PARTIAL IDENTITY OF HEART PURKINJE FIBRE SKELETIN AND NEUROFILAMENT PROTEIN

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1. Introduction

During the last two years, a number of investigations have been focused on filaments which are intermediate in diameter as compared with thick and thin myofilaments. From a morphological point of view such filaments seem to represent a more or less homogenous class of organelles, and have usually been called '100 Å filaments' or 'intermediate filaments' for this reason. There is however no general agreement concerning their terminology (for refs. see [1]).

Recent investigations have pointed out the biochemical similarities of intermediate-sized filaments from several tissues, suggesting that the filaments comprise a separate class of filamentous proteins, intentionally called 'skeletins' (for refs. see [1]). Immunohistochemical investigations have strengthened this hypothesis, although differences have also been demonstrated (for refs. see table 1). These observed differences have suggested that intermediate filament subclasses exist, such as epithelial, myogenic, (nonmyogenic) mesenchymal and neurogenic subclasses [2,3].

We have recently developed a technique enabling us to isolate cow heart Purkinje fibre skeletin [4]. Antisera raised to this polypeptide specifically react to the skeletin filaments of heart conducting cells [5]. Such antisera provided an appropriate tool to further investigate and elucidate the problem of skeletin ubiquity. We report here the results obtained with cultured neuroblastoma cells, which indicate a partial immunological identity between skeletin and neurofilament protein. This indicates a greater complexity in filament protein classification than has been proposed previously.

2. Experimental

2.1. *Antisera*

Antisera against Purkinje fibre skeletin were produced in rabbits using skeletin purified by AcA-44 gel filtration in buffer containing sodium dodecylsulphate (SDS) as in [5]. The immunoglobulin fraction was obtained by ammonium sulphate precipitation and ion-exchange chromatography. Antiskeletin antibodies were absorbed by incubation and continous agitation of 0.5 ml diluted immunoglobulin fraction (1:10 in PBS) with 0.1 g partially purified heart Purkinje fibre skeletin for 30 min at +37°C. The resulting immune precipitate was removed by centrifugation (9000 × *g*, 15 min). Non-immune sera were obtained from Dako-Patts, Copenhagen.

2.2. *Neuroblastoma cells*

The murine neuroblastoma cell line C-1300 was cultivated in MEM supplemented with 10% fetal calf serum with antibiotics in humidified air with 5% CO_2. The cells were grown in 50 mm plastic petri dishes.

For double diffusion tests, cells were removed from the petri dishes mechanically by the use of a bent Pasteur pipette. Subsequently, cells were suspended in 500 μl 0.2% SDS containing buffer [6] and homogenized by ultrasound (Sonifier cell disruptor B-30 equipped with special microtip) for 5 min. The opalescent homogenate was directly applied in the double diffusion wells.

For indirect immunofluorescent staining the cells were grown on sterile glass cover slips in petri dishes Some cell populations were treated with vinblastine sulphate (10 μg/ml) for 4 h before processing for immunofluorescent staining

2 3 *Indirect immunofluorescence staining*

Cells grown on cover slips were rinsed in PBS, immersed in cold (−38°C) absolute acetone for 5 min and air-dried The cells were incubated with either rabbit antiskeletin (1 25–50 in PBS), absorbed antiskeletin, or non-immune serum for 1 h at +37°C in a humidified atmosphere Cover slips were washed in PBS, incubated with fluorescein-labeled goat anti-rabbit globulin (GARG, diluted 1 25 in PBS) and again washed in PBS Cells were finally mounted in PBS–glycerol Control studies were also performed with cells incubated in GARG alone Examination was performed in a Leitz Orthoplane light microscope using epifluorescent optics Photomicrographs were recorded on Tri-X 135 film

2 4 *Immunodiffusion tests*

Double diffusion tests were performed in 1% agarose (Behring-Werke) in Tris–barbitone buffer at pH 8 6 The antigens were applied in their wells in soluble form in 0 1% SDS containing buffer This discontinous detergent concentration has been found not to significantly change the precipitation pattern [6] The specificity of the antiskeletin antisera in the reaction with the neurofilament protein was checked by application of skeletin in the antiserum well 30 min prior to application of the antiserum Staining was performed with Coomassie brilliant blue

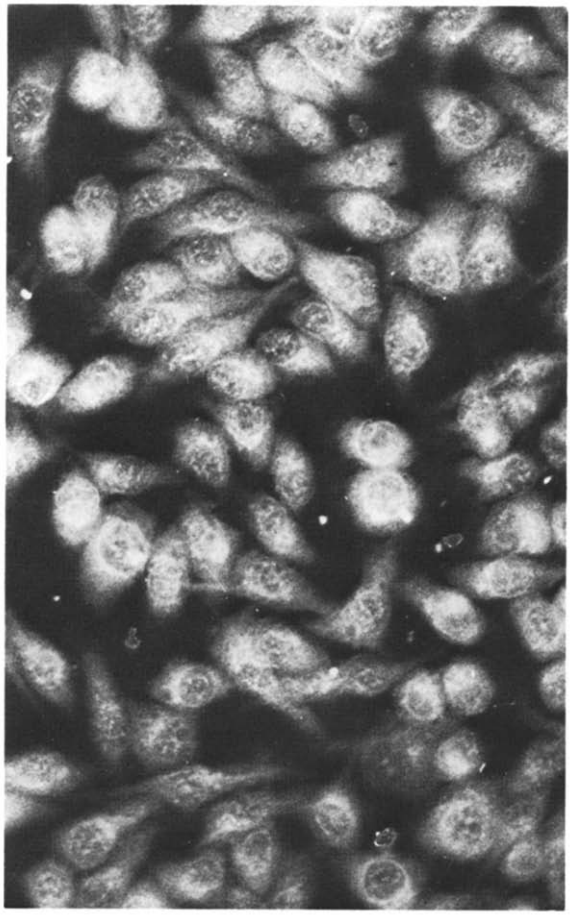

Fig 1 Neuroblastoma cells reacted with antiskeletin by the use of the indirect immunofluorescence technique Fluorescent antibodies are located mainly in the perinuclear region and in cell processes, but diffuse staining of the cytoplasm is also present × 400

3 Results

By phase-contrast microscopy the cultivated neuroblastoma cells were recognized as polygons with long extending processes Figure 1 shows that, in immunohistochemically treated preparations, the fluorescence was confined to a delicate network concentrated in the perinuclear region but also present in the cell periphery and projecting into the cell processes In vinblastine-treated cells, the staining was confined to perinuclear caps and intracytoplasmic coils as shown in fig 2 Tubulin paracrystals were readily identified by interference microscopy but did not show any fluorescence Control studies with non-immune and absorbed antisera gave no specific staining of caps or coils but only a faint diffuse non-specific staining of the cytoplasm

As shown in fig 3, homogenates of neuroblastoma cells formed a faint but discrete single immunoprecipitation line with rabbit anti-cow Purkinje fibre skeletin antibodies in immunodiffusion Prediffusion with skeletin prior to the application of the neuroblastoma cells significantly reduced the precipitate formation between neurofilament protein and antiskeletin A slight modification in the form of the precipitation

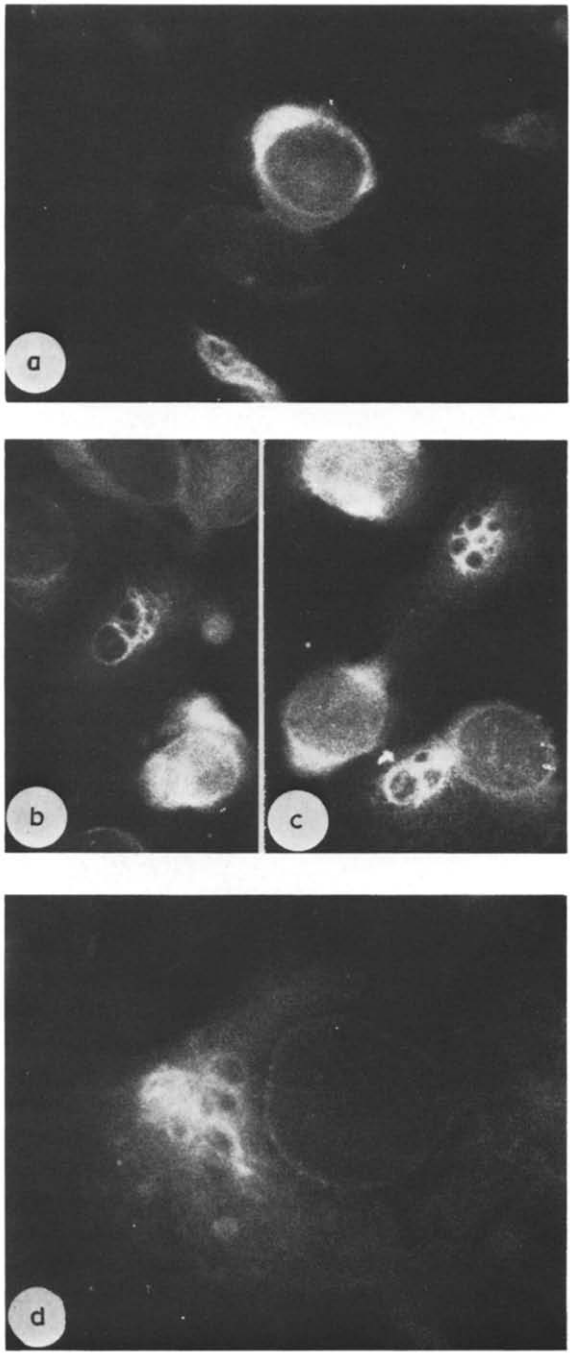

Fig.2. Indirect immunofluorescence of vinblastine-treated neuroblastoma cells. The specific formation of perinuclear caps and coils (a–d) is seen. In (d) the nuclear membrane is seen as a narrow but discrete fluorescent margin. (a–c) × 625. (d) × 1000.

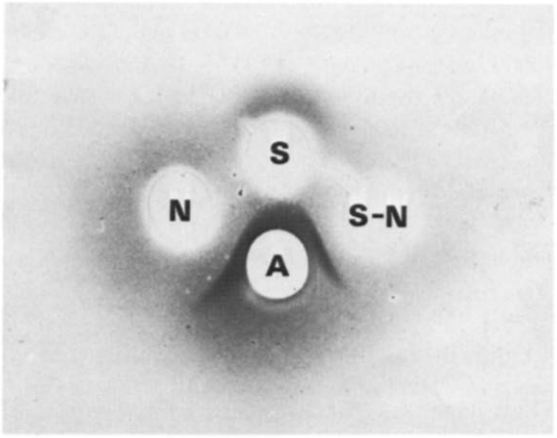

Fig.3. Immunodiffusion tests of antiskeletin and neuroblastoma cells. Fusion is seen between precipitation lines formed by antiskeletin (A) and neuroblastoma cells (N, S–N) and that formed by antiskeletin and skeletin (S). However, small modifications in the precipitation line are seen at the joint points. In other experiments, the tendency to spur formation was somewhat more pronounced. Prediffusion of skeletin in the neuroblastoma well (S–N) gives a marked reduction in the formation of precipitation as compared with that obtained in the absence of prediffusion (N). In this case the same amounts of neuroblastoma cells were applied in the two wells, N and S–N. If smaller amounts of neuroblastoma cells were applied, the formation of precipitates could be totally inhibited by skeletin prediffusion.

line was observed at the jointpoints of skeletin–antiskeletin and neurofilament protein–antiskeletin precipitates. Non-immune sera did not form precipitates with any of the filament preparations.

4. Discussion

The results presented indicate that an antigen immunochemically related to cow heart Purkinje fibre skeletin is present in detectable amounts in cultured murine neuroblastoma cells. By indirect immunofluorescence examination, the subcellular distribution of the fluorescent reaction product was similar to that of the intermediate-sized neurofilaments as observed by electron microscopy, i.e., in the perinuclear region and in the cell processes, parallel to the long axes of the processes (cf. [7]). The staining of intracytoplasmic coils after treatment with microtubule inhibitor

confirmed that the fluorescence was confined to the neurofilaments, since the formation of coils is specific for this class of filaments [8–10] A faint but discrete staining was also found along the nuclear membrane, compatible with electron microscopic observations of intermediate filaments in the vicinity of the nuclear membrane [9]

The appearance of the precipitation lines in the double diffusion tests indicates that the immunological similarity between the neuroblastoma and the heart Purkinje fibre skeletin is partial The ability of skeletin to reduce the reaction between antiskeletin and neurofilament protein as well as the modification of the precipitation line with a tendency to spur phenomena are compatible with the hypothesis that some determinants are shared between the two proteins but that they both contain specific structures It can not be excluded that this partial identity may depend on species differences The experiments also show that the antiskeletin antibodies are precipitating antibodies, in contrast to, e g , the antibodies to glial fibrillary acidic protein when reacted with cultured fibroblasts [11]

The present investigation is the first to show a partial immunological identity of muscular skeletin and neurofilament protein A review of previous immunological investigations on intermediate filament proteins is presented in table 1 From this table it is obvious that crossreactivity exists between filaments of different origin, but also that non-reactivity occurs, depending on tissue, species and possibly methodological differences Quite a few of these investigations have used antibodies to neurofilament protein and to glial fibrillary acidic protein, the latter claimed to be the integral part of the astroglial fila-

Table 1
A review of immunochemical investigations (references indicated by their arabic numerals) on intermediate filament relatedness using immune and autoimmune sera

Type of primary antigen		Target tissue: Epithelial	Mesenchymal	Muscular	Neuronal	Glial
Epithelial	+					
	–		[2,18,19]	[2]	[2,20]	[2]
Mesenchymal	+	[2,18]		[21,22]	[2,21–23]	[2,21,22]
	–	[21]		[2]		
Muscular	+	[24]	[5]		[24], this investigation	[22]
	–	[3,10]	[10,25]	[3][a]	[3,25]	[3,25]
Neuronal	+	[26]	[8,16,26]	[8,27]		[15,16,26, 28–32]
	–	[20]		[22]	[33][a]	[34,35]
Glial	+		[11]		[12,21,29–31]	
	–	[13,36,37]	[13,21,37]	[21,37]	[13,16,36–42]	[38,43][a]
Autogenic	+	[44,45]	[9,44–46]	[9,44,47]	[9,44,45]	[48]
	–					

+ = crossreactivity
– = nonreactivity

The investigations are arranged according to type of primary antigen and type of target tissue tested The table does not include papers restricted to target tissue of the same type as the primary antigen except when nonreactivity was demonstrated, which in all cases was due to species differences ([a])

ments. However, both these antigens have been critisized for impurities [12–16] and as the results obtained are indeed divergent, the character of these antigens must be considered doubtful. It is thus obvious that wider conclusions concerning skeletin ubiquity require additional investigations also using antibodies to filaments from other tissues. In such a recent study, evidence is presented for 4 immunologically different types of intermediate filament proteins, viz., epithelial, non-muscle mesenchymal, muscle mesenchymal and brain [2]. In another recent study, another division is suggested, viz., muscular, glial, neuronal and epidermal filament proteins [3]. Our results are not consistent with any of these proposed schemes as crossreactivity is demonstrated between muscular and neuronal filaments. Interestingly, this may be correlated with the neuronal properties of the conducting cells, though they are myogenic. The antiskeletin antibodies, however, also show crossreactivity to muscular filaments, e.g., to filaments in the vicinity of myocardial Z and intercalated disks and to the filamentous masses of myotubes in culture and in sectioned muscle tissue (in preparation). It is thus obvious that intermediate filament relatedness must be more complex than proposed in [2,3], and possibly two or more types of intermediate filaments occur in the same cell [17].

It is suggested that Purkinje fibre antiskeletin antibody can be used as a valuable tool for the further elucidation of the matter of filament protein relatedness, which at present obviously presents many questions.

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