THE NERVOUS SYSTEM SPECIFIC PROTEIN D2 IS INVOLVED IN ADHESION AMONG NEURITES FROM CULTURED RAT GANGLIA

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1. Introduction

The rat nervous system specific protein D2 is an integral membrane protein present on all bodies and neurites of cultured neurons from brain fetuses [1–3]. In adult brain, D2 is found mainly on the outside of synaptic membranes [4–6]. It has been hypothesized that this protein may be involved in the recognition mechanism during the early stage of synaptogenesis [5,6].

Here we have examined the effect of anti-D2 antibodies on the formation of neurite fascicles from cultured rat sympathetic ganglia. The results indicated that D2 may be involved in neurite–neurite interaction, a property which has been reported for the cell adhesion molecule (CAM) isolated from chick embryo neural tissue [7,8].

This property and similarities in molecular weight, membrane distribution and localization raise the possibility that D2 and CAM may be evolutionarily related proteins. This hypothesis is strengthened by the finding, that anti-CAM antibodies crossreact with D2 protein.

2. Materials and methods

The procedures for purification of CAM from chick embryo retina, production of anti-CAM antibodies in rabbits and preparation of monovalent $F_{ab'}$ fragments have been detailed in [7,9]. Immunized with excised D2 immunoprecipitates, monovalent antibodies against rat brain D2 were produced in rabbits [5]. The IgG-fraction and the $F_{ab'}$ fragments of the anti-D2 serum and of a serum from normal rabbits were prepared as described for anti-CAM [9]. Two different batches of anti-D2 were used.

Sympathetic ganglia were excised from day 17–19 rat fetuses. The ganglia were cultured in 35 mm NUNC tissue culture dishes as described for chick ganglia [10] in a medium containing 90% Dulbecco's modified Eagle medium, 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 5 U/ml nerve growth factor. After the ganglia had adhered to the dishes, antibodies and $F_{ab'}$ fragments solubilized in PBS (phosphate buffered saline) in a range of 0.03–0.3 g/l, or just PBS were added.

The ganglia were cultured for 48 h at 38°C in a humidified atmosphere containing 5% CO_2 and the outgrowing neurites were examined microscopically and photographed.

The sympathetic ganglia were washed in PBS and fixed in 3.7% formaldehyde in PBS for 30 min. Indirect labelling of the D2 molecules on the surfaces was initiated by incubation with anti-D2 IgG in PBS (1:50) for 30 min followed by fluorescein-conjugated sheep anti-rabbit IgG (1:100) (l'Institut Pasteur, Paris) [11]. Each step was preceeded by washing in PBS for at least 10 min. The labelled ganglia were embedded in glycerol and examined in a epifluorescence microscope.

The anti-CAM and the anti-D2 were compared by a modified line-immunoelectrophoresis [12] using a rabbit anti-rat brain synaptosomal membrane antiserum as reference. The immunoelectrophoresis was performed on a glass plate support in a gel containing 1% Agarose HSA (Litex, Denmark), 0.6% Triton X-100, 73 mM Tris, 24 mM barbital buffer at pH 8.6, and 10 KIE/ml aprotinin. Furthermore, the antibody gel (4 × 7 cm) contained 14 $\mu l/cm^2$ of a polyspecific rabbit antiserum against rat brain synaptic membranes

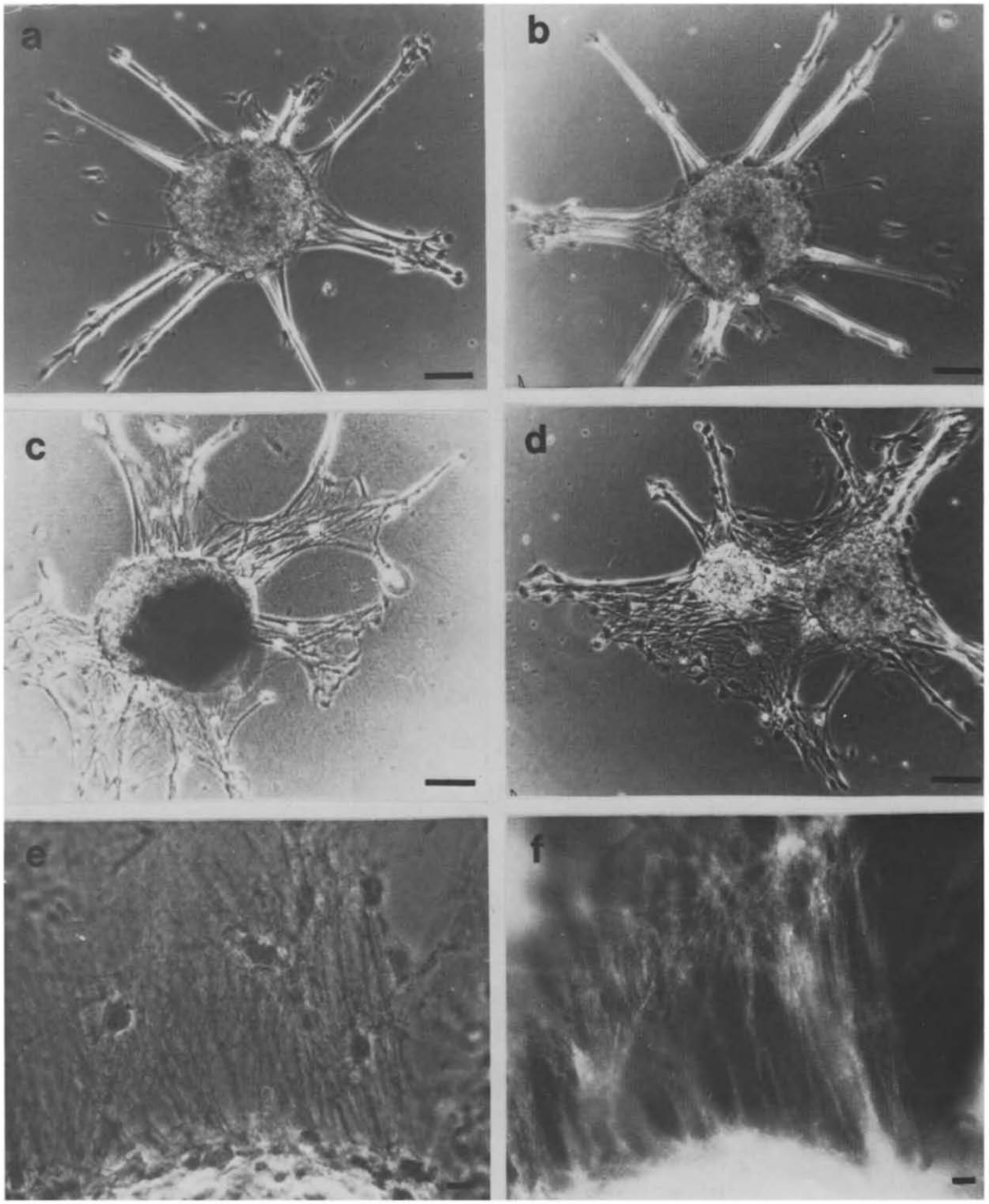

Fig.1. Phase contrast micrographs of cultured rat sympathetic ganglia. The ganglia were cultured in the presence of no addition (a), normal rabbit $F_{ab'}$ (0.3 g/l final concentration) (b), anti-D2 IgG (0.1 g/l) (c), and anti-D2 $F_{ab'}$ (0.03 g/l) (d). Neurites of an anti-D2 $F_{ab'}$-treated ganglion are shown in phase contrast in (e) and by epifluorescence in (f). The bars on a–d are 100 μM; the bars on e,f are 10 μm.

and the antigen gel (2 × 7 cm) contained 0.24 mg Triton X-100 solubilized rat brain membrane proteins. In the antigen gel was punched wells (1 × 0.2 cm) which contained the monospecific antibodies to be compared. Electrophoresis was run for 1 h at 1 V/cm, then overnight at 2.5 V/cm. The plate was then washed and stained by Coomassie brilliant blue R.

3. Results

After 48 h culture, fascicles of neurites extended from the rat sympathetic ganglia (fig.1a). Both anti-D2 IgG (fig.1c) and anti-D2 $F_{ab'}$ (fig.1d) decreased the number of fascicles of large diameter and increased the number of processes containing one or a few neurites. Similar effects were obtained over 0.03–0.3 mg/ml of two different batches of antibodies. However, in addition to inhibition of fascicle formation, we have observed that a dissociation of the ganglion occurred at 0.3 g/l of anti-D2 $F_{ab'}$, resulting in an outward cell migration. Addition of normal rabbit IgG or $F_{ab'}$ (fig.1b) was without effect and resembled the control (fig.1a). D2 is both present on the ganglion neurites in cultures made in the presence of normal rabbit $F_{ab'}$ and in the presence of anti-D2 $F_{ab'}$ (fig.1f).

Anti-CAM $F_{ab'}$ has been described to inhibit the formation of neurite fascicles of large diameter from chick embryo dorsal root ganglia [10] and therefore we investigated the crossreactivity between anti-CAM and rat D2. In order to detect crossreactivity of anti-CAM with only a few or even just a single antigenic site of D2 we used a modification of rocket immunoelectrophoresis [12,13] in which the anti-CAM was tested for its possible ability to inhibit the precipitation of rat brain synaptic membrane D2 by a polyspecific antiserum. A deviation, in casu dipping, of the line is taken as an indication of crossreactivity. As shown in fig.2, the D2-line dipped against the wells containing anti-D2 IgG. The much smaller dipping

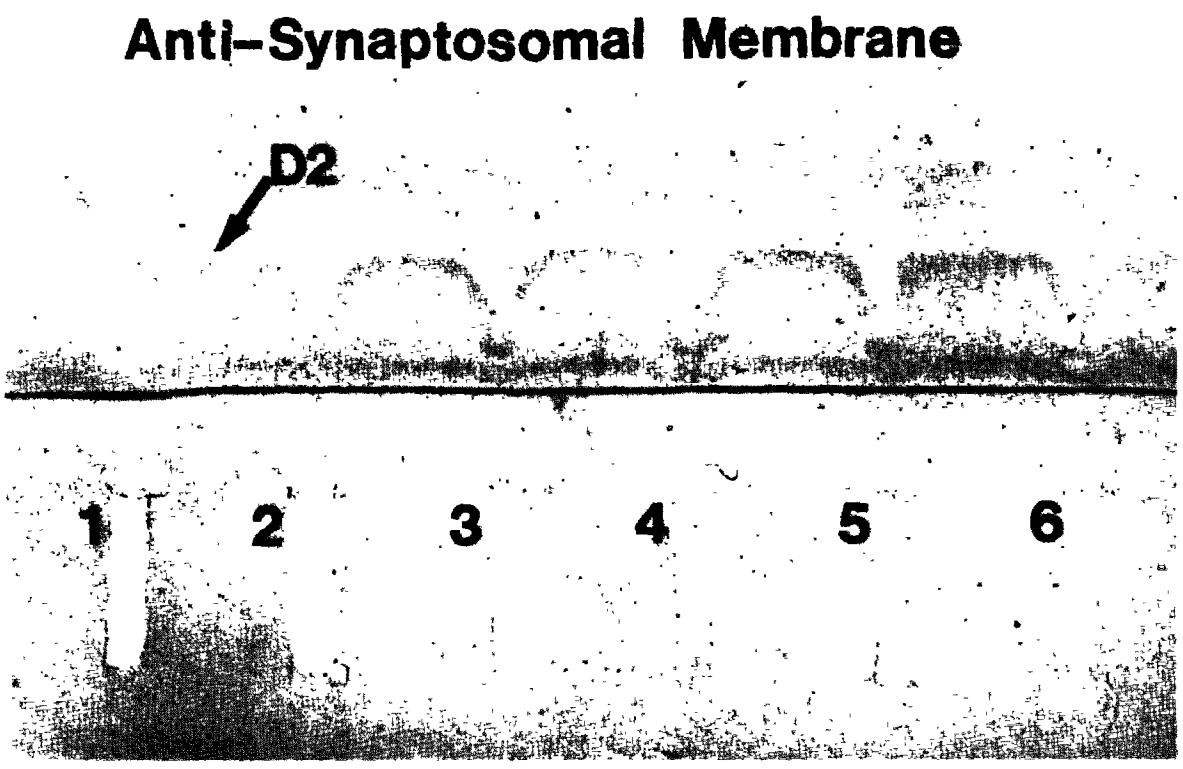

Fig.2. Immunoelectrophoretic comparison of anti-D2 IgG and anti-CAM IgG with anti-rat brain synaptosomal membrane in the antibody gel. The antigen gel, on the lower half of the figure contained solubilized rat brain membranes and 6 wells. In wells 1, 6 20 μl normal rabbit IgG were placed; in wells 2, 5, 20 μl anti-D2 IgG; in well 3, 8 μl anti-CAM IgG; in well 4, 20 μl anti-CAM IgG.

against the wells containing normal rabbit IgG was the result of simple dilution of rat D2 by the solution in the wells. Anti-CAM IgG also caused a dip in the rat D2-line. Neither antibodies disrupted the two other lines below the D2-line. These results therefore indicate a crossreactivity between D2 and anti-CAM.

4. Discussion

Like the nervous system specific protein CAM of the chick [7,8] the nervous system specific protein D2 of rat brain [1] appears to be implicated in neurite–neurite interaction. We found that anti-D2 antibodies and $F_{ab'}$ fragments of these antibodies inhibited the fasciculation of neurites in rat sympathetic ganglia in culture. Similar observation has been made in the case of chick embryo dorsal root ganglia grown in the presence of anti-CAM $F_{ab'}$. A dramatic decrease in the number of fascicles with a large diameter has been found accompanied by an increase in the number of processes composed of one or a few neurites [10].

Besides the inhibition of neurite fascicles, CAM from the chick and D2 from the rat display several similarities: The apparent molecular weight of the CAM polypeptide is 140 000 [7], that of D2 is 139 000 [14]. A soluble form of CAM is released from cultured retina into the medium [15] and a form of D2 is present in brain extracellular fluid [4]. Both proteins are found on the outside of neural plasma membranes all over the nervous system [5,6,8,16]. Furthermore, in the present study we have by immunoelectrophoresis found that anti-CAM crossreacts with rat D2. We suggest that chick CAM and rat D2 are related molecules occurring in different species. Definitive proof, however, must await detailed protein chemical characterization including peptide mapping, carbohydrate analysis, and amino acid sequence comparisons.

Moreover, because CAM has already been shown to be involved in the initial formation of cell–cell bonds, the similarities between CAM and D2 and the inhibition of neurite fascicles caused by anti-D2 antibodies support the hypothesis that D2 is associated with nerve membrane recognition.

Acknowledgements

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