Neurogenesis in Postnatal Rat Spinal Cord: A Study in Primary Culture

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Spinal cord injuries result in paralysis, because when damaged neurons die they are not replaced. Neurogenesis of electrophysiologically functional neurons occurred in spinal cord cultured from postnatal rats. In these cultures, the numbers of immunocytochemically identified neurons increased over time. Additionally, neurons identified immunocytochemically or electrophysiologically incorporated bromodeoxyuridine, confirming they had differentiated from mitotic cells in vitro. These findings suggest that postnatal spinal cord retains the capacity to generate functional neurons. The presence of neuronal precursor cells in postnatal spinal cord may offer new therapeutic approaches for restoration of function to individuals with spinal cord injuries.

Neurogenesis occurs postnatally in tissue from certain brain regions (*1–3*). However, generation of newly differentiated neurons in rat spinal cord is thought to cease by embryonic day 17 (*4*). Furthermore, although spinally transected neonatal rats (0 to 12 days old) recover substantial motor function mediated by spinal cord caudal to the site of injury, limited behavioral recovery is seen following transection on postnatal day 15 (P15) or later (*5*). We investigated whether postnatal spinal cord contains cells with a capacity to generate functional neurons.

Cervical spinal cord tissue was harvested from rats at P15 to P16 and cultured on laminin-coated cover slips (*6, 7*). Neurons and glia were identified immunocytochemically with antibodies directed against two different neuronal proteins and one glial-specific protein: class III neuron-specific β-tubulin (TuJ1), neuron-specific enolase (NSE), and glial fibrillary acidic protein (GFAP). The TuJ1 antibody is directed against class III β-tubulin, a tubulin isotype expressed only by neurons (*8, 9*) early in, during, or directly after the final mitotic division (*10*). The antibody to NSE used for these studies is directed against the γγ epitope of NSE and selectively identifies neurons (*11*).

Cells immunopositive for TuJ1 or NSE, but not GFAP, were identified in several cultures on various days [4 to 31 days in vitro (DIV)] after plating. The number of these presumptive neurons increased with advancing time in culture (Fig. 1). However, it is conceivable that increased expression of neuronal proteins by neurons already present at the earliest time point examined may have accounted for this result. We investigated this possibility with the following experiment.

Several cultures, plated at the same density, were incubated either with or without the mitotic inhibitor 5-fluoro-2-deoxyuridine (FUDR; 15 μg/ml) from 4 to 12 DIV. At 12 DIV, cultures maintained without FUDR exhibited a marked increase in TuJ1- and NSE-immunopositive cells compared with FUDR-treated cultures (Fig. 2). This result contrasts with the mutual increase that would have been expected in FUDR-treated cultures if increased labeling was due to up-regulation of neuronal protein expression. Despite differences in neuronal numbers that we attribute to variability in composition of conditioned media, we observed this result consistently in each trial. These data confirm that cell division is necessary for the increase of TuJ1- and NSE-immunopositive cells to occur. However, this experiment does not determine that mitotic cells exclusively differentiate into neurons. The possibility exists that generation of nonneuronal cells stimulates neurons to up-regulate neuronal protein expression. Therefore, two experiments were performed to determine if mitotic cells differentiated to become neurons in culture.

Postnatal cultures were incubated with bromodeoxyuridine (BrdU, 1 μM), a thymidine analog that is incorporated into DNA during the S phase of the cell cycle (*12*). Because cells incorporating BrdU during this phase of DNA replication subsequently undergo mitosis, detection of BrdU in a cell confirms it is the progeny of a mitotic division in vitro. After 7 to 20 days of incubation with BrdU, cultures were double-immunostained for BrdU content and either class III β-tubulin or NSE. Subsequent cell counts demonstrated colocalization of BrdU in 10 of 42 TuJ1-positive cells, consecutively identified on one cover slip (Fig. 3, A and B), indicating that these presumptive neurons differentiated from cells born in culture. Additionally, on average, 20% of anti-NSE–labeled cells were also immunopositive for BrdU (Fig. 3, C through E), confirming results obtained with TuJ1/BrdU double-immunocytochemistry.

Collectively, these immunocytochemical studies provide evidence that postnatal spinal cord contains cells that retain the capacity for neurogenesis in vitro. However, although TuJ1 identifies a neuron-specific tubulin isotype (*8, 10, 13*), several studies report that reactive glia may express other neuronal proteins such as microtubule-associated protein 2 (MAP2) and NSE (*14*). Furthermore, because glial cells proliferate in spinal cord postnatally (*15*), we addressed the possibility that some NSE/BrdU double-immunostained cells were glia. To confirm that at least a subset of spinal cord cells incorporating BrdU were functional neurons, we electrophysiologically studied 60 cultured postnatal cells between 20 and 27 DIV, using whole-cell patch clamp (Fig. 4).

Most cells exhibited some level of elec-

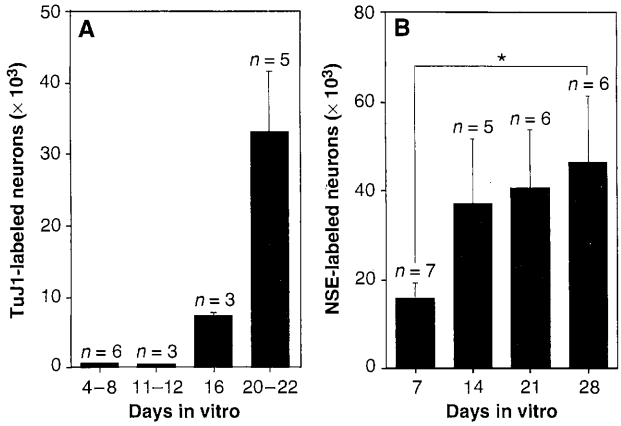

Fig. 1. Numbers of cells immunopositive for neuronal markers increase with time in vitro (*27*). (**A**) TuJ1-immunopositive cell numbers are shown at various times in vitro (mean + SEM). Analysis of variance: $P < 0.005$, $F(3,13) = 10.0$. (**B**) A number of NSE-immunopositive cells are shown on various DIV [mean + SEM; $^*P < 0.05$ (Student's *t* test)]. Anti-GFAP immunocytochemistry demonstrated that <3% of cells were astrocytes.

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trical excitability as demonstrated by the ability of depolarization to elicit voltage-gated currents. However, only 20 cells responded to depolarizing current steps by generating voltage spikes (*16*). These cells were iontophoretically injected with 0.2% dextran fluorescein dye and subsequently immunoreacted for BrdU content.

Application of depolarizing current evoked three types of voltage traces from the cells' resting membrane potentials (RMPs) in 18 cells filled sufficiently for post hoc identification. First, all-or-none action potentials (APs) were generated in three BrdU-positive (Fig. 4, A through C) and three BrdU-negative neurons (*16*). Second, graded APs were elicited from six BrdU-positive (Fig. 4, D through F) and two BrdU-negative neurons (*16*). These recordings are comparable to those described in cultured small hippocampal neurons (*17*) and developing neurons derived from a multipotent precursor cell line (*18*). Finally, four BrdU-positive cells (Fig. 4, G through I) exhibited small rudimentary voltage spikes of the type reported in neonatal hypoglossal motorneurons (*19*), developing neurons (*18*, *20*), and computer-modeled immature neurons (*20*).

AP-like responses are reported in pancake astroglia from cultured P0 spinal cord (*21*). However, the cells we studied electrophysiologically differed in two important ways. First, unlike flat nonprocess-bearing pancake astrocytes, cells in this study were phase-bright with two or more projections. Secondly, pancake astrocytes generate AP-like responses only after removal of Na^+-channel inactivation by hyperpolarization to potentials negative to –70 mV. Because these cells rest at around –40 mV, it is unlikely that AP-like responses would occur spontaneously in vivo (*22*). In contrast, APs in the present study were elicited from each neuron's RMP (–32 to –69 mV). This finding carries particular significance because it suggests that APs could be elicited in newly generated postnatal spinal cord neurons under physiologic conditions in vivo.

The generation of neurons raises the question of their source. The subventricular zone (SVZ) of mature mammalian brain contains precursor cells capable of differentiating into neurons (*2*). Although this area contributes primarily to gliogenesis (*23*), in vivo and in vitro studies have demonstrated an SVZ region containing essentially all TuJ1-immunoreactive cells (*13*). Like the cerebral ventricles, the spinal cord central canal derives from a cavity within the embryonic neural tube (*24*) and is lined with neuroepithelial cells that divide and differentiate into central nervous system neurons and glia. Conceivably, central canal cells in these postnatal cultures may have given rise to the newly generated neurons we observed.

This study confirms neuronal function (AP generation) in mammalian postnatal spinal cord cells identified with a proliferative immunomarker (BrdU). The observation of neuronal proliferation in the presence of preexisting neurons, unlike implantation of peripheral nerve or isolated neuronal precursors (*25*), suggests that spinal neurogenesis may be induced in situ (*16*). Indeed, patch-clamp studies conducted on more than 131 cultured spinal cord cells at P15 and P16 demonstrate functionality of neurons for up to 32 DIV, including the apparent capacity to form synaptic connections (*6*). Collectively, these findings support the utility of postnatal rat spinal cord cultures as a practical system to investigate factors involved in neurogenesis of functional postnatal spinal cord neurons. Parallel in vivo studies could lead to the design of innovative strategies for treating injured spinal cord. This would represent an important clinical advance given the severity of sequelae to cervical spinal cord injuries, including paralysis and dependence on ventilator-assisted breathing (*26*).

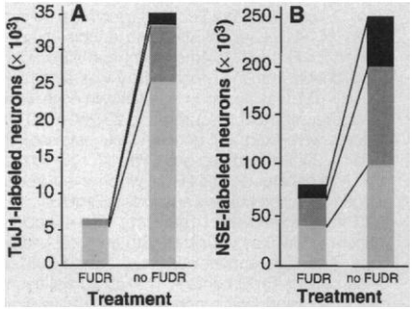

Fig. 2. FUDR inhibited the increase of immunocytochemically identified neurons over time (*28*). Shown are (**A**) TuJ1-immunopositive cell counts and (**B**) NSE-immunopositive cell counts. Stacked bar graphs represent total TuJ1- or NSE-immunopositive cell numbers at 12 DIV after an 8-day incubation in either neuronal media (no FUDR) or neuronal media with FUDR. This experiment was performed three times with different sets of cultures, plated on three different days. Comparisons were only made between cover slips plated on the same day, as indicated by bar graph segments with matching shades of gray or black. Note that in each case neuronal cell counts were greater when cultured without FUDR.

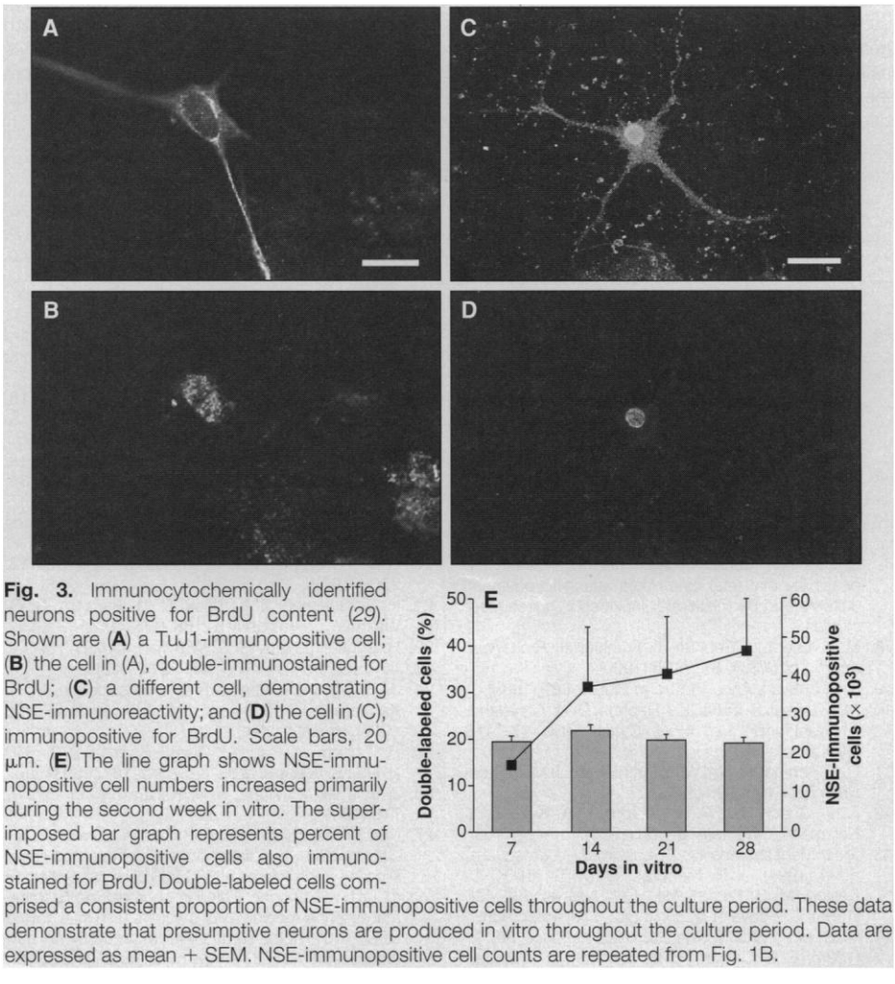

Fig. 3. Immunocytochemically identified neurons positive for BrdU content (*29*). Shown are (**A**) a TuJ1-immunopositive cell; (**B**) the cell in (A), double-immunostained for BrdU; (**C**) a different cell, demonstrating NSE-immunoreactivity; and (**D**) the cell in (C), immunopositive for BrdU. Scale bars, 20 μm. (**E**) The line graph shows NSE-immunopositive cell numbers increased primarily during the second week in vitro. The superimposed bar graph represents percent of NSE-immunopositive cells also immunostained for BrdU. Double-labeled cells comprised a consistent proportion of NSE-immunopositive cells throughout the culture period. These data demonstrate that presumptive neurons are produced in vitro throughout the culture period. Data are expressed as mean + SEM. NSE-immunopositive cell counts are repeated from Fig. 1B.

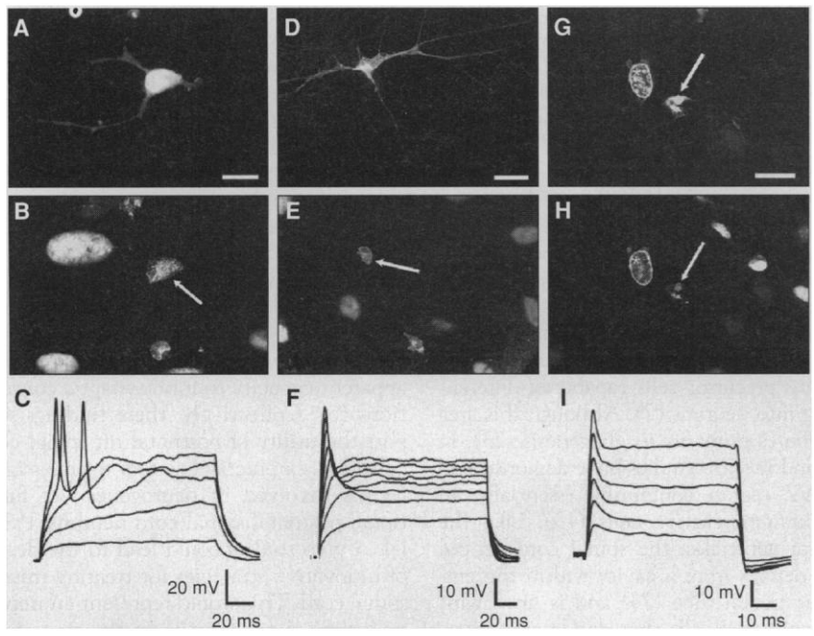

Fig. 4. BrdU-immunoreactivity in electrophysiologically identified neurons (*30*). Shown are (**A**) a dextran fluorescein–injected neuron from which all-or-none APs were elicited and (**B**) BrdU-immunoreactivity of the cell in (A) (arrow). (**C**) All-or-none APs were elicited from the neuron in (A) and (B) by incremental 20-pA depolarizing current steps from RMP (–66 mV). (**D**) A second dextran fluorescein–injected cell from which graded APs were generated and (**E**) BrdU-immunoreactivity of the cell in (D) (arrow) are shown. (**F**) Graded APs were generated by the neuron in (D) and (E) to incremental 50-pA depolarizing current steps from RMP (–51 mV). Shown are (**G**) a dextran fluorescein–filled cell (arrow) which generated rudimentary voltage spikes and (**H**) BrdU-immunoreactivity of the cell in (G). The white asterisk (G and H) indicates a dead cell stained by fluorescein, released during pipette withdrawal from the filled cell. (**I**) Small voltage spikes were generated by the cell in (G) and (H) to incremental 150-pA depolarizing current steps from RMP (–35 mV). Scale bars, 20 μm.

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27. Rat cervical spinal cord at P15 and P16 was dissociated. Cells were plated onto laminin-coated cover slips in six-well plates at 300,000 cells per milliliter (3 ml per well) and incubated in neuronal media (minimal essential medium with D-valine, 10% fetal bovine serum, 10% horse serum, 44 mM $NaHCO_3$, 33 mM glucose, 0.8 nM 7S nerve growth factor, and 50 μM N^{ω}-nitro-L-arginine methyl ester) conditioned by overnight incubation with astrocyte-rich fetal cortical monolayers. (Fetal monolayers were cultured in minimal essential medium with D-valine and 10% fetal bovine serum. After cells were confluent, monolayers received the following treatments before each biweekly media change: 1 mM L-leucine methyl ester for 2 hours to inhibit potentially neurotoxic microglia, followed by 100 μM glutamate for 20 min to reduce neuronal numbers, thereby promoting growth factor release by astroglia.) Between 4 to 31 DIV, cover slips were fixed and immunoreacted with the following primary antibodies: TuJ1 (1:500) and rabbit anti-GFAP (1:40; Boehringer-Mannheim) or rabbit anti-NSE (1:200; Dakopatts) and monoclonal anti-GFAP (1:40; Boehringer-Mannheim). After three washes and blocking in 10% goat serum, cover slips were incubated with fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit, or fluorescein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse secondary, respectively (1:40; all secondaries from Boehringer-Mannheim). Cells immunopositive for the neuronal marker and immunonegative for the glial marker were considered neurons. Multiple controls conducted with each experiment supported antibody specificity. Cover slips with less than ~1000 immunopositive cells were counted in their entirety. For cover slips with greater than ~1000 immunopositive cells, 6 to 40 random fields [0.64 mm^2 (0.45 mm radius); 200× magnification] were quantified and averaged to the entire cover slip. Differences in numbers between TuJ1- and NSE-immunopositive cells may be attributed to differential protein expression or different antibody sensitivity.
28. A six-well culture plate of P16 rat spinal cord cells was established without fetal monolayers. At 4 DIV, two cover slips were selected; the attached cells were fixed, immunoreacted with TuJ1 or anti-NSE, and counted (*25*). Of the four remaining wells, two received supplements of FUDR (15 μg/ml) with each media change. At 12 DIV, FUDR-treated and control cover slips (no FUDR) were immunoreacted with TuJ1 and anti-NSE. Cells were counted and comparisons were made between neuronal cell numbers in FUDR-treated versus control cultures (no FUDR). Negative controls were performed as described (*6*).
29. NSE/BrdU double-immunocytochemistry was carried out as follows: P15 to P16 spinal cord cultures, without monolayers, were incubated with 1 μM BrdU (added with media changes every 2 to 3 days) for 7 days during the first, second, third, or fourth week in vitro. Cultures were fixed on the seventh day of each week (7, 14, 21, or 28 DIV) and double-immunostained for BrdU (1:1; Amersham) and NSE. TuJ1/BrdU double-immunocytochemistry was as follows: BrdU (1 μM) was added to cultures with each media change (every 2 to 3 days) from 1 to 20 DIV. At 20 DIV, cultures were fixed and double-immunostained with TuJ1 (1:250) and sheep anti-BrdU (1:100; Fitzgerald). Acid denaturation (2N HCl), which is required for antigen detection with this antibody to BrdU, reduced TuJ1-immunoreactivity. Therefore, TuJ1/BrdU-immunoreactivity was visualized with confocal microscopy. Two independent observers scored TuJ1-labeled cells for BrdU content. TuJ1/BrdU cell counts performed with fluorescence microscopy on separate cover slips at 20 and 15 DIV yielded quantitatively similar results. Controls conducted with each experiment were as follows. TuJ1: mouse TuJ1 followed by anti-sheep secondary, sheep anti-BrdU followed by anti-mouse secondary, and TuJ1 primary omitted followed by co-application of anti-sheep and anti-mouse secondaries. NSE: rabbit anti-NSE followed by anti-mouse secondary, mouse anti-BrdU followed by anti-rabbit secondary, and anti-NSE primary omitted followed by co-application of anti-mouse and anti-rabbit secondaries. Control studies supported antibody specificity.
30. P15 to P16 cultures were established in six-well plates as described (*6*). Tissue culture inserts (0.45 μm per pore; Becton Dickinson) containing previously prepared fetal monolayers were placed in each well to condition media while ensuring fetal cells could not attach to postnatal cultures. To confirm fetal cells

would not pass through insert membranes, 12 control wells were prepared without postnatal tissue. At 20 to 23 DIV, cover slips were tested for trypan blue exclusion. No viable cells were found to have passed through insert membranes. Postnatal cells were patch-clamped at 20 to 27 DIV. Those generating APs were injected with 0.2% dextran fluorescein (Molecular Probes; −500 pA, 10 Hz, 30 min) for later identification. Then cells were fixed, treated with 2N HCl (37°C for 30 min), 0.1 M borate buffer (25°C for 10 min), and immunoreacted with sheep anti-BrdU (1:100; Fitzgerald), followed by rhodamine-conjugated secondary antibody (1:40; Jackson ImmunoResearch). Fluorescein-filled cells were identified with confocal microscopy and evaluated for BrdU-immunoreactivity by two independent observers.

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Control of Inflammation, Cytokine Expression, and Germinal Center Formation by BCL-6

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The gene encoding the BCL-6 transcriptional repressor is frequently translocated and mutated in diffuse large cell lymphoma. Mice with a disrupted BCL-6 gene developed myocarditis and pulmonary vasculitis, had no germinal centers, and had increased expression of T helper cell type 2 cytokines. The BCL-6 DNA recognition motif resembled sites bound by the STAT (signal transducers and activators of transcription) transcription factors, which mediate cytokine signaling. BCL-6 could repress interleukin-4 (IL-4)–induced transcription when bound to a site recognized by the IL-4–responsive transcription factor Stat6. Thus, dysregulation of STAT-responsive genes may underlie the inflammatory disease in BCL-6–deficient mice and participate in lymphoid malignancies.

Diffuse large cell lymphoma is a common and aggressive subtype of B cell non-Hodgkin's lymphoma that frequently harbors genetic alterations in the BCL-6 gene: Up to 45% of these lymphomas contain BCL-6 translocations and 73% have mutations in a putative 5′ regulatory region of the gene (*1*). Because these genetic changes invariably spare the BCL-6 coding region, the contribution of BCL-6 to lymphomagenesis is likely to be a subversion of its role in nontransformed cells. Consistent with this possibility, BCL-6 protein is expressed at the highest levels in germinal center B lymphocytes, which are the cells from which diffuse large cell lymphomas may arise (*2–5*). BCL-6 is a potent transcriptional repressor, but its natural target genes have not been identified (*6–8*). To determine the normal biological function of BCL-6, we disrupted BCL-6 in the mouse germ line.

Using embryonic stem cell methodology (*9*), we deleted a portion of the BCL-6 locus encoding the zinc finger DNA binding domain of the protein (*10*) (Fig. 1A) and confirmed the structure of the mutant locus by Southern (DNA) blot analysis of tail DNA (Fig. 1B). No BCL-6 protein derived from the targeted locus could be detected in either heterozygous (+/−) or homozygous (−/−) BCL-6 mutant mice (*11*) (Fig. 1C).

BCL-6$^{+/-}$ mice appeared normal and BCL-6$^{-/-}$ mice were born with a normal Mendelian frequency and size. However, beginning a few days to 3 weeks after birth, BCL-6$^{-/-}$ mice displayed variable degrees of growth retardation and ill health. About half of the BCL-6$^{-/-}$ mice were sickly and died before 5 weeks of age. Roughly 20% of BCL-6$^{-/-}$ mice appeared grossly healthy and were similar to wild-type littermates with respect to flow cytometric analysis of bone marrow, splenic, and thymic lymphocyte populations (*12*).

Pathological examination of the BCL-6$^{-/-}$ mice revealed a prominent myocarditis and pulmonary vasculitis that probably contributed to the animals' illness and death. Myocarditis was observed in 82% of the BCL-6$^{-/-}$ mice examined, and 73% of the mice had evidence of pulmonary vasculitis (Fig. 2), but neither pathology was observed in wild-type littermates. The cellular infiltrates in the hearts and lungs were composed of mononuclear cells and polymorphonuclear cells, virtually all of which were eosinophils (Fig. 2). Although inflammatory disease was generally correlated with ill health

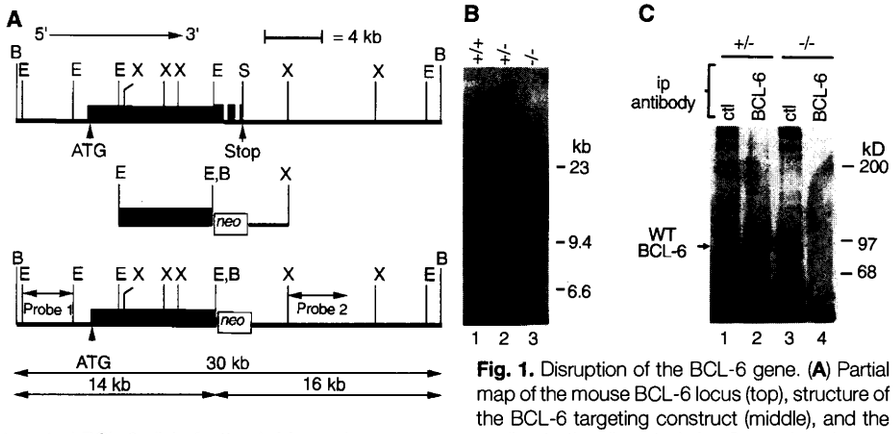

Fig. 1. Disruption of the BCL-6 gene. (**A**) Partial map of the mouse BCL-6 locus (top), structure of the BCL-6 targeting construct (middle), and the targeted BCL-6 allele (bottom). Mapped exons corresponding to the disrupted zinc finger domains are indicated by black boxes; the unmapped coding region is indicated by a grey box. The mutated BCL-6 locus would encode a truncated protein in which four of the six BCL-6 zinc fingers are disrupted and which cannot bind DNA in vitro (*16*). Restriction enzyme sites: B, Bam HI; E, Eco RI; S, Spe I; X, Xho I. *neo* = PGK-neomycin^r cassette. (**B**) Southern blot analysis of mice derived from intercrossing BCL-6$^{+/-}$ mice. Bam HI–digested tail DNA from wild-type mice (+/+) and mice heterozygous (+/−) or homozygous (−/−) for the disrupted BCL-6 allele was analyzed with a genomic flanking probe [probe 2 in (A)]. The structure of the targeted BCL-6 allele was also confirmed using probe 1 (A). (**C**) Analysis of wild-type and mutated BCL-6 protein expression. Spleen cells from immunized BCL-6$^{+/-}$ and BCL-6$^{-/-}$ mice were assayed for BCL-6 protein expression by immunoprecipitation followed by protein immunoblotting (*11*). Wild-type BCL-6 migrates at 90 to 100 kD (*2–5*) (lane 2); the truncated BCL-6 protein encoded by the targeted BCL-6 allele, predicted to be 12 kD smaller than the wild-type protein, was not detectable. ctl, control.

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