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CENTRAL AUTONOMIC CONTROL OF THE BONE MARROW: MULTISYNAPTIC TRACT TRACING BY RECOMBINANT PSEUDORABIES VIRUS

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Abstract—Bone marrow is the primary place of hematopoiesis, where the development, survival and release of multipotent stem cells, progenitors, precursors and mature cells are under continuous humoral and neural control. Dense network of nerve fibers, containing various neurotransmitters is found in the bone marrow, however, the central neuronal circuit that regulates the activities of the bone marrow through these fibers remained unexplored. Transsynaptically connected neurons were mapped by virus-based transneuronal tracing technique using two isogenic, genetically engineered pseudorabies viruses, Bartha-DupGreen and Ba-DupLac expressing green fluorescent protein and β-galactosidase, respectively. Bartha-DupGreen was injected into the femoral bone marrow of male rats and the progression of infection was followed 4–7 days post-inoculation. Virus-labeled cells were revealed in ganglia of the paravertebral chain and in the intermediolateral cell column of the lower thoracic spinal cord. Neurons were retrogradely labeled in the C1, A5, A7 catecholaminergic cell groups and several other nuclei of the ventrolateral and ventromedial medulla, the periaqueductal gray matter, the paraventricular and other hypothalamic nuclei, and in the insular and piriform cortex. Nerve transections and double-virus tracing from the bone marrow and the surrounding muscles were used to confirm the specific spreading of the virus. These results provide anatomical evidence for the CNS control of the bone marrow and identify putative brain areas, which are involved in autonomic regulation of the hematopoiesis, the release of progenitor cells, the blood supply and the immune cell function in the bone marrow.

Key words: autonomic nervous system, sympathetic innervation, hypothalamus, brain stem, spinal cord, green fluorescent protein.

The bone marrow is a richly vascularized organ containing the principal hematopoietic tissue that generates various blood cells, which are constantly replaced throughout the life. Hematopoiesis occurs in association with a complex marrow stroma comprising a heterogeneous population of cells including fibroblasts, adipocytes, osteoblasts and other cellular elements (Mayani et al., 1992; Bianco and Riminucci, 1998). The bone marrow also contains cells that meet the criteria for stem cells of non-hematopoietic tissues (Prockop, 1997; Gronthos et al., 2003). These marrow stromal stem cells may be released in response to various challenges and are involved in the replacement or the repair, tissues of mesenchymal origin (Bianco et al., 2001; Huang et al., 2001; Hofstetter et al., 2002).

The proliferation and activity of bone marrow cells are regulated by local and systemic humoral factors. By presentation of cytokines, growth factors and membrane-bound adhesion molecules, stromal cells locally modulate leukocyte production, activation, migration and release (Yoder and Williams, 1995; van Buul et al., 2002; Cassese et al., 2003). It has also been shown that circulating steroid and peptide hormones affect blood cell proliferation, and thereby the immune function. The best known example is the immunomodulatory effect of stress-induced adrenal corticosteroids and catecholamines (Dhabhar et al., 1995; McEwen, 2000).

It has been clearly demonstrated that, in addition to hormonal regulation, the neural control plays a prominent role in the regulation of hematopoiesis (Maestroni and Conti, 1994a,b; Afan et al., 1997; Broome and Miyan, 2000; Maestroni, 2000a). The bone marrow, similarly to other lymphoid organs, is controlled by the autonomic nervous system. Myelinated and non-myelinated fibers originate from the nerves that enter the bone along with blood vessels through the nutrient foramina and give rise to smaller branches that spread over the periosteum while other branches penetrate into the bone marrow cavity. Nerve fibers may cross the parenchyma of the bone marrow or terminate on sinusoid walls, vascular elements and on perivascular stromal cells (Calvo, 1968; Yamazaki and Allen, 1990; Mach et al., 2002). Nerve fibers containing calcitonin gene-related peptide (CGRP), substance P

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Abbreviations: ASP, putative antisense promoter; BDG, Bartha-DupGreen virus; BDL, Bartha-DupLac virus; BNST, bed nucleus of the stria terminalis; CAN, central autonomic nucleus; CGRP, calcitonin gene-related peptide; DAB, diaminobenzidine; β-gal, β-galactosidase; GFP, green fluorescent protein; IE, immediate early; IML, intermediolateral cell column; ir, immunoreactivity; KPBS, potassium phosphate-buffered saline; LC, locus coeruleus; LPGi, lateral paragigantocellular nucleus; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; OVLT, organum vasculosum of the lamina terminalis; PAG, periaqueductal gray matter; PFU, plaque-forming unit; PRV, pseudorabies virus; PVH, hypothalamic paraventricular nucleus; SP, substance P; SPN, sympathetic preganglionic neurons; TH, tyrosine hydroxylase.

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(SP), neuropeptide Y (NPY), tyrosine hydroxylase (TH) and dopamine (DA) were identified in the bone marrow (Tabarowski et al., 1996; Elenkov et al., 2000; Mach et al., 2002) and neuropeptide/neurotransmitter receptors are present on the surface of several hematopoietic cell types (Liebl et al., 1991; McGillis et al., 1991; Santambrogio et al., 1993; Petitto et al., 1994).

However, the exact localization and organization of the CNS circuit that provide relevant inputs to those cells that innervate the bone marrow remained unknown.

In order to reveal these circuits, we applied a multisynaptic tract-tracing method using pseudorabies virus (PRV), a neurotropic herpesvirus, which is a commonly used tool for delineation of hierarchically organized neuronal pathways (Card et al., 1990; DeFalco et al., 2001; Gerendai et al., 2001; Boldogkoi et al., 2002; Enquist and Card, 2003). After being uptaken by axon terminals, viral nucleocapsids travel retrogradely to the cell body, replicate, and the newly assembled mature virions infect functionally connected neurons across the synapses. We injected Bartha-DupGreen (BDG), a recombinant PRV strain with Bartha background (Boldogkoi et al., 2002, 2004) into the femoral bone marrow in order to identify the neuronal elements which are involved in the innervation of this structure.

EXPERIMENTAL PROCEDURES

Viruses

Two isogenic recombinant PRVs, BDG and Bartha-DupLac (BDL) (both are Bartha virus derivatives), with modified neuroinvasiveness, were used in these experiments. The spreading characteristics of the viruses were altered by insertion of the LacZ (BDL) or the green fluorescent protein (GFP) (BDG) gene expression cassettes into the putative antisense promoter (ASP) located at the inverted repeat region of the virus. Both reporter genes were put under the control of the human cytomegalovirus major immediate early (IE) one promoter. These mutants label autonomic neurons in a slow manner and infect fewer neurons. Due to the slow spread of viruses, local immune cells effectively isolate infected neurons, whereby preventing local (non-specific) spread of infective viral particles that might be released from compromised cells at late stage of viral infection. The slow transsynaptic passage of Ba-Dup viruses allows applying longer survival for the analysis of higher-order brain structures. In addition, the early appearance of the reporter proteins (GFP or β-galactosidase (β-gal)) during viral replication allows early identification of infected neurons and co-localization of phenotypic markers by immunofluorescence (Boldogkoi et al., 2002).

Viruses were grown in porcine kidney (PK)-15 cells to a titer of 6×10^8 plaque-forming units (PFU)/ml. BDG was further concentrated to 1.5×10^{10} PFU/ml by ultracentrifugation at $70{,}000\times g$. Stocks were aliquoted and stored at −80 °C. Aliquots were thawed immediately prior to injection and stored at 4 °C during surgery.

Animals

Adult, male Wistar rats (Toxi-coop, Budapest, Hungary) weighing 250–350 g were maintained under temperature-, humidity- and light-controlled conditions (21±1 °C, 65% humidity, 12-h light/dark cycle, with lights on at 7 AM). The animals had free access to food and water. After virus inoculation the rats were housed individually and monitored twice daily. All efforts were made to minimize their suffering. The rats ate and drank normally and exhibited normal behavior.

The procedures were conducted in accordance with the guidelines set by the European Communities Council Directive (86/609 EEC) and Hungarian Government directive 243/98 and approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Medicine.

Virus injections

Animals were anesthetized with a mixture of anesthetic solution: 50 mg/kg ketamin (Richter, Budapest, Hungary), 10 mg/kg xylazin (Spofa, Czech Republic) and 5 mg/kg prometazin (EGIS, Budapest, Hungary) by i.p. injection.

Twenty six animals were used for inoculation of bone marrow with virus. The titer of the virus and the volume of injected virus suspension were experimentally determined in pilot studies. Injection of 2×10^6 PFU of BDG into the bone marrow did not infect effectively any of the injected five animals. One out of four and two out of five animals were infected when rats were injected with inoculums containing 1.2×10^7 and 2×10^7 PFU of BDG, respectively. Finally, when 16 μl of 1.5×10^{10} PFU virus recombinants were injected into the bone marrow, 90% of the animals became infected. This dose was then further used to infect bone marrow in our mapping studies. The left femur was exposed, the bone surface was cleaned using 3% H_2O_2 and two holes were burred into the distal epiphysis. To avoid any viral contamination during injections, the femoral bone was isolated from the surrounding tissue using a cotton wool impregnated with 3% H_2O_2. A suspension (2×8 μl) of BDG virus (2.4×10^8 PFU) was injected into the bone marrow using a 10 μl Hamilton syringe. The needle was kept in place for 5 min to avoid the reflux of the inoculums along the needle track. The place of injections was stamped with Ethicon bone wax and the surface of the bone was wiped with 70% ethanol. The muscles surrounding the femur were sutured, and the skin was closed. Different survival times were used to evaluate temporal progression of the virus: 4 days ($n=4$), 5 days ($n=6$), 6 days ($n=7$), 7 days ($n=5$).

In four additional rats, the virus was inoculated into the bone marrow of the proximal epiphysis.

To infect the bone periosteum, 2 μl suspension of BDG virus (1.5×10^{10} PFU/ml) was dropped onto the isolated surface of the bone ($n=3$), kept in place for 10 min, wiped and the wound was closed. We investigated in former studies whether this infection time and the amount of the inoculum is sufficient for the virus to invade nerve endings and to develop productive infection. The same amount (1.5×10^{10} PFU/ml) of BDG was dropped onto the surface of femoral muscles and wiped off 10 min later. Five days afterward, virus-labeled neurons were found in the brain stem and in the hypothalamic pre-autonomic nuclei (PVH). One animal was killed 6 days after virus injection because of visible signs of infection. These experiments verified that this infection timing is sufficient for the attachment of the virus particles to the nerve endings and resulted in productive infection in case of muscular elements.

An additional group of six rats was used to infect the femoral muscles. Ten times 1 μl BDG injections were given to different places of the femoral muscles (musculus vastus lateralis and musculus adductor brevis). The titer of the virus was 6×10^8 PFU/ml ($n=3$) or 1.5×10^{10} PFU/ml ($n=3$).

To examine the specificity of BDG transport to the CNS following bone marrow injections, femoral, sciatic and obturator nerves were transected in six rats, immediately before inoculation of the ipsilateral bone marrow. The postinoculation time was 5 days.