Molecular Cloning and Expression of Neuroleukin, a Neurotrophic Factor for Spinal and Sensory Neurons

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A novel 56,000-dalton growth factor found in mouse salivary gland was purified, molecularly cloned, and expressed in monkey COS cells. The protein is a neurotrophic factor and also, surprisingly, a lymphokine product of lectin-stimulated T cells. The factor was therefore named neuroleukin. Neuroleukin promotes the survival in culture of a subpopulation of embryonic spinal neurons that probably includes skeletal motor neurons. Neuroleukin also supports the survival of cultured sensory neurons that are insensitive to nerve growth factor, but has no effect on sympathetic or parasympathetic neurons. The amino acid sequence of neuroleukin is partly homologous to a highly conserved region of the external envelope protein of HTLV-III/LAV, the retrovirus that causes acquired immune deficiency syndrome.

THE PRESENCE OF DENERVATED OR PARALYZED MUSCLE fibers within a muscle induces terminals of healthy motor axons on adjacent muscle fibers to sprout fine, neuritic processes (*1*). Components of the muscle fiber surface such as acetylcholine receptors and neural cell–adhesion molecules are modulated by denervation and paralysis. Each has been postulated to play a role in rendering the muscle fiber surface susceptible to reinnervation (*2, 3*). Motor axon terminals normally are covered by an overlying Schwann cell and by basal lamina, so they are shielded from direct contact with changes occurring on the surface of adjacent, denervated muscle fibers (*4*). Since the Schwann cell and basal lamina are not barriers to the diffusion of soluble proteins (for example, horseradish peroxidase) into the synaptic cleft, a soluble signal that induces axon terminals to sprout or that is chemotropic for the outgrowth of sprouted processes is likely to pass from denervated to innervated muscle fibers.

We sought to examine the hypothesis that sprouting of motor axon terminals may depend, in part, on soluble, muscle-derived factors. We identified a 56,000-dalton polypeptide antigen that is released by denervated muscle grown in organ culture. Polyclonal antisera or monoclonal antibodies directed against this protein partially inhibit the induction of terminal sprouting in the mouse gluteus muscle that would normally be caused by muscle paralysis (*5, 6*). We now report the presence of the 56-kD protein antigen in mouse salivary gland and its purification and molecular cloning from that tissue.

To explore further the biological activity of the 56-kD protein, we performed a series of experiments with cultured spinal neurons in an attempt to model the events leading to neuritic sprouting by motor axon terminals in vivo. Neuritic sprouting by cultured neurons is influenced primarily by the culture substratum. Substrates of insolubilized laminin or fibronectin induce neurite extension (*7*), whereas soluble polypeptide factors, such as nerve growth factor, are generally assayed by their ability to promote the survival of responsive neurons in culture (*8, 9*). In view of the implied involvement of the 56-kD protein in sprouting in vivo, we expected to find that it would promote neuritic sprouting by cultured spinal neurons. Instead, we found that the 56-kD protein is a soluble, neurotrophic factor that acts to promote the survival in culture of a subpopulation of spinal neurons. The factor also acts on a subpopulation of sensory neurons, but not on sympathetic or parasympathetic neurons. We subsequently found that the 56-kD factor is a potent lymphokine (*10*), and therefore we propose the name neuroleukin.

Purification of neuroleukin from salivary gland. Neuroleukin was purified from 200 salivary glands obtained from male BALB/c mice (retired breeders). The factor was purified by sequential dye-ligand matrix affinity chromatography, gel filtration, hydroxylapatite chromatography, and high-performance anion-exchange chromatography (Fig. 1 and Table 1). Fractions obtained in the purification were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with the monoclonal antibodies to neuroleukin (*11*) (Fig. 2). The purified factor retained immunoreactivity with the monoclonal antibodies tested (B109.9 and B130.6). It was not cross-reactive with polyclonal rabbit antibody to nerve growth factor (NGF) or with a rat monoclonal antibody (MCB-1) to NGF.

Neuroleukin behaved as a single species in each chromatographic fractionation. In the final step of the purification, approximately 800 μg of the purified factor eluted isocratically as a single symmetrical peak on chromatography over a Q300 column (Fig. 1). The purified factor chromatographed as a single species over a TSK3000SW high-performance liquid chromatography (HPLC) size-exclusion column or a Vydac C_{18} reversed-phase column and also migrated as a single band when analyzed by SDS-PAGE (Fig. 2). Determinations of the factor's apparent molecular size by gel filtration over AcA54 or TSK3000SW (approximately 60 kD) and by SDS-PAGE (approximately 56 kD) were in good agreement. It focused as a sharp band on isoelectric focusing between *p*H 8.5 and 9.0. Thus, neuroleukin behaved on purification as a basic, monomeric protein.

Purified neuroleukin was blocked to sequencing from the amino terminal. Tryptic peptides were prepared by digesting 200 μg of reduced, alkylated neuroleukin with tosylamide phenylethyl chloro-

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methyl ketone (TPCK)–treated trypsin (1:100). The digest was fractionated by reversed-phase chromatography. Amino terminal sequences were obtained from nine tryptic peptides as indicated in Fig. 3.

Molecular cloning of a cDNA-encoding neuroleukin. The amino terminal sequence obtained from T-36 (Phe-Ala-Ala-Tyr-Phe-Gln-Gln-Gly-Asp-Met-Glu-Ser) was chosen for the synthesis of an oligonucleotide probe as a guess to the deduced nucleotide sequence. A 33-nucleotide probe was prepared (5′-CTCCATGT-CACCCTGCTGGAAGTAGGCAGCAAA) by means of a DNA synthesizer (Applied Biosystems, model 380A).

A complementary DNA (cDNA) library was prepared in λgt10 phage with oligo(dT)-primed double-stranded cDNA synthesized from male BALB/cJ salivary gland polyadenylated [poly(A)$^+$] messenger RNA (mRNA) by the method of Gubler and Hoffman (*12*) and then ligated with linkers into the Eco RI site of the vector as described in Toole *et al.* (*13*). The T-36 oligonucleotide labeled at its 5′ end with γ-^{32}P-labeled adenosine triphosphate ([γ-^{32}P]ATP) (New England Nuclear) was used to screen the salivary gland cDNA library by a modification of the in situ amplification protocol described originally by Woo *et al.* (*14*).

Approximately 100,000 phage were screened; eleven independent phage were found to hybridize to the probe, and five of them contained inserts of roughly equivalent size. DNA from each of the five phage was digested with Eco RI and subcloned into M13 for DNA sequence analysis by the dideoxynucleotide chain termination method (*15*). The sequences of two clones (designated C2 and C19) were determined completely and revealed a single open reading frame that precisely codes for all of the amino terminal tryptic peptide sequences obtained from mouse neuroleukin.

Table 1. Neuroleukin was purified from 200 male BALB/c salivary glands weighing 18 g (wet weight). The neuroleukin activity present in the fractions was not detectable until the second PEG step. On the basis of the recovery of total activity calculated for the second PEG fraction, the activity of the fraction must be underestimated by at least a factor of 10. One unit (U) of activity is defined as the concentration of protein (in milligrams per milliliter) that elicits a half-maximal increase in spinal neuron survival in the culture assay. Abbreviations: SMG sup; salivary gland supernatant; RA, Red agarose; HT, hydroxylapatite; N.D., not detectable; N.T., not tested.

Fraction	Protein (mg)	Activity (mg/ml)	Total activity (U)
SMG sup	3.0×10^3	N.D.	
1st PEG	1.3×10^3	N.D.	
RA pool	4.7×10^2	N.D.	
2nd PEG	72	1.3×10^{-3}	5.5×10^4
AcA54 pool	18.6	7.6×10^{-6}	2.4×10^6
HT pool	5.2	N.T.	
Q300 pool	0.76	7.0×10^{-7}	1.1×10^6

Deduced amino acid sequence of neuroleukin. The complete nucleotide sequence and deduced amino acid sequence of the neuroleukin cDNA is shown in Fig. 3. The sequence contains 2063 nucleotides that terminate in a 3′ poly(A)$^+$ tract. The length of the cDNA agrees well with the length that we estimate for the message represented by these clones. Primer extension with the T-36 oligonucleotide failed to reveal significant extension of the sequence in

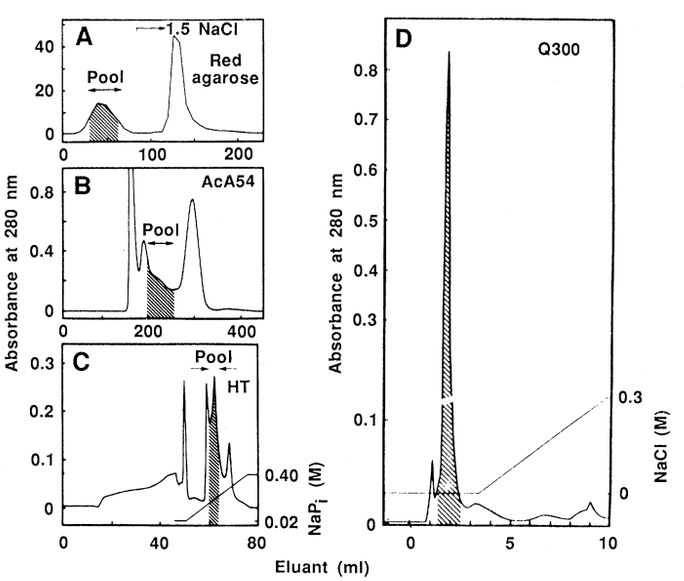

Fig. 1. Chromatograms obtained with different separation media during the course of the purification. Neuroleukin was purified from a pool of 18 g of salivary glands obtained from 100 male BALB/c retired breeders. The shaded region on each chromatogram indicates the fractions that were pooled. For the first polyethyleneglycol 8000 (PEG 8000) precipitation, the frozen salivary glands were thawed in 100 ml of 50 m*M* sodium phosphate buffer (NaP_i), 5 m*M* EDTA, 0.5 m*M* EGTA, and 1 μ*M* leupeptin (*p*H 7.5) and homogenized on ice in four aliquots (Brinkmann Polytron). The homogenate was clarified by centrifuging for 40 minutes at 20,000 rpm in a Sorvall SS-34 rotor, and then centrifuged for 60 minutes at 40,000 rpm in a Beckmann type 70ti rotor. The supernatant was then brought to 24 percent PEG by addition of 60 percent (weight to volume) PEG 8000 in 10 m*M* NaP_i (*p*H 7.5). The solution was kept on ice for 30 minutes and then centrifuged for 30 minutes at 15,000 rpm in the SS-34 rotor. The pellet was suspended in 30 ml of 10 m*M* NaP_i (*p*H 7.5) and then centrifuged for 30 minutes at 15,000 rpm in the SS-34 rotor to remove insoluble material. (A) The first PEG fraction was applied to a 1.6 by 25 cm column of Red agarose (Amicon) which was equilibrated with 10 m*M* NaP_i (*p*H 7.5). The column was eluted at a flow rate of 30 ml/hour until the absorbance at 280 nm was below 1, and then washed with 1.5*M* NaCl in 10 m*M* NaP_i (*p*H 7.5). The factor was not retained by Red agarose. Material that did not bind to the column was pooled and adjusted to 24 percent PEG by addition of 60 percent (weight to volume) PEG 8000 in 10 m*M* NaP_i (*p*H 7.5). The precipitate remained on ice for 30 minutes and then was collected by centrifuging for 1 hour at 20,000 rpm in the SS-34 rotor. The second PEG precipitate was then suspended in 3 ml of 150 m*M* NaCl and 10 m*M* NaP_i (*p*H 7.5). (B) The second PEG precipitate was applied to a 2.2 by 160 cm column of AcA54 Ultrogel (LKB) equilibrated to 150 m*M* NaCl and 10 m*M* NaP_i (*p*H 7.5), and eluted at 19.5 ml/hour. The column was calibrated with thyroglobulin, bovine serum albumin, ovalbumin, and carbonic anhydrase as molecular size standards. Fractions (6.5 ml) were analyzed by SDS-PAGE. Neuroleukin eluted with an apparent molecular size of 6.0×10^4 to 5.5×10^4 daltons. The fractions were pooled as indicated. (C) The AcA54 pool (58 ml) was applied to a 1 by 25 cm column of Bio-Gel hydroxylapatite (HT) (Bio-Rad) at a flow rate of 1 ml/min in 150 m*M* NaCl and 10 m*M* NaP_i (*p*H 7.5). The column was then washed with 20 m*M* NaP_i until the absorbance at 280 nm returned to baseline. The column was eluted with a 150-ml gradient from 20 m*M* NaP_i to 400 m*M* NaP_i. The fractions (2 ml) were analyzed by SDS-PAGE. Fractions containing the factor (20 ml) were pooled, concentrated against solid PEG to 5 ml and dialyzed overnight against 2 liters of 5 m*M* NaP_i (*p*H 7.5). (D) The dialyzate was concentrated to 1 ml by repetitive centrifugation in a microconcentrator (Centricon 30, Amicon). The material was then injected onto a 4.1 by 100 mm Q300 column (Synchrom) equilibrated to 5 m*M* NaP_i (*p*H 7.5). The column was eluted at a flow rate of 0.25 ml/min by HPLC (Beckman 342 system) and then washed with a linear gradient of 0.3*M* NaCl in NaP_i (*p*H 7.5). Neuroleukin was the major peak that eluted isocratically from the column. The bioactivity of purified neuroleukin proved to be difficult to stabilize. Generally, the factor was frozen at −70°C in aliquots at a concentration of 1 mg/ml in PBS containing 5 m*M* ascorbate and 10 percent maltose. Proteolysis of the factor upon storage was not observed and therefore is unlikely to contribute to the loss of bioactivity. Reduction with dithiothreitol, alkylation, trypsin digestion, and chromatographic purification of the tryptic peptides followed previously published protocols (*49*). Amino acid sequencing was performed with a gas-phase sequenator (Applied Biosystems, model 470A).

the 5′ direction. Although the long open reading frame encoding neuroleukin extends to the 5′ end of the sequence, the first ATG in this reading frame begins at nucleotide 50 and is embedded in a canonical sequence for eukaryotic translation initiation sites [CC$^{A}_{G}$C-CAUG(G)] (*16*). We believe that translation initiates at this codon. From the ATG beginning at nucleotide 50, the open reading frame continues until it is terminated by a stop codon beginning at nucleotide 1750.

The sequence encodes a protein of 558 amino acids, which corresponds to a total molecular size of 62,803 daltons. The established protein sequence begins very near the amino terminal of the deduced amino acid sequence and continues to within 46 amino acids of the carboxyl terminal of the deduced sequence. Three potential *N*-linked glycosylation sites (Asn-X-Thr or Asn-X-Ser) are predicted by the deduced amino acid sequence. However, at present, we have no biochemical evidence indicating that glycosylation of the factor occurs.

The deduced amino acid sequence of mouse neuroleukin was compared with sequences in data banks at the National Biomedical Research Foundation and GenBank. No strong homology to any of the sequences of known polypeptide growth factors, proteases, or digestive enzymes present in the salivary gland was found. The best alignment obtained was between neuroleukin and the deduced amino acid sequence of the envelope protein of HTLV-III/LAV (*17, 18*). The partial sequence homology extends over codons 403 to 447 of neuroleukin and codons 238 to 282 of the deduced envelope protein sequence [nucleotides 6514 to 6648 (*17*)]; 30 percent identity is seen over 47 amino acids. The segment of HTLV-III/LAV that is homologous to neuroleukin overlaps a region of the external envelope protein gp120 (codons 233 to 274) that is highly conserved among all HTLV-III/LAV isolates sequenced (*19*). The biological significance of the homology is unknown.

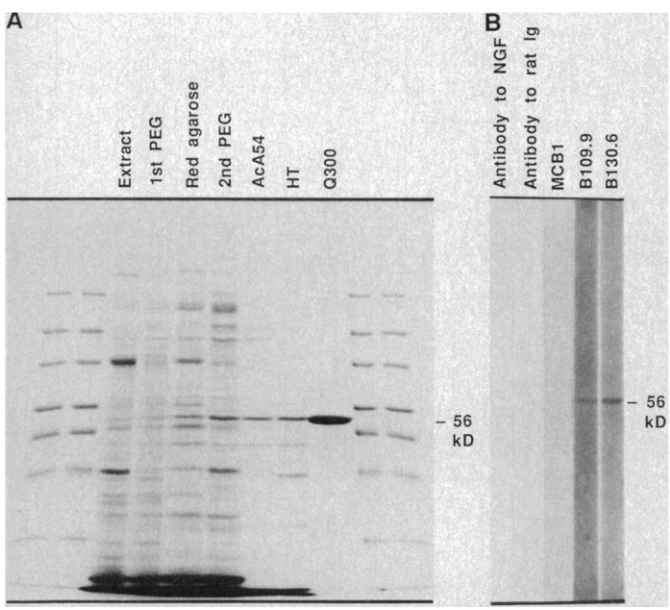

Fig. 2. Analysis of fractions from the purification. (A) Samples were analyzed by SDS-PAGE on a 7.5 percent polyacrylamide gel, which then was fixed and stained with Coomassie blue (*11*). Shown on the gel are the following samples: (from the left) molecular size standards; salivary gland extract (50 μg of protein analyzed); the first PEG fraction (50 μg of protein); the Red agarose pool (50 μg of protein); the second PEG fraction (50 μg of protein); the AcA54 pool (10 μg of protein); the Bio-Gel hydroxylapatite pool (10 μg of protein); the Q300 fraction (10 μg of protein). (B) Immunoblots of the Q300 fraction analyzed by SDS-PAGE on 10 percent polyacrylamide gels (1 μg of protein analyzed per lane) reacted with antibody to NGF, antibody to rat Ig, MCB-1, a rat IgG2b antibody to NGF, and two monoclonal antibodies to neuroleukin (B109.9 and B130.6). Both react with the purified factor.

Neuroleukin promotes the survival of cultured chick spinal neurons. The effects of neuroleukin on chick spinal neurons were assayed by dissociating spinal cords of day 5 embryos into single-cell suspensions with trypsin and then seeding the cells at 10,000 cells per culture well (16 mm) (*20*). In initial experiments, we tested neuroleukin-coated culture substrates for neurite-promoting activity. The culture wells were coated with purified neuroleukin at concentrations ranging from 1 to 100 ng of factor per milliliter in either saline or 150 m*M* borate buffer, *p*H 9.6. The wells were then washed, seeded with 10,000 spinal cord cells, and maintained at 37°C for 24 hours. Alternatively, the wells were coated with poly-L-ornithine (pORN) at 100 μg/ml and then incubated with neuroleukin as above. In either case, neuroleukin-coated substrates failed to promote neurite extension by cultured spinal neurons, whereas wells coated with pORN and laminin supported neurite extension by 50 to 60 percent of the cultured cells. In view of the involvement of neuroleukin in neuritic sprouting from axonal terminals in vivo, the failure of insolubilized neuroleukin to promote neurite extension was unexpected. We also examined the possibility that neuroleukin promoted neurite extension when spinal cord cells were plated on plastic, as was reported for myotube-conditioned medium (*21*). Again, the results were negative.

In subsequent experiments, spinal neurons were seeded in culture wells that had been incubated with pORN at 100 μg/ml and then with purified laminin at 5 μg/ml (*22*). For these cultures, we used twice the amount of laminin required to elicit maximum neurite extension (scored as the percentage of cells with neurites after 24 hours of culture) as determined in a dose-response series. Inclusion of neuroleukin in the culture medium led to a doubling of the number of spinal neurons present at the end of the 24-hour culture period (*23*) (Fig. 4A). A doubling of neuron number was obtained consistently in many separate experiments performed with neuroleukin preparations of various degrees of purity (Table 1), with purified neuroleukin obtained from salivary glands (Fig. 4A), and with recombinant neuroleukin expressed from the C19/p91023(B) construct (Fig. 4C). Other neurotrophic factors, such as NGF, or the serum-free medium supplements of Bottenstein and Sato (*24*) did not promote an increase in spinal neuron number in the assay system.

The spinal cord cultures are thought to contain a variety of neuron subtypes. Therefore, if neuroleukin affects only some subtypes of spinal neurons, a doubling of the total number of neurons obtained may represent a large effect on only some subtypes. We do not know whether the neuroleukin-responsive neurons include spinal motor neurons.

Since most of the chick spinal cord on embryonic day 5 is filled by proliferative neuroblasts within the germinal zone, we examined the possibility that neuroleukin is a neuroblast mitogen. To assess DNA synthesis by [^{3}H]thymidine autoradiography, we seeded 35-mm wells with 40,000 spinal cord cells and incubated them with 0.5 μCi of [^{3}H]thymidine (67 Ci/mmol) for the 24-hour culture period. The labeling of neurons with [^{3}H]thymidine was negligible, and treatment of the cultures with neuroleukin did not change the labeling index of neurons or of nonneuronal cells (Table 2). Thus, neuroleukin lacks mitogenic activity as shown by this assay. The increase in the number of spinal neurons reflects enhanced spinal neuron survival, not proliferation.

The neurotrophic activity of a neuroleukin preparation was quantitated by determining the concentration of protein required to promote a half-maximal increase in spinal neuron survival in the assay system (Table 1). When purified neuroleukin was used, half-maximal activity was consistently obtained with this factor at 0.7

ng/ml (Fig. 4A), which corresponds to a concentration of $1.25 \times 10^{-11}M$ for a 56-kD protein. Thus, the activity of neuroleukin compares favorably to the activity of purified NGF or other polypeptide factors. These similarly act in the range of $10^{-11}M$ to $10^{-12}M$.

Inhibition of survival activity by monoclonal antibodies to neuroleukin. To demonstrate that bioactivity and antigenicity resided in a single polypeptide chain, we tested the ability of monoclonal antibodies to neuroleukin to inhibit the bioactivity of purified neuroleukin in the culture assay. Monoclonal antibodies B86.3, a γ-immunoglobulin 2b (IgG2b), B109.9 (an IgM), B216 (an IgG2a), and B70.10 (also an IgM) all react with neuroleukin, as shown by

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                                                  CAATTCCGCTTCCGAGCACGTC CTGCTCCGTGTACCTCTCGGGTCCCTCGCC

ATGGCTGCGCTCACCCGGAACCCGCAGTTC CAGAAGCTCCTGGAGTGGCACCGCGCGAAC TCTGCCAACCTCAAGCTGCGCGAACTTTTT
MetAlaAlaLeuThrArgAsnProGlnPhe GlnLysLeuLeuGluTrpHisArgAlaAsn SerAlaAsnLeuLysLeuArgGluLeuPhe  30

GAGGCGGATCCGGAGCGCTTCAACAACTTC AGCTTGAACCTCAACACCAACCATGGGCAT ATTCTGGTGGACTACTCCAAGAACCTTGTG
GluAlaAspProGluArgPheAsnAsnPhe SerLeuAsnLeuAsnThrAsnHisGlyHis IleLeuValAspTyrSerLysAsnLeuVal  60

AACAAGGAGGTGATGCAGATGCTGGTGGAG CTGGCCAAGTCCAGAGGCGTGGAGGCTGCA CGGGACAACATGTTCAGTGGTTCCAAGATC
AsnLysGluValMetGlnMetLeuValGlu LeuAlaLysSerArgGlyValGluAlaAla ArgAspAsnMetPheSerGlySerLysIle  90
      ---------------------------------- T56
AACTACACCGAGGATCGGGCGGTGCTGCAT GTGGCCCTTCGGAACCGGTCCAACACACCC ATCAAGGTGGACGGCAAAGATGTGATGCCG
AsnTyrThrGluAspArgAlaValLeuHis ValAlaLeuArgAsnArgSerAsnThrPro IleLysValAspGlyLysAspValMetPro 120
                  -----E/W H/W---------- T32
GAGGTGAACAGGGTTCTGGACAAGATGAAG TCTTTCTGCCAGCGGGTCCGGAGTGGTGAC TGGAAAGGGTACACTGGCAAATCCATCACG
GluValAsnArgValLeuAspLysMetLys SerPheCysGlnArgValArgSerGlyAsp TrpLysGlyTyrThrGlyLysSerIleThr 150

GACATCATCAACATCGGCATCGGGGGCTCT GACCTGGGACCCCTCATGGTGACTGAAGCT CTCAAGCCTTACTCGAAAGGAGGTCCCCGT
AspIleIleAsnIleGlyIleGlySer AspLeuGlyProLeuMetValThrGluAla LeuLysProTyrSerLysGlyGlyProArg 180

GTCTGGTTTGTCTCTAACATTGATGGGACC CACATTGCCAAAACACTGGCCAGCTTGTCC CCTGAGACTTCCCTCTTTATAATCGCCTCC
ValTrpPheValSerAsnIleAspGlyThr HisIleAlaLysThrLeuAlaSerLeuSer ProGluThrSerLeuPheIleIleAlaSer 210
------------S/T--------- T44              ------------------------ ? ------ ?  S  T58/59
AAGACCTTCACCACCCAGGAGACCATCACC AATGCAGAGACAGCAAAGGAGTGGTTTCTC GAAGCGGCCAAGGATCCATCTGCAGTTGCA
LysThrPheThrThrGlnGluThrIleThr AsnAlaGluThrAlaLysGluTrpPheLeu GluAlaAlaLysAspProSerAlaValAla 240
                                                  ------------------------------------------T42
AAGCACTTTGTCGCCCTGTCTACGAACACG GCCAAAGTGAAAGAGTTTGGAATTGACCCT CAAAACATGTTCGAGTTCTGGGATTGGGTA
LysHisPheValAlaLeuSerThrAsnThr AlaLysValLysGluPheGlyIleAspPro GlnAsnMetPheGluPheTrpAspTrpVal 270
      ---------------------------- T26
GGTGGCCGCTATTCGCTGTGGTCAGCCATT GGACTTTCCATTGCTCTGCATGTAGGTTTT GACCACTTCGAGCAGCTGCTGTCCGGGGCT
GlyGlyArgTyrSerLeuTrpSerAlaIle GlyLeuSerIleAlaLeuHisValGlyPhe AspHisPheGluGlnLeuLeuSerGlyAla 300

CACTGGATGGACCAGCACTTCCTCAAGACG CCCCTGGAGAAGAATGCCCCCGTCCTGCTG GCTCTACTGGGCATCTGGTACATCAACTGC
HisTrpMetAspGlnHisPheLeuLysThr ProLeuGluLysAsnAlaProValLeuLeu AlaLeuLeuGlyIleTrpTyrIleAsnCys 330

TACGGCTGTGAGACCCACGCCTTGCTGCCC TATGACCAGTACATGCACCGCTTTGCTGCC TATTTCCAGCAGGGTGACATGGAGTCCAAC
TyrGlyCysGluThrHisAlaLeuLeuPro TyrAspGlnTyrMetHisArgPheAlaAla TyrPheGlnGlnGlyAspMetGluSerAsn 360
                                                  ------------------------------------ T36
GGAAAGTACATCACCAAGTCCGGGGCCCGT GTGGACCACCAGACAGGCCCCATCGTGTGG GGGGAACCAGGGACCAATGGTCAACATGCA
GlyLysTyrIleThrLysSerGlyAlaArg ValAspHisGlnThrGlyProIleValTrp GlyGluProGlyThrAsnGlyGlnHisAla 390
                                  ------ ? --- ? ------------ ?  I/G  T51
TTCTACCAGCTCATCCACCAAGGCACCAAG ATGATACCCTGTGACTTTCTCATCCCTGTC CAGACCCAGCACCCCATACGGAAAGGTCTG
PheTyrGlnLeuIleHisGlnGlyThrLys MetIleProCysAspPheLeuIleProVal GlnThrGlnHisProIleArgLysGlyLeu 420

CATCACAAGATCCTCCTGGCTAACTTCTTG GCCCAGACTGAGGCCCTGATGAAGGGGAAG TTGCCTGAAGAGGCCAGGAAGGAGCTCCAG
HisHisLysIleLeuLeuAlaAsnPheLeu AlaGlnThrGluAlaLeuMetLysGlyLys LeuProGluGluAlaArgLysGluLeuGln 450

GCTGCCGGAAAGAGCCCAGAAGACTTGGAG AAACTCTTGCCACACAAGGTCTTTGAAGGA AACCGCCCGACCAACTCTATTGTGTTTACC
AlaAlaGlyLysSerProGluAspLeuGlu LysLeuLeuProHisLysValPheGluGly AsnArgProThrAsnSerIleValPheThr 480
                                                  ------------------------------- T34
AAGCTGACACCCTTCATTCTGGGGGCCTTG ATTGCCATGTATGAGCACAAGATCTTTGTT CAGGGCATCATGTGGGACATCAACAGCTTC
LysLeuThrProPheIleLeuGlyAlaLeu IleAlaMetTyrGluHisLysIlePheVal GlnGlyIleMetTrpAspIleAsnSerPhe 510

GACCAGTGGGGAGTGGAGCTGGGGAAGCAG CTGGCCAAGAAAATTGAGCCGGAGCTGGAG GGCAGCTCTGCTGTGACCTCCCATGATTCC
AspGlnTrpGlyValGluLeuGlyLysGln LeuAlaLysLysIleGluProGluLeuGlu GlySerSerAlaValThrSerHisAspSer 540

TCCACTAACGGACTGATCAGCTTCATCAAG CAACAGCGGGACACCAAACTAGAATAACTC CAGCCGCGGCCCTACTGACTGGTCCTCCGT
SerThrAsnGlyLeuIleSerPheIleLys GlnGlnArgAspThrLysLeuGlu                                        558

GTCCCTTCTCACCATATGCACTGCATGGTC CTGCCCCTCCCTGCCCAGAGCGCACCACCG GTAGTTGGCCTGGACTACAAGGCTGTTGGG
AGAAGCTGGTCTGGAACTGCCATCCACCCA CTACGCACCCTCCCTGTTGAAGCTGATGGA AGGGCTTTGACGTGTCATGTTGTTCTGACC
TGTATTTCACACCCCAGCTAGAATAAAGAC ACCTAGAGGAGGCAAAAAAAAAAAAAAAAA AAAAAAA
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Fig. 3. Deduced amino acid sequence of mouse neuroleukin. The complete sequence of the C19 neuroleukin cDNA was determined by the dideoxynucleotide chain termination method following subcloning of fragments into M13 vectors (*15*). The predicted amino acid sequence of neuroleukin is given below the nucleotide sequence. The deduced sequence contains all of the NH_2-terminal amino acid sequences obtained from the neuroleukin tryptic peptides (underlined).

Western blotting (*6*) (Fig. 2). The panel of monoclonal antibodies to neuroleukin reacts with at least two different epitopes on the factor (*6*). The monoclonal antibodies were prepared in ascites fluid (*25*) and were added directly to the spinal neuron culture medium at a 1:80 dilution together with neuroleukin (30 ng/ml). B86.3 and B109.9 both completely blocked the effect of the added neuroleukin, whereas B70.10 and B216 had no effect (Fig. 4B). Since B109.9 and B70.10 are both IgM-type antibodies, the inhibition obtained with B109.9 is specific to the monoclonal antibody and not a nonspecific effect due to inclusion of either an IgM or mouse ascites fluid in the culture medium. The same argument holds for the differential effect of B86.3 and B216, both of which are IgG-type antibodies.

B109.9 strongly inhibits neuritic sprouting by axon terminals of spinal motor neurons in vivo, B86.3 and B70.10 are both slightly inhibitory, and B216 does not inhibit terminal sprouting (*6*). B109.9 also inhibits the lymphokine activity of neuroleukin, whereas B216, once again, has no effect (*10*). Presumably, B109.9 inhibits terminal sprouting by binding to an epitope near a functionally important domain of the neuroleukin polypeptide, and the same region of the factor is important for both its neurotrophic and lymphokine activities.

The neuroleukin cDNA expresses biologically active neuroleukin. To show directly that the neuroleukin polypeptide was solely responsible for the promotion of spinal neuron survival in the culture assay, we transiently expressed the neuroleukin cDNA in monkey COS-1 cells (Fig. 5). The serum-free culture supernatant collected from COS-1 cells transfected with the C19/p91023(B) construct (designated C19) contained immunoreactive neuroleukin polypeptide as shown by Western blotting (Fig. 5). Control cultures (designated Mock) transfected with the p91023(B) vector alone did not produce detectable neuroleukin.

The C19-transfected cell supernatant promoted survival of chick spinal neurons, whereas no increase in neuron survival was observed in spinal cord cultures treated with control supernatants collected from the Mock transfectants (Fig. 4C). To demonstrate that the biological activity present in C19 was due to neuroleukin, we added C19 to spinal cord cultures together with B109.9 or B216. Ascites fluid containing B109.9 abolished the promotion of neuron survival elicited by C19, whereas B216 was not inhibitory. Thus, the biological activity present in C19-transfected cell supernatants is due to neuroleukin.

Table 2. [^{3}H]Thymidine incorporation by neurons and nonneuronal cells in culture as determined by autoradiography. DMEM cultures were grown in basal medium (22). Neuroleukin cultures were grown in medium that contained 30 ng of purified neuroleukin per milliliter. Cells (40,000) were plated in a 35-mm well and incubated with 0.5 μCi of [^{3}H]thymidine (67 Ci/mmol) for the 24-hour culture period. The cultures were fixed with 4 percent *p*-formaldehyde in PBS, extracted with 5 percent trichloroacetic acid, and then washed with 100 percent ethanol and air dried. For autoradiography, the bottom of the well was coated with diluted Kodak NTB2 liquid autoradiographic emulsion; the emulsion was exposed for 2 days, and then developed with Kodak D-19. Cells were scored as labeled if the number of silver grains over the cell nucleus was more than five. Background labeling was less than one grain per nucleus. Values are means ± SEM.

Culture	Spinal neurons		Nonneuronal cells	
	Cells per field	Percent labeled	Cells per field	Percent labeled
DMEM	27 ± 4	0	33 ± 4	33 ± 7
Neuroleukin	51 ± 6	2 ± 2	34 ± 5	35 ± 6

Sensory neurons, but not sympathetic or parasympathetic neurons, also respond to neuroleukin. The availability of recombinant neuroleukin enabled us to test the major populations of peripheral neurons for responsiveness to the factor. Sensory neurons were obtained from dorsal root ganglia (DRG) of 10-day and 16-day chick embryos; parasympathetic neurons were obtained from ciliary ganglia of 8-day chick embryos; and sympathetic neurons were obtained from superior cervical ganglia (SCG) of neonatal rats. Ganglia were dissociated into single-cell suspensions with trypsin and then plated on tissue culture plastic to remove most of the adherent nonneuronal cells. The nonadherent neurons were collected, seeded at 5000 cells per culture well (16 mm) on pORN-laminin, and then cultured for 48 hours (*26*).

Neuroleukin promoted the survival of a subpopulation of chick sensory neurons in both 10-day and 16-day embryonic DRG. Optimal concentrations of C19-transfected cell supernatant promoted the survival of approximately 55 percent of the sensory neurons cultured from the chick DRG (Fig. 6), and the effect was blocked by the addition of ascites fluid containing B109.9, but not B216, to the

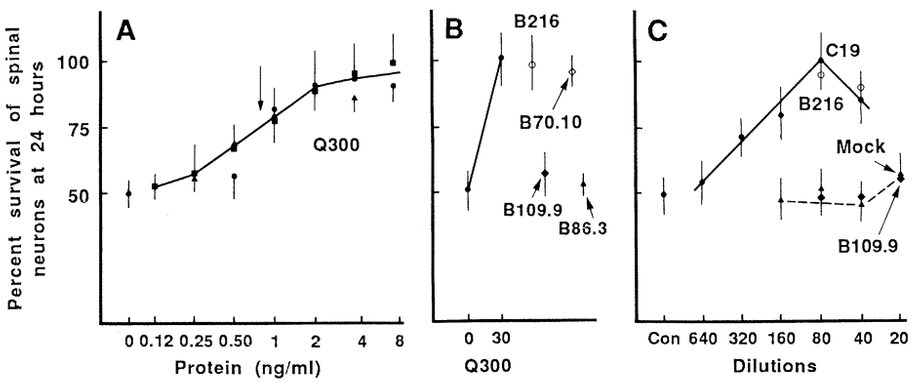

Fig. 4. Stimulation of spinal neuron survival by purified and recombinant neuroleukin. (A) Purified neuroleukin. (B) Inhibition of neurotrophic activity of purified neuroleukin by monoclonal antibodies (mAb's) to neuroleukin. (C) Recombinant neuroleukin. Purified neuroleukin (A) was obtained in the Q300 fraction (Fig. 1). Control cultures were in DMEM with 10 percent low-mitogen serum and glucose at 6 mg/ml (*23*). Cultures were maintained for 24 hours and examined for cells with neurites that were scored as neurons. Three separate experiments were performed (●, ■, ▲). The error bars indicate the standard error of the mean for six replicate wells determined in each experiment. The half-maximal survival of spinal neurons (arrow) was obtained consistently with purified neuroleukin at 0.7 ng/ml. In (B), purified neuroleukin (Q300) at 30 ng/ml was added to the indicated cultures together with 1:80 dilutions of ascites fluids containing mAb's B109.9 (◆), B86.3 (▲), B216 (○), or B70.10 (◇). B109.9 and B70.10 are both IgM's, B216 is an IgG2b, and B86.3 is an IgG2a. B109.9 and B86.3 inhibit the neurotrophic effect of the added neuroleukin, whereas B70.10 and B216 do not. Thus inhibition of neuroleukin bioactivity is a property of particular mAb's to neuroleukin and presumably is due to the particular neuroleukin epitope recognized by the mAb. The inhibition is not a nonspecific effect due to inclusion of ascites fluid or particular subclasses of rat immunoglobulin in the assay medium. In (C), neuroleukin expressed from the mouse salivary gland neuroleukin cDNA is shown to be biologically active. C19 (●) and Mock (▲) are serum-free conditioned mediums from monkey COS-1 cells transfected with the C19/p91023(B) construct or the p91023(B) expression vector, respectively; C19 contains neuroleukin (Fig. 5). Each was added to the indicated cultures at serial dilutions ranging from 1:20 to 1:640. C19 promotes a doubling of spinal neuron survival at optimal concentrations, whereas the Mock control has no effect. The neurotrophic activity of C19-transfected cell supernatants is due to neuroleukin, as shown by its inhibition with B109.9 added at a 1:80 dilution (◆). B216 was not inhibitory in the assay (○).

culture medium. The dose-response of chick sensory neurons to increasing concentrations of C19 was equivalent to the response of spinal neurons within the precision of the assay.

Interpretation of the effects of recombinant neuroleukin on sensory neurons was slightly complicated by the finding that the Mock control promoted a slight increase in the survival of sensory neurons. The neurotrophic effect of the Mock control was not inhibited by monoclonal antibodies to neuroleukin, however, and Mock supernatants did not have an effect on the survival of spinal neurons. Presumably, monkey COS-1 cells secrete a substance distinct from neuroleukin that also promotes some survival of sensory neurons under these culture conditions.

Nerve growth factor also promotes the survival of a subpopulation of sensory neurons in culture (*27*). NGF and C19 support the survival of roughly equivalent numbers of sensory neurons (Fig. 6) in DRG cultures from 10-day embryo tissues. When NGF is added together with C19, the effect of NGF is additive at all concentrations of C19 tested. Although ascites fluid containing B109.9 inhibits the effect of C19 on sensory neurons, it does not inhibit the effect of NGF. These results are consistent with the hypothesis that NGF and neuroleukin act on different populations of sensory neurons. Experiments with DRG from 16-day chick embryos reinforce this view. Neurons responsive to NGF are absent from the 16-day DRG, whereas neurons responsive to neuroleukin are still present (Fig. 6B). Differences in morphology were also observed in the two types of neurons cultured from DRG of 10-day chick embryos. Neurons responsive to C19 tended to be small, phase-dark cells with highly branched neurites, whereas the neurons responsive to NGF tended to be larger, phase bright, and to have longer, unbranched neurites.

Addition of C19 to cultures of dissociated chick ciliary neurons or rat SCG neurons had no effect on neuronal survival. When chick ciliary neurons were cultured in medium alone, or in the presence of recombinant neuroleukin, 2 to 5 percent survived, whereas 100 percent of the ciliary neurons survived for 48 hours when treated with an optimal 1:40 dilution of chick embryo eye extract (*9*). SCG neurons cultured in medium alone, or with added neuroleukin, had a 4 to 6 percent survival rate, whereas 85 to 95 percent of the cultured neurons survived when treated with an optimal NGF concentration of 50 ng/ml.

Tissue distribution of neuroleukin. Although neuroleukin was identified initially in salivary gland and muscle, its tissue distribution is widespread in mouse. Analysis of tissue extracts by SDS-PAGE and Western blotting with a polyclonal rabbit antiserum to neuroleukin (*28*) (Fig. 7A) revealed that the relative tissue contents of the factor were skeletal muscle > brain >> heart, kidney, testes > liver and salivary gland. Low but detectable amounts of factor were observed in serum, lung, thymus, spleen, ovary, adrenals, and pancreas. Northern blot analysis with a single-stranded probe complementary to neuroleukin mRNA (*29*) revealed a single hybridizing mRNA of about 2 kilobases expressed in skeletal muscle, brain, liver, salivary gland, and kidney (Fig. 7C). Levels of message expression parallel the tissue content of neuroleukin.

The expression of neuroleukin by muscle, the inhibition of neuritic sprouting by motor axon terminals after application of antibodies to neuroleukin to the adult neuromuscular junction in vivo, and the finding of a neuroleukin-responsive subpopulation of cultured spinal neurons are all consistent with the hypothesis that neuroleukin is a target-derived trophic factor for spinal motor neurons. However, the tissue distribution of neuroleukin and the known targets of its action (spinal neurons, sensory neurons, and lymphocytes) indicate that it is not solely a motor neuron growth factor.

Since partial denervation of muscle induces terminal sprouting by the remaining motor axons, we examined the possibility that neuroleukin content was modulated by muscle denervation. Increased neuroleukin expression or secretion after denervation could conceivably be the signal that induces terminal sprouting. Since the amount of sprouting induced by partial denervation varies among species and varies with the type of fiber in the muscle, we examined regulation of neuroleukin content in both rat diaphragm and mouse soleus muscle. The mouse soleus contains predominantly slow muscle fibers, and its axon terminals sprout more briskly than those of the rat diaphragm in response to partial denervation. However, we obtained the same result with both types of muscle. No difference in neuroleukin protein content between normal and denervated muscle was apparent on Western blots (Fig. 7B).

Role of neuroleukin in vivo. Muscle-derived signals have been postulated to regulate survival of embryonic spinal neurons (*30*) and sprouting by adult motor axon terminals (*1, 31*). Previous experiments have not succeeded in determining whether the signals for sprouting and survival are the same or different. Our findings suggest that the two phenomena share a common mechanism; yet the finding that neuroleukin functions as a survival factor for spinal neurons in culture assays does not directly translate into understanding of its role in sprouting at the adult neuromuscular junction.

The apparent differences in the action of neuroleukin in culture and at the neuromuscular junction may simply reflect the pleiotropic nature of the factor (as also is indicated by its novel lymphokine activity). Nerve growth factor, for example, also exhibits divergent biological activities; in some assays, it is shown to act as a chemoattractant for neuritic sprouting (*32*), and in other assays it promotes neuron survival (*8*).

Various experiments in vivo indicate that embryonic and adult

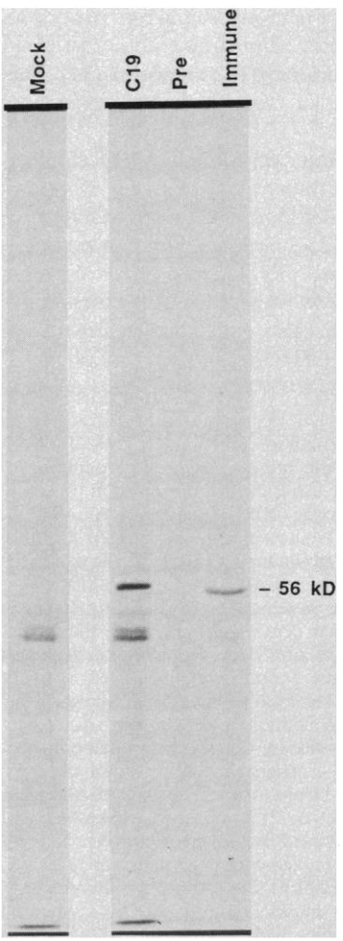

Fig. 5. Expression of recombinant neuroleukin. The structure of the expression vector is described by G. Wong *et al.* (*50*). C19 was inserted into the Eco RI site of p91023(B). DNA (5 μg) was used to transfect monkey COS-1 cells in a 10-μm dish by the use of DEAE-dextran–mediated DNA transfection with additional chloroquine treatment (*51*). A control was provided by transfection with the p91023(B) vector only (designated Mock). The transfectants were cultured for 30 hours in DMEM containing 10 percent fetal bovine serum and then were transferred into serum-free DMEM. The serum-free supernatant was collected after 24 hours and used in these experiments. Recombinant neuroleukin was not purified further. [^{35}S]Methionine-labeled supernatants from Mock or C19 transfectants were analyzed by SDS-PAGE and by immunoprecipitation (*10*). A prominent 56-kD secretory product is present in supernatants collected from C19 transfectants (C19), but is absent from Mock supernatants (Mock). The 56-kD polypeptide is immunoprecipitated specifically by rabbit antiserum to neuroleukin (*28*) (Immune), but not by the rabbit antiserum collected before immunization (Pre). No immunoprecipitable material is present in the Mock-transfected cell supernatant.

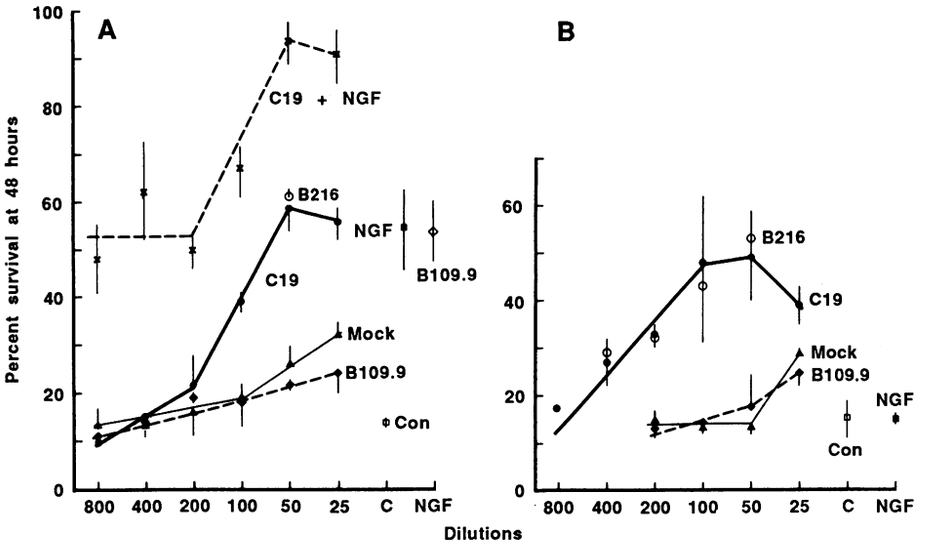

Fig. 6. Neuroleukin promotes the survival of cultured sensory neurons from (A) 10-day DRG and (B) 16-day DRG from chick embryos. Serial dilutions of C19 (●) and Mock (▲) ranging from 1:25 to 1:800 were added to the cultures as indicated; C19 contains neuroleukin (Fig. 5). Ascites fluid (*25*) containing mAb B109.9 (◆) or mAb B216 (○) was added at a final dilution of 1:100 to the indicated cultures, which had also received serial dilutions of C19. NGF (■) was added to the indicated cultures at 50 ng/ml and was also challenged with B109.9 (◇) at a 1:100 dilution in some cultures. Control cultures (□) were in DMEM with 10 percent (by volume) heat-inactivated horse serum and added glucose at 1.5 mg/ml (*26*). Cultures were maintained for 48 hours and examined for neurons. Error bars on the graphs are the range for two to four separate experiments for each condition. For the 10-day DRG (A) a 1:50 dilution of C19 supported the optimal survival of 55 percent of the neurons cultured. NGF also supported the survival of an equivalent number of 10-day DRG neurons. The effects of C19 and NGF were additive with maximal stimulation of 95 percent survival; at lower concentrations of C19, the promotion of survival was solely due to NGF. Addition of B109.9 to C19 abolished the neurotrophic activity of C19, making it equivalent to Mock; addition of B216 had no effect. B109.9 did not inhibit the effect of NGF; this result demonstrates that the mAb specifically inhibits neuroleukin-dependent sensory neuron survival. In (B), NGF-responsive neurons are absent from the 16-day DRG. In control cultures, or with the addition of NGF at 50 ng/ml, 15 percent of the neurons survived, while C19 continued to promote the survival of 50 percent of the sensory neurons cultured. Addition of B109.9 to C19 again made C19 equivalent to Mock, while addition of B216 had no effect.

motor neurons differ in their requirements for continued survival and in their propensity to undergo sprouting. The effect of limb amputation is immediate and drastic in the embryo but has little effect upon the continued survival of adult motor neurons (*30, 33*). An opposite pattern of developmental modulation is seen with respect to axonal sprouting of motor neurons. Refractory periods in the response of perinatal spinal motor neurons to stimuli that elicit axonal sprouting in juveniles or adults have been reported (*34*). The reason for the refractoriness of neonatal motor neurons to induction of axonal sprouting is not known.

We have no evidence that neuroleukin is a survival factor for spinal motor neurons in the adult. Short-term treatment of mouse muscles with antibody to neuroleukin does not cause retraction or disruption of motor terminals, nor does prolonged immunization of adult rats against neuroleukin cause motor neuron loss (*6*). The immunized rats, however, do show weight loss, weakness, and impaired grooming behavior. We also find that treatment of bulbocavernosus muscles of the newborn rat with monoclonal antibody to neuroleukin, in amounts sufficient to suppress terminal sprouting in adults, has no effect on the survival of the afferent population of perinatal spinal motor neurons (*35*). This finding contrasts with the destruction of the sympathetic nervous system caused by treatment of newborn mice with antiserum to NGF (*36*).

We incline toward the hypothesis that the action of neuroleukin as a survival factor is limited to a critical period during development and that thereafter the factor modulates aspects of neuronal growth or function but it is not required for continued viability of the responsive neuronal population. The critical period for neuroleukin-

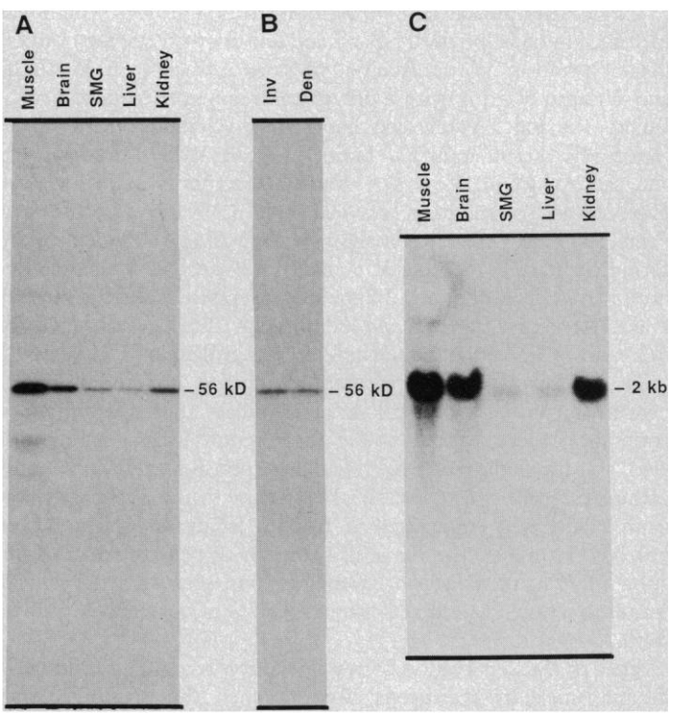

Fig. 7. Tissue distribution of neuroleukin protein and mRNA. In (A), protein extracts were prepared from the indicated tissues of adult male BALB/c mice (SMG, salivary gland) by using the homogenization protocol described in Fig. 1. Protein from each tissue (100 μg) was analyzed by SDS PAGE and Western blotting (*11*). Neuroleukin was detected on the nitrocellulose transfer by reaction with rabbit antiserum to neuroleukin used at 1:1000 (*28*). Neuroleukin content in muscle (B) is not modulated by denervation. Rat diaphragms were hemi-denervated by cutting the left phrenic nerve (*5*); the mouse soleus was denervated by cutting the sciatic nerve. At intervals of 1, 2, 3, or 4 days after denervation, the denervated muscle was harvested together with the contralateral innervated control. Tissue extracts were prepared and analyzed as in (A) with the exception that the antiserum to neuroleukin was used at a dilution of 1:4000. No difference between innervated and denervated muscles was observed in any experiment, as illustrated for innervated (Inv) and 3-day denervated (Den) mouse soleus muscle. In (C), total RNA was prepared from the indicated tissues by use of a guanidine thiocyanate procedure. RNA, 25 μg from each tissue, was analyzed on a Formalin-agarose gel followed by Northern blotting. Neuroleukin mRNA was detected with a complementary single-stranded DNA probe synthesized from a C19/M13 template (*29*). A strongly hybridizing message of about 2 kilobases is seen in muscle, brain, and kidney, whereas lower levels of expression are seen in salivary gland and liver.

dependent survival presumably coincides, at least in part, with the developmental period of naturally occurring motor neuron death (*37*). An example from a very different literature is the effect of androgen on neuron number within the zebra finch song system. Brief treatment of newly hatched female zebra finches masculinizes (increases) the number of neurons in some telencephalic nuclei of the song system (*38*). In the adult, androgen no longer influences neuron number; instead it modulates song system function and allows the male to sing (*39*). Androgen similarly influences survival of motor neurons within the spinal nucleus of the bulbocavernosus (*40*).

We reported earlier that neuroleukin is associated with amyotrophic lateral sclerosis (ALS), a progressive, degenerative disease of the motor neuron (*41*). Clinical features of the disease include weakness in more than one limb and hyperreflexia (*42*). Autopsy reveals loss of motor neurons of the ventral horn, upper motor neuron involvement, and axonal swellings. As death of motor neurons proceeds, denervation of skeletal muscle and consequent atrophy become apparent. Serum samples from about one-third of the ALS patients tested inhibited sprouting by motor axon terminals in the in vivo bioassay. In one case the inhibition was associated with immunoglobulin, and three of the ALS sera reacted by Western blotting with a 56-kD protein present in rat muscle–conditioned medium (*41*). We believed that the 56-kD protein was neuroleukin, but the same three ALS sera have not been found to react with recombinant mouse neuroleukin by Western blotting (*43*). The findings that neuroleukin acts on sensory neurons and is a lymphokine also weaken its importance for ALS, since sensory involvement is conspicuously absent (*42*), and no immune dysfunction has been found (*44*).

In addition to NGF and epidermal growth factor (EGF), several other biological activities have been found in mouse salivary gland. These include a mesodermal growth factor, a thymocyte-transforming factor, and an uncharacterized colony-stimulating factor (*45*). NGF, EGF, and the other less well-characterized factors in salivary gland have molecular sizes of 15 to 30 kD, as do most other polypeptide growth factors such as the interleukins, transforming growth factors, and platelet and fibroblast growth factors. Therefore, the factor that we obtained from salivary gland is unusual in that the biological activity resides in a single polypeptide chain of 56 kD. Biologically active substances of about 50 to 60 kD, which similarly promote the survival of cultured spinal neurons, have been partially characterized in muscle extracts or in medium conditioned by cultured myotubes (*21, 46*) and in fetal bovine serum (*47*) and may represent neuroleukin.

In addition to its action on neurons, neuroleukin is also a lymphokine. B-lymphocyte growth factors of 50,000 to 60,000 daltons have been described (*48*). One of the factors referred to may be neuroleukin, since we find that neuroleukin is a lectin-stimulated T-cell product that induces polyclonal B-cell maturation to immunoglobulin secretion (*10*). Thus, neuroleukin is a novel growth factor with potent effects on both neurons and lymphocytes.

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23. Spinal cord cells were plated at 10^4 cells per well and cultured in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) containing glucose (6 mg/ml) and 10 percent low-mitogen serum (Zeta Sera-D, processed adult bovine serum; Sigma). Cultures were incubated in a humidified atmosphere containing 5 percent CO_2 at 37°C. Samples containing neuroleukin were assayed in six replicate wells. Cultures were fixed with 10 percent glutaraldehyde in phosphate-buffered saline (PBS) 24 hours after plating and then were examined under phase contrast at a magnification of ×100. Neurons were scored as cells with neurites longer than two cell diameters. The neurites of such cells were separately shown to be immunoreactive for the 200-kD and 140-kD neurofilament proteins (T. Stitt and M. E. Gurney, unpublished data). The number of surviving neurons was counted in two or three microscope fields that covered the center of each well (1/145 of the well was viewed per field). Cells were shown to be viable by exclusion of trypan blue and propidium iodide and by metabolism of dimethylthiazol diphenyltetrazolium bromide (MTT) [T. Mosmann, *J. Immunol. Methods* **65**, 55 (1983)]. An *F* test of the variance of individual measurements per well against the variance of the average per well across the six replicate wells revealed no significant difference. Data have been expressed as the percentage of maximum neuron survival after 24 hours in culture. With the inclusion of neuroleukin in the culture medium, 80 to 95 percent of the cells initially plated survived for 24 hours; 60 percent of the cells present were neuronal and the rest were nonneuronal. The nonneuronal cells lacked neurites, were vimentin-positive, and 30 to 40 percent were labeled with [^{3}H]thymidine during the culture period. By those criteria, the nonneuronal cells were tentatively identified as mitotically competent neuroblasts obtained from the spinal cord germinal zone [S. J. Tapscott, G. S. Bennet, Y. Toyama, F. Kleinbart, H. Holtzer, *Dev. Biol.* **86**, 40 (1981)]. No cells in the cultures expressed the glial markers, glial fibrillary acidic protein, or galactocerebroside. Neuron number varied by a factor of 2 in the presence and absence of neuroleukin, so data were normalized between experiments by letting the number of spinal neurons surviving in the absence of neuroleukin represent 50 percent of the maximal neuronal survival we expected to obtain.
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26. All ganglia were dissected under sterile conditions, incubated in CMF-HBSS for 20 minutes at 37°C, digested in 0.1 percent trypsin in CMF-HBSS for 20 minutes at 37°C, washed in culture medium, and then triturated into a single-cell suspension. Cell suspensions were plated in 10-cm plastic tissue culture dishes for 3 hours at 37°C; nonadherent cells were harvested and then seeded at 5000 cells per 16-mm well. Each well was coated with pORN-laminin (*22*). Cells were cultured in DMEM containing 10 percent heat-inactivated horse serum and glucose (1.5 mg/ml). Cells with neurites were scored as neurons. Identification of the cells as neurons was confirmed by showing reactivity on the neurites with monoclonal antibodies to 160-kD and 200-kD neurofilaments (Amersham International).
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highly homologous to that from mouse. Three immunizations of the rabbit were performed with 50 μg of purified neuroleukin emulsified in Freund's adjuvant. Complete adjuvant was used for the first immunization and incomplete adjuvant for the second and third immunizations. The emulsion was injected subdermally at multiple sites.
29. Synthesis of a single-stranded DNA probe was primed from the 3′ end of the neuroleukin coding sequence (cloned into M13mp9) nucleotides 1685 to 1699, with the oligonucleotide CTGTTGCTTGATGAA used at a 5:1 molar ratio. The synthesis was performed with the Klenow fragment of DNA polymerase I (New England Biolabs) and [α-^{32}P]dATP (410 Ci/mmol). M13 sequences were trimmed from the probe with Eco RI and then the probe was denatured and purified by electrophoresis through a low melting temperature agarose gel. Hybridization with the probe at 10^6 cpm was performed in 5× standard saline citrate (SSC), 1× Denhardt's solution, 0.1 percent SDS, and salmon sperm DNA (Sigma) (100 μg/ml) at 55°C. Filters were washed first in 5× SSC and 0.1 percent SDS at 55°C, then in 0.1× SSC and 0.1 percent SDS at 55°C. The filters were then exposed to Kodak XAR5 film at −70°C.
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Neuroleukin: A Lymphokine Product of Lectin-Stimulated T Cells

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Neuroleukin is a lymphokine product of lectin-stimulated T cells that induces immunoglobulin secretion by cultured human peripheral blood mononuclear cells. Neuroleukin acts early in the in vitro response that leads to formation of antibody-secreting cells, but continued production of immunoglobulin by differentiated antibody-secreting cells is neuroleukin-independent. Although the factor is not directly mitogenic, cellular proliferation is a late component of the response to neuroleukin. Neuroleukin does not have B-cell growth factor (BCGF) or B-cell differentiation factor (BCDF) activity in defined assays. Neuroleukin-evoked induction of immunoglobulin secretion is both monocyte- and T cell-dependent.

IMMUNE RESPONSES OF B LYMPHOCYTES, INCLUDING ACTIVATION, clonal expansion, antibody secretion, and immunoglobulin heavy chain class switching are regulated by an unknown number of T cell–derived lymphokines (*1*). Interleukin-1 (IL-1), interleukin-2 (IL-2), and γ-interferon (IFN-γ) all modulate B-cell function, and another dozen or so factors in T-cell or lymphoblastoid-cell supernatants have been partially characterized (*1, 2*). The T cell–derived regulators of B-cell immune responses are of particular interest because of their potential clinical use in treating humoral autoimmunity or immunodeficiency.

So far only a single T cell–derived lymphokine regulator of B-cell function has been characterized at the molecular level (*3*). The factor, termed IL-4 (*3*), elicits three different in vitro responses by purified mouse splenic B cells. IL-4 directly stimulates quiescent B cells to increase surface expression of Ia; it is mitogenic for B cells costimulated with antibody to immunoglobulin (Ig), and it induces a switch from synthesis of IgG3 to IgG1 by B cells costimulated with lipopolysaccharide (LPS), a T cell–independent, polyclonal B-cell activator. The B-cell responses measured in the three in vitro assays are so different that IL-4 was earlier thought to be three different lymphokines (*3–5*).

That a single lymphokine acting on B cells can have such

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