The results indicate that an antigen immunochemically partially identical to brain GFA is present in human fibroblasts, as demonstrated by an immunohistochemical procedure. The fibroblast antigen was able to bind GFA antibodies leading to a decreased titre of the GFA antiserum as demonstrated by crossed immunoelectrophoresis with intermediate gel. However, the antigen–antibody complexes seemed to be soluble as no immunoprecipitate was obtained by means of double diffusion technique or ordinary crossed immunoelectrophoresis. A possible explanation is that the extracted fibroblast antigen only share one or a few antigenic determinants with human brain GFA, as revealed by the present antiserum. Whether the fibroblast antigen is a part of the 8–10 nm filaments present in this cell type has to be verified by demonstration of specific binding of the antiserum at the ultrastructural level.

## Acknowledgements

The financial support by P. Carl Petersens Foundation and the Danish Cancer Society is gratefully acknowledged.

## References

[1] Shelanski, M. L., Yen, S.-H. and Lee, V. M. (1976) in: Cell Motility (Goldman, R., Pollard, T. and Rosenbaum, J. eds) pp. 1007–1020, Cold Spring Harbor Laboratory, NY.
[2] Cooke, P. H. and Chase, P. H. (1971) Exp. Cell Res. 66, 417–425.
[3] Uehara, Y., Campbell, G. R. and Burnstock, G. (1971) J. Cell Biol. 50, 484–497.
[4] Goldman, R. D. and Knipe, D. M. (1973) Cold Spring Harbor Symp. Quant. Biol. 37, 523–534.
[5] Cooke, P. (1976) J. Cell Biol. 68, 539–556.
[6] Ishikawa, H., Bischoff, R. and Holtzer, H. (1968) J. Cell Biol. 38, 538–555.
[7] Buckley, I. K. (1974) Tissue Cell 6, 1–20.
[8] Eng, L. F., Vanderhaeghen, J. J., Bignami, A. and Gerstl, B. (1971) Brain Res. 28, 351–354.
[9] Bignami, A., Eng, L. F., Dahl, D. and Uyeda, C. T. (1972) Brain Res. 43, 429–435.
[10] Yen, S.-H., Dahl, D., Schachner, M. and Shelanski, M. L. (1976) Proc. Natl. Acad. Sci. USA 73, 529–533.
[11] Dahl, D. and Bignami, A. (1976) FEBS Lett. 66, 281–284.
[12] Dahl, D. and Bignami, A. (1975) Biochim. Biophys. Acta 386, 41–51.
[13] Møller, M., Ingild, A. and Bock, E. (1977) Brain Res. in press.
[14] Sewell, M. M. H. (1967) Science Tools 14, 11–12.
[15] Ouchterlony, O. (1968) in: Handbook of Immunodiffusion and Immunoelectrophoretic Techniques, Ann Arbor Science Publishers, Ann Arbor, Mich.
[16] Weeke, B. (1973) in: A Manual of Quantitative Immunoelectrophoresis. Methods and Applications (Axelsen, N. H., Krøll, J. and Weeke, B. eds) pp. 47–56, Universitetsforlaget, Oslo.
[17] Axelsen, N. H. (1973) in: A Manual of Quantitative Immunoelectrophoresis Methods and Applications. (Axelsen, N. H. Krøll, J. and Weeke, B. eds) pp. 71–77, Universitetsforlaget, Oslo.