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Promotion of neuronal survival in vitro by thermal proteins and poly(dicarboxylic)amino acids

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Evaluating molecules for their ability to promote survival and growth of neurons, we tested *thermal proteins* on cultures of dissociated fetal rat forebrain neurons (Thermal proteins are polyamino acids formed when mixtures of amino acids with minimal proportions of glutamic or aspartic acid are heated) Thermal proteins, added to low-density cultures in serum-free medium, stimulated neurite outgrowth and induced the formation of neuronal networks which survived for 6–10 days Neurons in control cultures failed to grow and degenerated completely within 2–4 days Effective concentrations (EC_{50}) of thermal proteins ranged from 3 to 100 μg/ml They were equally effective when present in the medium during the culture time or after precoating of the culture dishes A single preparation which contained only aspartic and glutamic acid was effective, and similar survival promoting actions were then found for polyglutamic acid and mixed polyamino acids containing glutamic or aspartic acid Thermal proteins and polyglutamic acid acted in a specific manner since, under the same experimental conditions, many control peptides, proteins and growth hormones failed to promote survival of neurons Furthermore, their effects were antagonized by heparin, but not heparan sulfate nor chondroitin sulfate These findings suggest that sequences of successive dicarboxylic amino acid residues are able to promote survival and neurite elongation of cultured neurons and that such sequences are responsible for the survival promoting action of thermal proteins They invite the speculation that sequences of successive dicarboxylic amino acids, which occur in many proteins and show a high degree of evolutionary conservation, may have functional role in molecular recognition processes during neuronal development

INTRODUCTION

Evaluating various substances for their ability to stimulate survival, growth and differentiation of neurons, a group of compounds called *thermal proteins* were tested These thermal proteins are complex polyamino acids formed abiotically and are believed to be similar to proteins that arose during protobiotic evolution[16,17,43] They are formed when mixtures of amino acids including aspartic acid or glutamic acid are warmed in the presence of small proportions of water[17] Most of the thermal proteins display functions similar to those of modern proteins Some were shown to exhibit enzymatic activities and to assemble into spheroidal structures containing membranes[17]

MATERIALS AND METHODS

Cell cultures

Cultures were prepared from dissociated fetal forebrain cells taken at embryonic day E16–E17 Pregnant rats (Wistar) were anesthetized with Nembutal, the fetuses were removed with forceps and transferred into cold sterile Dulbecco's phosphate buffered saline (PBS) The forebrain was dissected by cutting between diencephalon and mesencephalon, transferred into a culture dish containing dissociation medium (minimal essential medium), and cut into pieces of less than 1 mm^3 with a scalpel Dissected pieces were then transferred into sterile tubes The dissociation medium was removed by aspiration and 1 ml of a trypsin solution (1% trypsin (Gibco) in PBS) and 1 ml of a DNAse solution (0 1% DNAse (type 1, Sigma)) were pipetted into the tubes They then were incubated for 15 min at 37 °C The supernatant was removed after incubation, and the tissue pieces were washed two times with dissociation medium After the final wash, 5 ml of dissociation medium and 0 1 ml of 0 1% DNAse solution were added to the tissue pieces Cells were then dissociated by mild trituration with a fire-polished Pasteur pipet Cells were pelleted at 200 g (5 min), supernatants were discarded and the dissociated cells were resuspended by mild trituration in 1–2 ml of growth medium (cf below) Cells were counted in a hemocytometer (using Trypan blue exclusion as criterion for viability) and plated in previously prepared culture dishes of 16 mm diameter normally at a plating density of 50,000 cells/cm^2 (100,000 cells/well) Culture dishes normally were coated with polylysine (Sigma, 0 1 mg/ml in 0 15 M sodium borate buffer pH 8 3) In some experiments, polylysine was replaced by polyethyl-

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eneimine (PEI, 1 mg/ml in 0 15 M sodium borate buffer pH 8 3) which was earlier shown to be equally effective as culture substrate as polylysine[39] After coating, dishes were washed twice with PBS, and growth medium was then added to them The growth medium consisted of a modified L-15 medium[21] It was prepared by adding to basal L-15 medium (purchased from Gibco) 11 3 g/liter glucose, 1 1 g/liter glutamine, 5 mg/liter β-alanine, 15 mg/liter aspartic acid, 15 mg/liter cystine, 15 mg/liter glutamic acid, 5 mg/liter *p*-aminobenzoic acid, 51 mg/liter ascorbic acid, 10 mg/liter choline chloride, 25 mg/liter fumaric acid, 3 mg/liter glutathione, 60 mg/liter imidazole, 10 mg/liter inositol, 0 5 mg/liter α-lipoic acid and 2 mg/liter vitamin B_{12} Eighty ml of this modified L-15 medium was mixed with 20 mol of 0 15 M $NaHCO_3$ Cells were grown at 37 °C in 5% CO_2

Thermal proteins

Thermal proteins were initially dissolved in 0 1 M NaOH (10 mg/ml) These solutions were then immediately neutralized by adding an equal amount of 0 1 M HCl This solution was further diluted in culture medium and thermal proteins were added to the culture at final concentrations of 1–1000 μg/ml For most of the studies the thermal protein, TP9, produced by heating equimolar proportions of glutamic acid, aspartic acid, and tryptophan was used TP9 was prepared by grinding 0 3 mol amounts of each amino acid together and heating them under N_2 at 170 °C for 2 h and at 200 °C for 4 h After being cooled, the solid polymer was slurried with 500 ml of distilled water and the slurry was filtered The resulting precipitate was dried Other thermal proteins were prepared in a similar way TP9 was hydrolyzed for analysis with methanesulfonic acid according to Simpson et al [45] An aliquot sample contained 192 nmol Asp, 169 nmol Glu, and 120 nmol Trp, corresponding to a ratio of 1 0 88 0 62 as compared to the ratio of these amino acids in the mixture heated (1 1 1) References to proportions of amino acids, as given in Fig 6, denote proportions of amino acids in the mixture heated when preparing the thermal proteins, not the proportion of amino acids in the resulting polymer

The synthesis of thermal proteins can be done in many variations Temperatures below 200 °C are normally used Similar results are obtained by heating an amino acid mixture for 4 h at 180 or at 60 °C for 2 weeks The vast majority of the backbone bonds in thermal proteins are peptide bonds Small proportions of pigments formed during the thermal polycondensation of amino acids have been found to consist of flavin and pterin Although L amino acids are normally used as starting materials the use of DL amino acids instead of L forms makes little or no difference because of racemization during the polymerization process The thermal proteins formed exhibit limited heterogeneity Their molecular weights typically vary from 1000 to 10,000 Da in various preparations The term thermal proteins is synonymous with 'proteinoid' and 'thermal copolyamino acids' For detailed discussion of thermal proteins, see refs 16, 17 and 44

Cell counting

Cultures were analyzed every day by microscopical analysis (phase contrast microscopy using a Leitz Labovert microscope) For counting of surviving cells, cultures were washed with PBS and the number of cells were assessed by counting several visual fields corresponding together to 5–10% of the total area In time course studies, neuronal survival was first counted 2 h after plating (day 0) The total number of surviving cells at this time normally ranged from 20 to 40% of the number counted immediately after trituration Normally, no attempt was made to exclude non-neuronal cells during counting, since by identifying neuronal cells with neurofilament immunocytochemistry in representative cultures we found that neurons comprised more than 95% of all cells in these cultures (see Fig 5)

Measurement of protein content

High density cultures (800,000 cells/16 mm dish) were washed with PBS and homogenized by sonication in 250 μl of a 50 mM Tris-HCl buffer, pH 6 0, containing 0 1% Triton X-100 Aliquots of the homogenate were taken for the measurement of protein according to the method of using bovine γ-globulin (Bio-Rad) as standard[8]

Attachment assay

Culture dishes were precoated with polylysine Half of the dishes then received an additional coating of TP9 (100 μg/ml, dissolved in medium, applied for 2 h) After TP9 coating, dishes were washed, and medium without TP9 was added to them A total of 100,000 dissociated rat forebrain cells per culture dish were then plated One hour after plating, the number of cells adhering to the culture dish was counted in cultures not receiving any further treatment (before wash) and in cultures washed 3 times with PBS (after wash)

Immunocytochemistry

Cultures were fixed with a mixture of 3 parts of acetone and two parts of ethanol For visualization of glial fibrillary acid protein (GFAP) they then were incubated for 12 h at 4 °C with PBS containing 5% bovine serum albumin, 5% sucrose, 0 1% Triton X-100, and a 1 500 dilution of rabbit antiserum to human GFAP (Dakopatts) and normal goat serum (1 100) For neurofilament immunocytochemistry, the incubation solution contained a monoclonal anti-neurofilament antibody (RT 97 1 1000 ascites fluid) and rabbit serum (1 100) Dishes were then stained by the successive addition of biotin-conjugated secondary antibodies, a complex of avidin and biotinylated peroxidase, and diaminobenzidine (Vactastain) Washes between steps were done with PBS

Materials

Chemicals of analytical grade were purchased from Sigma, if not otherwise stated Media and sera for tissue cultures were obtained from Gibco Mouse nerve growth factor (NGF) was purified from adult mouse submandibular glands Protein S-100β was a gift from Dr D R Marshak Submaxillary gland epidermal growth factor (EGF) and bovine pancreas insulin were purchased from Sigma Human recombinant basic fibroblast growth factor (bFGF) was a gift from DuPont de Nemours Co (Wilmington, DE) Cell-tak was purchased from BioPolymers, Inc (Farmington, CT) The RS/1 data analysis system (BBN Software Products, Cambridge, MA) was used for statistical analysis

RESULTS

Initial observations

In pilot experiments, a selected group of thermal proteins were added to cultures of dissociated forebrain cells plated at high density and in serum-containing medium, i e at standard conditions in our laboratory to grow cultures of dissociated septal and mesencephalic neurons[21,40] Thermal proteins were found not to change the visual appearance of these cultures, however, some of the thermal protein preparations produced a mild elevation in the protein content of the cultures These tentative findings prompted us to test thermal proteins under less favorable culture conditions and it was found that, in serum-free medium, some of the thermal proteins reliably elevated the protein content of cultures of dissociated forebrain cells We then initiated a detailed study of trophic effects of TP9, the thermal protein most efficient in elevating the protein content of cultures TP9 was prepared from equimolar amounts of aspartic acid, glutamic acid, and tryptophan (see Materials and Methods)

Characterization of neurotrophic actions of TP9

TP9 stimulated survival and growth of dissociated forebrain neurons in culture This action was highly dependent on the initial plating density When plated at densities less than 400,000 cells/16 mm dish, dissociated neurons failed to develop in serum-free modified L-15 medium Under these control conditions, all cells failed to show significant neurite outgrowth and degenerated completely within 2–4 days At these low plating densities, the addition of TP9 resulted in cellular survival and neurite outgrowth, and produced well-developed cultures viable for 6–10 days (Fig 1) At higher plating densities (>400,000 cells/dish), neurons survived and developed in control medium, however, the addition of TP9 resulted in a higher number of surviving cells counted in phase contrast microscopy The extent of this stimulation decreased with increasing plating density The finding that dissociated neurons require high plating densities or the presence of a trophic molecule for survival in culture is in agreement with earlier findings by other authors[5]

Given the strong influence of plating density, further experiments were carried out at low density (100,000 plated cells/dish) At this plating density, most neurons added to dishes with control medium degenerated shortly after plating Some of the cells extended fibers during the first 2 days, however, they all degenerated within 4 days after plating The addition of TP9 supported the survival of approximately 25% of the originally plated cells and promoted elongation of fibers in these cultures (Figs 2 and 3) After 4 days, when control cultures had completely degenerated, the number of surviving cells was only slightly reduced and all surviving cells had numerous fibers The vast majority of the cells appeared to be neurons, since they had spherical cell bodies and thin fibers of uniform diameter Using immunocytochemical visualization to identify neurons, we found that neurons constituted at least 95% of the cells in these cultures (see below) Four days after plating, the number of surviving cells started to decline gradually in treated cultures, and complete degeneration occurred 8–10 days after plating

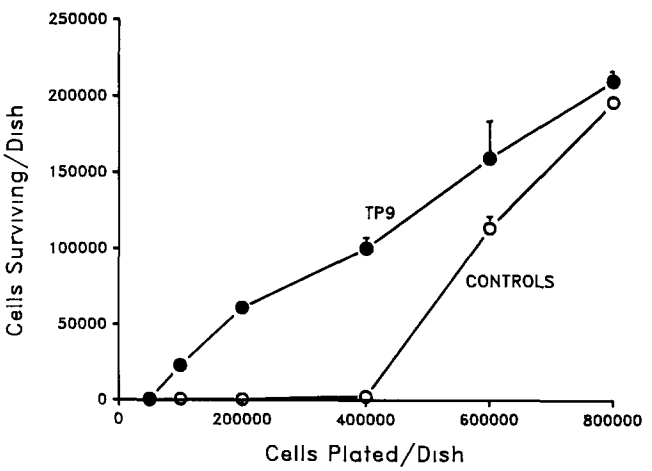
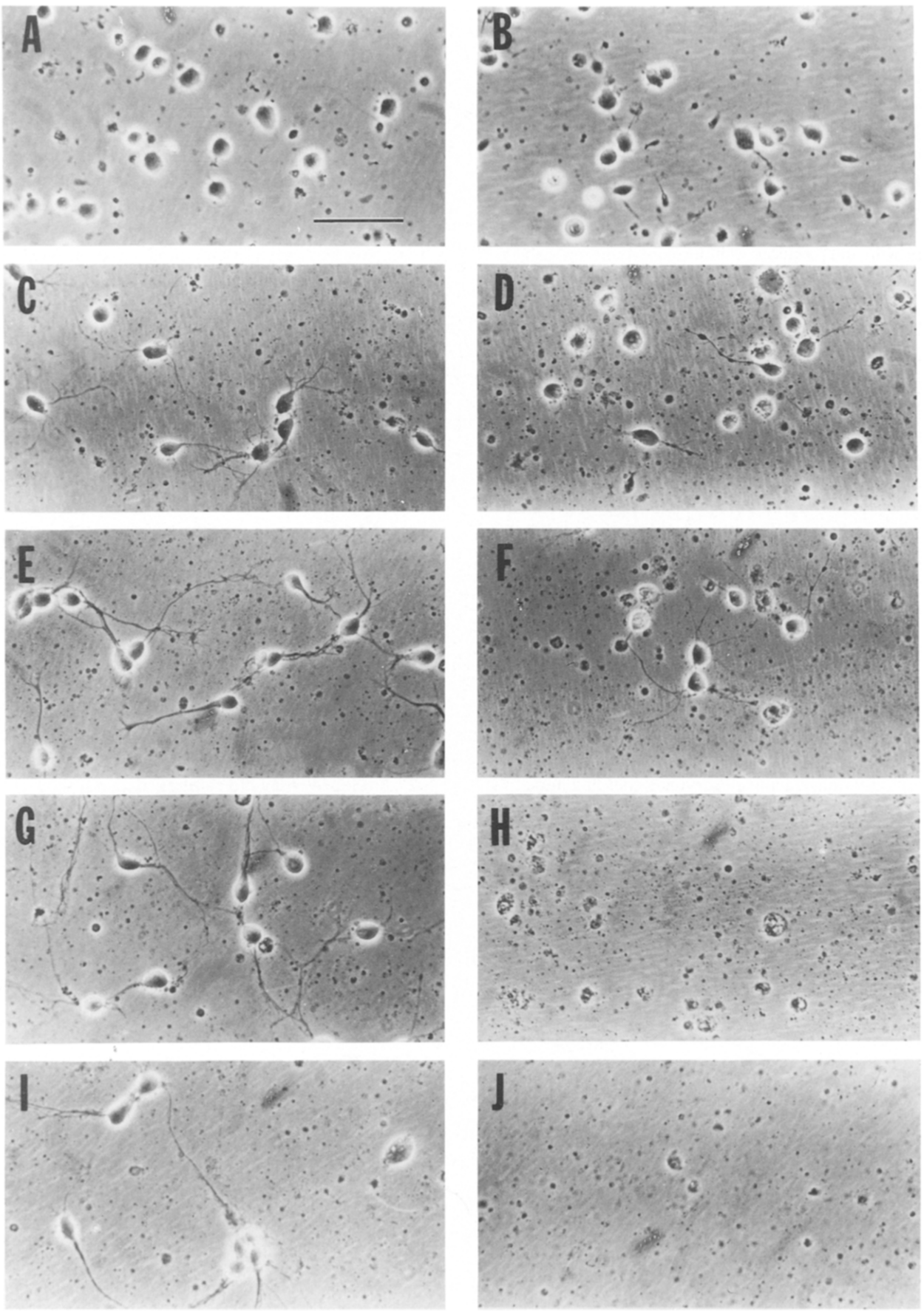


Fig 1 Promotion of neuronal survival by TP9 at various plating densities Dissociated fetal rat forebrain neurons were plated in 16-mm dishes precoated with polylysine and containing modified L-15 medium TP9 (50 μg/ml) was present during the entire culture time Four days after plating, the number of surviving cells was counted in phase contrast microscopy Values represent means ± S E M of 5–10 culture dishes

The survival promoting effect of TP9 was evident over a wide range of culture conditions Similar results were obtained when poly-L-lysine, which was normally used for coating of the dishes, was replaced by poly-D-lysine or by PEI, a polymer shown earlier to support survival of cultured neurons[39] The survival promoting effects of TP9 required precoating of culture dishes with a cationic polymer, since cells failed to survive when plated in culture dishes not subjected to pretreatment and in medium containing TP9 TP9 was equally effective in promoting neuronal survival in our modified L-15 medium and in DMEM medium However, no survival-promoting activity was observed in cultures grown in Ham's F-10 medium or in Barrett's N5 medium[25] Addition of TP9 to N5 medium resulted in precipitation of amorphous material Similar survival-promoting actions were observed in cultures of dissociated cells from brain areas other than the forebrain and from the spinal cord (data not shown)

Effective doses of TP9 ranged from 10 to 100 μg/ml of culture medium (Fig 4) Concentrations equal to or higher than 300 μg/ml were cytotoxic, as indicated by the fact that such concentrations resulted in degeneration of fully developed 6-day-old cultures (data not shown) This toxicity was mediated by TP9 itself, since the carrier solution failed to exert comparable toxic actions Two identical syntheses of TP9 were used for the studies, results obtained with the two preparations were also identical

To test whether TP9 was able to promote neuronal survival after binding to the surface of culture dishes, L-15 medium containing TP9 was added to dishes precoated with polylysine After 2 h, this medium was removed, the dishes washed with PBS, and L-15 medium without TP9 was then added Such TP9-coated dishes supported an equal number of surviving neurons as dishes in which TP9 was present during the growth of the neurons (Fig 4) This finding suggested that TP9 acted, at least in part, by providing an adequate substrate for neuronal survival and growth However, the survival promoting effect of TP9 was not due to differential attachment of neurons to the surface The cell attachment assay used, which tests for the ability of cells to attach to the substrate 1 h after plating, failed to show a difference between dishes coated with polylysine alone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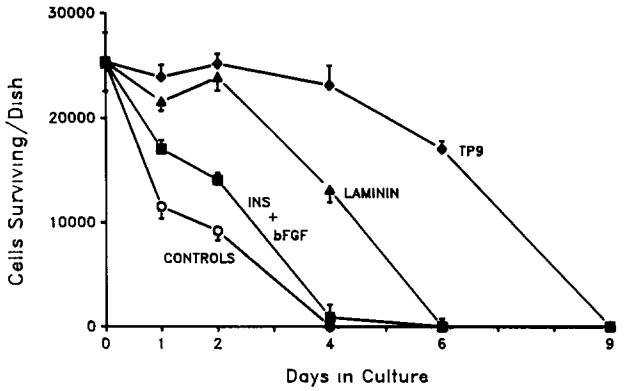

Fig 3 Time course of survival of dissociated cells in the presence of TP9, laminin, and bFGF + insulin Dissociated forebrain cells were plated at a density of 100,000 cells/culture dish Dishes were precoated with polylysine TP9 (100 μg/ml), laminin (10 μg/ml), or insulin (5 μg/ml) plus bFGF (100 ng/ml) were added to the medium and were present during the entire culture time Values represent means ± S E M of 5–10 culture dishes

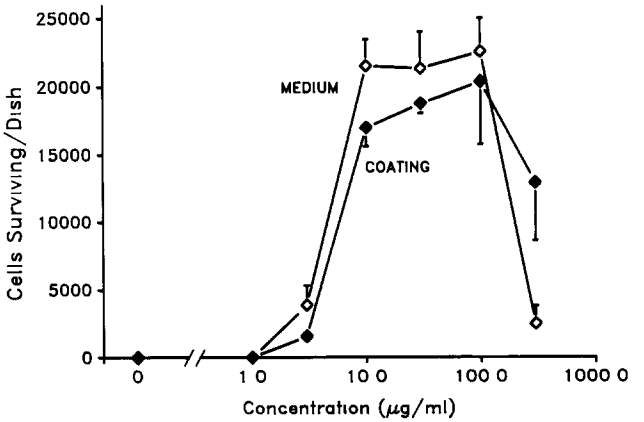

Fig 4 Dose–response curve of effect of TP9 and effect of precoating of culture substrate A total of 100,000 dissociated forebrain cells were plated per 16-mm dish grown during 4 days TP9 was either present during the entire culture time or dishes were coated with this compound For coating, culture dishes were precoated with polylysine and then exposed for 2 h to growth medium containing the indicated concentrations TP9 Thereafter, dishes were rinsed 3 times with normal growth medium and fresh medium without any additions was added to the dishes Values represent means ± S E M of 5–10 culture dishes

and dishes receiving an additional coating with PT9 (Table I)

Specificity of action

The survival-promoting effect of TP9 was not due to the presence of free amino acids, which may limit growth or stimulate neurons by acting on transmitter receptors[30], since the individual components of TP9 aspartic acid, glutamic acid and tryptophan, when added alone or together, failed to promote the survival of dissociated cells under our culture conditions (Table II) Chemically related molecules or short peptides containing aspartic acid or glutamic acid were ineffective This was also true for peptides and proteins with a high percentage of dicarboxylic amino acids (fibrinopeptide B, δ-sleep inducing peptide, cholecystokinin, mellitin)

Serum is well known to strongly enhance survival of dissociated neurons in culture[19 25] Accordingly, the addition of fetal bovine serum to our culture system improved neuronal survival (Table II) However, cultures grown in the presence of serum, in contrast to those grown in presence of TP9, contained a large proportion of flat non-neuronal cells We used antibodies that specifically bind to neurofilaments or GFAP to identify neurons and astrocytes, respectively (Fig 5) This study confirmed that, in cultures grown in presence of TP9, less than 5% of the cells were non-neuronal In contrast, in cultures grown in the presence of fetal calf serum, astrocytes proliferated rapidly and, after 4 days of growth, represented approximately one-third of the total number of cells These findings suggest that TP9 is unique inasmuch as its effects are not identical to those of serum However, the data are compatible with the view that TP9 promotes neuronal survival by mechanisms similar to those of some of the effective ingredients of serum

Like TP9 and related polymers, gangliosides contain lipophilic and hydrophilic moieties in the same molecule[4] Since gangliosides were reported to promote neuronal survival and fiber elongation[11,14 46,47], we compared the effects of TP9 with those of gangliosides in our culture system The ganglioside GM_1 failed to promote neuronal survival in the cultures used (Table II) suggesting that the mechanism of action of thermal proteins is unrelated to that of gangliosides

Additional compounds reported to promote survival of cultured neurons are catalase[57,58], transferrin[2], and ACTH analogs[54] None of these compounds was effective in our culture system (Table III) The addition of high concentrations of K^+, which is well documented to prolong neuronal survival in some culture systems[42,48] was also ineffective

Several naturally occurring neurotrophic factors pro-

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Fig 2 Promotion of survival of dissociated fetal rat forebrain cells by TP9 Neurons were plated at a density of 100,000 cell/16-mm culture dish in a serum-free modified L-15 medium Left panels cultures grown in the presence of TP9 (50 μg/ml) Right panels control A, B dissociated forebrain neurons immediately after plating C, D cultures grown for 1 day in medium containing TP9 and controls, respectively E, F cultures grown for 2 days G, H cultures grown for 4 days I, J cultures grown for 6 days Bar represents 50 μm

mote survival of certain population neurons in culture These include NGF[51], ciliary neurotrophic factor[33 43], and brain-derived neurotrophic-factor[6] Furthermore, general neurotrophic effects on populations of CNS neurons have been reported for a subfraction of serum proteins[25], insulin[2,38], EGF[35,36], and bFGF[35,36 58 59] In our cultures, insulin, NGF and EGF were ineffective (Table II) bFGF alone was ineffective, however, when combined with insulin, resulted in a significant elevation of the number of surviving neurons (Table II and Fig 3) Our findings corroborate those of Morrison et al [35,36] and Walicke[58,59], who showed that bFGF, when added to serum-free 'N2' medium of Bottenstein and Sato[7] containing a large concentration of insulin, promotes neuronal survival

Besides being affected by soluble neurotrophic factors, neuronal survival and fiber elongation are influenced by proteins forming the substrate for neuronal attachment These compounds include laminin, fibronectin, and collagen, which are all constituents of the basal lamina[22] [34] In our culture system, laminin produced a similar effect to TP9 (Table II and Fig 3) Fibronectin was marginally effective, collagen was ineffective when tested under our culture conditions

Identification of the active principle

The presence of aspartic acid or glutamic acid is required for the formation of thermal proteins Besides TP9, which was prepared from aspartic acid, glutamic acid, and tryptophan, several other thermal protein preparations containing other amino acids instead of tryptophan were effective in promoting neuronal survival However, in several cases, preparations starting from identical original amino acid compositions but polymerized using different conditions yielded effective or ineffective polymers A polymer derived from tryptophan alone was ineffective, whereas one of the prepara-

TABLE I

Effect of TP9 on adhesion of dissociated forebrain neurons

Culture dishes were precoated with polylysine Half of the dishes then received an additional coating of TP9 (100 μg/ml, dissolved in medium, applied for 2 h) Thereafter, dishes were washed, medium without TP9 was added to them, and 100,000 dissociated rat forebrain cells were plated per culture dish One hour after plating, the number of cells adhering to the culture dish was counted in cultures not receiving any further treatment (before wash) and in cultures washed 3 times with PBS (after wash) Numbers indicate means ± S E M of 8 dishes per group

	Cells/dish
Before wash	
controls	28,900 ± 2,200
TP9 coating	35,300 ± 1,600
After wash	
controls	26,600 ± 1,300
TP9 coating	30,800 ± 800

TABLE II

Comparison of TP9 with chemically related compounds, natural growth and adhesion factors and other compounds reported to promote neuronal survival of cultured neurons

A total of 100,000 cells were plated in PEI-coated dishes Compounds were added to the medium and were present during the entire culture time Surviving neurons were counted 4 days after plating

	Cells/dish (% of TP9)
TP9 (100 μg/ml)	100%[a]
Free amino acids	
Aspartic acid (3–300 μg/ml)	0
Glutamic acid (3–300 μg/ml)	0
Tryptophan (3–300 μg/ml)	0
Asp + Glu + Trp (3–300 μg/ml each)	0
Peptides and proteins with high content of dicarboxylic amino acids	
D-Glu-Gly (1–300 μg/ml)	0
α-Asp-Phe (1–300 μg/ml)	0
Arg-Gly-Asp-Ser (10–100 μg/ml)	0
L-Aspartic acid-α,β-di-t-butylester (3–300 μg/ml)	0
Human fibrinopeptide B (10–100 μg/ml)	0
δ-Sleep inducing peptide (3–300 μg/ml)	0
Cholecystokinin (100 μg/ml)	0
Mellitin (100 μg/ml)	0
Additions promoting neuronal survival in vitro	
Fetal bovine serum 1%	72 0±4 4%
Fetal bovine serum 10%	66 0±5 4%
Catalase (50 μg/ml)	0
Transferrin (50 μg/ml)	0
KCl (25 mM)	0
Ganglioside GM_1 (10^{-7}–10^{-5} M)	0
ACTH	0
$ACTH_{(4-10)}$	0
'N2' additions[b]	0
Growth and adhesion factors	
Insulin (1–50 μg/ml)	0
bFGF (3–30 μg/ml)	0
Insulin (5 μg/ml) + bFGF (100 ng/ml)[c]	2 4±1 6%
EGF (1–10 μg/ml)	0
NGF (0 1–10 μg/ml)[d]	0
Protein S-100β[e] (0 001–1 μg/ml)	0
Collagen[f]	0
Laminin (10 μg/ml)	68 5±23 9%
Fibronectin (100 μg/ml)	4 5±1 3%

[a] The average number of surviving neurons in TP9-treated control cultures was 23,100 ± 9300 (mean ± S D , $n = 10$)

[b] N2 additions according to ref 7 0 5 mg/liter insulin, 10 mg/liter transferrin, 1 6 mg/liter putrescine, 0 63 μg/liter progesterone, 0 52 μg/liter sodium selenite

[c] The survival promoting action of insulin + bFGF was more pronounced 1–3 days after plating (see Fig 3)

[d] NGF-responsive forebrain cholinergic neurons represent a very minor percentage of the plated neurons making it unlikely that a selective survival effect on this population can be detected

[e] Neurite promoting factor

[f] 1 mg/ml collagen (Sigma) was dissolved in 0 1% acetic acid, 100 μl of this solution was dried on the culture surface

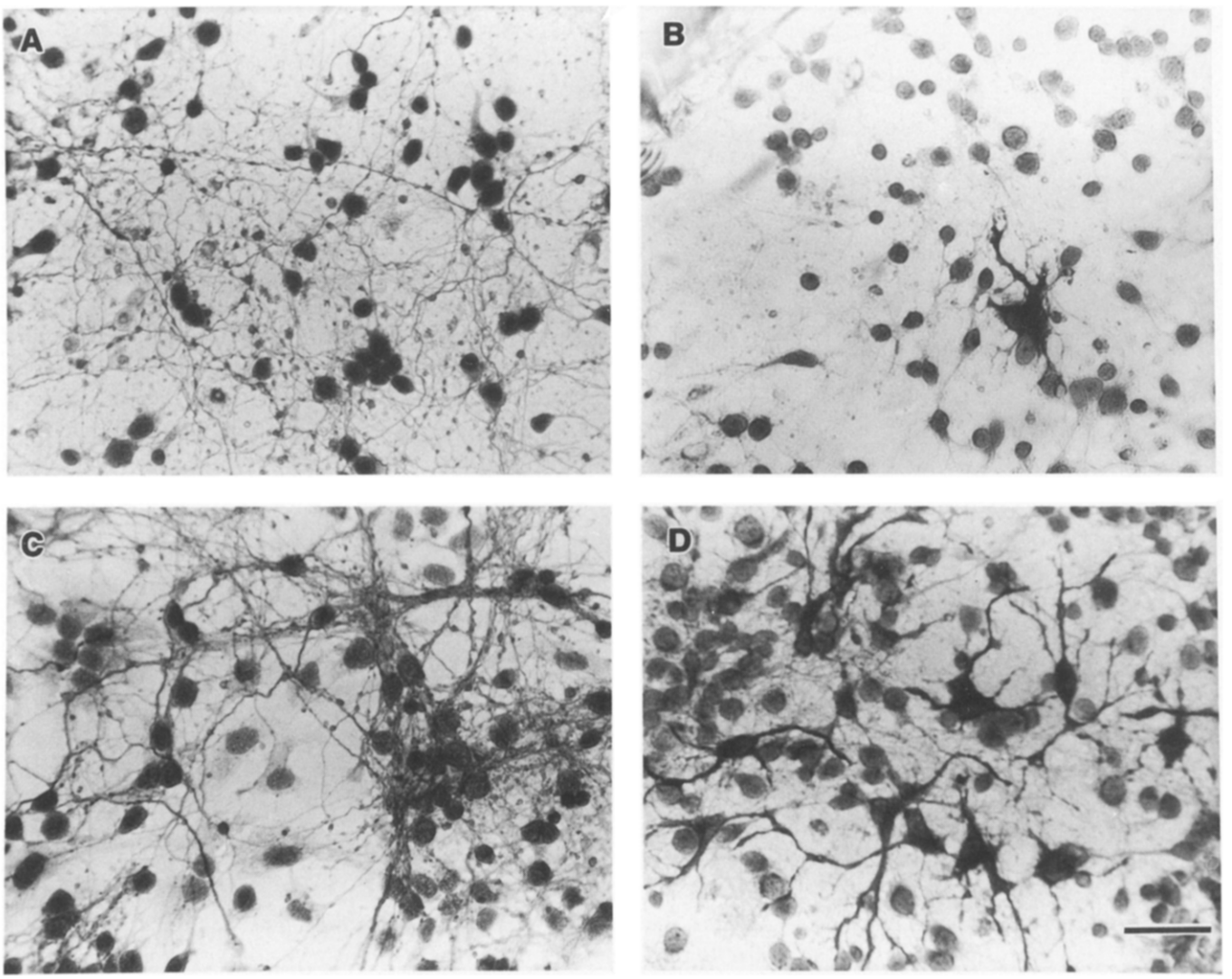

Fig 5 Comparison of cultures grown in the presence of TP9 with those grown in serum-containing medium A, B cultures of dissociated forebrain cells grown in the presence of TP9 (50 μg/ml) and taken for visualization of neurofilaments (A) or GFAP (B) using specific antibodies C, D cultures grown in medium containing 1% fetal calf serum and taken for neurofilament (C) or GFAP (D) immunocytochemistry Bar represents 50 μm

tions containing aspartic acid and glutamic acid but no other amino acid was effective Effective thermal proteins varied, in a narrow range, in their potencies and efficacies TP63, a thermal protein produced from equimolar amounts of aspartic acid and tryptophan, was the most potent preparation and supported neuronal survival at concentrations from 3 μg/ml However, TP63 supported only half the number of neurons supported by TP9 TP64, produced from equimolar amounts of aspartic acid, glutamic acid and lysine, was the most effective but least potent thermal protein (Fig 6)

The comparative analysis of various thermal proteins showing that a preparation from only aspartic and glutamic acid was effective suggested that the dicarboxylic amino acids were of crucial importance for the survival promoting activity of the thermal proteins However, since thermal proteins contain a small amount of pig-

TABLE III

Inhibition of TP9 and polyglutamic acid induced neuronal survival by glycosaminoglycans

A total of 100,000 dissociated cells were plated in polylysine-coated dishes containing L-15 medium TP9 (100 μg/ml) or polyglutamic acid (10 μg/ml) and various glycosaminoglycans Surviving neurons were counted 4 days after plating

	Cells/dish
TP9	18,100 ± 1,900
+ heparin 5 μg/ml	19,300 ± 1,100
+ heparin 50 μg/ml	0
+ heparin 200 μg/ml	0
+ heparan sulfate 200 μg/ml	28,900 ± 2,600
+ chondroitin sulfate 200 μg/ml	32,900 ± 2,900
Polyglutamic acid	25,600 ± 3,400
+ heparin 50 μg/ml	0
+ heparan sulfate 50 μg/ml	32,900 ± 3,100
+ chondroitin sulfate 50 μg/ml	31,100 ± 2,900

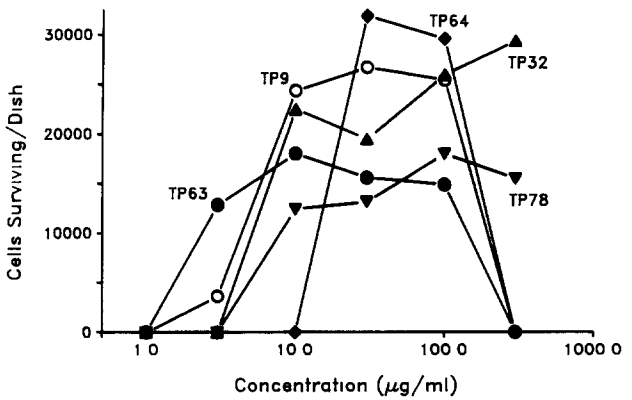

Fig 6 Comparison of various survival-promoting thermal proteins Dissociated forebrain cells were plated at a density of 100,000 cells/16-mm culture dish Dishes were precoated with polylysine and thermal proteins were added at concentrations indicated and were present during the entire culture time of 4 days TP9 = (Asp-Glu Trp, 1 1 1), TP32 = (Asp Glu Val, 1 1 1), TP63 = (Asp Trp, 1 1), TP64 = (Asp Glu Lys, 1 1 1), TP78 = (Asp Glu, 1 1) The chemistry of thermal proteins is described in Materials and Methods Values represent means ± S E M of 5–10 culture dishes

ments and some non-peptide bonds (see description of chemical properties of thermal proteins in Materials and Methods), these findings did not conclusively show that polypeptides containing only aspartic acid and glutamic acid were responsible for the trophic actions of TP9 To test this hypothesis, we obtained commercially available polyamino acids prepared from carboxylic anhydrides of amino acids These experiments revealed that several polyamino acids containing aspartic or glutamic acid effectively supported neuronal survival similar to TP9 Poly-L-glutamic acid was chosen for further detailed analysis Poly-L-glutamic acid was slightly more potent than TP9 and supported neuronal survival at concentrations ranging from 1 to 300 μg/ml (Fig 7) Similar to TP9, poly-L-glutamic acid required precoating of the culture dishes with polylysine or a similar substrate to be effective, since dishes coated with polylysine or polyglutamic acid alone failed to support neuronal survival under our standard conditions Poly-L-glutamic acid preparations of various molecular sizes and various batches were effective

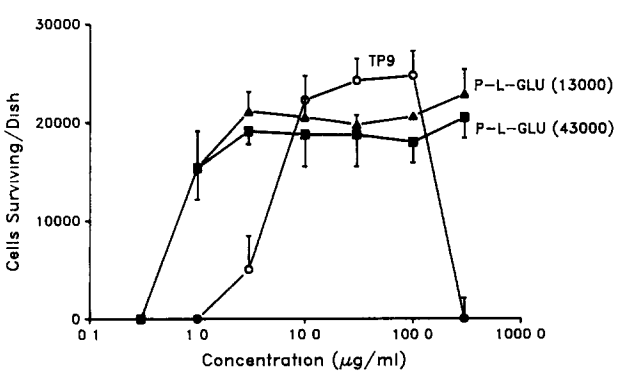

Fig 7 Dose–response curve of effect of polyglutamic acid on neuronal survival A total of 100,000 dissociated forebrain cells were plated per 16-mm dish grown during 4 days Dishes were precoated with polylysine Polyglutamic acid or TP9 were added to the medium and were present during the entire culture time Triangles and squares represent cultures grown in the presence of poly-L-glutamic acid of 13,000 and 43,000 Da, respectively Values represent means ± S E M of 5–10 culture dishes

Inhibitory action of heparin

Since the actions of thermal proteins and polyglutamic acid bore some similarity with those of laminin, and since heparin has been reported to interfere with the survival promoting activity of laminin[3,15], we tested heparin in our culture system Heparin concentrations which prevented neuronal survival produced by laminin were equally effective in inhibiting the effects of TP9 and polyglutamic acid (Table III) This action was highly specific, since other glycosaminoglycans, heparan sulfate and chondroitin sulfate, were ineffective at the same concentrations

DISCUSSION

The principal finding of this study is that some thermal proteins rich in dicarboxylic amino acids and polypeptides uniquely composed of dicarboxylic amino acids are able to promote survival and growth of dissociated fetal rat neurons in serum-free medium in vitro

The survival-promoting effects of thermal proteins and polyglutamic acid required precoating of culture dishes with polylysine or related cationic polymers Basic polymers are routinely used in the preparation of neuronal cultures[39,60] and do not receive much attention It is often believed that cationic polymers favor the interaction with anionic groups present at the surface of neuronal cells and belonging to a variety of different molecules Accordingly, this interaction is often considered 'unspecific' and referred to as a 'charge phenomenon' Similarly, effective thermal proteins and polyglutamic acid might interact with various cationic molecules on the neuronal surface Adding anionic polypeptides to a surface coated with a cationic polymer may result in 3-dimensional molecular arrangements which incidentially mimic specific binding domains of cell surface molecules Therefore, the relevance and physiological meaning of our findings is not clear and it cannot be excluded that the findings represent phenomena only observed under artificial cell culture conditions However, the fact that heparin but not heparan sulfate and chondroitin sulfate inhibit the survival-promoting actions of thermal proteins and polyglutamic acid suggests that there is some degree of specificity in this interaction Addition of heparin has been shown to inhibit trophic

TABLE IV

List of proteins containing sequences of successive dicarboxylic amino acids

Proteins were identified using the polypeptide sequence bank of the IBI Pustell Sequence Analysis Program Numbers given represent amino acids flanking the sequence of successive dicarboxylic amino acids

Protein	*Ref*	*Sequence of successive dicarboxylic acids*
N-myc transforming protein (mouse)	49	$_{256}$DDEDDEEEDEEEE$_{270}$
Homeobox gene product (mouse)	13	$_{85}$DEEEEEEEEEEEEEEE$_{STOP}$
Drosophila serendipity locus gene product	54	$_{149}$DEEEEEDDDDDDE$_{STOP}$
Amyloid precursor protein (human)	24	$_{242}$DDDEDDED$_{251}$ [a]
Laminin binding protein (chick)	12	$_{373}$DDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD$_{STOP}$
Laminin (nuclear envelope protein, human)	32	$_{560}$EDDEDED$_{568}$
Bacteriophage T4 prehead core protein	56	$_{50}$EDEDEDEDDED$_{62}$
Adenovirus hexon protein	23	$_{156}$DEEEE$_{162}$
Sodium channel protein (electric eel)	37	$_{941}$EEEEEEEEE$_{951}$
bFGF receptor (chick)	28	$_{65}$EDDDDEDD$_{74}$
RNA polymerase sigma subunit (*E coli*)	10	$_{190}$DDDEDEDEED$_{201}$
Ubiquinol-cytochrome c reductase (yeast)	52	$_{49}$EDEDEDEDEDDDDDDDEDEEEEEE$_{74}$

[a] Longest uninterrupted sequence of polydicarboxylic amino acids in a sequence of 31 amino acids composed of 17 glutamic acids, 7 aspartic acids, and 7 other amino acids

actions of laminin on epithelial cells and PC12 cells[3,15] Chondroitin sulfate, a related glycosaminoglycan was not effective on PC12 cells[3] Heparin may directly interfere with the binding of cells to thermal proteins and polyglutamic acid or sterically hinder this interaction by binding to other membrane molecules

Our findings seem significant when considering the fact that sequences of successive dicarboxylic amino acids exist in a number of proteins A list of such proteins is presented in Table IV It includes several proteins involved in growth regulation, as well as a laminin binding protein, a nuclear envelope protein, the amyloid precursor protein, viral capsid proteins, an ion channel protein, basic fibroblast growth factor receptor, RNA polymerase, and ubiquinol-cytochrome c reductase In ubiquinol-cytochrome c reductase and the N-myc transforming factor, sequences of successive dicarboxylic amino acid show a higher degree of evolutionary conservation than other parts of the amino acid sequence[49, 52], suggesting that these sequences are functionally important The amyloid precursor protein contains an evolutionary well conserved anionic sequence of 31 amino acids composed of 24 dicarboxylic and 7 other amino acids[24,31] Based on our findings it may be speculated that anionic amino acid sequences play an important role in recognition processes Speculations for a functional role of poly(dicarboxylic)amino acid sequences can be extended to the postulation of receptor molecules specifically recognizing these sequences The recently cloned macrophage scavenger receptor may represent an example of such postulated receptors since it exhibits a very broad specificity and recognizes a large number of polyanions[9] The survival-promoting activities described in the present report probably are not mediated by the macrophage scavenger receptor, since binding of its ligands is not inhibited poly-D-glutamic acid or heparin

Since thermal proteins are believed to be similar to primitive 'protoproteins', sequences of successive dicarboxylic amino acids, forming clusters of negative charges interacting with clusters of positive charges, may represent primitive recognition signals conserved during evolution In an evolutionary context it seems noteworthy that the first two nucleotides of the codons for aspartic acid and glutamic acid, which are functionally equivalent, are identical While possibly coincidental, this fact can be used to search for novel proteins containing sequences of successive dicarboxylic amino acids

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