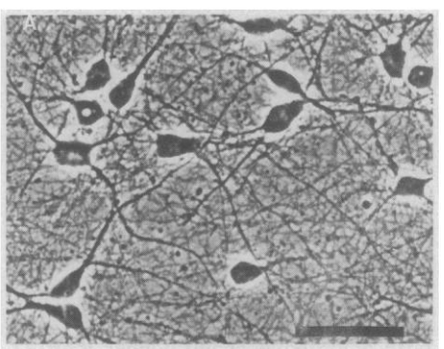
Serum Factor Supporting Long-Term Survival of Rat Central Neurons in Culture

Abstract. *Gel filtration of serum at* p*H 3.6 yielded a fraction that supported long-term (months) survival of dissociated rat central neurons in monolayer culture more reliably than the traditionally used unfractionated serum. The cultures remained neuron-rich, because this fraction did not support the proliferation of glia and fibroblasts that occurs in whole serum. With an apparent molecular weight of 55,000 and an isoelectric point of 5.6, the active factor (or factors) in this fraction is distinct from any well-defined growth factor.*

Neurons in peripheral sensory and sympathetic ganglia require a particular trophic protein, nerve growth factor, for their survival during embryonic development (*1*). Most mammalian central neurons are not affected by nerve growth factor, but their survival in vitro does depend on serum components (*2*). The isolation of these essential serum components may reveal factors critical for neuronal survival both in vitro and in vivo.

In tissue culture studies of mammalian central neurons, the required serum components are usually provided by adding whole serum to the basic nutrient medium. However, use of whole serum has disadvantages: promotion of neuronal survival varies widely among serum lots, and whole serum stimulates proliferation of glia and fibroblasts which overwhelm the nondividing neurons (*2*). We report a single-step serum separation procedure that yields a fraction that reliably supports long-term neuronal survival in vitro and excludes factors mitogenic to glia and fibroblasts.

Most assays for neuronal survival promoting activity were performed on cultures of spinal cord neurons prepared from fetal (day 13 to 14) Sprague-Dawley rats. Spinal cords were freed of meninges and dorsal root ganglia, mechanically dissociated in a basic nutrient medium (Dulbecco's modified Eagle's medium, DMEM), plated into 16-mm diameter culture wells previously coated with collagen and poly-L-lysine (*2*, *3*), and maintained at 35°C in an atmosphere of 10 percent CO_2 in air. Cells were plated at a density of about 850 per square millimeter, of which about 65 percent (550 cells per square millimeter) survived the first hour in vitro. Two hours after plating, the medium in the wells was replaced with the serum fractions, whole serum, or other samples to be bioassayed, all equilibrated in DMEM. For all subsequent cell feedings, the basic nutrient medium used was a modification of DMEM that more closely resembled cerebrospinal fluid (CSF) (*4*). Cultures were fed by replacing three-fourths of the medium every 5 to 7 days.



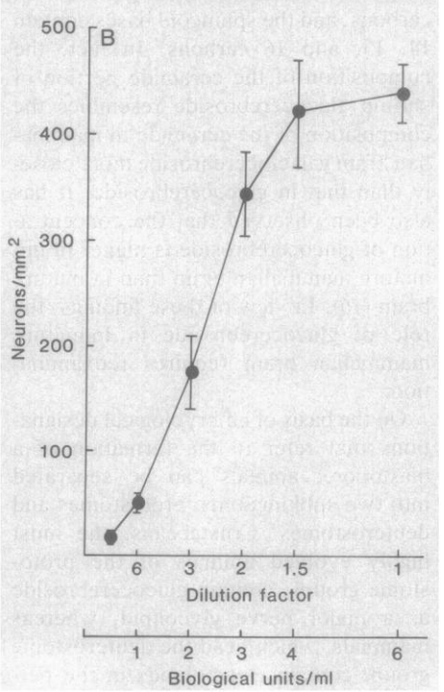


Fig. 1. (A) Photomicrograph of rat spinal cord cells maintained for 3 weeks in vitro. Culture medium contained the serum fraction indicated by the horizontal bar in Fig. 2A. Calibration bar, 50 μm. (B) Titration of neuronal survival promoting activity in an active fraction from fetal calf serum (see Fig. 2A). Each point represents the average density of surviving neurons in nine low-power (×150) fields in three separate wells after 3 weeks in culture. Vertical bars represent ± 1 standard deviation. The maximum dilution of this fraction that would support the survival of 50 neurons per square millimeter was sixfold; thus, this fraction contained 6 BU/ml. The scale on the lower abscissa converts dilution to survival factor concentration for this fraction. Survival promoting activity usually saturated at about 4 BU/ml (450 neurons per square millimeter).

Neuronal survival was assayed 21 days after the cells were plated by counting cells with neuron-like morphology (rounded soma with phase-bright halo and long branching processes) (Fig. 1A) in three representative low-power fields (0.72 mm^2) in each culture well. Intracellular electrophysiological recordings demonstrated that 48 out of 50 sampled cells with this morphology fired action potentials (*5*).

To quantitate the neuronal survival promoting activity in the various serum fractions, we defined one biological unit (BU) per milliliter in terms of the maximum degree to which the sample could be diluted and still support the survival of 50 neurons per square millimeter for 21 days in culture. Dilution curves such as that in Fig. 1B were constructed for each bioassayed fraction. Each fraction was assayed at a minimum of three dilutions, with at least three culture wells per dilution. Each experiment was repeated at least three times.

Figure 2A plots the elution profile of neuronal survival promoting activity after gel filtration of fetal calf serum using citrate-buffered saline at *p*H 3.6 as the running buffer. Neuronal survival was confined to serum fractions with apparent molecular weights ranging from 50,000 to 80,000, with peak activity at 55,000 (*6*). There was no apparent proliferation of nonneuronal cells in cultures grown in these active fractions (*7*). After 3 weeks in vitro most cells (92 percent in this experiment) exhibited a morphology typical of neurons (Fig. 1A). Cultures maintained in the active serum fractions survived and remained neuron-rich for several months. Neither DMEM nor the CSF-like medium, alone or with added bovine serum albumin or fetuin (0.1 to 5.0 percent), supported neuronal survival beyond 3 days, indicating that long-term neuronal survival specifically required the active serum factor.

The pooled active serum fraction indicated by the horizontal bar in Fig. 2A supports neurons in vitro more reliably than unfractionated sera. Best survival with whole serum was obtained with 5 percent horse serum, in which an average of 152 ± 80 neurons per square millimeter (± 1 standard deviation) (28 percent of the original 550 viable cells per square millimeter) survived for 3 weeks. In comparison, sister cultures in the active serum fraction (6 BU/ml) averaged 80 percent survival (442 ± 24 neurons per square millimeter).

When whole fetal calf serum was gel

filtered in neutral- rather than acid-buffered saline, maximum neuronal survival promoting activity still eluted at an apparent molecular weight of 55,000. However, many of the fractions from the neutral column (molecular weight, 40,000 to 100,000) stimulated proliferation of nonneuronal cells, such that in long-term cultures neurons were overwhelmed by dividing cells. Thus, acidic conditions are needed to separate the serum factor, or factors, promoting neuronal survival from factors promoting nonneuronal cell proliferation.

Some fractions of serum that eluted during the acid gel filtration procedure were toxic to cultured spinal cord neurons. Even in the presence of the survival promoting serum fraction, those fractions with apparent molecular weight greater than 100,000 or around 35,000 caused neuronal death. The substances underlying this toxicity might account for part of the variability of neuronal survival in unfractionated serum.

The neuronal survival promoting activity of the active serum fraction is relatively stable to temperature and *p*H changes. Heating to 56°C for 30 minutes reduced activity by only 29 ± 8 percent (mean ± 1 standard deviation). Two successive freeze-thawings produced no significant activity loss. Acidification to *p*H 2.6, followed by reneutralization, also produced no activity loss, but treatment at *p*H 1.6 for 30 minutes destroyed all activity. Unlike many known growth factors, the survival promoting factor described here does not dissociate into small active subunits at low *p*H (*8*): when the active serum fraction was ultrafiltered at *p*H 2.6 (Amicon PM-30, molecular weight cutoff, 30,000), 73 ± 3 percent of the activity was retained by the ultrafilter and no activity passed through the filter.

Preparative isoelectric focusing of the active serum fraction in a granulated gel (LKB Ultrodex) with the use of Pharmacia ampholines (*p*H gradient ranges 3 to 10 or 4 to 6.5) focused the neuronal survival promoting activity between *p*H 5 and 6, with peak activity at *p*H 5.6 (Fig. 2B). The most active isofocused fraction demonstrated a 9.6-fold increase in specific activity over the sample applied to the focusing bed. Recovery of activity averaged 82 percent of that in the applied sample.

The acid gel-filtration step (Fig. 2A) that isolates the neuronal survival promoting activity of fetal calf serum, and removes factors stimulating nonneuronal cell proliferation, produces similar results when applied to serum from other animals. Maximum activity eluted at an apparent molecular weight of 55,000 in all sera tested. The total survival promoting activity per milliliter of the original serum and specific activity in the active fraction were: fetal calf serum (lot 1), 86 BU/ml, 4.9 BU/mg; (lot 2), 74 BU/ml, 7.6 BU/mg; newborn calf serum, 23 BU/ml, 1.7 BU/mg; adult horse serum (lot 1), 612 BU/ml, 23.1 BU/mg; (lot 2), 487 BU/ml, 17.6 BU/mg; adult human serum, 333 BU/ml, 7.9 BU/mg. Thus in practical terms, acid gel-filtration of 1 ml of horse serum followed by dilution of the active fraction (Fig. 2A) to 50 to 60 ml will yield a solution with about 10 BU of neuronal survival promoting activity per milliliter, more than adequate to support good long-term neuronal survival.

The active serum fraction generated by acid gel-filtration also supports the survival in vitro of other cell types, again without stimulating proliferation. Myoblasts and fibroblasts dissociated from rat limb muscle (day 18 to 19 of gestation) survived for a month in DMEM containing the active fraction, but these cells did not proliferate and the myoblasts did not fuse into myotubes (as would have occurred in unfractionated serum), until whole serum was added. Rat cortical, striatal, hippocampal, cerebellar, and brainstem neurons also survived several months in vitro when maintained in medium containing this serum fraction. In contrast, other biological factors known to enhance certain aspects of neuronal development (for example, conditioned media, muscle extracts, and β nerve growth factor) (*9*) were not sufficient to support 3-week survival of dissociated spinal cord neurons. The serum-free, defined N1 com-

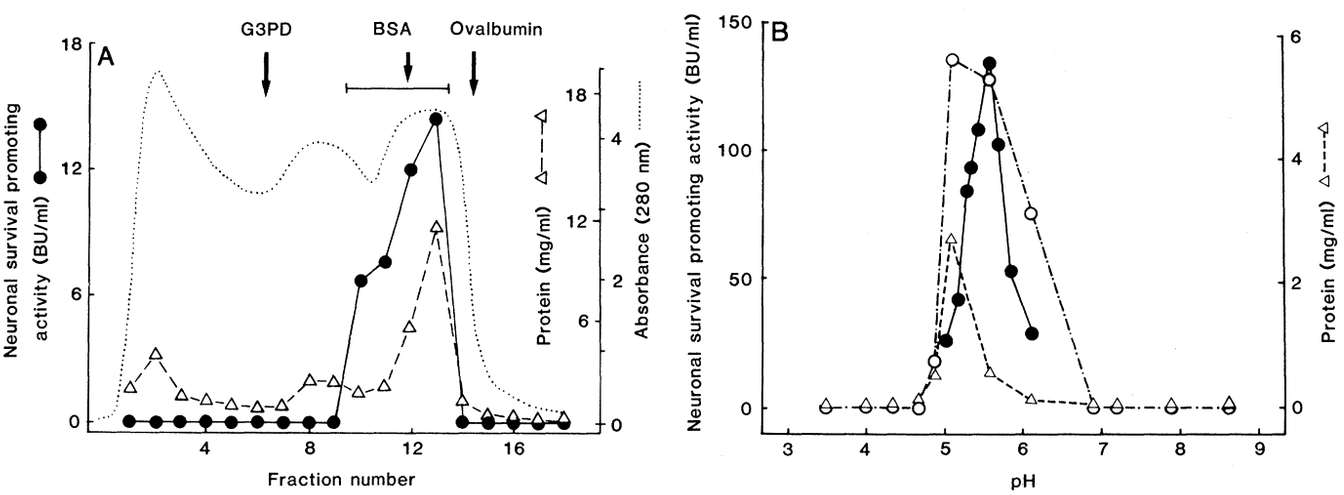


Fig. 2. (A) Elution profile of neuronal survival promoting activity (left ordinate, circles). Fetal calf serum (17 ml) was run at 15 ml/hour through an LKB Ultrogel AcA 44 column (2.5 × 100 cm) in a buffer solution of 150 m*M* NaCl and 5 m*M* citrate (*p*H 3.6). Fractions were neutralized, ultrafiltered (Millipore PTGC, molecular weight cutoff, 10,000) and portions brought up to their final dilution in either DMEM or the CSF-like medium, for bioassay. Neuronal survival promoting activity and protein content (*14*) (triangles) in the fractions are expressed per unit volume of whole serum loaded on the column. Dotted line plots absorbance of the eluate at 280 nm. Vertical arrows indicate elution positions of standards: glyceraldehyde 3-phosphate dehydrogenase (*G3PD*, 140,000), bovine serum albumin (*BSA*, 67,000), and ovalbumin (40,000). Fractions from about molecular weight 25,000 down to the completely included volume are not shown; they were bioassayed and showed no survival promoting activity. Active fractions (horizontal bar) were pooled for all further characterizations. (B) Distribution of survival promoting activity after isoelectric focusing with broad-range ampholines (*p*H 3 to 10, open circles) or narrow-range ampholines (*p*H 4 to 6.5, closed circles). The active fraction from the acid gel-filtration of horse serum was concentrated and desalted by ultrafiltration, applied to a 25 by 5.5 by 0.4 cm focusing bed (after a 1-hour prefocus period), and focused for 14 hours at 8 W of constant power. Protein content (triangles) was measured in the fractions obtained with broad-range ampholines. Ordinates are calibrated to the volume of whole serum before gel filtration.

ponents of Bottenstein *et al.* (*10*) supported spinal cord neuronal survival during the first week in culture, as also observed by Skaper *et al.* (*11*), but not beyond 2 weeks. Addition of N1 components to the active serum fraction did not alter the relation between fraction concentration and neuronal survival measured at 21 days.

In conclusion, acid gel-filtration of serum from a variety of mammals yields a fraction that reliably supports the long-term survival of rat central neurons and several other cell types in vitro, without concomitantly stimulating cellular proliferation. These properties are of obvious practical importance to studies requiring neuron-rich cultures, especially since the metabolic inhibitors traditionally used to limit nonneuronal cell proliferation can alter neuronal properties (*12*). Biochemical and biological characteristics of the active serum factor do not appear to correspond to those of any well-defined growth factor (*13*). Since the concentration of this factor in the sera we tested was more than sufficient to support neuronal survival in vitro, it is likely that the factor contributes to neuronal survival in vivo.

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3. Costar 24-well cluster dishes were coated with a thin layer of rat tail collagen dissolved in 1 m*M* acetic acid, air dried, washed for 30 minutes with a solution of poly-L-lysine (10 μg/ml, Sigma type 1-B) buffered to *p*H 9.0, and finally rinsed with water [E. Yavin and Z. Yavin, *J. Cell Biol.* **62**, 540 (1974)].
4. Culture medium modified from DMEM by: (i) the deletion of glycine, glutamine, pyruvate, and ferric nitrate; (ii) reduction of phenylalanine to 2 mg/liter, tryptophan to 2 mg/liter, and glucose to 40 mg/liter; (iii) addition of alanine (150 mg/liter), proline (40 mg/liter), cyanocobalamin (1 mg/liter), carnitine (1 mg/liter), *p*-aminobenzoic acid (0.5 mg/liter), succinate (10 mg/liter), citrate (20 mg/liter), α-β-glycerophosphate (100 mg/liter), galactose (200 mg/liter), fructose (100 mg/liter), and mannose (50 mg/liter). Motivations for this change of nutrient medium included elimination of potential neurotransmitters and provision of a more complete supply of small nutrient molecules normally found in CSF. Use of this CSF-like medium avoided a late neuronal die-off that frequently occurred after 15 days in vitro when the cells were maintained throughout the experiment in the active serum fractions (see text) equilibrated in DMEM.
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6. To check our molecular weight estimate for maximum neuronal survival promoting activity, we rechromatographed the active fraction from the acid column described above on a high-performance liquid chromatography column (LKB, TSK G 3000 SW) at *p*H 7.0. Again, peak activity eluted at an apparent molecular weight of 55,000.
7. The lack of proliferation of nonneuronal cells in medium containing the active serum fraction (horizontal bar, Fig. 2A) is not due to toxic effects, since adding whole serum to medium containing this fraction results in rapid proliferation of glia and fibroblasts.
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15. We thank E. Barrett for help in preparing the manuscript and D. Nonner and S. Barca for technical help. Supported by NIH grants NS12207 and NS07044 and the National Parkinson Foundation.

4 March 1983

Simple Repeat Array in Epstein-Barr Virus DNA Encodes Part of the Epstein-Barr Nuclear Antigen

Abstract. *The size of the Epstein-Barr virus (EBV) nuclear antigen (EBNA) in cells infected with different EBV isolates varies directly with the size of the EBV triplet repeat array, IR3. The isolate with the largest IR3 fragment has approximately 170 more codons than the isolates with the smallest IR3 fragment; it encodes an EBNA which is approximately 17,000 daltons larger than the smallest EBNA. The EBV IR3 encodes part of a 2-kilobase exon of a latently infected cell messenger RNA which must be translated into a repetitive amino acid domain of EBNA.*

Epstein-Barr virus (EBV) infection induces proliferation of human lymphocytes (*1*). The infected lymphocyte is frequently nonpermissive for the expression of early or late viral replicative functions. An Epstein-Barr intranuclear antigen (EBNA), presumed to be specified by EBV, can be detected in growth-transformed cells with human immune serum (that is, from infected patients) by means of anticomplementary immunofluorescence (ACIF). The antigen is also present in cells from Burkitt's lymphoma (*2, 3*) and nasopharyngeal carcinoma (*4*), two human malignant diseases in which EBV may be an etiologic agent. The entire virus genome persists in the growth-transformed cell as circular episomes (*5, 6*) and as integrated viral DNA (*7*); it consists of single-copy (unique, U), direct terminal repeat (TR) elements, and direct internal repeat (IR) elements in the order TR-U1-IR1-U2-IR2-U3-IR3-U4-IR4-U5-TR (from left to right) (*8*). Messenger RNA's [3.0 and 3.7 kilobases (kb)] are transcribed from left to right from the IR1–U2 and U3–IR3–U4 regions of EBV DNA; and a 2.7-kb RNA is transcribed from right to left from U5 (*8–12*). The sequence IR3 consists of direct repeats of GGG, GCA, GGA (G, guanine; A, adenine; C, cytosine) in two nonanucleotides or one hexanucleotide repeat unit and encodes part of a 2-kb exon of the 3.7-kb latently infected cell RNA (*11*). The unusual triplet symmetry of this repeat through 708 base pairs (bp) and the transcription of this sequence in latently infected cells suggested that this repeat might encode part of a protein in a latently infected cell (*11*).

The nuclear antigen, EBNA, is the most conspicuous protein from a latently infected cell as judged by immunoblots (*13*) with ACIF-positive EBV immune human serum (*14–16*). These serums identify a 65,000-dalton (65K) protein in Raji cells and a 72K protein in B95-8 cells (*15, 16*). Since the EBV Bam HI K fragment that contains the IR3 simple triplet nucleotide repeat array is 0.3 kb shorter in Raji EBV DNA than in B95-8 EBV DNA, the EBNA protein could vary in size among samples of EBV as a consequence of variation in the length of the repeat (*11, 17*). We have been able to map with reasonable precision the IR3 exon and directly test the relation between IR3 and EBNA by determining the sizes of the IR3 repeat region and the EBNA protein in B95-8, in IB4 (which is a latently infected B95-8 transformant) (*18*), in Raji, and in two other cell lines carrying viruses whose IR3 regions differ substantially from those of B95-8 and Raji (*11*).

The relevant restriction endonuclease sites in EBV DNA are indicated in Fig. 1. In previous mapping of the 2-kb exon of the 3.7-kb RNA, separated strands of the Sal I F fragment of EBV DNA were utilized (*11*). In our experiments, the Bam HI K fragment was cloned into pKH47 to map the exon. The EBV DNA strand R (5′ end to the right in the map shown in Fig. 1) was chromatographed on oligodeoxyadenylate cellulose, and it was then hybridized to RNA from the IB4 cell line. The size of the Bam HI K DNA segment protected by IB4 cytoplasmic polyadenylated RNA is 2 kb and is identical in size to the Sal I F segment