

Minor Immunoreactivity in GDNF-, BDNF-, or NT-3-Treated Substantia Nigra Allografts

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SUMMARY

Glial-cell-line-derived neurotrophic factor (GDNF) stimulates the survival of dopaminergic neurons. Little is known, however, about the possible immune sequelae of GDNF exposure or of exposure to other putative trophic factors. To address these questions, pieces of mesencephalic tissue, substantia nigra, from 15-day-old donor embryos were transplanted into the anterior chamber of the eye of adult male Sprague-Dawley recipient rats. At 5-day intervals, an aliquot (0.5 µg) of GDNF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or cytochrome-C (CC) was injected into the anterior chamber of the eye of the recipients, and the sizes of the transplants were measured. GDNF increased transplant survival and growth. On day 42, all rats were sacrificed, and the grafts were evaluated by cresyl-violet staining and by immunohistochemistry using antibodies raised against neurofilament (NF), tyrosine hydroxylase, or glial fibrillary acidic protein (GFAP), as well as the following monoclonal antibodies: OX-38 anti-CD4, OX-8 anti-CD8, OX-18 anti-MHC class I, OX-6 anti-MHC class II, OX-42 anti-CD11b, R-73 anti- α and anti- β T-cell receptor, and ED1 raised against monocytes/macrophages. BDNF-treated

grafts showed only weak immunoreactivity, and even weaker reactions were seen in grafts treated with NT-3, GDNF, or CC. No single immune system marker was significantly elevated in grafts from any treatment group. We used OX-42 and ED1 to study possible alterations of microglial components. Ramified microglial cells were found in GDNF-treated grafts and to a lesser extent in NT-3 and BDNF-treated grafts. ED1-labeled reactive microglial components were found in NT-3- and BDNF-treated grafts. Additionally, large and rounded OX-42-positive phagocytic cells were found in NT-3-treated grafts. Together with our previous finding that GDNF treatment of spinal cord transplants activates immune responses and leads to microglial activation, our data demonstrate that although treatment with GDNF and to some degree with BDNF can enhance immune responses to immunogenic grafts, such as fetal spinal cord grafts, but the trophic factors *per se* do not elicit any marked response in non-immunogenic grafts like substantia nigra.

KEY WORDS

transplantation immunology, glial-derived neurotrophic factor, brain-derived neurotrophic factor, neurotrophin-3, TGF- β , microglia,

INTRODUCTION

Glial-cell-line-derived neurotrophic factor (GDNF) shows trophic effects not only on ventral mesencephalic dopaminergic neurons [4,6,11,18, 37,44,48] but also on spinal cord motoneurons

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/30,54/, but little is known about the possible immune sequelae of host and graft exposure to GDNF or to other putative trophic factors. In allogeneic but not syngeneic intraocular fetal spinal cord transplants, GDNF upregulates the expression of major histocompatibility (MHC) loci antigens and activates graft-derived microglial cells /40/. GDNF mRNA in the cortex and hippocampus is upregulated in such pathological conditions as experimentally induced status epilepticus in the hippocampus and striatum /38/.

GDNF is a member of the transforming growth factor- β (TGF- β) superfamily /24/ of proteins that are produced and secreted by a variety of immunocytes /52/. The TGF- β proteins are well-known immunomodulators having the following immune functions: (a) inhibit the proliferation of thymocytes, T-cells, B-cells, and natural killer (NK) cells; (b) inhibit the generation of cytotoxic macrophages; (d) inhibit IgG and IgM production; (e) switch B-cells from IgG to IgA production; (f) modulate the production of cytokines; and (g) serve as chemoattractants for monocytes and neutrophils /52/. TGF- β proteins, however, have also been reported to elicit strong immune responses (reviewed in /10,15,28,50/).

Intraocular transplantation of central nervous system (CNS) tissue provides a unique method for examining the *in vivo* effects of trophic factors. Such proteins can be selectively administered into the anterior chamber, thus minimizing remote or indirect drug effects /6,18,44,49/. For example, fetal substantia nigra tissue grafted to the anterior chamber of the eye responds to GDNF /44/. Of particular relevance for immunological studies is that allogeneic grafts, even when manifesting good survival and growth, contain significant amounts of immunoreactive elements. In addition, area-specific differential immune responses are elicited after grafting /39/.

In the study presented here, we used embryonic substantia nigra transplants and an *in oculo* graft model as appropriate targets for GDNF activity to determine whether the TGF- β superfamily trophic molecule, in addition its stimulatory effects, might induce or interact with immune elements. To monitor the various elements of the immune system after GDNF administration, we used monoclonal antibodies (mAbs) raised against the following

T-cell, B-cell, or monocyte/macrophage cell-surface antigens: rat CD4 (OX-38), rat CD8 (OX-8), rat MHC class I (OX-18); rat MHC class II (OX-6). MAb (OX-42) raised against rat CD11b identifies cellular complement-receptor type 3 (CR3), which is found on the surface of classical (ramified) and amoeboid and/or reactive microglial subtypes /14,36,45/. MAb ED-1, raised against rat monocytes and macrophages, is a cytoplasmic marker of macrophages or activated microglia or both /8/ that localizes only to cells of the monocytic lineage /9,45/. We used allogeneic grafts to vary the basal immune status of the CNS tissue. For purposes of comparison, we tested other trophic factors from the neurotrophin family, including brain-derived neurotrophic factor (BDNF), which has survival-promoting activity on substantia nigra /17,21/, and neurotrophin-3 (NT-3), which influences immune reactions in grafted CNS tissue /40/.

MATERIALS AND METHODS

Intraocular grafting procedures

Young adult male Sprague-Dawley rats (SD rats, B & K, Sweden) weighing 150 g each were used as recipients of intraocular grafts. Fetuses from pregnant female rats were used as donors of embryonic mesencephalic tissue grafts. On the 15th embryonic-day (E15), pieces (1–3 mm wide) of substantia nigra were dissected out from the fetuses and bilaterally grafted, under ether anesthesia, to the anterior eye chamber of the eye of the adult hosts. To prevent prolapse of the iris, the eyes were pretreated with a drop of 1% atropine solution. The grafts were placed on the anterior surface of the iris through a small opening in the cornea as previously described /29/. Every 5th day after grafting (up to day 40), the volume of each transplant was estimated by stereomicroscopic observation, measuring the longest diameter multiplied by the diameter perpendicular to it. Every 5th day, each eye was injected with 5 μ L of a solution containing 0.5 μ g of one of the following factors: (a) GDNF (100 μ g/mL), (b) cytochrome C (CC, 100 μ g/mL), (c) BDNF (100 μ g/mL), (d) NT-3 (100 μ g/mL) /49/. Donor material from any one dam was equally distributed among all treatment groups. The

measured sizes of the grafts correlate well with the actual weight of the grafts at sacrifice /5/.

Immunohistochemistry

Forty-two days after transplantation, the host animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused via the ascending aorta with 50 mL of calcium-free tyrode solution (37 °C) and then with 300 mL of ice-cold fixative (4% paraformaldehyde in phosphate buffer). Transplants attached to host irises were post-fixed by immersion for 30–60 min in the same fixative, rinsed in 10% sucrose, frozen, and then sectioned on a cryostat at 14 μ m. Sections at periodicities of 8 to 12 were processed for glial fibrillary acid protein (GFAP, Sigma[®]) (1:100), neurofilament (NF, a kind gift from Prof. Doris Dahl (1:400), and tyrosine hydroxylase (TH, Pel Freeze[®]) immunohistochemistry (1:00), or stained for cresyl violet. MAbs raised against rat CD4–clone OX-38, rat CD8–clone OX-8, rat CD11b–clone OX-42, rat MHC class I–clone OX-18, and rat ED-1 raised against monocytes and macrophages were purchased from Serotec[®]; rat MHC class II–clone OX-6 was obtained from Pharmingen[®]. Primary MAbs were diluted 1:1000, except for OX-42 (1:200) and ED-1 (1:300). All tissues were stored for 48 hr at 4 °C in a humidified chamber. For OX-38, OX-8, OX-42, and OX-18, the secondary antibody was Fluorescein-linked rabbit anti-mouse IgG. For GFAP, NF, and TH, Fluorescein-isothiocyanate (FITC)-labeled goat anti-rabbit IgG served as the second antibody /39,41/. For each graft, a “total immunological score” was calculated by a summation of the scores for CD4, CD8, MHC classes I and II. Immunological scores were based on cell counts as follows: scores of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 were given to grafts where the respective average numbers of immunoreactive cells/field of view, using a primary magnification of 20 \times were 0–2, 3–7, 8–12, 13–17, 18–22, 23–27, 28–32, 33–37, 38–42, 43–47, and > 48. When in addition to cells, immunoreactive glial or fibrous processes or both were also noted, the scores were incremented by 0.5, 1, or 2 for small, moderate, or large amounts of MHC-I or MHC-II-positive material. Scores of OX-42 (CD11b) and ED-1 were 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 given to

grafts where these elements were found in 0–4, 5–14, 15–24, 25–34, 35–44, 45–54, 55–64, 65–74, 75–84, 85–94, or >95% of the area of the graft /40/. In addition, the TH-positive volume was estimated by scoring the percentage of the TH-positive area at three different levels of the transplant and then calculating the averages. We used the Mann-Whitney U test to determine the differences between paired groups.

RESULTS

Effect of growth factors on graft growth

Figure 1a shows the growth of intraocular substantia nigra grafts treated with different neurotrophic factors. GDNF treatment led to significantly larger graft volumes than CC ($p < 0.05$). Figure 1b shows the volume of TH-positive regions. GDNF-treated grafts had a significantly larger TH-positive volume compared with NT-3 and CC ($p < 0.05$). Neither BDNF nor NT-3 had a significant effect on either graft growth or volume of TH-positive areas in the grafts.

Overall immune response to growth factors

Figures 2–4 summarize the total immunological score and scores for each immune element. Although BDNF-treated allografts contained somewhat larger mean numbers of immune-related cells, including lymphocytes and antigen-presenting cells (Figs. 5,8b), the differences were not significant when compared with the control group (Figs. 2–4). Occasionally, NT-3 and BDNF treatments led to higher amounts of immune-related elements (Figs. 6,8c). GDNF treatment did not appear to cause any significant immune reactivity. All transplants contained very low numbers of OX-38-positive elements (data not shown).

Immunologic patterns for individual growth factors

GDNF-treated transplants showed excellent neuronal survival (Figs. 7,8). MHC Class I-positive, ramified microglial cells could be identified, but only a few such cells were seen in the grafts. In addition, GDNF-treated grafts showed well-developed

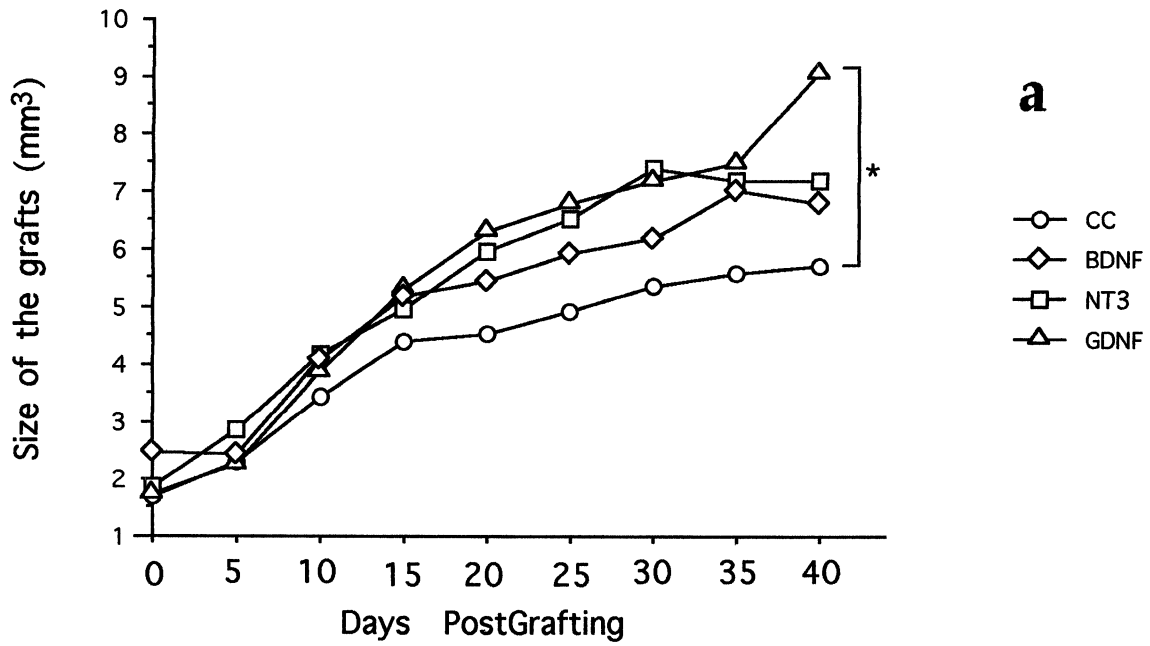


Fig. 1. a: Growth curves of intraocular transplants given GDNF (triangle) or BDNF (diamond), NT-3 (□), and cytochrome-C (○) I Sprague-Dawley rats. GDNF-treated transplants showed increased growth (mean±s.e.m., * = $p < 0.05$) compared with control, cytochrome-C treatment.

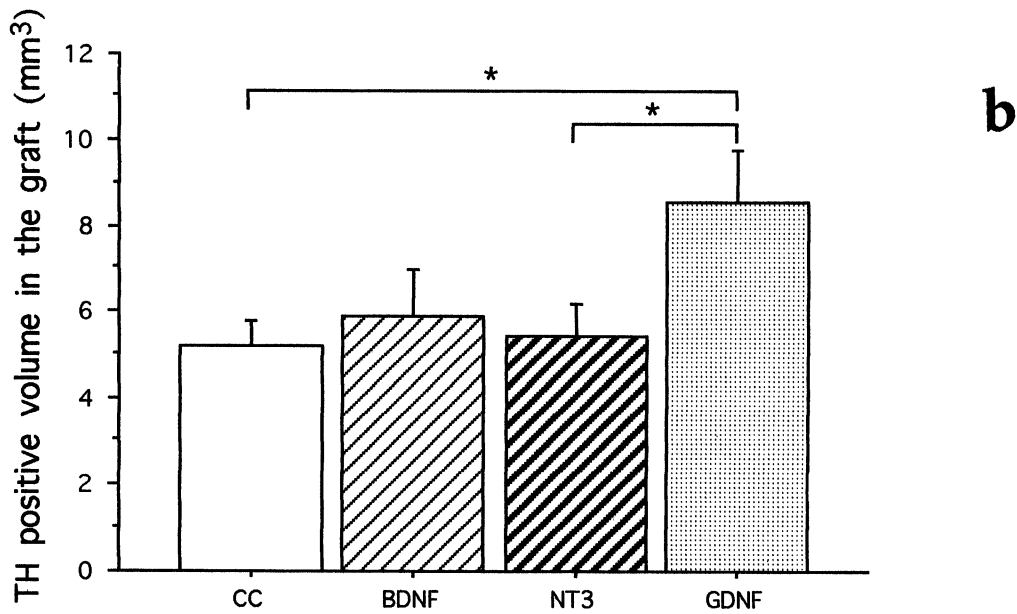


Fig. 1. b: TH-positive volume in grafts. GDNF treatment led to significantly larger TH-IR areas than BDNF, NT-3, or cytochrome-C treatment (mean±s.e.m., * = $p < 0.05$).

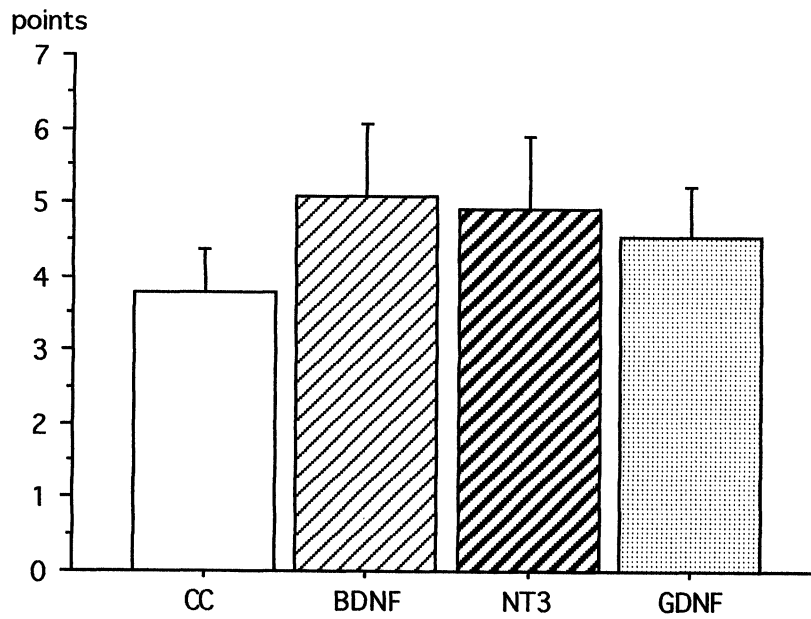


Fig. 2. Total immunological scores. BDNF, NT-3, and GDNF treatments causes only minor changes in mean scores. Differences were not significant.

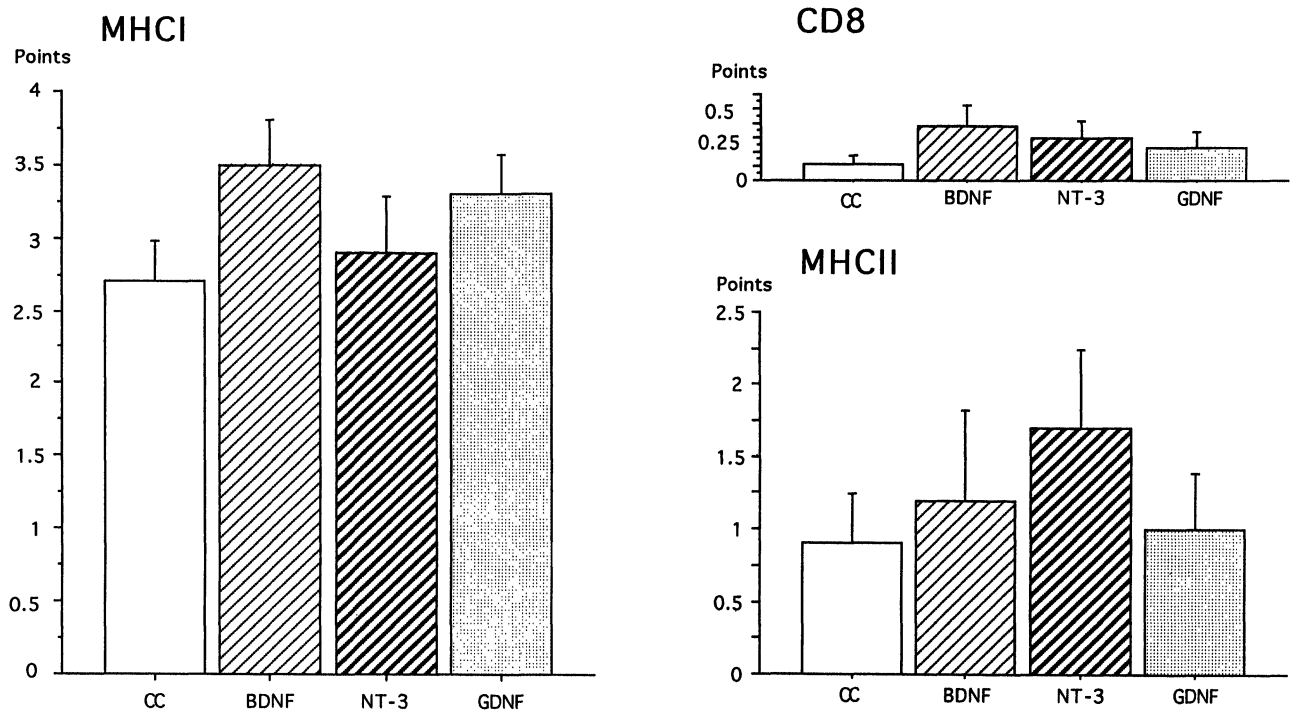


Fig. 3. Scores for individual immune markers. Scores of CD4 were excluded because they were close to zero. The NT-3 treatment group had a higher mean MHC class II expression score, but the difference was not significant.

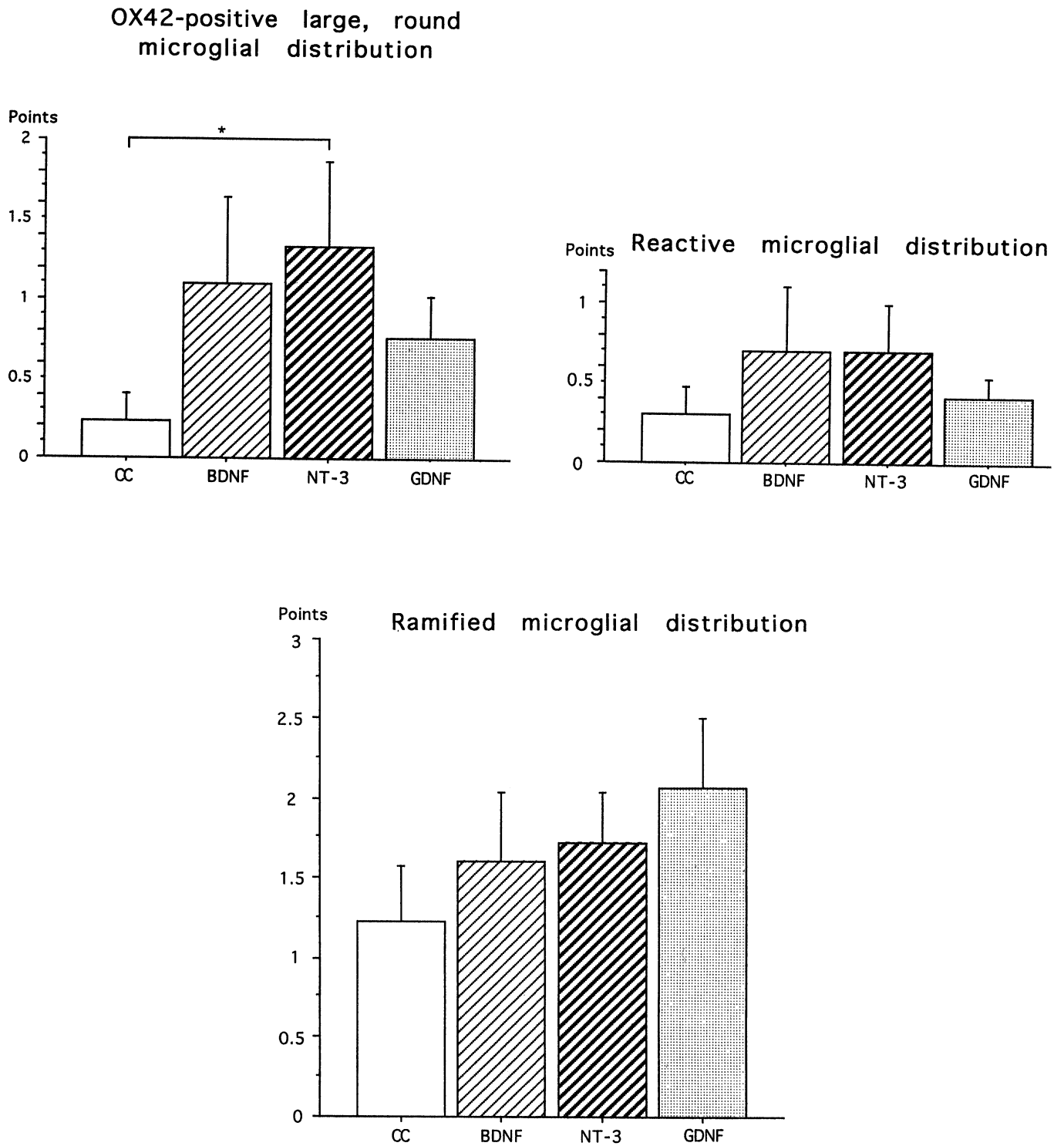


Fig. 4. Microglial alterations. GDNF-treated grafts had well-developed ramified microglial cells and fewer reactive microglial cells. NT-3-treated grafts had significantly higher numbers of OX-42-positive large and round microglial cells than the cytochrome-C-treated grafts ($p < 0.05$).

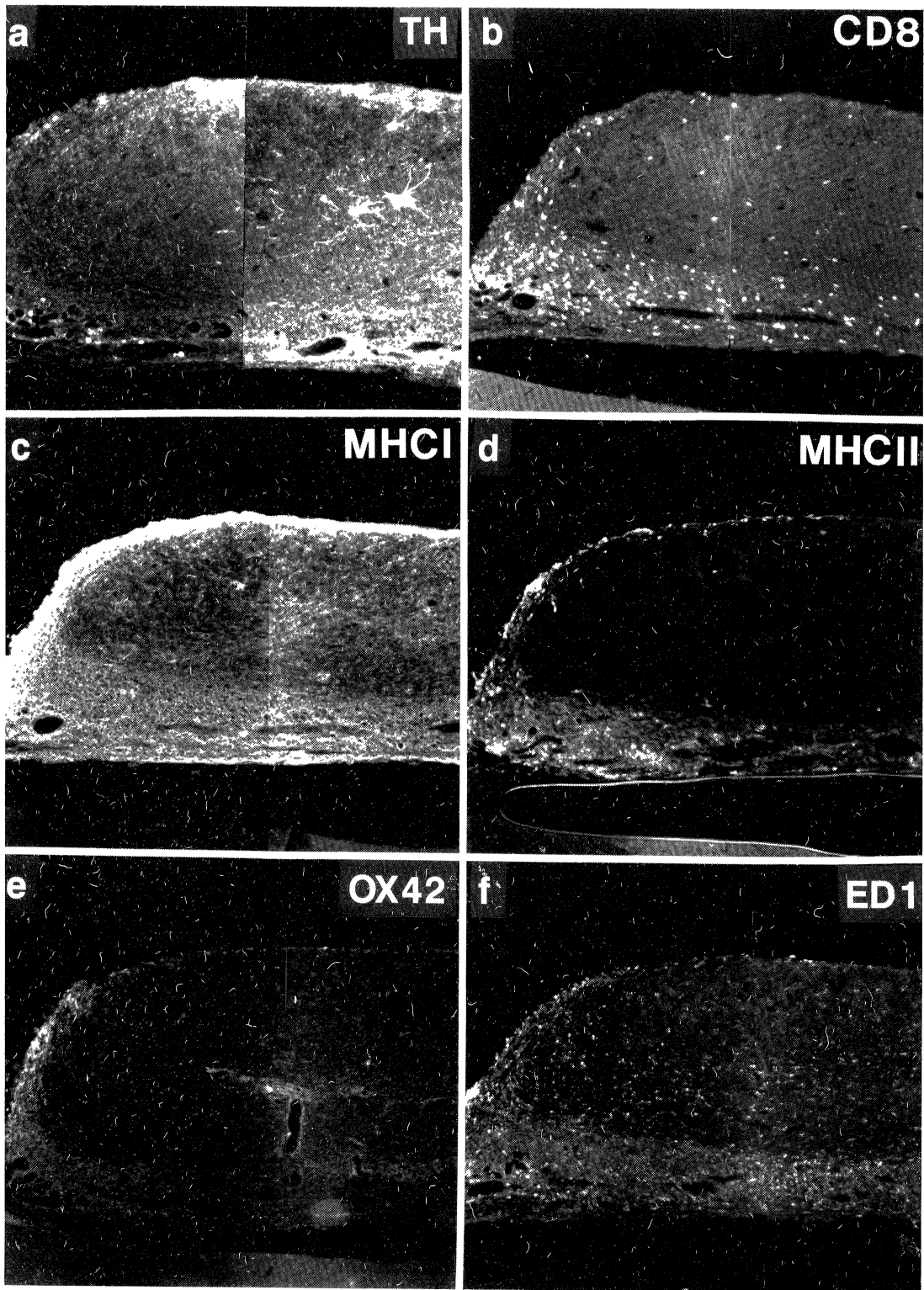


Fig. 5. Appearance of a transplant after BDNF treatment ($\times 106$). This particular transplant has an above-average number of immunoreactive cells. (a) TH, (b) CD8, (c) MHC I (OX-18), (d) MHC II (OX-6), (e) CD11b (OX-42), and (f) ED1-1. Higher numbers of immunocytes can be seen with OX-3 antibodies, and OX-42-positive large and round microglial cells are seen in areas of transplants close to the iris. ED-1-positive reactive microglia are also present.

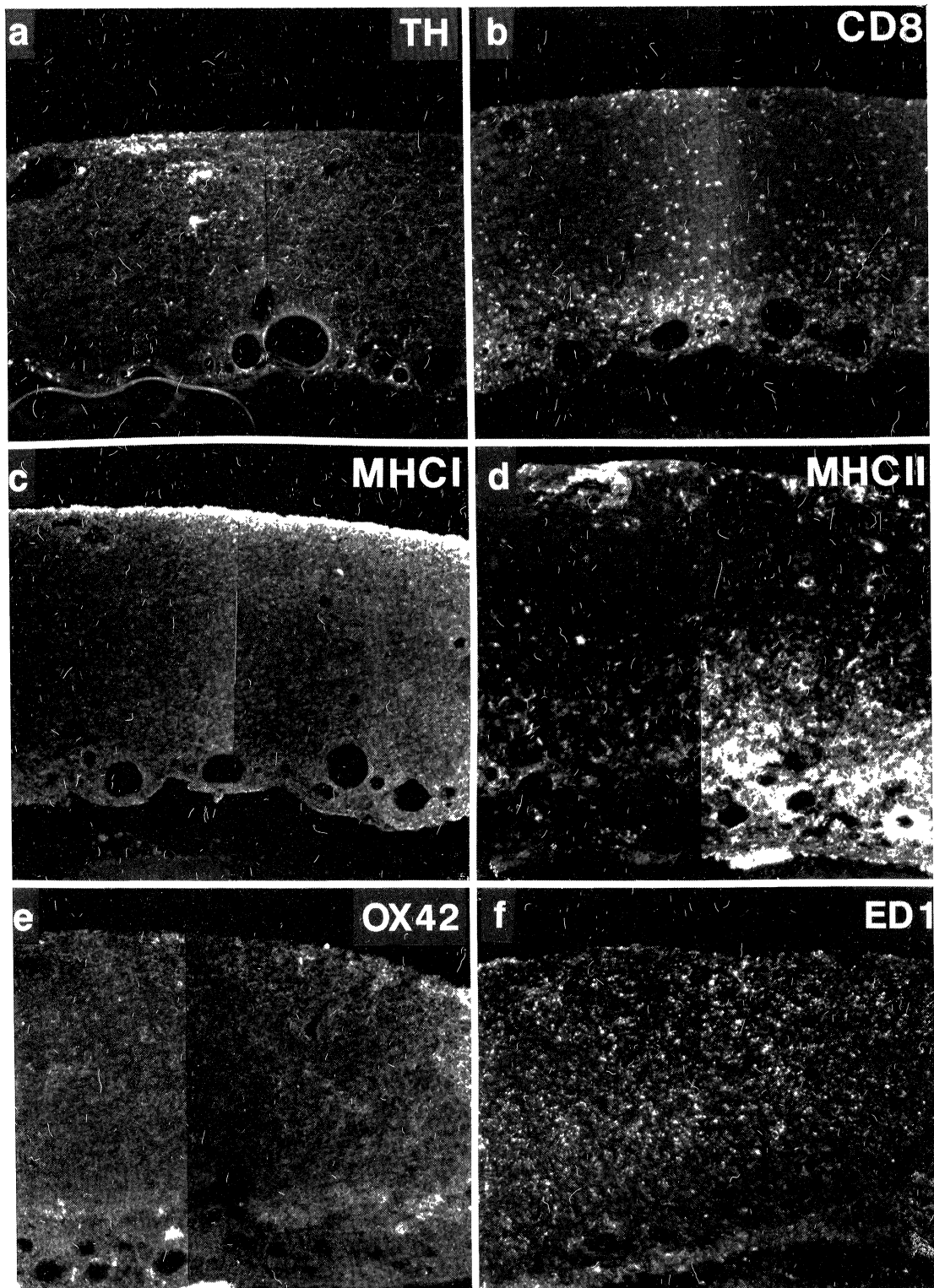


Fig. 6. Appearance of a transplant typical of those that showed severe immune reactivity after NT-3 treatment ($\times 106$). (a) TH, (b) CD8, (c) MHC I (OX-18), (d) MHC II (OX-6), (e) CD11b (OX-42), and (f) ED1-1. Some TH-positive neurons can be seen, as well as CD8+ lymphocytes, MHC class II-expressing cells (especially perivascular lesions), ED-1-positive reactive microglial cells, and OX-42-positive large and round cells.

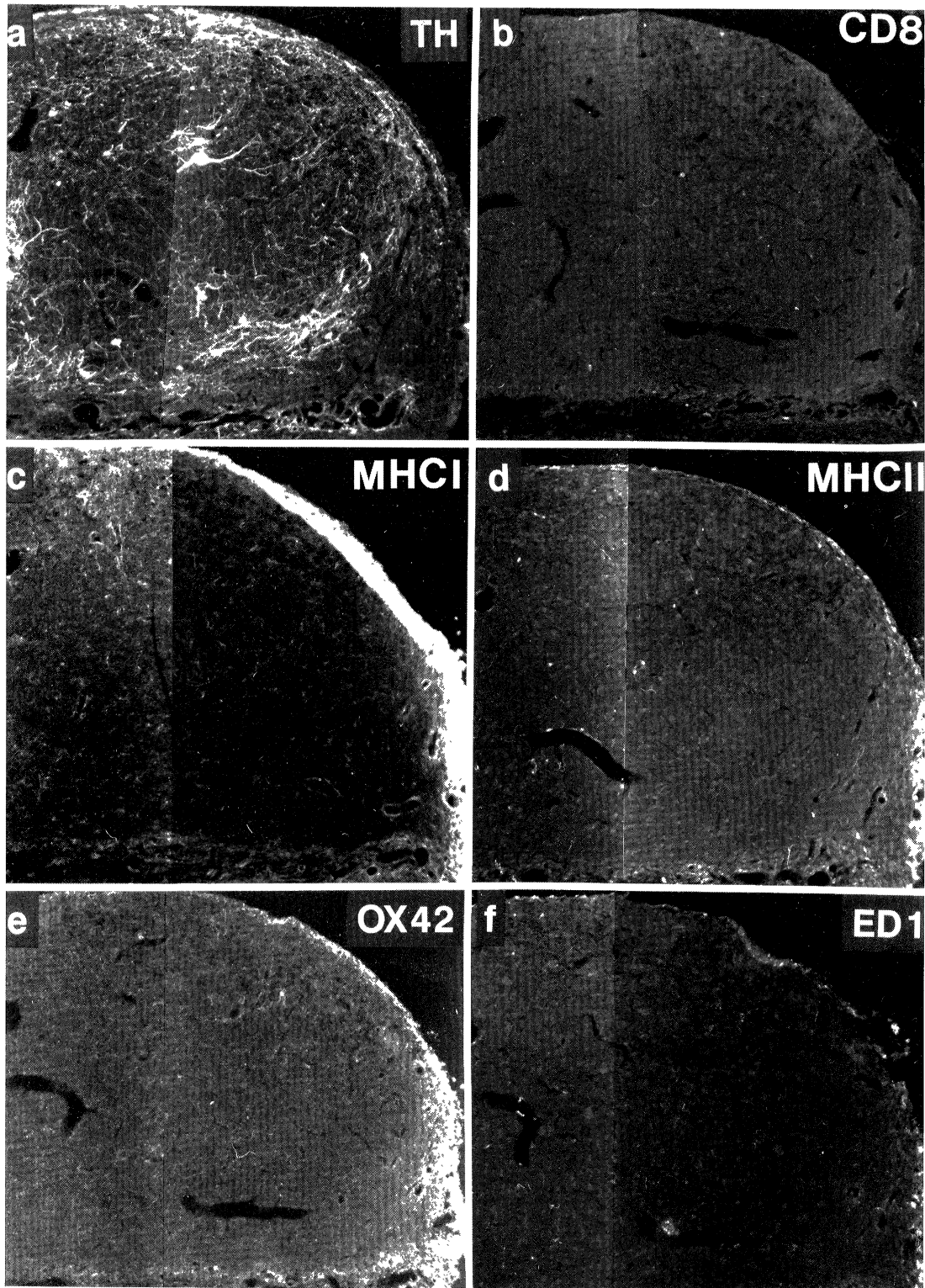


Fig. 7. Typical appearance of transplants after GDNF treatment ($\times 106$). (a) TH, (b) CD8, (c) MHC I (OX-18), (d) MHC II (OX-6), (e) CD11b (OX-42), and (f) ED1-1. Many TH-positive neurons can be seen in the grafts and quite a few immune-related elements, except MHC class I-positive microglia. OX-42 labeling shows only ramified microglial cells.

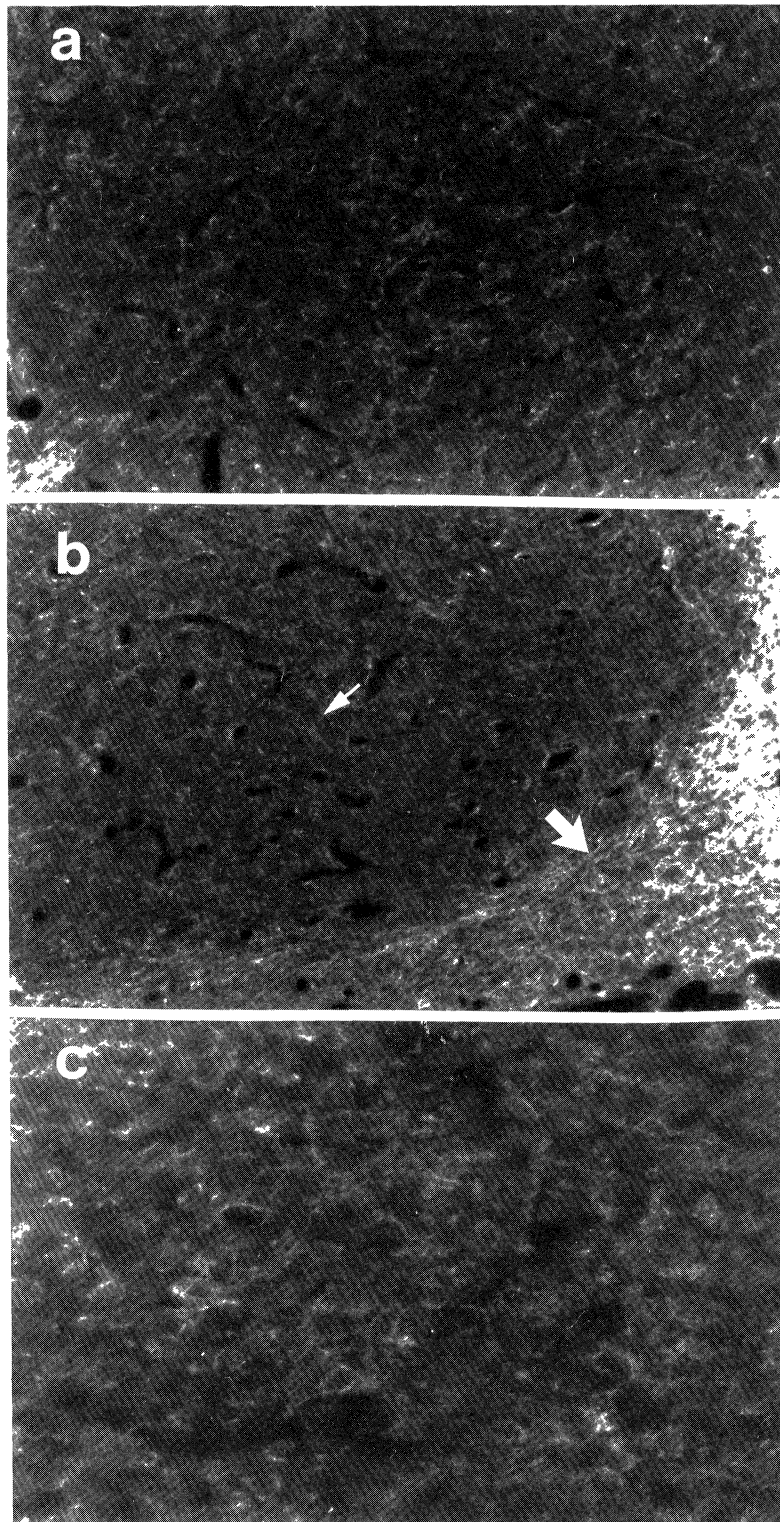


Fig. 8. Morphologic differences seen with OX-42 antibodies. (a) Close-up view of Fig. 5e. GDNF-treated, well-defined ramified microglial cells in the grafts. (b) Close-up view of Fig. 6e. BDNF-treated, partly activated (large and round) microglial elements. (c) Close-up view of Fig. 7e. NT-3-treated severe immunocompromised grafts showing large and round OX-42-positive cells in a wide area.

OX-42-positive ramified microglial cells and a few large, round microglial cells. In addition, GDNF-treated grafts contained a few microglial reactive ED-1-positive microglial cells (Figs. 4b, 4c). Overall, more immunologically compromised transplants were found in BDNF-treated (3 of 11) and in NT-3-treated groups (5 of 14) than in CC-treated (2 of 17) and GDNF-treated groups (3 of 17). Moreover, grafts with a marked infiltration of immune cells were found only in the BDNF- (1) and NT-3-treated (3) groups.

DISCUSSION

The combined data from this and other studies performed in our laboratories demonstrate that although treatment with GDNF and to some degree with BDNF can enhance immune responses to such immunogenic tissue as fetal spinal cord grafts, the trophic factors *per se* do not elicit a significant response in non-immunogenic grafts like substantia nigra. In the present study, although GDNF treatment led to significantly larger substantia nigra graft volumes than CC treatment, both GDNF and CC showed almost the same level of immune reactivity, except for MHC class I positive elements. GDNF-treated grafts also had the highest values of normal ramified microglial distribution, as well as lower numbers of immunoreactive microglial components and OX-42 positive round and large microglial cells.

The origin of microglial cells is controversial /20,35/42/, although most studies have suggested a mesodermal origin /7,25,27,33/. Three types of microglial cells are found in the CNS. Ramified microglia, prominent in the mature CNS, are derived from ameboid microglia, which, in turn, are present during late prenatal and early post-natal ages. Reactive microglia appear primarily in cases of CNS injury (reviewed in /19,27,33,45/). After 6-OHDA-induced dopaminergic denervation, microglial cells in substantia nigra have been shown to express MHC loci /1/, and ED1 and OX-42-positive microglial cells have also been found in the substantia nigra area (Shinoda, Lindqvist, and Olson, unpublished).

As mentioned before, GDNF belongs to the TGF- β superfamily /24/ of immunomodulators.

TGF- β is produced by a large number of different cell types, including various cells of the immune system, such as macrophages, peritoneal monocytes and neutrophils, as well as by T- and B-lymphocytes. In cases of injury (localized inflammation), platelet aggregation and degranulation, one of the earliest events in the inflammatory response, might occur and trigger the release of TGF- β . Activated macrophages release TGF- β and other cytokines (for example, interleukin-1 (IL-1), tumor necrosis factor (TNF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF)). The inhibition of T-cell proliferation by TGF- β may reduce the inflammatory response while promoting healing /51/. Interestingly, TGF- β 1 null/deficient mice show increased MHC Classes I and II gene expression /12/, as well as an increased inflammatory-cell infiltration of cardiac tissue that is related to allogeneic cardiac tissue rejection /53/.

We previously reported that in the Sprague-Dawley strain of rats, GDNF-treated intraocular allogeneic spinal cord grafts showed the highest cytotoxic immune responses when compared with BDNF- and CC-treated grafts. In contrast, we saw no immune upregulation after GDNF treatment in syngeneic Fisher strain spinal cord grafts /40/. Comparing fetal spinal cord grafts /40/ with the embryonic substantia nigra grafts to the eye chamber reported here reveals that the immune reactions elicited by spinal cord tissue tends to be more pronounced than those induced by ventral mesencephalic tissue. Two major reasons could account for the neuroimmunologic differences between cerebral gray matter and the spinal cord in development. First, normal mature spinal cord contains MHC Class I positive microglial cells /22/. Additionally, in P1 rats MHC Class I-positive microglial cells can be found on the surface of cervical and thoracic spinal cord, and they increase in number and appear to move into the white matter with time so that several weeks after birth, they become normal MHC Class I-positive microglial cells (Shinoda, Kobayashi, and Olson, unpublished). Substantia nigra, however, does not contain MHC Class I-positive microglia (Shinoda, Kobayashi, and Olson, unpublished). Second, the spinal cord also contains a large proportion of white matter, which contains more ameboid microglial cells during

development /16/. Because they can express MHC loci and Il-1 /13,26/, amoeboid and/or activated microglial cells may contain more immunomodulatory components.

Stromberg *et al.* /44/ reported using the same procedures, in which 0.1, 1 μ g, and 1 mg/eye GDNF were given to stimulate neuronal growth. In the present study, the final sizes of GDNF-treated substantia nigra transplants were more than 1.5-fold larger than those in Stromberg's experiments. The reasons for such differences are as follows: (a) We used 8 injections of GDNF at 5-day intervals, and the final period was 42 days after the first injection, whereas in the former experiments the final period was only 33 days.

Nerve growth factor (NGF) and other neurotrophins have been shown to produce several immunomodulatory effects, such as in mast cells /2,3,32/, B-cell stimulation /31,43,46/ and T-lymphocyte stimulation /47/. In the present study, NT-3, which had no effect on graft growth, elicited small immunomodulatory effects, particularly microglial activation. The significantly increased numbers of OX-42-positive large and round cells in NT-3-treated transplants suggests that NT-3 stimulates macrophage-related cytotoxic immunity in neuronal cells. NT-3 and NT-4 mRNA, as well as truncated trkB and trkC transcripts, have been found in rat thymus, thymic stroma (tissue depleted of mononuclear cells), spleen, and splenic stroma /23/. Concanavalin A-treated rat-thymus cells and lipopolysaccharide-treated rat-spleen mononuclear cells express a two-fold increase in NT-4 but not in NT-3 or BDNF. We have previously shown that BDNF has almost no immunomodulatory effect on fetal spinal cord grafts *in oculo* /40/. Thus it appears that BDNF does not elicit a strong immunoreaction *in vivo*.

In conclusion, neither GDNF nor BDNF enhanced immune reactivity in allogeneic embryonic substantia nigra grafts. GDNF may act to upregulate immune markers in allogeneic CNS tissue grafts where MHC Class I is strongly expressed, such as in spinal cord, but not in allogeneic CNS tissue in which MHC Class I is weakly expressed, such as in substantia nigra.

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