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Survival and maturation of heterotopic fetal brain stem xenografts after treatment with 2-chlorodeoxyadenosine and cyclosporine A

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Abstract. Brain stem halves from fetal rabbits were transplanted to the caudate nucleus area of adult rats. The animals were treated postoperatively with cyclosporine A (CsA) and 2-chlorodeoxyadenosine (CdA) for three days, and with CdA alone for the next 13 days. The treatment started at the day of implantation, and in some animals it was repeated starting at day 36 after grafting (at the time when signs of a light inflammatory reaction appeared in some grafts). Grafts survived and matured histologically, and no signs of acute rejection were observed up to the 90th day. In some grafts we recorded phasic neuronal activities similar to the respiratory-related neural activities characteristic for the adult brain stem. Immunosuppression with CdA and CsA deserves further evaluation in fetal brain grafting.

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INTRODUCTION

Grafting fetal neural tissues into adult brain has been explored as a possible treatment for neurodegenerative diseases (Brundin et al. 1990). Neural transplants could also be used for restoring neural tissue continuity, or substituting for lost neural activating systems (e.g., in the severed spinal cord) (Nornes et al. 1983).

While some clinical results of fetal allografting in the treatment of Parkinson's disease support the soundness of neural transplants as a therapeutic means, the use of human fetuses as donors of neural tissue is debated on both ethical and practical grounds (Brundin et al. 1990). A more acceptable solution would be the use of xenogeneic material, provided that graft rejection could be controlled without unacceptable side effects.

2-Chlorodeoxyadenosine (cladribine, CdA) is a selectively lymphocytotoxic compound succesfully used for treatment of some lymphoid malignancies (Beutler 1992). It is also effective in the short-term treatment of multiple sclerosis (Grieb et al. 1994b, Sipe et al. 1994). In humans the drug produces a long-lasting depletion of lymphocytes, most pronounced in the CD4+ subset (Beutler et al. 1994). *In vitro* it inhibits human B- and T-cell activation (Priebe et al. 1990, Górski et al. 1993a, b,) and prolongs skin graft survival in mice (Górski et al. 1993a). Its possible use as an adjunct treatment in xenografting has been suggested (Górski et al. 1993b). CdA is unique among cytotoxic and immunosuppressive drugs in that it causes no organ toxicity other than marrow depression (Beutler 1992, Beutler et al. 1994).

In the present experiments we grafted halves of rabbit fetal brain stem into the nucleus caudatus area of adult rats. Graft hosts were subjected to transient immunosuppressive treatment consisting of CdA and cyclosporine A (CsA). The fate of grafts was followed histologically and their maturation assessed from the development of neurone-specific enolase (NSE) and myelin basic protein (MBP) expression.

METHODS

General

The grafting experiments were performed with 28 adult rats of Wistar or DA strain, body weight 220-260 g, which served as graft recipients. Fetal brain stems were obtained from rabbit fetuses collected from 8 pregnant mongrel rabbits at 18 days of gestation (E18). Experimental procedures were in agreement with the rules of animal experiments accepted by the Ethical Committee of the Polish Academy of Sciences Medical Research Centre.

Sampling fetal brain stem for grafting

Fetal rabbit brains were collected under i.m. ketamine (30 mg/kg) and xylazine (6 mg/kg) anaesthesia, according to a technique described in detail by Kromer et al. (1983). The uterus was exposed and the individual fetuses were isolated sequentially. Usually three to four fetuses were obtained from one animal. Individual fetuses were placed in ice-cold Eagle 1959 medium supplemented with insulin, 40 i.u./l and dexamethasone, 16 mg/l, saturated with carbogen, and buffered with bicarbonate to pH 7.3. Under a dissection microscope the brains were excised from the fetuses and brain stems were dissected out. The sampled brain stem comprised the tissue block beginning at the caudal end of the pontine flexure and stretching 2 mm caudally (to the supposed caudal end of the medulla oblongata), as shown in Fig. 1A. Each brain stem was then split along the cleft spine and the excised half (volume ca. 1 mm^3) was introduced, together with ca. 10 µl of the medium, into a Pasteur micropipette (inner diameter 1.3 mm^2) with the help of light negative pressure imposed with a 1 ml syringe. The abovementioned procedure lasted not more than 10 min.

The technique of grafting

Graft recipients were anaesthetized i.m. with a mixture of ketamine, 90 mg/kg, and xylazine, 15

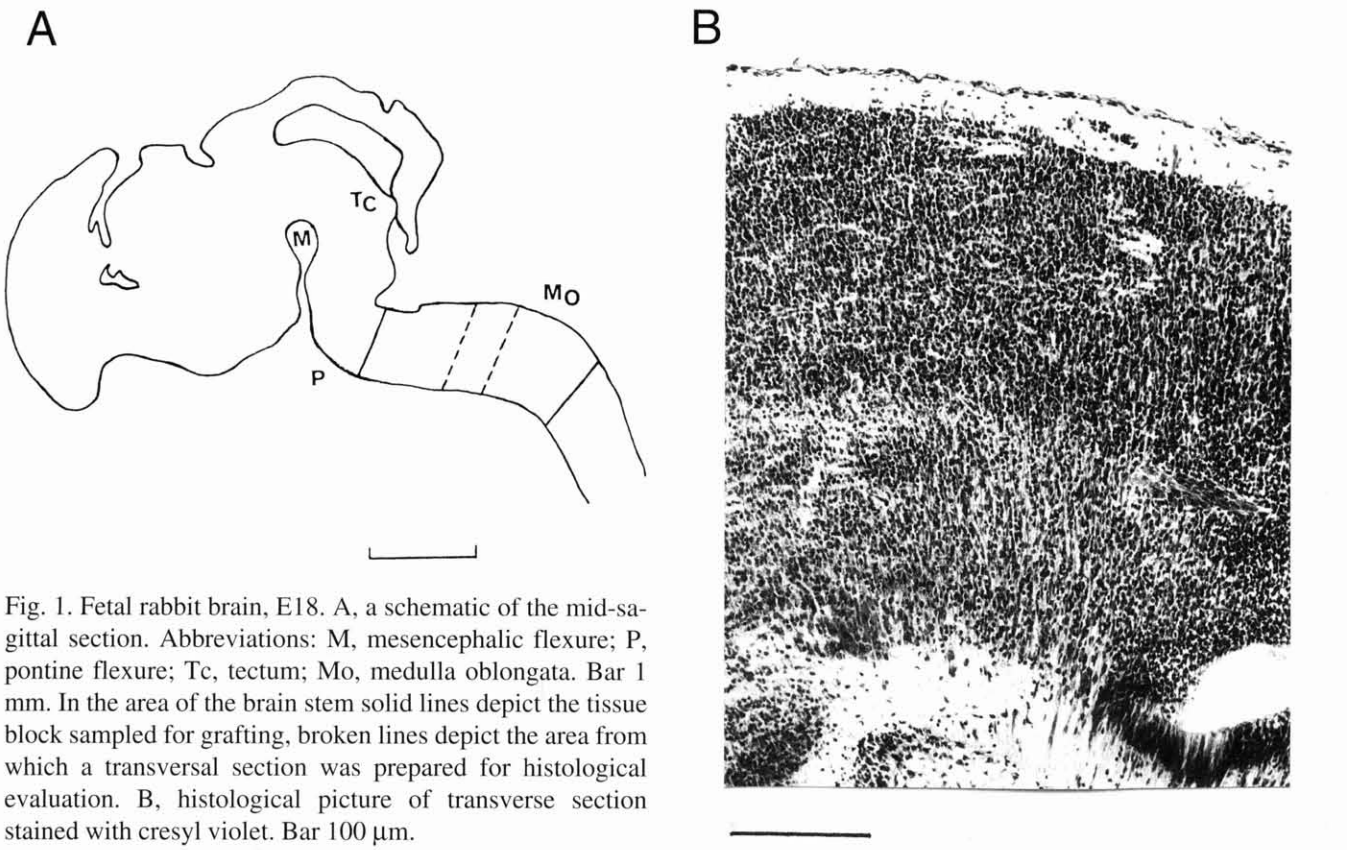

Fig. 1. Fetal rabbit brain, E18. A, a schematic of the mid-sagittal section. Abbreviations: M, mesencephalic flexure; P, pontine flexure; Tc, tectum; Mo, medulla oblongata. Bar 1 mm. In the area of the brain stem solid lines depict the tissue block sampled for grafting, broken lines depict the area from which a transversal section was prepared for histological evaluation. B, histological picture of transverse section stained with cresyl violet. Bar 100 μm.

mg/kg. Anaesthesized animals were fixed in a stereotactic frame and the skull was exposed by a skin incision. Bone around the point of 0.5 mm rostrally from bregma and 3 mm laterally from the medial sutura was removed with the help of a diamond dental drill (diameter 2.3 mm). The exposed dura was punctured with a 16-gauge needle. A micropipette containing a fetal brain stem tissue block was inserted to a depth of 6 mm below the brain surface with the use of a manual micromanipulator (Narishige, model 303). The implant was pushed out from the micropipette under minimal positive pressure applied with a 1 ml syringe. The pipette was slowly evacuated from the brain two minutes later. Hemostasis was established with Spongostane (Ethicon). The wound was then closed with bone wax (Ethicon) and the skin was sutured.

Postoperative pharmacological treatment

Immunosuppressive treatment was initiated at the day of surgery and consisted of daily doses of CdA given subcutaneously (4 mg/kg for the first two days, and 1 mg/kg for the subsequent 13 days) and CsA given orally (20 mg/kg for the first three days). Some animals were subjected to the identical second immunosuppressive treatment course starting at day 36 after grafting. Additional postoperative treatment consisted of five daily doses of antibiotic ceftriaxone, 200 mg/kg i.m., and a single dose of dexamethasone, 4 mg/kg i.m. before surgery, to counteract bacterial infections and to ameliorate brain oedema, respectively.

Host brain sampling and histological evaluation of grafts

Under ketamine-xylazine anaesthesia (dose as mentioned) the hosts were perfused transcardially with ca. 250 ml of PBS with 10% formaldehyde. Brains were excised under the dissection microscope and embedded in paraffin. A standard hematoxylin-eosin (HE) stain was used to visualise cells. Myelin basic protein (MBP) and neurone-specific

enolase (NSE) were visualised immunohistochemically with the use of an indirect immunoperoxidase staining technique (Stenberger et al. 1970), using anti-NSE and anti-MBP primary antibodies (Dakopatts).

Electrophysiology of grafted area

For electrophysiological examination host animals were anaesthetized as mentioned, bilaterally vagotomised, paralysed with d-tubocurarine and mechanically ventilated with oxygen to obtain end-tidal CO_2 of approx. 5%. The trunk of the right phrenic nerve was exposed through a mid-section neck approach, cut at the entry to the thorax and placed on a bipolar silver electrode. The skull was exposed and bone wax removed from the hole over the graft. The area of the graft was penetrated with glass microelectrodes filled with 3M KCl and connected to an NL 105 amplifier and a NL 125 filter (Digitimer). Neuronal activities from the graft area and simultaneous host phrenic activity (amplified and integrated) were digitalized and memorized with a dedicated IBM-compatible microcomputer.

RESULTS

General

Of 28 xenografted rats, 23 survived and were sacrificed as scheduled. Five animals died during the first week following the surgery. The deaths resulted from general infections (3 cases) and brain hematoma and oedema (two cases).

Histology

Histological evaluation of the fetal brain stem tissue sampled for grafting revealed a dense population of weakly differentiated cells (Fig. 1B.). Specific NSE and MBP immuno-stain was negative (data not shown).

For histological evaluation of the fate of xenografts, the hosts were sacrificed on the following days: 16 (3 animals), 32-40, (7 animals), 47 (two animals), 72-78 (3 animals) and 90 (two animals) after grafting, and their brains were processed as mentioned in the Methods section. Histological pictures are shown in Figs. 2-8.

Immunohistological documentation concerning time-dependent changes in NSE and MBP staining patterns in the grafts is presented uniformly in Figs. 2-7. As shown, a progressive increase in the amount of both NSE (Figs. 2, 3 and 4) and MBP (Figs. 5, 6 and 7) occurs inthe grafts. While the neighbouring host tissues show patterns of NSE and MBP immuno-staining characteristic for histology of particular adult brain areas, in grafts a different pattern gradually emerges with time (e.g., see clusters of myelinized axons in capsula interna and high density of myelinized axons in corpus callosum versus a network of myelinated fibres in the graft, Fig. 5).

Although maturation of the grafts seemed to be undisturbed, infiltrations of mononuclear cells were found in some of them at 35 - 40 days after implantation; they were located interstitially at the border of graft and host tissue (Fig. 8). However, evidence of massive graft destruction was never observed. Even at 90 days after implantation the histological picture revealed the presence of some highly differentiated cells which resembled large multipolar neurones characteristic for the adult brain stem reticular formation (Fig. 9).

Electrophysiology

In six graft recipients we succeded in recording extracellular neuronal activities in the area of the graft. The recordings were made at 36 (two rats), 45, 49 and 72 (two rats) days after grafting. In one case (45 day post-implantation) the activities recorded were only tonic. In the other five grafts, among tonic activities we were able to find tonic-phasically modulated, and also clearly phasic activities. Their frequency was usually lower than and not influenced by the respiratory activity of the host (Fig. 10).

Electrophysiological penetrations of the nucleus caudatus region in intact adult rats under the same anaesthesia and conditions failed to reveal phasic activities in this area.

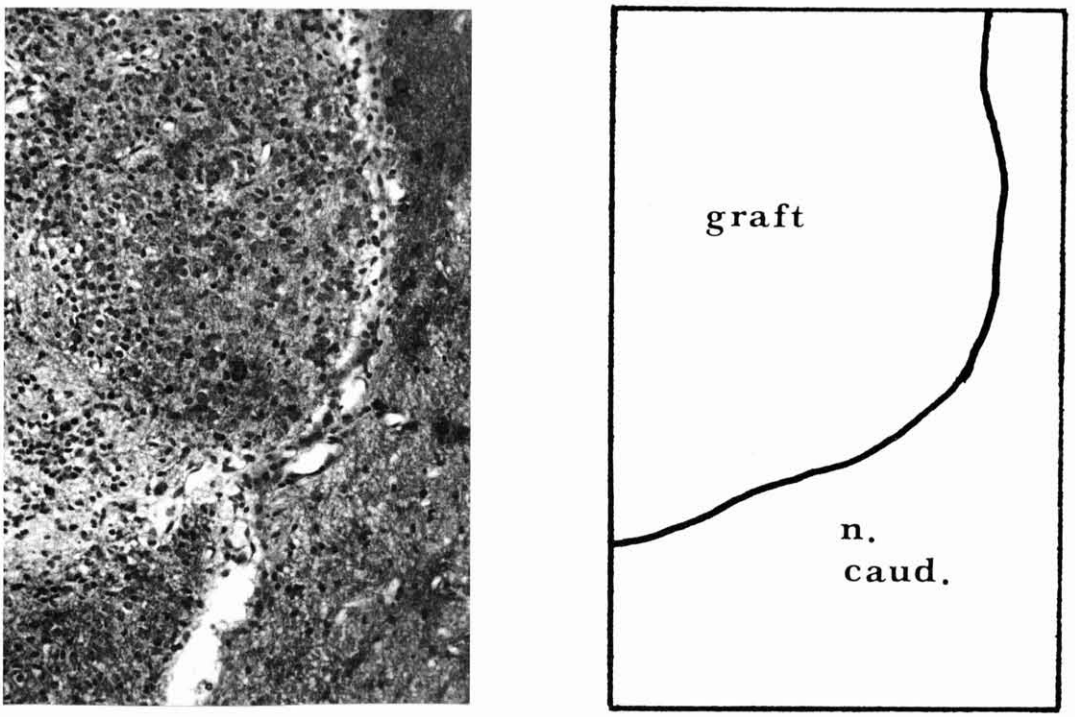

Fig. 2. Sixteen days after implantation. NSE immuno-stain reveals several dark-stained neurones in the grafted area. Left side, microscopic photograph; right side, corresponding topographical outline; n. caud., nucleus caudatus. Bar 50 μm.

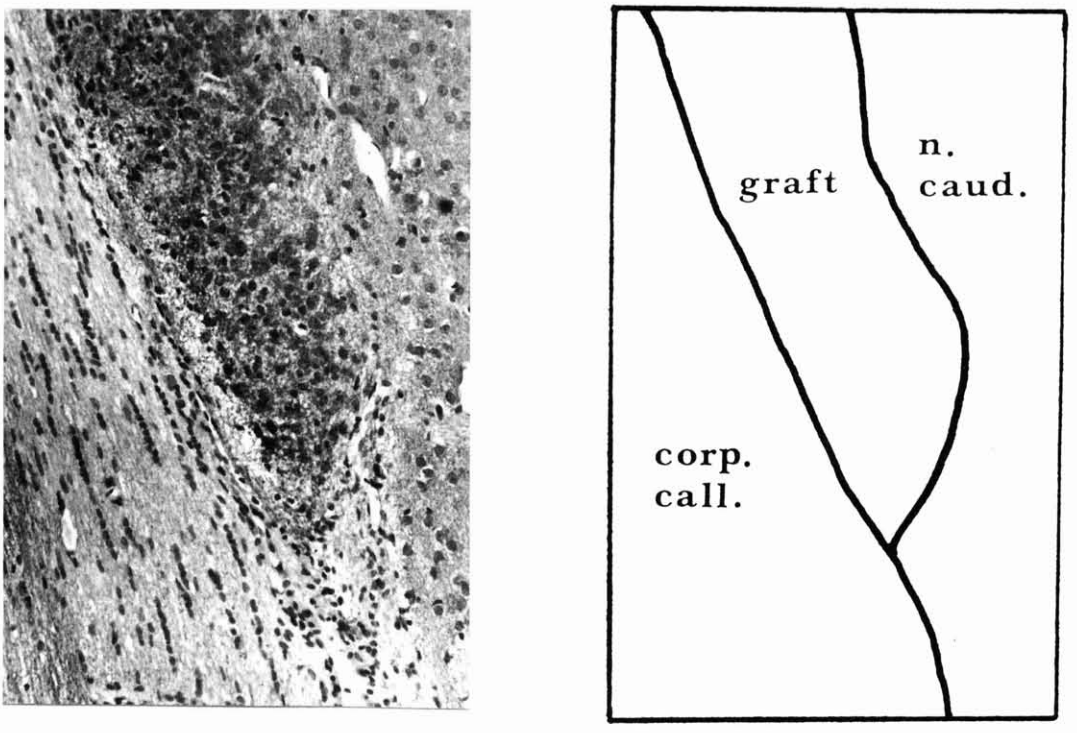

Fig. 3. Thirty five days after implantation. NSE immuno-stain reveals increased density of dark-stained neurones in the graft area. Left side, microscopic photograph; right side, corresponding topographical outline; n. caud., nucleus caudatus; corp. call., corpus callosum. Bar 50 μm.

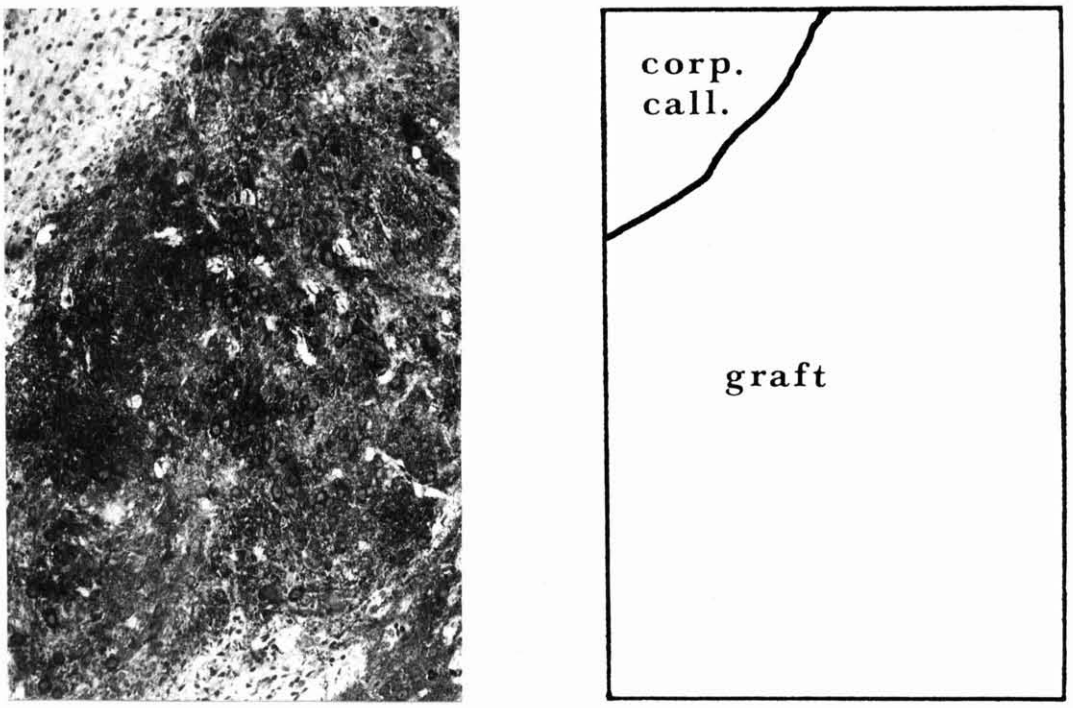

Fig. 4. Ninety days after implantation. NSE immuno-stain reveals very dense population of cells resembling in shape the matured brain stem neurones. Left side, microscopic photograph; right side, corresponding topographical outline; corp. call., corpus callosum. Bar 50 μm.

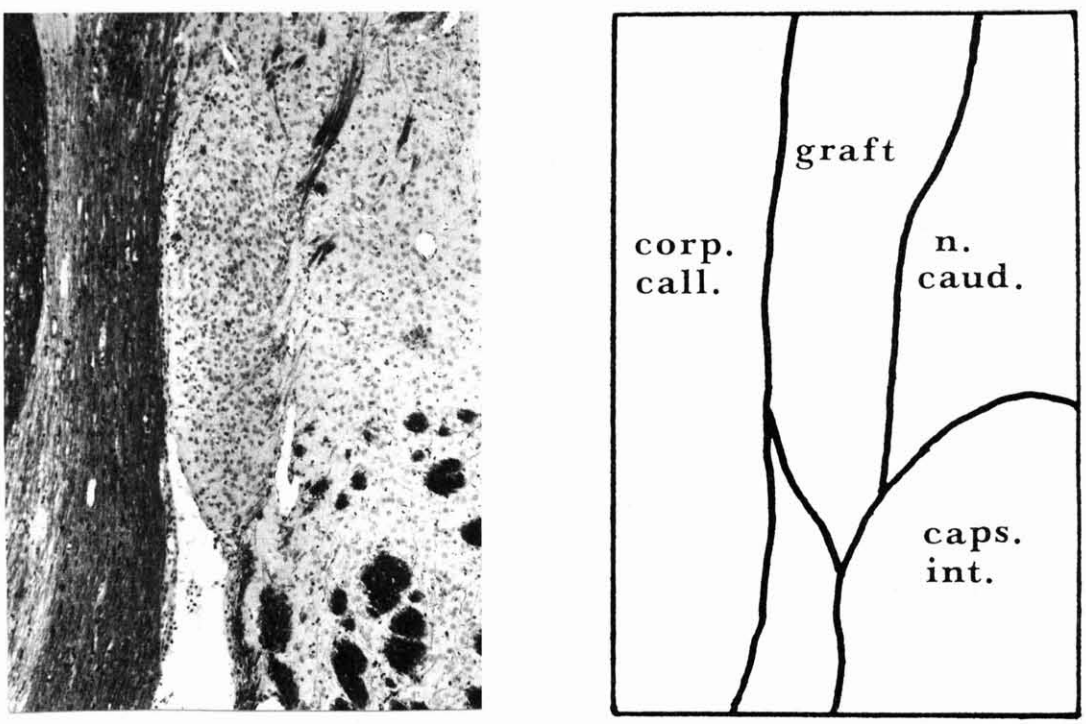

Fig. 5. Sixteen days after implantation. MBP immuno-stain reveals sparse myelinated fibres at the border of the graft. Left side, microscopic photograph; right side, corresponding topographical outline; n. caud., nucleus caudatus; corp. call., corpus callosum, caps. int., capsula interna. Bar 100 μm.

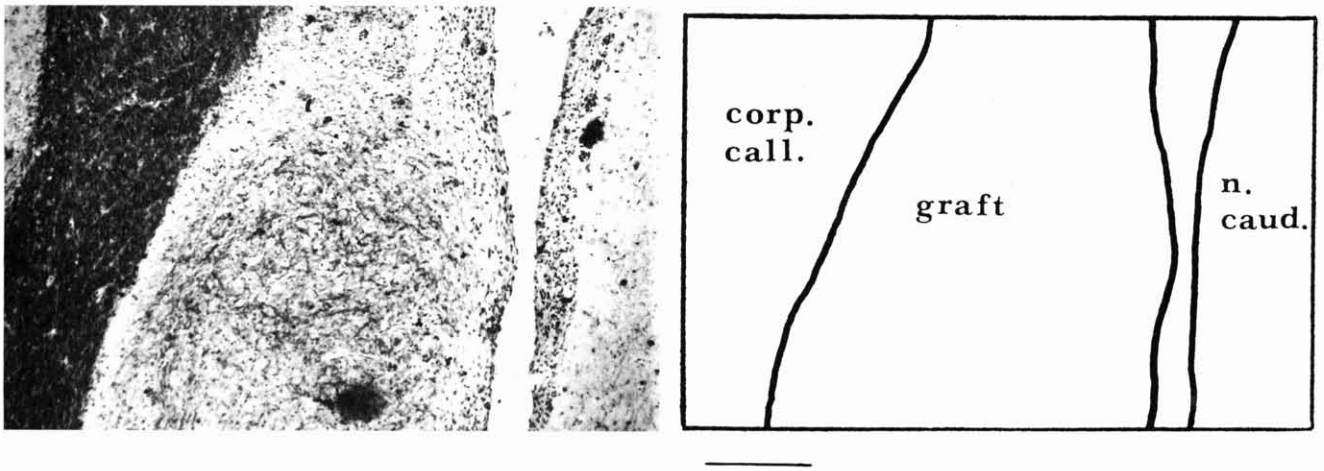

Fig. 6. Thirty five days after implantation. MBP immuno-stain reveals a delicate network of myelinated fibres spreading throughout the graft. Top, microscopic photograph; bottom, corresponding topographical outline; n. caud., nucleus caudatus; corp. call., corpus callosum. Bar 100 μm.

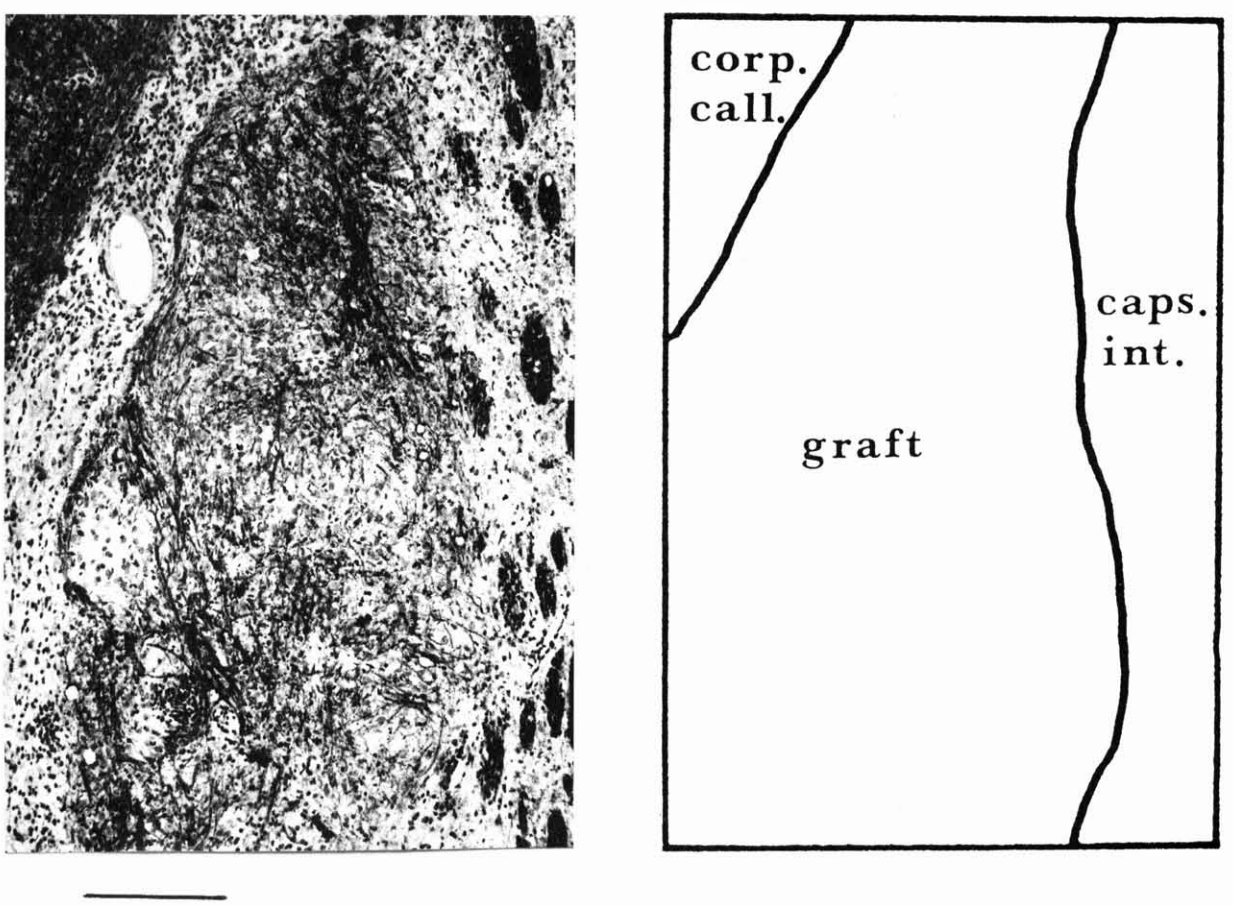

Fig. 7. Ninety days after implantation. MBP immuno-stain reveals a complex network of myelinated fibres, which spread across the graft with varying density. Left side, microscopic photograph; right side, corresponding topographical outline; corp. call., corpus callosum, caps. int., capsula interna. Bar 100 μm.

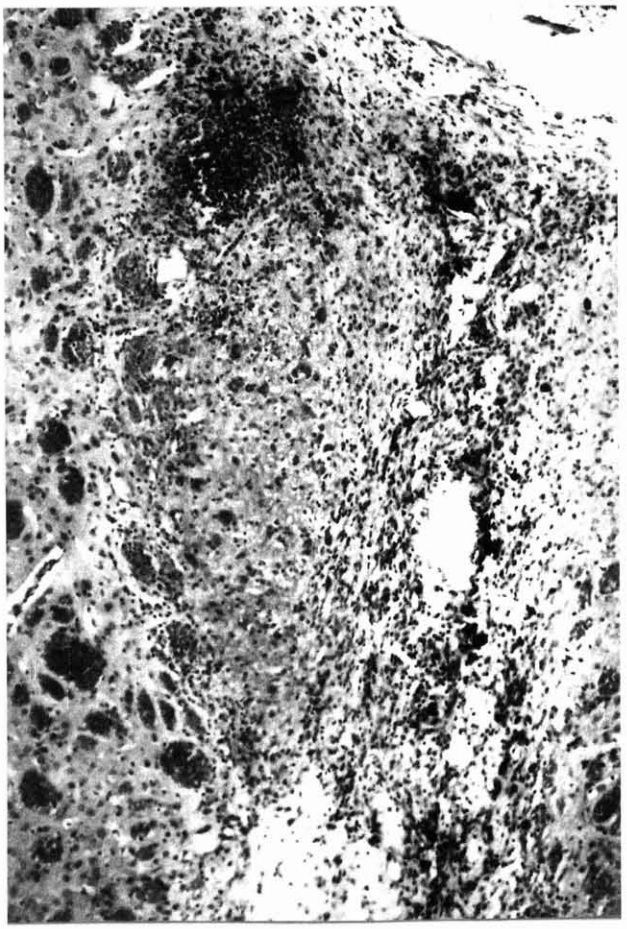

Fig. 8. Thirty five days after implantation, HE stain. Interstitial infiltration of mononuclear cells at the border of graft and host tissues. Bar 100 μm.

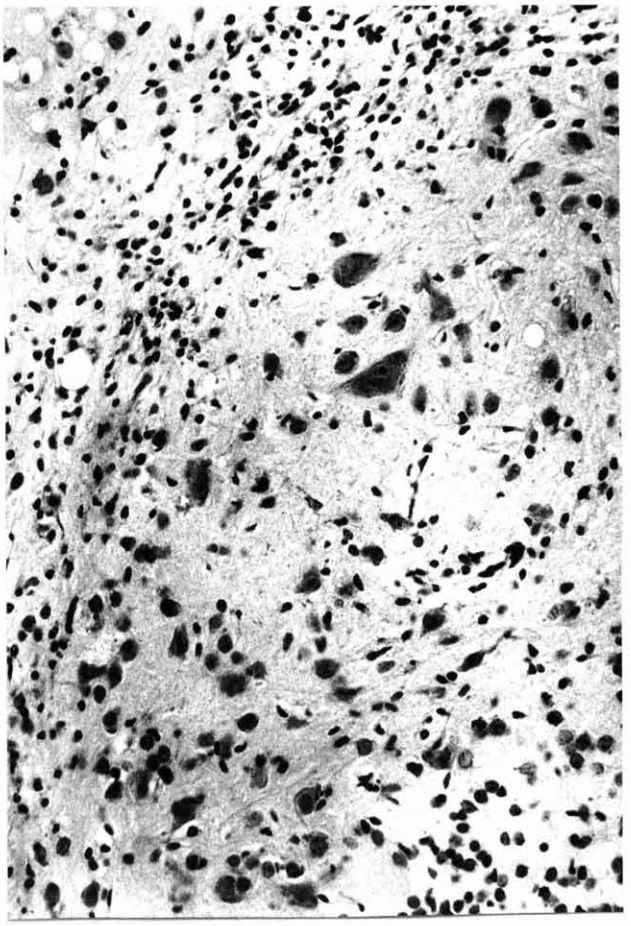

Fig. 9. Ninety days after implantation, HE stain. Left upper corner, high cellularity zone formed by glial cells at the graft border. Large multipolar neurones below. Bar 50 μm.

DISCUSSION

Can CdA combined with CsA induce tolerance to fetal neural xenografts?

Fetal neural xenografts (Mason et al. 1986, Freeman et al. 1988, Takei et al. 1990), including rabbit-to-rat grafts (Freeman et al. 1987), are generally quickly rejected, and continuous intense immunosuppressive treatment is necessary for prolongation of their survival. The usual picture of rejection consists of massive infiltrations of graft tissues with mononuclear cells, which begin perivascularly and within days penetrate interstitially, resulting in gradual disintegration and disappearance of the graft within a few weeks at the very latest. In the present experiments this was not found. Three weeks after the end of the first immunosuppressive treatment a few mononuclear infiltrates were found perivascularly or interstitially at graft borders in some grafts, but the bulk of grafted tissues seemed undisturbed. Following the second course of immunosuppressive treatment an inflammatory reaction was not seen up to day 90 post-implantation.

The mechanisms involved in rejection of fetal neural xenografts are not fully understood, but many observations suggest that the initiation of the host immune response is related to the local cellular damage during the transplantation procedure and to subsequent disruption of the blood-brain barrier

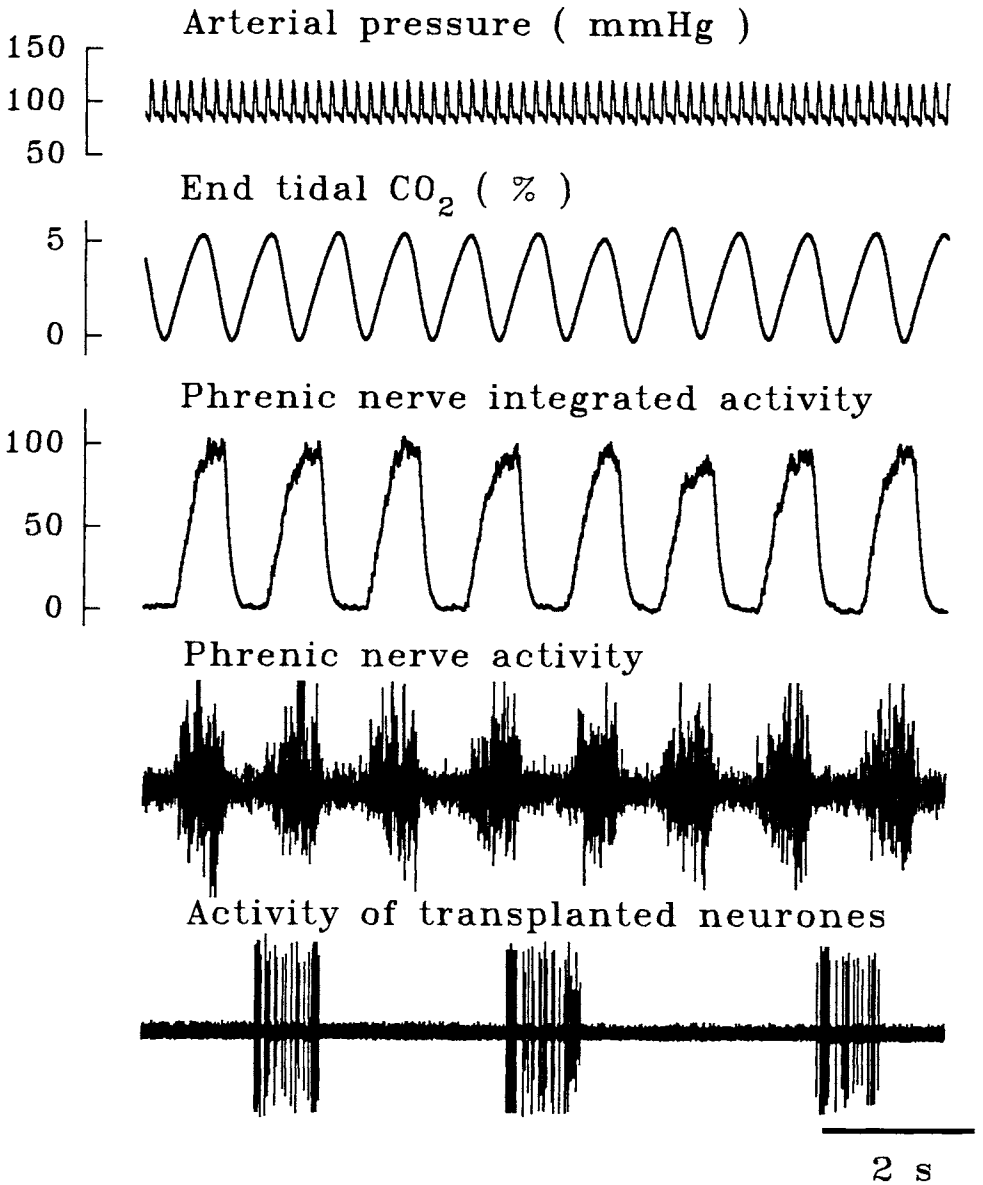

Fig. 10. Phasic neuronal activity recorded from the area of the graft (35 days after grafting).

(BBB). A light inflammatory reaction is found both in and around solid fetal brain tissue grafts within a few hours after transplantation; it quickly resolves in isografts, but in xenografts it usually initiates a cascade of events leading to graft rejection (Mason et al. 1986, Freeman et al. 1988, Finsen et al. 1990, Nicholas et al. 1990, Takei et al. 1990). Xenografted brain stem is rejected similarily to other parts of the brain (Nakashima et al. 1988).

Normal adult, as well as immature, brain cells have negligible constitutive expression of MHC antigens (Lampson 1987), but class I MHC antigens are induced and transiently expressed shortly after transplantation even in isografts (Mason et al. 1986, Freeman et al. 1988). In solid neural xenografts the MHC class II antigen, which plays a critical role in transplant rejection (Milton et al. 1986), is induced at the end of the first week after the transplantation, when host and graft vessels inosculate, and its expression is followed by the massive infiltration of the graft by lymphocytes (Takei et al. 1990).

When fetal mouse neural grafts are implanted into the brains of neonatal rats, 80% of the grafts survive for up to 4 months without immunosuppressive treatment (Lund et al. 1988). The explanation is that rats become fully immunocompetent 8-10 days after birth. Until this time immune reactions do not interfere with tissue healing. When transplantation-related trauma and BBB disruption resolve, the grafted tissues are "hidden" behind the BBB and "invisible" to the host immune system. The undisturbed repair of the BBB is critical, because the infiltration of even a small number of lymphocytes results in a complete breakdown of the barrier and graft rejection (Rao et al. 1988). Furthermore, the expression of MHC antigens in the graft area and graft rejection may be evoked by some immunoprovocative stimuli applied at locations distant from the graft. The authors hypothesize that the expression of MHC antigens is a prerequisite of graft rejection and, when it occurs, it is a secondary event resulting from prior activation of lymphocytes or microglia (Pollack et al. 1990).

If MHC expression in a xenograft area is secondary to immune activation, the rejection of a xenogeneic tissue grafted into the adult brain could perhaps be prevented and a state of apparent immunological tolerance induced also in a host fully immunocompetent at the time of grafting, provided that activation of the host immune system after the implantation would be very deeply suppressed for a relatively short time until the blood-brain barrier heals and shields the grafted tissues. The problem is how to induce such a state of very deep immunosuppression without prohibitive toxicity.

CdA is an immunosuppressant which has virtually no systemic toxicity. Furthermore, it not only produces a long-lasting immunosuppression by reducing the population of lymphocytes (in particular of CD4+ cells) in peripheral blood, but also inhibits lymphocyte activation and response to costimulation by extracellular matrix (ECM) proteins (collagens, fibronectin) (Górski et al. 1993a). This last observation may be of particular importance, because costimulation by ECM proteins is critical for guiding the traffic of lymphocytes from blood to their extravascular targets (Shimizu and Shaw 1991). The rationale for supplementing a short course of CdA with an additional very short parallel treatment with CsA came from the results of Nawrocki, Rowiński and collaborators (unpublished data) on heart allografts in rats. In these experiments CdA alone did not significantly prolong the survival of transplanted hearts; however, when a two-day treatment with CsA was added immediately after transplantation, the survival time of transplanted hearts was very markedly prolonged.

Our results seem to indicate that a 15-day treatment with CdA supplemented with CsA given in the first three days may provide conditions for a multiweek survival and apparently undisturbed maturation of fetal xenogeneic neural tissues grafted into fully immunocompetent adults. Furthermore, repeating such a treatment when signs of a mild inflammatory reaction in the graft area appear in some grafts prolongs the graft survival up to 90 days. While this does not mean that a true immunological tolerance of the xenogeneic tissues was established, it may indicate that conditions of virtual immunological "invisibility" of the graft were created. The possibility that after such treatment(s) xenografts will survive even longer (perhaps indefinitely) either without any further immunosuppression, or under significantly reduced and relatively well tolerated doses of conventional immunosuppressants, shall be the subject of further studies.

Phasic neural activities in the graft area

In preliminary experiments we were able to record phasic, respiratory-like activities in the area of nucleus caudatus after rabbit brain stem allografting (Grieb et al. 1994a). Since no rhythmic neurones are expected to be active there in an anaesthesized, paralysed animal (Vickens 1993), we hypothesized that these discharges originate in the graft and are produced by matured brain stem pacemaker neurones. Such neurones were identified in slices of neonatal rat brain containing a limited region of the medulla known as the pre-Bötzinger complex (Smith et al. 1991) and are suggested to be the pri-

mary source of respiratory rhythmicity in mammals.

In the present experiments we were able to confirm and extend our previous findings. The phasic activities apparently similar in appearance to the rhythmicity of respiratory-related brain stem neurones are also a frequent finding in xenografted brain stem. We did not explore electrophysiologically fetal rabbit brain stem and cannot prove that it generates rhythmic activities on day 18 of gestation. In a rat, the ability to generate rhythmic output from the brain stem and spinal cord preparation appears on day 17 of gestation (Greer et al. 1992). The possibility that respiratory oscillations in brain stem neurones not resulting in motor output are present at earlier ontogenetic stages was not investigated and cannot be excluded. However, E18 in the rabbit morphologically is equivalent to E14 in the rat. At this stage of ontogenesis neurones are not yet differentiated, and they lack both NSE and MBP.

According to Smith et al. (1991) brain stem pacemaker neurones are "conditional bursters" requiring depolarizing synaptic inputs to generate rhythmic activities. Such inputs may originate in the graft or may result from its functional integration with the host tissue. Our experimental design did not allow us to differentiate between these two possibilities. However, several investigators reported on the functional integration of homo- and heterotopic grafts with surrounding host tissues (Ebner et al. 1989, Bragin et al. 1990), and survival and growth of fetal brain stem neurones grafted into caudate nucleus has also been shown (Sladek et al. 1986). In any case, the presence of rhythmic activities in the grafted area seems to indicate that the grafts matured not only histologically, but also functionally. The idea of substituting for lost neural activating "command systems", including those generating rhythmicity, by grafting fetal neural tissues may also be worth further exploration.

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