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Biotin–dextran: a sensitive anterograde tracer for neuroanatomic studies in rat and monkey

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The properties of a new anterograde tracer, biotin–dextran (BD), are reported. Iontophoretic or pressure injections of BD in the spinal cord of rats and monkeys revealed terminal-like BD label in many regions of the brain. The BD label was visualized by an avidin–biotin reaction combined with diaminobenzidine, with and without nickel enhancement. This reaction resulted in permanent label and revealed the fine morphology of terminal boutons and en-passant endings. Biotin–dextran is an excellent anterograde tracer that can also be visualized at the electron microscopic level, used in combination with other retrograde and anterograde tracers and with immunohistochemical labeling of neurotransmitters.

Introduction

Extracellularly injected anterograde tracers have been used in the past to study the connectivity of the central nervous system. Perhaps the most popular anterograde tracer has been wheat germ agglutinin-coupled horseradish peroxidase (WGA–HRP). This tracer is transported quickly and reliably in rodents, cats and primates over long distances (Mesulam, 1978). However, WGA–HRP has a number of shortcomings; when WGA–HRP is processed with tetramethylbenzidine (the most sensitive reaction for WGA–HRP labeling), the morphology of terminal structures is masked by the large crystals of the reaction product. Also, WGA–HRP when used at high concentrations and with long survival times can result in transynaptic labeling (Apkarian and Hodge, 1989). The plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHAL), has recently become the tracer of choice for most anterograde studies, especially in rodents, since it reveals fine terminal morphology from small, localized injection sites (PHAL is injected iontophoretically; Gerfen and Sawchenko, 1984). The major disadvantage of PHAL is its unreliable transport in cats and monkeys (personal observations). Biocytin, a conjugate of biotin and lysine, can be used as an anterograde tracer, but its use is limited to studies requiring transport over short distances due to tracer degradation over time (within 36 h; King et al., 1989; Izzo, 1991; Norita et al., 1991). Studies have shown that dextran conjugated with various fluorescent markers, such as tetramethylrhodamine, can be used as anterograde tracers (Nance and Burns, 1990; Schmued et al., 1990; Nicolelis et al., 1991). These markers are sensitive and reliable anterograde tracers. However, the disadvantage of fluorescent dextrans is rapid fading, making data collection difficult.

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In this study, we report the properties of a new anterogradely transported tracer, dextran coupled with biotin (BD). Exploiting the selectivity and affinity between biotin and avidin, the avidin–biotin complex reacted with diaminobenzidine (DAB) resulted in a sensitive, permanent reaction that reveals the fine morphology of anterogradely labeled terminals. Following submission of this paper for publication, Veenman et al. (1992) published a description of the anterograde transport properties of BD in rat, pigeon and chick showing similar label quality.

Materials and methods

Three rats (395–480 g) and 3 squirrel monkeys (570–605 g) were used for this study. Animals were premedicated with atropine (0.05 mg/kg, i.m.) and Decadrone (0.5 mg/kg, i.m.). In addition, primates received Dilantin (2.5 mg/kg, i.m.) and Rocephine (75 mg/kg, i.m.). All surgical procedures were done under sterile conditions in strict compliance to the guidelines of the Institutional Animal Care and Use Committee. Animals were initially anesthetized with ketamine (20–40 mg/kg, i.m.), rats were maintained with 0.3–0.5% Metofane and monkeys with 0.5–1.5% halothane, both in combination with 2/3 oxygen and 1/3 nitrous oxide. In primates, blood pressure, expiratory CO_2 and temperature were monitored and kept within the physiological range.

Lysine-fixable BD (mol.weight 10,000; Molecular Probes, D-1956) was freshly prepared for each injection (10% solution in saline). Injections were done by pressure (tip diameter 50 μm, 0.2–0.5 μl volumes; Picospritzer II, General Valve Corp.) or iontophoretically (tip diameter 30 μm, 5–7 μA positive current, 0.1 Hz, for 20 min; Midgard CS-4). Extracellular BD injections were made in gracile nucleus, upper cervical spinal cord grey matter (C1–C2) or cervical enlargement (C5–T1) grey matter. After each injection, the micropipette was kept in place for 1 min. In 1 monkey, the spinal cord BD injection was combined with a somatosensory cortical injection of the retrogradely transported fluorescent dye diamidino yellow (DY). After surgery, the animals were returned to their cages and monitored for normal recovery. The monkeys received supplemental doses of antibiotics for 2 days and Torbogesic (0.05–0.1 mg/kg, i.m.) when needed. All animals were conscious and moving normally within 1 day and showed no signs of behavioral or locomotor disorders. Survival times for the rats were 7, 14 and 23 days, and for the squirrel monkeys 3, 5 and 7 weeks. Rats were overdosed by Nembutal (50 mg/kg), monkeys with ketamine (8 mg/kg) and Nembutal (15 mg/kg), and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M acetate buffer (pH 4.5, room temperature). All animals, except 1 rat and 1 monkey, received an additional liter of chilled 0.05 M borate buffer (pH 9.5) with 4% paraformaldehyde and 0.05% glutaraldehyde (4°C), followed by 10% sucrose in phosphate buffer (PB; pH 7.6, 4°C). The tissue was removed and stored in 20% sucrose in PB (4°C).

The tissue was normally cut (50-μm-thick sections, freezing microtome) and reacted within 1 week of fixation. One piece of tissue was sectioned and reacted 9 weeks after perfusion to determine reliability of reaction over longer time periods. The avidin–biotin reaction was performed at 37–40°C under constant agitation with the Vectastain Standard kit or the Elite kit (Vector). The reaction product was developed with the DAB or the nickel-enhanced DAB reaction.

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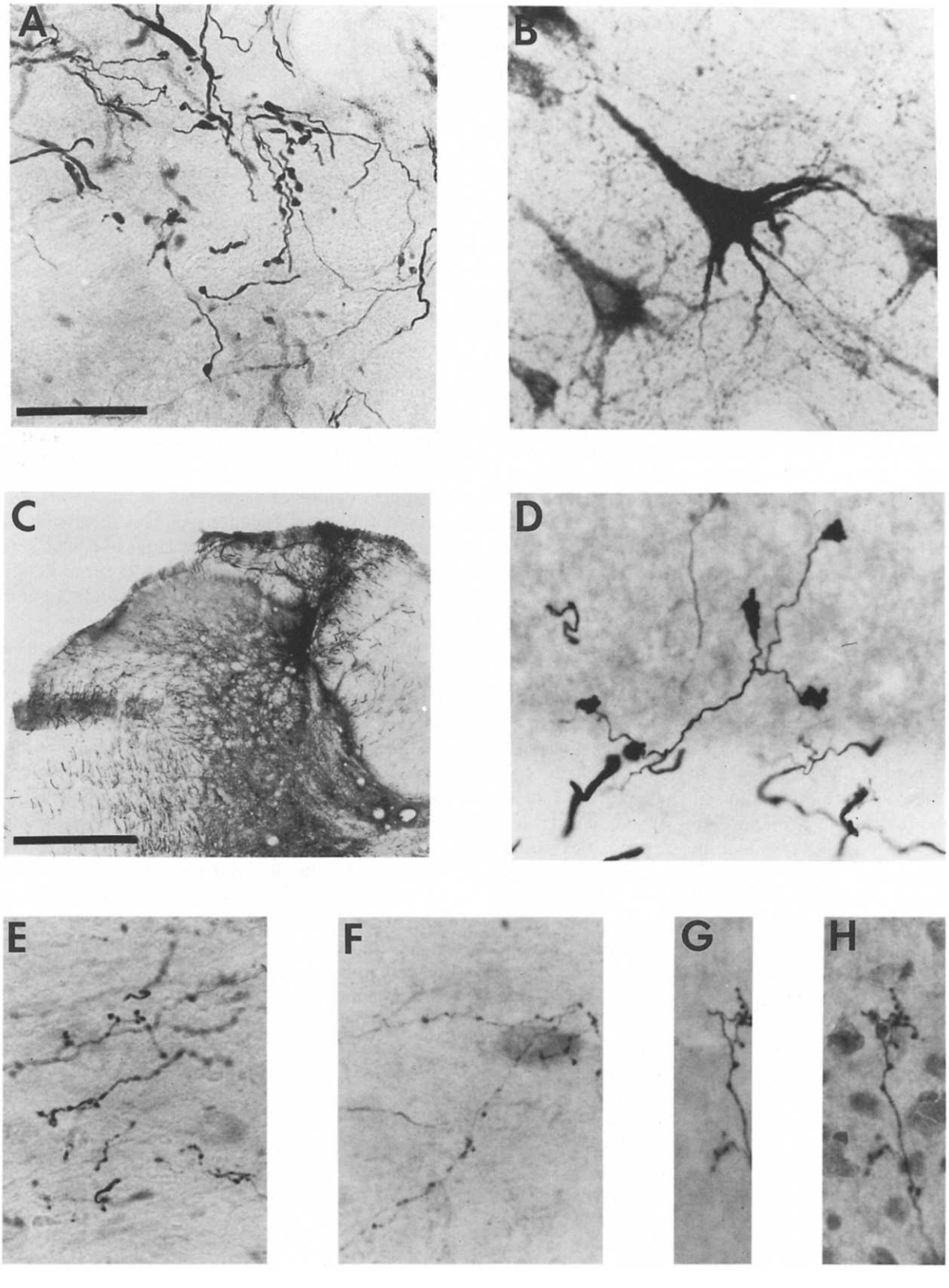
Fig. 1. Biotin–dextran injection site and anterograde terminal label in rat and monkey. A: photomicrograph of terminal label seen in VPL in the rat following an injection into the gracile n. This tissue was reacted with DAB only. B: retrogradely labeled cell in squirrel monkey cortex after BD injection into spinal C1–C2. C: a pressure injection site of BD in upper cervical spinal cord of a rat; the injection covers only the medial portion of laminae IV–V. D: mossy fibers labeled with BD in the cerebellum of a squirrel monkey. This tissue was reacted 9 weeks after perfusing the animal. E: terminal label in zona incerta. F: terminal label in the substantia innominata. G and H: terminal BD in the hypothalamus before and after thionin staining, respectively. D–H are from upper cervical spinal cord BD injections in a squirrel monkey. The reactions in B–H are all nickel-enhanced DAB. Calibration bar in A is 50 μm, calibration bar in C is 0.5 mm. Magnification in B, D–H is the same as in A.


A
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Sections were washed in 0.1 M PB saline with 0.5% Triton-X 100 for 5 min, incubated for 60 min with the Vectastain kit (concentration as recommended by Vector), washed twice (10 min each) and incubated in DAB or nickel-enhanced DAB for 10–20 min. The DAB reaction used 0.05% DAB and 0.005% H_2O_2 in Tris buffer (0.05 M, pH 7.6) (Smith et al., 1990); the nickel-enhanced DAB solution consisted of 0.4% nickel ammonium sulfate, 0.015% DAB and 0.005% H_2O_2 (combined methods of Wouterlood et al., 1990 and Matsushita and Yaginuma, 1990). After the tissue was rinsed in 0.1 M PB and mounted out of 0.033 M PB, sections were dehydrated, cleared and coverslipped. Multiple reaction procedures were performed per animal to optimize visualization of the anterograde label. After plotting the locations of the labeled terminals, the coverslips of some sections were removed and the tissue stained with thionin for cytoarchitectural determination of nuclear boundaries, using the atlas of Paxinos and Watson (1982) for the rat and Emmers and Akert (1963) for the squirrel monkey.

Results

Anterograde terminal BD labeling was observed in all the animals. In both rat and squirrel monkey, terminal and en passant BD-labeled boutons were seen in exquisite detail (Fig. 1A, D–H). Labeled terminal-like structures of various sizes and shapes were observed, some of which, due to the fine caliber of the structures, could only be seen at high magnification. Large numbers of labeled axons were seen filled both anterogradely (spinothalamic tract) and retrogradely (corticospinal tract). Retrogradely labeled cells were also observed (Fig. 1B). These cells were located around the injection sites, in the brain stem and the cortex in regions of known afferent input. Cells labeled with BD around the injection sites were small in number and some were located in the contralateral grey matter, similar to PHAL-labeled cells observed around spinal cord injections (personal observations). Large numbers of BD-labeled cells were seen in the brain stem and the cortex, although most labeled cells were lightly filled rendering many indistinguishable from background DAB reacted cells. For the majority of labeled cells the dendrites were not filled; a few cells, however, were intensely labeled exhibiting the details of their major dendritic branches.

The number of labeled terminals as well as their intensity of filling was best after long survival times (23 days in rats and 5 and 7 weeks in monkeys). For example, following BD injections in the cervical enlargement, anterograde terminal label was found as far anterior as the ventral portion of the globus pallidus (as recently described in the rat by Cliffer et al., 1991), in both rat and monkey. Pressure injection of BD into the gracile n. of 1 rat resulted in heavy labeling in the ventral posterior lateral n. (VPL) (Fig. 1A). Distinct regions and patterns of label were observed following injections of BD in gracile n., various laminae of the upper cervical spinal cord (Fig. 1C) or the cervical enlargement, the details of which will be described in the future (manuscript in preparation).

In the monkey with BD injections throughout the cervical enlargement (C5–T1), some of the injection sites were located in the spinal cord white matter, but there was no BD spread across midline. In this animal, thalamic anterograde BD label was found ipsilateral as well as contralateral to the injection. The contralateral BD label in VPL was confined to the hand portion of the nucleus, while the ipsilateral VPL labeling was confined to the leg portion. We interpret the ipsilateral BD label to be primarily due to axonal uptake from the white matter injections.

In the monkey with cortical DY injection, fluorescent retrogradely labeled cells were visualized in thalamic tissue processed for BD with the nickel-enhanced reaction. The quality of anterograde BD labeling was similar for iontophoretic and pressure injections; although pressure injections always resulted in larger injection sites and, thus, higher density of terminals. There was no difference in labeling quality after perfusion with or without glutaraldehyde and sucrose. However, the intensity and number of labeled terminals was

higher in sections reacted with the Elite kit as compared to the Standard kit, as described by Lachica et al. (1991). The Elite kit also produced a background staining helpful in determination of nuclear boundaries. Using the Elite kit, there was no observable difference between sections developed with DAB versus those developed with nickel enhanced DAB. The Vectastain working solution was reuseable for a 1-week period, and tissue blocks, kept in 20% sucrose, when cut and reacted 9 weeks after the perfusion showed no significant loss in label quality (Fig. 1D).

Additionally, when coverslips were removed from sections and tissue stained with thionin, the majority of the BD label was preserved (Fig. 1G and H).

Discussion

Biotin–dextran is an excellent anterograde tracer that reveals fine details of terminal structures and is readily transported over long distances both in rats and monkeys. Anterograde transport of BD, however, requires relatively long survival times. The reaction is fast and simple, and the resulting reaction product is permanent and compatible with many retrogradely transported fluorescent tracers (e.g., DY and rhodamine beads; see Steward, 1981). This technique can be combined with various immunohistochemical procedures (Veenman et al., 1992) and since the chromagen is DAB it is possible to visualize it at the electron microscopic level (Wouterlood and Jorritsma-Byham, manuscript submitted for publication). Since BD completely fills labeled axons both anterogradely and retrogradely, the interpretation of labeled axons requires caution. Also, since BD is readily taken up through white matter injections, it is likely that BD is anterogradely transported through fibers of passage from grey matter injections as well. Since large numbers of BD-labeled cells are only lightly filled, we do not find BD a useful retrograde tracer. We found no evidence for transynaptic transport of BD (e.g., there were no BD-labeled cells in the thalamus and the BD-labeled cells were located in regions known to project to the injected site) even with large, multiple injections and long survival periods.

The location of BD-label found throughout the brain in rats with upper cervical injections was very similar to the label we have observed in rats with PHAL injections in the same region (Apkarian and Brandt, 1991). Also, BD injections in the upper cervical spinal cord level in monkeys resulted in labeled terminals in the same locations as in the rats; PHAL injections in the same region in monkeys showed label in some of the same regions but at much reduced densities (manuscript in preparation). Cervical enlargement injections of BD in monkeys resulted in thalamic label similar to that we have observed in monkeys injected with WGA–HRP (Apkarian and Hodge, 1989). However, anterior to the thalamus, BD revealed fine terminals in areas where we have not previously seen anterograde labeling (manuscript in preparation). This tracer reveals fine terminal thalamocortical structures in cats, and primary afferent terminals in the spinal cord of the neonatal frog (personal communication, Vahle-Hinz and Stelzner, respectively, using our technique).

The anterograde transport properties of BD seem similar to the anterograde transport properties of dextran conjugated to tetramethylrhodamine. Whether the fluorescent dextran tracers have the same sensitivity and reproducibility as BD remains to be ascertained. More importantly, BD and the fluorescent dextran tracers can be used together to compare anterograde label from multiple sites (see e.g., Hazrati and Parent, 1992). Overall, BD is a superior anterograde tracer than WGA–HRP and PHAL; however, since it is avidly taken up by fibers of passage the anterograde label must be interpreted cautiously.

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