FUNCTIONAL ROLES OF MICROGLIA IN NEURAL TISSUE TRANSPLANTATION

By

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Transplantation of fetal neural tissue is being evaluated as a treatment for neuropathological disorders ranging from Parkinson's disease to spinal cord injury. This dissertation addresses some fundamental aspects of fetal transplant development with a primary focus on microglial cells.

First, fluorescently prelabeled microglia were traced for two weeks after injection into the adult rat brain. The labeled cells survived *in vivo* and differentiated into ramified microglia. This study showed that, unlike perivascular cells, resting endogenous microglia are not actively pino- or phagocytic. This finding sheds some doubt upon previous ideas of microglial immunocompetence.

Second, to test the hypothesis that donor microglia induce allo- and xenograft rejection, fetal rat and mouse suspensions were depleted of microglia prior to

transplantation into the either the injured spinal cord or the intact striatum. This treatment did not alter the pattern or time course of xenograft rejection. Both depleted and non-depleted allografts, however, survived for three weeks without signs of rejection. Between three and four weeks post-transplantation (*pt*) rejection started and proceeded rapidly with destruction of all grafts by 45 days. Rejection did not correlate with graft major histocompatability complex (MHC) antigen expression, suggesting that minor histocompatability antigens were involved. The ineffectiveness of microglial depletion in preventing rejection, along with *in vitro* results showing that allogeneic microglia are not functional antigen presenting cells, indicates that microglia do not initiate allograft rejection.

Finally, the development of microglia, astrocytes, and vasculature within fetal intrastriatal and intraspinal transplants was followed for the first month *pt*, as was the expression of angiogenic cytokines within the grafts. Microglia, of primarily host origin, invaded the grafts prior to the onset of neovascularization and were often associated with developing vessels. Astrocytes and blood vessels were intimately associated with each other during the early time points *pt*. The grafts expressed high levels of vascular endothelial growth factor (VEGF) throughout the first month *pt*, while basic fibroblast growth factor (bFGF) and transforming growth factor-beta (TGF-β) were primarily expressed after 21 days *pt*. These cytokines were not primarily expressed by glial cells, however, indicating that the relationship between glia and angiogenesis may be indirect.

CHAPTER 1
BACKGROUND

Neural Tissue Transplantation

Although it was once thought that damage to the central nervous system was irreversible and irreparable, it is now understood that the CNS's lack of regenerative ability is surmountable (Richardson et al., 1980; Savio and Schwab,1990; Davies et al., 1997). The use of fetal neural transplants is currently under investigation as a possible treatment for a wide range of neuropathies, ranging from neurodegenerative disorders such as Parkinson's Disease to traumatic disorders such as spinal cord injury (Bjorklund, 1991; Dunnett and Richards, 1990; Gage and Buzsaki, 1989; Gash and Sladek, 1988; Reier et al., 1992). In the case of Parkinson's disease, research demonstrating functional recovery in animals following fetal neural transplantation (Brundin et al., 1988; Freeman et al., 1995; Lindvall et al., 1990) has precipitated the movement of this treatment into clinical trials, with similar results (Freed et al., 1993; Kordower et al., 1997; Lindvall , 1991). The evidence for functional recovery following transplantation into the injured spinal cord is less abundant, although there are indications that recovery of function is possible (Reier et al., 1994; Anderson et al., 1995). In light of these indications, pilot studies are currently underway to determine the beneficial effects of intraspinal transplants in humans (Falci et al., 1997; D.K. Anderson, personal communication). However, there are still gaps in our basic knowledge of neural transplant biology. For

instance, although a great deal of attention has been paid to the phenomenon of transplant rejection (Widner and Brundin, 1988; Nicholas and Arnason, 1989; Lawrence et al., 1990; Theele et al., 1996), the mechanisms underlying this phenomenon are as yet poorly understood. Another example is the increased focus on the development of vasculature within allografts (for review, see Horner et al., 1994), although understanding of this process is still limited. The purpose of this dissertation was to further our knowledge of functional roles of microglial cells regarding these two aspects of neural transplant development.

Glial Cell Transplantation

Researchers have recognized the necessity of studying the interactions between neural transplants and cellular elements within the host CNS. However, the transplantation of fetal neural tissue involves a mixture of cellular elements, including various populations of neurons as well as the complete gamut of glial cells and mesodermal cells. In order to isolate the functions of defined cellular elements, purified populations of neuronal (Freed et al., 1992; Onifer et al., 1993; Rostaing-Rigattieri et al., 1997) or glial elements (Zhou and Lund, 1992; Gout and Dubois-Dalco, 1993; Vignais et al., 1993; Wang et al., 1995; Xu et al., 1995; Pundt et al., 1995) have been grafted and traced. The study of glial cell transplantation has been of particular interest.

One of the deleterious events which occurs following traumatic spinal cord injury, as well as in multiple sclerosis, is the loss of myelin in otherwise intact white matter tracts (Bunge et al., 1993; Waxman et al., 1994; Olby and Blakemore, 1996). Grafted populations of either oligodendrocytes or Schwann cells are able to remyelinate, to some

extent, regions of myelin loss. Transplanted oligodendrocytes can migrate considerable distances through the spinal cord towards demyelinating lesions, and can form new myelin sheaths (Rosenbluth et al., 1990; Gout and Dubois-Dalco, 1993), even when the cells are derived from a different species (Crang and Blakemore, 1991). Schwann cells are one of the glial cell populations which show the most promise in transplantation paradigms. These cells have been shown to remyelinate areas of demyelination following X-ray treatment (Blakemore et al., 1987). More importantly, Schwann cells have been shown to promote CNS neurite growth and regeneration both *in vitro* and *in vivo* (for review, see Guénard et al., 1993), a finding in keeping with their known role in peripheral nerve regeneration.

Another glial cell which has received a great deal of attention in transplantation experiments is the astrocyte. Although the astrocytic scar may be a barrier to regeneration (Reier et al., 1983; Reier et al., 1988), astrocytes may also play a beneficial role. *In vitro* studies have shown that astrocytes can create a permissive environment for axonal elongation (Noble et al., 1984; Fallon, 1985; Assouline et al., 1987; Ard et al., 1991) and that transplanted astrocytes can reduce scarring and increase neurite ingrowth into gelfoam implants (Wang et al., 1995). Co-grafting of striatal astrocytes with fetal mesencephalic neural tissue has been shown to have neurotrophic and neurotropic effects on dopaminergic neurons *in vivo* (Pierret et al., 1998), and astrocytic grafts have also been shown to reduce memory deficits caused by experimental lesioning of the rat septal regions (Bradbury et al., 1995). These investigations into the use of glial cell grafts have broadened our knowledge of the possible effects these cell populations might have within fetal neural transplants into the brain and spinal cord. Nevertheless, much work is still to

be done before we understand the interactions between these cells and the host environment.

Grafting of Microglia/Macrophages

Despite representing nearly 10% of the total cells in the adult CNS, the cell type that has been most often ignored in transplantation paradigms is the microglial cell. A great deal of study has focused on transplantation of neurons and glial cells such as astrocytes, oligodendrocytes, and Schwann cells (see above), but we know next to nothing about the role of grafted microglia. Although several studies of graft rejection have reported microglia/macrophage infiltration (Lawrence et al., 1990; Finsen et al., 1991; Duan et al., 1995; Poltorak and Freed, 1989), none have addressed a possible role for these cells beyond phagocytosis of debris and the speculation that they might be antigen presenting cells (APCs). There has, however, been a recent interest in the use of microglia/macrophages as tools to promote regeneration. Polymeric tubes filled with purified microglia, when placed into the acutely injured spinal cord, were shown have increased neuritic ingrowth over gelfoam alone or astrocytic grafts. Even neuritic ingrowth into tubes devoid of grafted microglia was associated with host macrophage infiltration (Rabchevsky and Streit, 1997). Microglia/macrophages transplanted into the injured spinal cord, in conjunction with fetal neural transplants, have been shown to increase the regeneration of DRG sensory fibers (Prewitt et al., 1997) Macrophages alone placed into the injured spinal cord had a similar effect that was attributed to their degradation of myelin debris and the promotion of neovascularization (Franzen et al., 1998). A similar study used injections of activated peripheral macrophages to overcome the inherent non-permissive nature of the optic nerve

for regeneration (Lazarov-Speigler et al., 1996). The role of macrophages in peripheral nerve regeneration has been known for some time (for review, see Griffin et al., 1993). However, until recently these attributes had not been tested within the CNS. Despite indications that grafted microglia/macrophages may potentially be beneficial to the injured CNS, however, there remained no clearly defined method for the reliable tracing of grafted cells. Chapter 2 details such a method, which should be of benefit to future studies regarding functional roles of transplanted microglial cells.

There are many benefits to working with transplants of purified microglial cells. However, to truly understand how these cells interact with fetal neural transplants as well as the host environment, studies must be designed to look at microglia within complete fetal grafts. As described in the following paragraphs, there is ample evidence to suggest beneficial roles for microglia in graft development as well as a detrimental role in the immunology of graft rejection. The research in this dissertation has attempted to delineate some of these functions.

Microglia as Immunocompetent Cells

Although astroglial cells have been shown to express properties similar to antigen presenting cells (APCs) *in vitro* (Fontana et al., 1984; Wong et al., 1985), the preponderance of evidence has led to the current belief that microglia are the resident immunocompetent cells of the CNS (Frei et al., 1987; Hickey and Kimura, 1988; Poltorak and Freed, 1989; Lawrence et al., 1990; Graeber and Streit, 1990). Numerous immunohistochemical studies, conducted independently by various laboratories, have examined the expression of antigens of the Major Histocompatability Complex (MHC) in

both normal and injured CNS tissue. These studies have shown conclusively that the principal cell type in the CNS expressing MHC antigens is the microglial cell (Matsumoto et al., 1986; McGeer et al., 1988; Konno et al., 1989; Streit et al., 1989; Morioka et al., 1992a; Popovich et al., 1993). These immunophenotypic studies on tissue sections have been confirmed by flow cytometric characterization of microglial cells from normal and diseased CNS tissue (Sedgwick et al., 1991). Furthermore, continuous systemic administration of interferon-gamma (IFN-γ), a potent MHC inducer, results in widespread MHC expression specifically on microglia and not astrocytes (Steiniger et al., 1988). This provides additional support for microglial immune competence. Similarly, upon treatment with IFN-γ, cultured microglia have been found to express MHC class II antigen and present antigen to MHC class II restricted T-cells (Frei et al., 1987).

An important finding in understanding microglial immunological activity concerns their secretory activity with regard to the elaboration of cytokines and growth factors. This work, which was largely derived from studies on microglia in tissue culture, and more recently from *in vivo* investigations (Kiefer et al., 1993), has provided evidence for a second critical component for defining microglial immunocompetence, namely the production and secretion of substances such as interleukin-1, interleukin-6, tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β) (Giulian et al., 1986; Remick et al., 1988; Sawada et al., 1989). Cytokines/growth factors are known to mediate a number of immunological cell-cell interactions, including antigen presentation and cytotoxicity, as well as T lymphocyte proliferation and activation. Many of the tissue culture studies on microglial cytokine production remain to be confirmed by *in vivo* localization studies.

Nevertheless, they have provided an essential counterpart to studies on MHC expression by revealing that both membrane surface constituents (MHC antigens), as well as secretory components (cytokines) facilitate the immune competence of microglia. In regards to functional roles of microglia in graft rejection, this evidence indicates that grafted microglia may be a major cellular source of cytokines which would normally contribute towards activating the host immune system. Conversely, the infiltration of a developing graft by host microglial cells provides a large population of cells capable of both antigen presentation and pro-inflammatory cytokine production.

New and conflicting evidence, however, paints a different picture of the state of microglial immunocompetence. Ford and colleagues (1995) have shown that microglia can be separated from other macrophages within the CNS, i.e. perivascular cells, by flow cytometry and that purified microglia do not function *in vitro* as antigen presenting cells. This finding was supported by another study which characterized microglia as poor APCs, similar to other tissue-specific macrophages during development and prior to full immunocompetence (Carson, 1998). This can be partly attributed to the lack of expression of appropriate costimulatory molecules, in particular B7.1. Microglia *in vitro* have been shown to express B7.1 at low levels which increase upon exposure to IFN-γ (Williams et al., 1994; De Simone et al., 1995), and *in vivo* in acute multiple sclerosis lesions (De Simone et al., 1995; Dangond et al., 1997). However, these same authors confirm that microglia *in vivo* do not express B7.1 in the normal, non-pathologic CNS nor following normal trauma. A more telling study has demonstrated that purified microglia actually induce apoptosis, or programmed cell death, in T-cells, rather than stimulating them to

divide as would be expected of an APC (Ford et al., 1996). The experiments outlined in Chapter 3 were designed to shed new light on the state of microglia immune function by examining their role in transplant rejection.

The Immunology of Transplantation to the CNS

Immune Privilege Within the CNS

The central nervous system has long been regarded as a likely target for transplantation because of it's perceived status as an immunologically privileged site (Medawar, 1948; Barker and Billingham, 1977). This term implies that the CNS is somehow shielded from the immune system in ways that other regions of the body are not. There are three primary observations which contributed to this conclusion. First, most cells of the CNS do not express antigens of the Major Histocompatability Complex (MHC), the primary molecules which define immunological self from non-self (Nicholas and Arnason, 1989). Second, it was thought that lymphocytes did not enter the CNS, eliminating both the possibility of detection of transplanted tissue and the effector cells needed to cause rejection (Widner and Brundin, 1988). Finally, and perhaps most importantly, it was thought that the brain had no lymphatic drainage (Yoffey and Courtice, 1970), effectively isolating the CNS from lymphatic tissue and thus the immune system. It is well known that transplants of neural and non-neural tissue grafted into the CNS survive better than they would be expected to based upon their histocompatability (Murphy and Sturm, 1923; Ridley and Cavanaugh, 1969; Mason et al., 1986; Widner et al., 1989), but it is also clear that the brain is not as well shielded from immune attack as was once thought.

It has been shown that although most cells of the CNS do not express MHC antigens under normal conditions (Widner and Brundin, 1988), many are capable of MHC expression under conditions such as trauma or inflammation (Traugott et al., 1985; Steiniger et al., 1988; Streit et al., 1989). Of greater interest is a subpopulation of microglia and perivascular cells which constitutively express MHC Class II antigens, constituting a possible pool of cells capable of antigen presentation (Graeber et al., 1992). It has also been shown that T-cells can in fact enter the CNS during pathological conditions such as experimental allergic encephalomyelitis (EAE) (Wekerle et al., 1986; Cross et al., 1990). Lastly, in regards to lymphatic drainage, it is clear that there is drainage from the CNS into the deep cervical lymph nodes, and that this drainage occurs primarily through the arachnoid villi and across the cribriform plate (Bradbury and Westrop, 1983; Widner et al., 1988; Harling-Berg et al., 1989). Together, these facts indicate that the immune privilege of the CNS is a relative and not absolute quantity, and that transplants placed into the brain and spinal cord are at risk of immunological rejection.

Graft Rejection

According to Lawrence et al. (1990), it is possible to distinguish at least three phases of the graft rejection process: 1) The immune induction phase (also known as the afferent limb of an immune response); 2) the phase of immune attack (efferent limb of immune response); and 3) a quiescent phase. During the induction phase it is believed that expression of MHC antigens on cells from within the grafted tissue represents the primary inducing stimulus. There are two primary pathways through which this may occur: direct and indirect antigen presentation (see Fig. 1-1). In the direct pathway, thought to be the

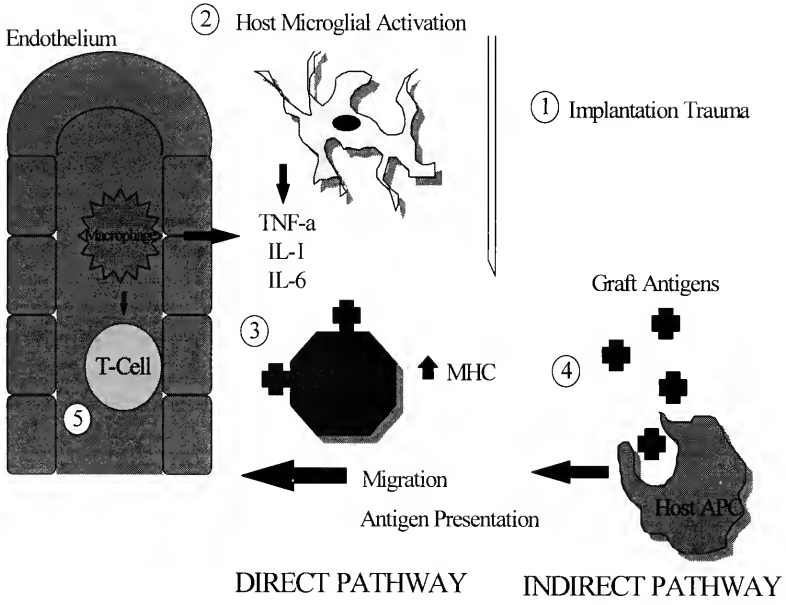
primary pathway for the initiation of allograft rejection (VanBuskirk et al., 1994), MHC-expressing cells acting as donor APCs migrate out of the graft and present foreign antigen directly to host T-cells. In the indirect pathway, foreign antigen within the graft, of which MHC antigens are a large part, is phagocytosed by host APCs and then presented to host T-cells. With this in mind, the removal of all donor cells capable of MHC expression should interrupt these pathways and prevent or at least delay immune induction (Nicholas and Arnason, 1989; Bartlett et al., 1990). It is important to point out, however, that minor histocompatability antigens alone are capable of inducing immunological rejection (Steinmuller, 1983; LaRosa and Talmage, 1985).

Since normal embryonic CNS tissue contains no cells that constitutively express MHC antigens, it is thought that MHC expression is induced following transplantation. From the literature regarding *in vivo* expression of MHC antigens, it is evident that MHC II expression in the rodent CNS is limited to two cell types: microglia and perivascular cells or pericytes (Streit et al., 1989a). Endothelial cells are the other cellular component within the rodent CNS capable of MHC expression, but this expression is limited to Class I antigen (Streit et al., 1989b). We know that microglial and endothelial cells are present in the developing CNS as early as E12 (Ashwell et al., 1989) while perivascular cells do not appear before the first postnatal week (Mato et al., 1985). Together with their MHC-expressing capabilities, this makes it very likely that transplantation-induced MHC expression occurs on just microglia and endothelial cells.

The research described in Chapter 3 is designed to test the hypothesis that the removal of the cell types capable of expressing MHC antigens, i.e. microglia and endothelial cells, will delay or prevent the phase of immune induction. By correlating the

Figure 1-1. The Induction of Graft Rejection. **1.** The implantation procedure and/or the inflammation resulting from a preexisting lesion causes a breakdown of the blood-brain barrier. **2.** This trauma activates host microglia and infiltrating macrophages, which produce pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. **3.** The cells within the graft capable of expressing MHC antigens, i.e. microglia, begin to do so. They may migrate out into peripheral lymphatic tissue and present foreign antigen directly to host T-cells. This is known as the direct pathway. **4.** Foreign antigen, either shed from living cells or debris from dead cells, is taken up by host APCs and presented to host T-cells. This is the indirect pathway. **5.** The activated T-cells initiate the rejection cascade.

Graft Rejection Cascade



temporal expression of donor MHC Class II antigens with graft rejection, this study will shed further light on the relationship between MHC expression and graft rejection. This experiment also has clinical implications. Currently, immunosuppressive agents, in particular cyclosporin A and FK 506, are widely employed to prevent the immune response against a neural transplant. There are at least two major problems associated with the use of these agents: increased susceptibility to secondary infections and rejection of the graft upon withdrawal of the immunosuppressant. Thus, the administration of immunosuppressants during the early post-transplantation period does not provide long-lasting protection from immune attack but only delays the onset of rejection. Immunosuppression is probably the least desirable regimen for a human transplant patient who is very susceptible to numerous infections. This research will explore a potential new method of inducing tolerance of allo- and xenografted tissue without otherwise compromising the immune system of the host.

Angiogenesis

The survival of any adult tissue depends on the presence of a vascular network. The availability of nutrients, oxygen, and waste disposal through the blood is crucial once tissues reach a certain minimum size. This is illustrated by the lack of viability of animals deficient in factors that regulate angiogenesis (Beck and D'Amore, 1997) and in tumors in which angiogenesis has been inhibited (Folkman, 1990). In addition, the efficacy of the immune system depends on the ability of bloodborne leukocytes to reach their targets by way of the vasculature, as well as on the ability of endothelial cells to express the necessary adhesion molecules for leukocyte binding and migration (Abbas et al., 1994). For these reasons, the development of new blood vessels within developing neural

transplants is an important topic, both for its role in graft survival as well as for its contribution to graft rejection.

Blood vessel formation during development is carried out by a combination of two different processes: vasculogenesis and angiogenesis. Vasculogenesis is a process in which endothelial cell precursors, termed angioblasts, migrate into developing tissues and form vessel tubes. Most endodermal tissues are vascularized by vasculogenesis. The development of vasculature within the central nervous system, as with most tissues of ectodermal origin, is carried out by angiogenesis, or the formation of vessels by budding off of already existing vessels (Beck and D'Amore, 1997). One important exception to this rule is the developing retina, which is thought to be vascularized through vasculogenesis despite being a part of the CNS (McLeod et al., 1987; Beck and D'Amore, 1997). Angiogenesis is also the process by which healing wounds, growing tumors, and developing neural transplants form new blood vessels (Iruela-Arispe and Dvorak, 1997; Horner et al., 1994). The common role of angiogenesis in the formation of new vessels both in neural transplants and in the developing CNS illustrates the usefulness of fetal transplantation as a model for recapitulating development, in addition to its potential therapeutic value.

There are several distinct processes involved in angiogenesis The first is the degradation of extracellular matrix by released proteases. The next steps are the induction of endothelial cell proliferation and the migration of the cells towards the angiogenic stimulus. Following migration, the cells differentiate and form capillary tubes, a new basement membrane, and recruit pericytes to the perivascular space (Pötgens et al., 1995). All of these processes are controlled through various angiogenic factors and cytokines.

Three major cytokines known to be involved in angiogenesis are examined in this dissertation; vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-beta (TGF-β).

Angiogenic Cytokines (VEGF, bFGF, and TGF-β)

Vascular endothelial growth factor (VEGF; also known as Vascular Permeability Factor) is expressed as a 34-46 kDa homodimeric glycoprotein. Alternative splicing of the VEGF mRNA gives rise to four splice variants of 121, 165, 189, and 206 amino acids. However, the 206 and 189 aa variants are rare, and remain bound to the expressing cells by heparin-like molecules or bound to heparin in the ECM, being released only by plasmin or heparinase cleavage. These variants have little or no mitogenic activity on cultured endothelial cells (Houck et al., 1991), and their biological significance is not known. The more common 165 and 121 aa variants have little and no affinity, respectively, for heparin and are freely secreted by the producing cells (Pötgens et al., 1995). Binding of VEGF is restricted to the high and low affinity VEGF receptors (*flt* and *flk-1*), which are primarily restricted to endothelial cells (Ferrara et al., 1992; Pötgens et al., 1995). VEGF is potently mitogenic exclusively for vascular endothelial cells, in contrast to bFGF which also induces the proliferation of a wide range of additional cells. VEGF is, by itself, capable of initiating all of the events necessary for blood vessel formation (see above; Ferrara et al., 1992). VEGF production by astrocytes is thought to be responsible for vascular development in the retina (Stone et al., 1995) as well as lesion-induced neovascularization following brain injury (Papavassiliou et al., 1997).

Basic fibroblast growth factor (bFGF) is expressed as a 16-24 kDa protein characterized by its strong ability to bind to heparin. bFGF also exists as several different splice variants, although the 155 aa variant predominates. All the variants have similar or identical biological properties. bFGF is a pleiotropic molecule which induces proliferation of a number a cells, from astrocytes to smooth muscle cells to fibroblasts. More to the point, it affects proliferation, migration, and differentiation of vascular endothelial cells and is potently angiogenic *in vitro* and *in vivo* (Pötgens et al., 1995). Interestingly, it also induces neuronal differentiation (Vlodavsky et al., 1991). bFGF also acts through high and low affinity receptors (*flg* and *bek*). Like the VEGF receptors, these are both transmembrane receptor tyrosine kinases. It is thought that bFGF can only act through simultaneous binding of both the high and low affinity receptors (Pötgens et al., 1995).

In *in vitro* models of angiogenesis, VEGF and bFGF act synergistically, with much stronger effects than either has alone (Pepper et al., 1992). Both induce expression of proteases required for ECM degradation (i.e. collagenase and constituents of the plasminogen activator pathway) as well as induce migration and capillary tube formation in collagen gels (Pötgens et al., 1995). However, despite bFGF's unquestioned potency in inducing angiogenesis in model systems, its role in normal angiogenesis *in vivo* is a matter of some dispute (Horner et al., 1994; Beck and D'Amore, 1997).

Transforming Growth Factor - beta (TGF-β), in active form, is a disulfide-linked homodimer with two chains of 112 amino acids. There are five isoforms of TGF-β, three of which (TGF-β1-3) are expressed in mammals, and all of which are 65-80%

homologous with the same basic structure (Flanders et al., 1998). TGF-β is normally secreted in an inactive form, bound to a so-called latency associated protein (LAP), and the *in vivo* method of activation is unclear. TGF-β binds to a heteromeric receptor complex consisting of two subunits termed RI and RII, which are serine/threonine kinases. TGF-β also binds with high affinity to the type III receptor, a high molecular weight protein with no signaling domain that is thought to aid in the binding of TGF-β to RII (Flanders et al., 1998). TGF-β has diverse functions ranging from immunosuppression to up-regulation of NGF production by astrocytes (Horner et al., 1994). It also is involved in the inhibition of endothelial cell proliferation, the deposition of matrix, and in vascular differentiation (Horner et al., 1994; Beck and D'Amore, 1997; Flanders et al., 1998). TGF-β1 is thought to be expressed by microglia in the adult rat brain, while TGF-β2-3 are expressed in neurons and astrocytes (Flanders et al., 1998). Its inhibitory effects, in contrast to the angiogenic effects of VEGF and bFGF, may make it an important player in the overall regulation of neovascularization.

Angiogenic Properties of Microglia

The idea that macrophages are involved in wound healing and inflammatory angiogenesis is not a new one. Leibovitch and Ross discovered in 1975 that macrophages were necessary for undisturbed wound repair. Other groups showed both a temporal correlation between endothelial DNA synthesis and mononuclear cell infiltration (Polverini et al., 1977a) and that macrophages isolated from wounds induced neovascularization *in vitro* and in *in vivo* assays (Clark et al., 1976; Greenburg and Hunt, 1978). It was soon discovered that macrophage conditioned medium alone promoted

vascular proliferation (Polverini, 1977b), implicating some diffusable substance. Macrophages have also been implicated in tumor angiogenesis. Mice depleted of monocytes showed decreased tumor vascularization (Evans, 1977), and neoplastic tissue explants demonstrate angiogenic properties only when macrophages are present (Mostafa et al., 1980). We now know that activated macrophages produce a number of angiogenic cytokines, the most potent among them being VEGF, bFGF, TNF-α, TGF-β, and IL-1β (Sunderkötter et al., 1994), although IL-1β and TNF-α likely act through indirect, pro-inflammatory pathways.

Regardless of other functions attributed to them, it is well established that microglia represent the brain's tissue-specific macrophage population. Although the subject remains controversial, there is ample evidence that microglia are derived from fetal macrophages which migrate into the developing CNS at early prenatal timepoints and give rise to the adult microglial population (Hurley and Streit, 1996). Following injury, resting microglia become activated and upregulate typical macrophage cell surface molecules, such as MHC antigens. In pathological situations resulting in cell death, activation of microglia results in the formation of brain macrophages, phenotypically indistinguishable from blood derived macrophages (Graeber et al., 1988; Streit et al., 1988, 1989a). In tissue culture, microglia have been demonstrated to produce bFGF (Shimojo et al., 1991) and TGF-β (Lindholm et al., 1990). It is not known whether microglia are also capable of VEGF production, although peripheral macrophages are known to be a source of VEGF (Sunderkötter et al., 1994). Horner and colleagues (1994) speculate that microglia play a large role in intraspinal transplant neovascularization, but

there has been surprisingly little work done to demonstrate this phenomenon. Chapters 4 and 5 examine the hypothesis that microglia are a principal mediator of graft neovascularization. The relationship between microglia and vasculature is examined both morphologically and from the standpoint of cytokine production.

CHAPTER 2
TRACING OF FLUORO-GOLD PRELABELED MICROGLIA INJECTED INTO THE ADULT RAT BRAIN

Introduction

Since microglial cells were first identified as a distinct glial population by del Rio-Hortega (1932), the prevailing view of microglial function has been that they serve as macrophages of the central nervous system (CNS) (Rio-Hortega, 1932; Giulian and Baker, 1986; Kreutzberg, 1996). It is well known that injury to the CNS resulting in neuronal death induces resting microglia to become phagocytic cells that are morphologically indistinguishable from blood-derived macrophages (Streit et al., 1988). In recent years, this traditional view of microglial function has been expanded by studies showing that microglia secrete neuronal growth factors and angiogenic cytokines *in vitro* (Mallat et al., 1989; Elkabes et al., 1996; Lindholm et al., 1990; Shimojo et al., 1991). This suggests that microglia have important functions in supporting neuronal health and promoting vascular development in the normal as well as the pathological CNS (Horner et al., 1994; Streit and Kincaid-Colton, 1995; Pennell and Streit, 1997). Additional support for such beneficial activities of microglia is derived from studies using transplanted peripheral macrophages and microglial cells, indicating that both of these cell populations can enhance the limited regenerative capabilities of injured CNS neurons

(Lazarov-Speigler et al., 1996; Rabchevsky and Streit, 1997). Taken together, these findings paint a picture of microglial function that is different from their traditional role as the brain's scavenger cell and suggest that microglia perform functions critical for the repair and reconstruction of injured CNS tissue.

The recent interest in transplantation of microglial cells and macrophages raises the question of a reliable method for tracing the grafted cells and distinguishing grafted microglia from resident microglia. One common method for the labeling of glial cells prior to transplantation is the use of fluorescent labels such as fluorescein-conjugated lectin (Goldberg and Bernstein, 1987) and vital dyes such as DiI, Hoechst 33342, and Fluoro-Gold (Rabchevsky and Streit, 1997; Xu et al., 1995; Andersson et al., 1993). In the present study, we have investigated the use of Fluoro-Gold (FG) to prelabel cultured microglial cells before injecting them into the normal rat brain.

Materials and Methods

Preparation of Purified Microglial Cultures

Isolated whole brains (cerebellum and tectum removed) from newborn Wistar rats (Harlan Sprague-Dawley, Inc.) were stripped of meninges in a 35 mm Petri dish while immersed in "Solution D" (0.8% NaCl, 0.04% KCl, 0.1% Glucose, 2.2% Sucrose, 0.01% Streptomycin sulfate, 0.025% Fungizone, and 0.006% Penicillin G in dH_2O). Clean fragments were mechanically minced, transferred to a 15 ml conical tube, and incubated under bi-directional rotation in 0.05% trypsin in "Solution D" for 20 minutes at 37°C. The suspension was then triturated, DNAase added to a final concentration of 5800 units/ml, and the suspension incubated for an additional 10 min. at 37°C. An equal volume of

complete medium (DMEM + 10% FBS) was added to the suspension to quench the reaction, and the tissue was triturated again and passed through a 130 μm Nitex filter before being pelleted (400 x g, 10 min.). The pellet was then resuspended in 10 ml of complete medium and passed through a 40 μm Nitex filter before being plated in poly-L-lysine coated 75 cm^2 flasks. The plating density was one brain per 1.5 flasks. Cultures were incubated in a 37°C incubator set at 8% CO_2 for 3 days. The medium was then changed and the cultures incubated for an additional 7 days after which microglia were harvested every three or four days. The flasks were shaken on an orbital shaker in a 37° incubator for 1 hour at 200 rpm. After shaking, the supernatant yielded a high number of microglia which were pelleted, resuspended in complete medium, and plated on 100 mm Petri dishes. The medium was changed after one hour to remove non-adherent cells. Microglia were plated at a density of 5 x 10^6 cells/100mm dish, and maintained in culture overnight prior to FG-labeling and injection.

Labeling and Transplantation of Microglia

Five ml of 0.1% Fluoro-Gold (Fluorochrome International) in DMEM + 10% FBS was added to the cultured microglia followed by incubation at 37° for 2 hours. Dishes were washed with fresh medium several times to remove excess FG. Labeled microglia were removed from the dish using a sterile rubber cell scraper, after which the cells were pelleted, resuspended in complete media, and counted prior to injections.

Under ketamine/xylazine anesthesia, cells were injected stereotactically into either the right corpus callosum or the right lateral ventricle of adult male Wistar rats as a total volume of 3 μl (50,000 cells/μl in DMEM + 10% FBS), using a 25 gauge Hamilton syringe.

Injections were made slowly over a period of several minutes, and the needle allowed to remain in place for several minutes after injection to prevent reflux. Control rats received injections of 1 μl of 2% FG in either sterile H_2O or DMEM + 10% FBS into the right corpus callosum. Stereotaxic coordinates were as follows: 2 mm lateral of Bregma, 3 mm ventral to the dural surface.

Tissue Processing

Following survival times of 1, 2, 7, and 14 days, rats were sacrificed by transcardiac perfusion with 100 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde in phosphate buffered saline, pH 7.4. Brains were removed and post-fixed in 4% paraformaldehyde for at least 48 hours prior to sectioning on a vibratome at 60 μm thickness. Using a slightly modified procedure from previously described protocols (Streit, 1990), selected sections were incubated with biotinylated isolectin from *Griffonia simplicifolia* (Sigma L-2140; 10 μg/ml in PBS containing 0.4% Triton X-100 and 0.1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$) for 3 hours at room temperature, followed by streptavidin-Texas Red (Molecular Probes S-872; 10 μg/ml in PBS) for 45 min. at room temperature. Sections were mounted on gelatin-coated slides, allowed to air dry, and then either dehydrated in ascending ethanols and coverslipped with Permount (FG only) or coverslipped with fluorescent mounting media.

Results

Following a two hour incubation with 0.1% FG, all microglial cells in a given culture dish were brightly labeled (Fig. 2-1A). The cells maintained the typical

morphology of microglia in culture, having either a rod-like or flattened shape with few processes.

Twenty-four hours after injection, the FG prelabeled cells were seen grouped together in a small cavity created by injection into the corpus callosum (Fig. 2-1B). Grafted microglia remained brightly labeled *in vivo*, with no apparent labeling of endogenous ramified cells. Twenty-four hours after control injections of free FG there was extensive labeling of axons within the corpus callosum, as well as retrograde labeling of neurons near the injection site. There appeared to be abundant free FG at the injection site, as evidenced by hazy staining that was not localized to any particular cell type. Importantly, there was no apparent labeling of resident microglial cells.

Seven days after injection into the right lateral ventricle, bright FG-labeled cells were found within the choroid plexus and ependymal layers of both lateral ventricles. These cells extended processes (Fig. 2-1C), and were easily distinguishable against an unlabeled background. Seven days after control injection of free FG into the corpus callosum, there was no hazy staining indicative of free FG at the injection site, suggesting that most of the FG had been cleared from the extracellular space. Neurons throughout both hemispheres were labeled, and brightly labeled perivascular cells were evident throughout the injected hemisphere (Fig. 2-2). Perivascular cell labeling was brightest near the injection site and became less pronounced farther away, where it was found primarily in white matter containing FG-labeled axons (Fig. 2-2B). Brains injected with free FG in DMEM + 10% FBS also showed labeling of cells with oligodendrocyte morphology (not shown). However, this labeling was present only in the corpus callosum at the injection site. There was no detectable labeling of oligodendrocytes in the animals

injected with FG in H_2O. Resident microglia that were visualized with fluorescent lectin staining revealed no FG labeling in these control experiments (Fig. 2-3).

Two weeks post-injection FG-labeled microglia were still brightly labeled *in vivo*, and some had migrated out along the corpus callosum, although most of them were still clumped together at the injection site. Some of the grafted cells had also migrated into the surrounding gray matter and extended processes, resembling normal resting microglia (Fig. 1D). There was moderate staining of neurons and perivascular cells in the immediate area of the injection site, indicating that these cells had taken up the FG released from prelabeled microglia that died after the injection.

Discussion

FG has traditionally been used as a fluorescent tracer for the mapping of axonal projections in the PNS and CNS (Schmued and Fallon, 1986; Zhou and Rush, 1995). It is retrogradely transported by axons, and has several advantages over other fluorescent dyes. The human retina has its highest sensitivity at the wavelength emitted by FG (Thanos et al., 1994), and FG does not fade appreciably over time even under frequent exposure to light. It is ideal for double-labeling studies as the dye is visible in vibratome, frozen, paraffin, and plastic sections, and the light emitted does not cross over into the wavelengths of fluorescein or rhodamine (Schmued and Fallon, 1986). It has also been demonstrated in neuronal labeling studies that the dye does not leak from healthy, intact cells (Rinaman et al, 1991; Crews and Wigston, 1990). However, it can be expected that after transplantation a certain percentage of the cells die and release FG into the

Figure 2-1. Fluoro-Gold (FG) labeling and tracing of cultured microglial cells. **(A)** Microglia *in vitro* following a 2 hour incubation with 0.1% FG. All cells in the field are labeled and display the characteristic morphology of cultured microglial cells. x 112. **(B)** FG labeled microglia 24 hours after injection into the adult rat corpus callosum. Notice that despite robust labeling of the injected cells there is very little background labeling of neurons by free FG. x 112. **(C)** FG prelabeled microglial cells are present in the ependymal layer of an adult rat one week after intraventricular injection of prelabeled cells. **v** = ventricular space. x 225. **(D)** FG prelabeled microglial cell exhibits ramified morphology two weeks after injection into the adult rat corpus callosum. This shows that cultured cells with an ameboid morphology differentiate into process-bearing cells when exposed to the CNS environment. x 560.

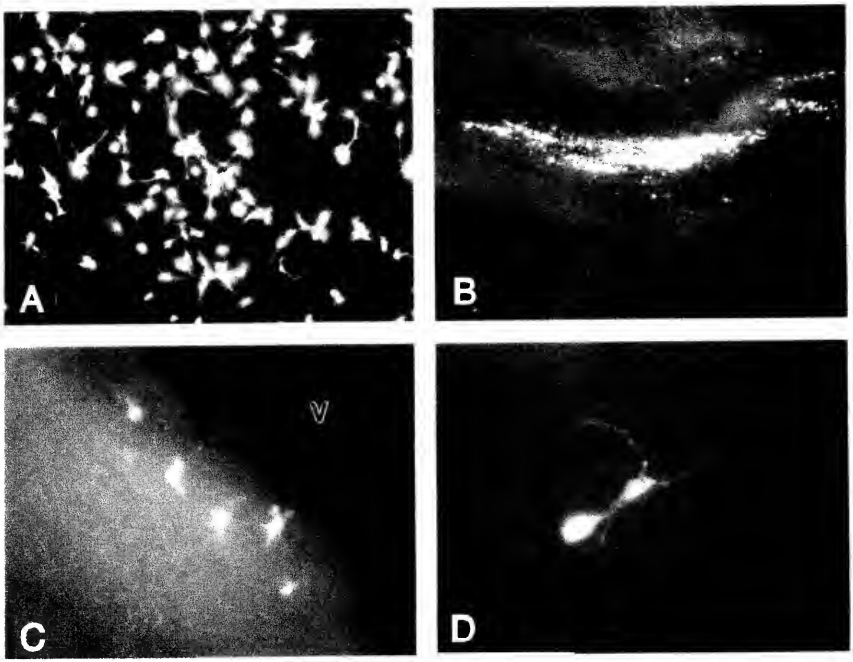


Figure 2-1

Figure 2-2. Cellular labeling one week following injection of free FG into the adult rat corpus callosum. **(A)** Numerous FG labeled cells are evident, notably perivascular cells (arrows) and neurons. No cells with microglial morphology are labeled. x 125. **(B)** Higher power micrograph demonstrates specific labeling of perivascular cells by free FG (arrow). FG is also evident within axons passing through the corpus callosum. x 250. **(C)** Pyramidal neurons immediately dorsal to the injected corpus callosum exhibit bright labeling. Note that there are no non-neuronal cells labeled, demonstrating that the dye is retained within healthy neurons. x 250

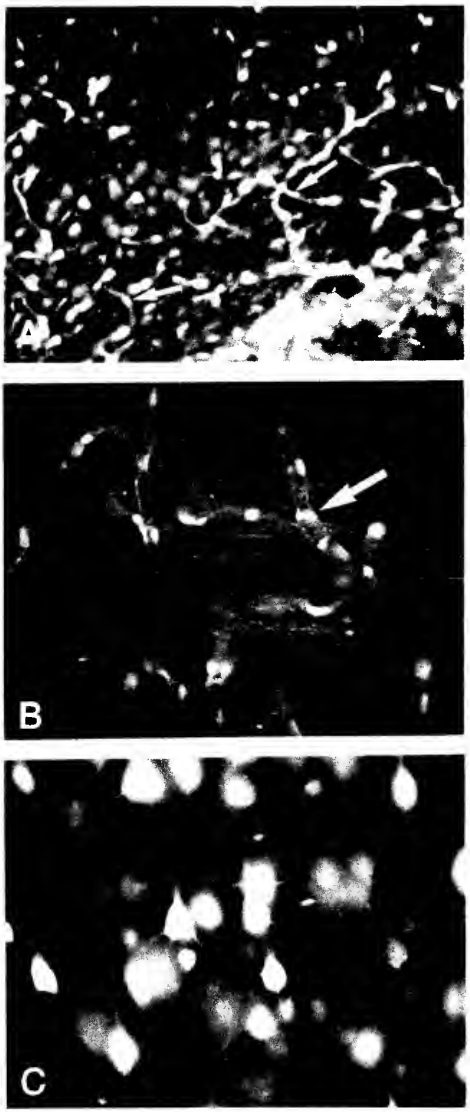


Figure 2-2

Figure 2-3. Control brain injected with 2% FG in DMEM + 10% FBS, one week after injection. The section was stained with biotinylated GS I-B_4 and streptavidin-Texas Red to visualize resident microglial cells. **(A)** FG labeled neurons in the cortex near the injection site. **(B)** The same field shows Texas Red-labeled resident microglial cells (arrows show location of microglial cell nucleus). Note the lack of FG labeling of resident microglial cells. x 800

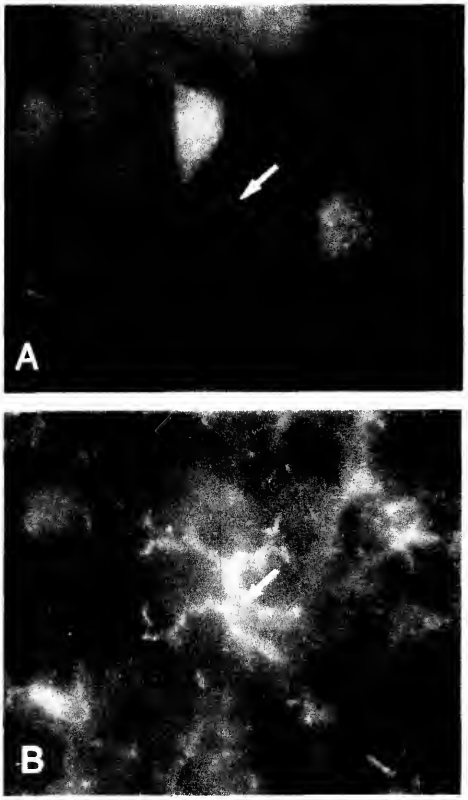


Figure 2-3

extracellular space. It was therefore necessary to determine which cells in the CNS are capable of taking up free FG.

Based on both morphology and double labeling with GS I-B_4, we have demonstrated that resting, endogenous microglia do not take up free FG, and thus do not seem to be actively pino- or phagocytic. This finding is in contrast to the known phagocytic activity of activated microglia, illustrating the functional plasticity of microglial cells and providing direct *in vivo* evidence that ramified microglia are functionally distinct from reactive or phagocytic microglia (Giulian and Baker, 1986; Bocchini et al., 1988; Streit at al., 1988). Ward and colleagues (1991) hypothesized that ramified microglia in the brain served the function of interstitial fluid cleansing, cleaning out excess neurotransmitters and unwanted wastes. This hypothesis was based on the selective pinocytotic activity of microglia in whole-brain cultures. Our current findings, however, argue against such a function *in vivo*, as resident microglia did not sequester free FG in the manner of cultured microglia. Instead it appears that perivascular cells are the predominant pinocytotic cells of the uninjured CNS, as has been reported previously (Kida et al., 1993; Streit and Graeber, 1993). In addition, our findings suggest that oligodendrocytes may become pinocytotic under certain conditions, although their capacity for uptake of FG was much less than that of perivascular cells.

Aside from leakage, a concern with the use of any diffusable substance as a cellular label is the problem of dilution following repeated division of cells. Although microglia are known to proliferate following neuronal injury (Graeber et al., 1988), they do so only minimally under normal conditions (Korr et al., 1983). Furthermore, when fluorescently prelabeled microglia respond to optic nerve transection by phagocytosing

dying retinal ganglion cells, they remain specifically labeled afterwards, indicating that even injury-induced proliferation is insufficient to dilute out their fluorescence (Thanos, 1991). Another concern involves the duration of FG-labeling. Although we did not follow the FG-labeled cells longer than two weeks post-transplantation, previous studies have shown that microglia labeled through phagocytosis of retrogradely FG-labeled neurons are identifiable as long as six weeks after labeling, the longest time point examined (Crews and Wigston, 1990). Studies using other fluorescent dyes have traced microglia *in vivo* for over twelve months (Thanos et al., 1994), demonstrating that microglia are not readily replaced in the adult brain, and suggesting that microglia labeled with a dye as persistent as FG would be traceable for extended periods of time (Schmued and Fallon, 1986).

In summary, we have demonstrated that cultured microglial cells can be robustly labeled with FG prior to transplantation, that FG prelabeled microglia are readily identifiable within the host brain up to two weeks, and that some of these transplanted cells undergo differentiation into ramified microglia. We have also shown that ramified microglia *in vivo* do not take up free FG, emphasizing their resting nature and supporting the concept of functional plasticity. Our findings indicate that FG pre-labeling is appropriate for the tracing of transplanted microglia.

CHAPTER 3
SINGLE-CELL SUSPENSION GRAFTS OF FETAL CNS TISSUE UNDERGO DELAYED REJECTION WHICH IS NOT ALTERED BY DEPLETION OF MICROGLIA

Introduction

Fetal neural transplants are currently being tested as a possible treatment for neurodegenerative disorders such as Parkinson's Disease as well as traumatic disorders such as spinal cord injury (Bjorklund, 1991; Dunnett and Richards, 1990; Gage and Buzsaki, 1989; Gash and Sladek, 1988; Reier et al., 1992). Since the use of isogeneic neural tissue is not clinically feasible, investigators and clinicians have focused instead on allogeneic and xenogeneic donor tissue, which raises the question of histocompatability and the possibility of graft rejection (Nicholas and Arnason, 1989).

To date, the majority of strategies used to prevent immunological rejection involve either immunosuppression (Duan et al., 1996; Pederson et al., 1997; Nicholas and Arnason, 1989) or the alteration of the host immune system in such a way as to induce tolerance (Theele and Reier, 1996; Okura et al., 1997; Wood et al., 1996). An alternative approach would be to alter the donor tissue in some way as to make it unrecognizable to the host immune system. It has been speculated that the presence of antigen presenting cells (APCs) within the donor tissue is the primary stimulus for the onset of graft rejection, as these cells may present foreign antigen directly to host T-cells (Lawrence et al., 1990; Nicholas and Arnason, 1989; VanBuskirk et al., 1994; Hirota et al., 1997).

These cells are primarily defined as cells which express Class II antigens of the major histocompatability complex (MHC II), as well as appropriate co-stimulatory molecules such as B7 (Abbas et al., 1994) . From the literature regarding *in vivo* expression of MHC antigens, it is evident that MHC II expression in the rodent CNS is limited to two cell types: microglia and perivascular cells (Streit et al., 1989a). Endothelial cells are the other cellular component within the rodent CNS capable of MHC expression, but only Class I antigen (Streit et al., 1989b). As microglial and endothelial cells are present in the developing rodent CNS as early as E12 (Ashwell et al., 1989) while perivascular cells do not appear in significant numbers before the first postnatal week (Mato et al., 1985), it is very likely that donor-derived MHC II expression occurs primarily on microglia within fetal transplants. Thus, if donor-derived MHC II expression were truly responsible for initiating immunological rejection, the removal of microglia from the donor tissue prior to transplantation should delay or prevent this response. The removal of endothelial cells may also be beneficial by removing an additional source of foreign antigen, i.e. MHC Class I antigens.

In the present study, we describe the use of single-cell suspensions of microglia and endothelia-depleted fetal neural tissue in two common transplantation paradigms in which rejection has been well characterized: xenografts of E14 mouse neural tissue placed into the intact rat striatum (Takei et al., 1990; Finsen et al., 1991; Duan et al., 1995); and allografts of E14 rat neural tissue (RT1.A/B^a MHC haplotype) placed into the injured spinal cords of histo-incompatible rats (RT1.A/B^u MHC haplotype) (Theele et al., 1996). The depletion of microglia and endothelial cells from fetal cell suspensions has been previously described (Pennell and Streit, 1996), using the specific affinity of the B_4-isolectin from *Griffonia*

simplicifolia for these cell types and negative cell selection with magnetic beads. Both the growth and survival of these grafts as well as the pattern of immunological rejection is followed over time both morphologically and immunohistochemically. In addition, to ascertain the immunogenic potential of the allogeneic donor tissue both cultured microglia and whole and microglia-depleted fetal suspensions from donor rats were tested for their ability to present antigen to host T-cells *in vitro* using a modified mixed lymphocyte reaction.

Materials and Methods

Preparation of Whole and Microglia-Depleted Fetal CNS Suspensions

The preparation of fetal CNS single-cell suspensions and the microglia depletion procedure have been previously described (Pennell and Streit, 1997). Briefly, E14 fetuses were obtained from either timed-pregnant female ACI rats or ICR mice (Harlan Sprague-Dawley, Inc.). Isolated fetal CNS (cortex, brainstem, and spinal cord) was stripped of meninges in a 35 mm Petri dish while immersed in 2 ml of "Solution D" (0.8% NaCl, 0.04% KCl, 0.1% Glucose, 2.2% Sucrose, 0.01% Streptomycin sulfate, 0.025% Fungizone, and 0.006% Penicillin G in dH_2O). Clean fragments were then incubated in 0.05% trypsin in "Solution D" for 20 min., triturated, and incubated an additional 10 min.. in DNAase (5800 units/ml). An equal volume of complete medium (DMEM + 10% FBS) was then added to stop the reaction and the suspension was filtered through a 130 μm Nitex filter. The filtrate was pelleted (400 x g, 10 min..), resuspended in 5 ml of complete medium, and filtered through a 40 μm Nitex filter. The cells were then counted using a hemocytometer

and their viability and degree of dissociation assessed using an acridine orange/ethidium bromide viability stain (live cells fluoresce green while dead cells fluoresce red; Fig. 3-1c).

GS I-B_4 was conjugated to tosylactivated Dynabeads® (Dynal, Inc.) according to the manufacturer's specifications. Fetal CNS suspensions ($1x10^7$ cells) were added to $4x10^7$ GS I-B_4-conjugated Dynabeads® in 5 ml test tubes. The tubes were then incubated at 4°C under bi-directional rotation for one hour, after which the tubes were placed in a magnetic particle concentrator which pulls bound cells to the side of the tube. The suspension was transferred to a new tube and the process repeated a second time. The freshly depleted suspension, as well as a control, undepleted (whole) suspension, was smeared on gel-subbed slides, allowed to dry, and stained with GS I B_4-HRP (Sigma L539I). By forming a ratio of GS I-B_4-positive cells in experimental suspensions and GS I-B_4-positive cells in control suspensions, we obtained a measure of depletion effectiveness (Fig. 3-1a-c).

Grafting Procedures

A total of 79 adult female Wistar-Furth (WF) rats (Harlan Sprague-Dawley, Inc.) weighing 200-250 g were used as graft recipients. For the striatal xenografts, approximately 1×10^6 cells (10 µl total volume) from whole or depleted murine suspensions were injected stereotactically into the intact right striatum under ketamine/xylazine anesthesia, using a 25 gauge Hamilton syringe. The stereotaxic coordinates were 1 mm rostral to bregma, 3 mm lateral to bregma, and 4.5 mm ventral to the dural surface. The injection was performed slowly over a period of five minutes and the needle was allowed to remain in place for

several min. afterwards to avoid reflux. The number of animals and time points examined are described in Table 1.

Allograft recipients received low thoracic spinal contusion injuries under ketamine/xylazine anesthesia. Spinal cord injury was induced using the New York University (NYU) weight drop contusion device (Gruner et al., 1992). Seven to ten days after injury, approximately 1 x 10^6 cells (15 µl total volume) from whole or depleted fetal rat suspensions were injected into the lesion cavity at the T11 spinal cord level, using a 25 gauge Hamilton syringe. The number of animals used and time points examined is described in Table 1.

Tissue Processing

The animals were sacrificed by transcardiac perfusion following the indicated survival periods after transplantation (see Table 1). Two animals from each group were perfused with 100 ml of 0.9% saline and the tissue frozen in cold isopentane for immunocytochemistry. The remaining animals at each timepoint were perfused with 100 ml saline followed by 200 ml Bouin's fixative. The Bouin's-fixed tissue was post-fixed for 24 hours and embedded in paraffin.

Immunostaining was carried out on the fresh-frozen tissue using a panel of monoclonal antibodies (Harlan Sera-Lab). F4/80 (1:100 dilution) was used to visualize murine microglia within the xenografts, OX-6 (1:2000 dilution) was used to visualize MHC Class II antigens from both donor and host cells, F17-23-2 (1:1000 dilution) was used to visualize allograft donor (ACI) haplotype-specific MHC Class II antigens, and W3/25

(1:150 dilution) was used to visualize $CD4^+$ T-cells, macrophages, and microglia. Frozen sections were cut on a cryostat at a thickness of 25 μm, mounted onto gel-subbed slides, and allowed to air dry for 30 min.. The sections were fixed for 5 min. at room temp. using a modified periodate-lysine-paraformaldehyde (0.75% paraformaldehyde) fixative (McLean and Nakane, 1974; Theele et al., 1996), followed by 2 min. in cold acetone. After rinsing in PBS, the sections were blocked in 10% normal goat serum for one hour at room temp., rinsed briefly, and incubated overnight at 4° C in primary antibody diluted in PBS + 1% BSA + 0.2% Triton X-100. Sections were then rinsed three times in PBS and incubated for two hours at room temp. with biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Inc.; 1:300 dilution in PBS). The sections were rinsed three times in PBS and incubated for 45 min. at room temp. with avidin D-HRP (Vector; 1:400 dilution in PBS). The sections were then rinsed three times with PBS and the antibody binding sites visualized using diaminobenzidine-H_2O_2 (DAB; Sigma) as a peroxidase substrate. The sections were dehydrated in an ascending series of ethanols, cleared in xylene, and coverslipped in Permount. Some sections from each specimen were stained with cresyl violet.

To elucidate general graft morphology, paraffin embedded tissue was cut on a microtome at a sectional thickness of 7 μm for hematoxolin/eosin (H&E) staining.

Antigen Presentation Assays

The spleens from adult female WF (host/responder) and ACI (donor/stimulator) rats were removed and homogenized in 5 ml of "Solution D" using a 15 ml Dounce

homogenizer. 5 ml of homogenate was layered onto 3 ml of Ficoll solution (Histopaque 1083, Sigma) and centrifuged at 800 x g for 30 min. at 4°C. The interface was collected, spun down (250 x g, 10 min.), and resuspended in Iscove's modified Delbecco's medium (IMDM) containing 10% FBS. To enrich the responder T-cell population, the WF splenocytes were panned to remove B-cells using anti-rat IgG-coated 100 mm Petri dishes. The cells were added to the coated dishes and the IgG-positive cells were allowed to bind for 30 min. at room temp., after which the process was repeated twice using fresh plates. The remaining non-adherent cells were collected and counted for antigen presentation assays. The ACI-derived splenocytes were counted and used as positive control stimulator cells.

Purified microglial cultures were prepared as previously described (Pennell and Streit, 1998). Stimulated microglia were treated with 100 units/ml of recombinant rat IFN-γ (R&D Systems, Inc.) and 10 μg/ml LPS (Sigma) in complete medium for 72 hours, while unstimulated microglia were cultured in complete medium alone. Stimulated and unstimulated microglia were scraped from their culture dishes using a disposable rubber cell scraper, pelleted, resuspended in IMDM + 10 % FBS, and counted.

Stimulator cells were irradiated with ^{137}Cs to prevent proliferation. ACI splenocytes as well as whole and depleted fetal rat CNS suspensions were exposed to 1500 rad of radiation, while microglial cells were exposed to 2000 rad. 4×10^5 stimulator cells were added to 8×10^5 WF splenocytes, in triplicate, in round bottomed 96 well plates. WF splenocytes alone served as a control for non-specific proliferation. The cells were maintained in an incubator with 8% CO_2 at 37° C for three days before being pulsed with 1

μCi/well of [^{3}H]-methylthymidine (NEN, Inc.). Approximately 18 hours after being pulsed, the cells were collected using a semi-automated cell harvester and the incorporation of [^{3}H]-methylthymidine was assessed using a liquid scintillation counter.

TABLE 3-1
Timepoints and Number of Animals

	XENOGRAFTS		ALLOGRAFTS	
Time (days)	Depleted	Non-depleted	Depleted	Non-Depleted
7	3	3	6	6
14	5	5	3	3
21	5	5	3	4
30	-	-	4	4
35	5	5	-	-
45	-	-	5	5

Results

Fetal Cell Suspension and Microglial Depletion

Suspensions of E14 rat and mouse brain consisted of single cells with a few scattered clumps of five cells or fewer (Fig. 3-1d). Viability stains consistently demonstrated >95% viability of whole and depleted suspensions. Lectin staining of non-depleted suspensions indicates that microglia and endothelial cells together make up no more than 1% of the total cells within the suspensions. As has been previously described (Pennell et al., 1995; Pennell and Streit, 1997a), we were able to remove approximately 98% of microglia from the depleted suspensions (Fig. 3-1c). This represents approximately one microglial or endothelial cell per 5,000 total cells within the suspensions.

Figure 3-1. Analysis of the fetal CNS suspensions. **(A)** Whole (non-depleted) E14 mouse CNS suspension, stained with GS-1-B_4-HRP. Notice the darkly stained cells (arrows), which represent donor microglia and/or endothelial cells. **(B)** Depleted E14 mouse CNS suspension, also stained with the lectin. Notice lack of stained cells. x 200 **(C)** Acridine orange/ethidium bromide viability stain of living whole E14 mouse CNS suspension, illustrating both the high degree of dissociation and the high viability. **(D)** Graphical representation of microglial depletion from both E14 rat and mouse CNS suspensions. Approximately 98% of lectin-positive cells have been removed from the depleted suspensions. Student's t-test indicates significance of this depletion with a $p<0.0001$ ($n=3$ for the mouse and 4 for the rat suspensions).

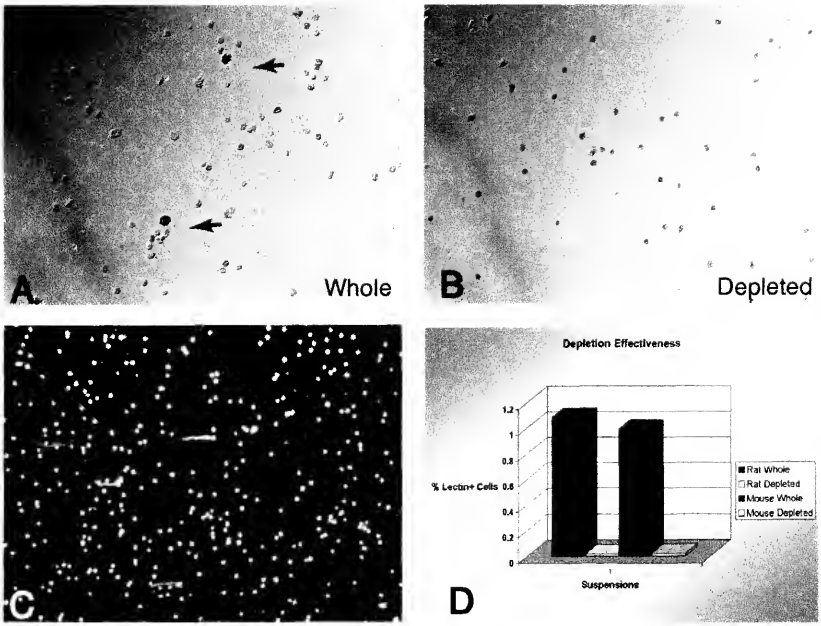

A
Whole
B
Depleted
C
Depletion Effectiveness
% Lectin+ Cells
1.2
1
0.8
0.6
0.4
0.2
0
Suspensions
Rat Whole
Rat Depleted
Mouse Whole
Mouse Depleted
D

Figure 3-1

Analysis of Intrastriatal Xenografts

By 7 days post-transplantation (*pt*), intrastriatal xenografts in all animals appeared as large areas of undifferentiated cells (Fig. 3-2a,b). Perivascular cuffing, the accumulation of mononuclear cells within the perivascular space and a hallmark of graft rejection, was evident in blood vessels within grafts of both whole and microglia-depleted tissue. There were no F4/80-positive cells within the grafts in either group, indicating a lack of donor microglial cells within the grafts. OX-6 immunostaining indicated that grafts from both groups were populated with MHC Class II-positive cells with the ramified morphology typical of activated microglia. Perivascular cells within and around the grafts were also positive for OX-6. There was little or no W3/25 immunoreactivity within the seven day grafts, although CD4-positive microglia and perivascular cells were evident throughout the rest of the brain.

By 14 days *pt* the grafts were larger and more organized, with many cells within the grafts taking on a distinctly neuronal morphology (Fig. 3-2c,d). Perivascular cuffing was more prominent than at seven days within both whole and depleted grafts, with most inflammatory cells localized to the periphery of the grafts. There were still no F4/80-positive cells within any of the grafts examined. All grafts were filled with OX-6-positive cells with both ramified and rounded morphology. W3/25 staining indicated that both CD4-positive microglia and a scattering of lymphocytes were present within all grafts by this time.

By 21 days after transplantation, the grafts were much larger than at 14 days, with small islands of apparently healthy tissue scattered among larger areas of clearly rejecting graft (Fig. 3-2e,f). Both whole and depleted grafts were filled with mononuclear cells, and

even in regions of surviving graft the neurons often appeared shrunken and unhealthy. No F4/80-positive cells were apparent in any graft at this time, and OX-6 immunoreactivity was spread evenly throughout the grafts, making identification of individual cells difficult. CD4-positive lymphocytes were present in large numbers in all grafts examined, indicating the presence of a large antigen-specific immune response.

By 35 days *pt,* there was no identifiable surviving graft tissue in any animal examined (Fig. 3-2g,h). Surrounding microglia were still OX-6 and W3/25-positive, but there were no lymphocytes remaining within the area of the grafts.

Intraspinal Allografts

At 7 days *pt,* the grafts in both groups were small and appeared relatively undifferentiated (not shown). The grafts were located at the periphery of the lesion site, often at the edges of cystic cavities formed as a result of the contusion injury. There were numerous macrophages in and around the grafts, but no signs of perivascular cuffing in the surrounding blood vessels. There were numerous OX-6-positive cells throughout the injured spinal cord, mostly activated microglial cells and macrophages, and there were numerous large, round MHC II-positive cells within the grafts. There were no donor-derived MHC II-positive cells within the whole or depleted grafts, as evidenced by F17-23-2 immunostaining. CD4-positive cells were limited to the injured spinal cord surrounding the grafts.

By 14 days *pt,* the grafts were large, filling most of the center of the lesioned spinal cords and extending rostral and caudal of the injury site (Fig. 3-3a,b). Both whole and depleted graft tissue appeared more organized and less densely cellular than at 7 days,

Figure 3-2. H&E stained sections of whole and depleted intrastriatal xenografts at 7, 14, 21, and 35 days *pt.* **(A, B)** At 7 days *pt* both whole and depleted grafts were fairly large. Notice that large blood vessels near each graft contain numerous darkly staining mononuclear cells i.e. perivascular cuffing. **(C, D)** At 14 days *pt* the grafts are larger and contain many neurons. Both whole and depleted grafts are bordered by blood vessels containing inflammatory cells, but the grafts themselves are relatively free of immune cells. **(E, F)** By 21 days *pt* all grafts in both groups are undergoing rigorous rejection, with many foci of densely packed mononuclear cells in each graft. **(G, H)** By 35 days *pt* there was no recognizable graft left in any animal examined, although the site of injection was readily visible, sometimes surrounded by pockets of inflammatory cells. Scale bar=110 μm.

Figure 3-2

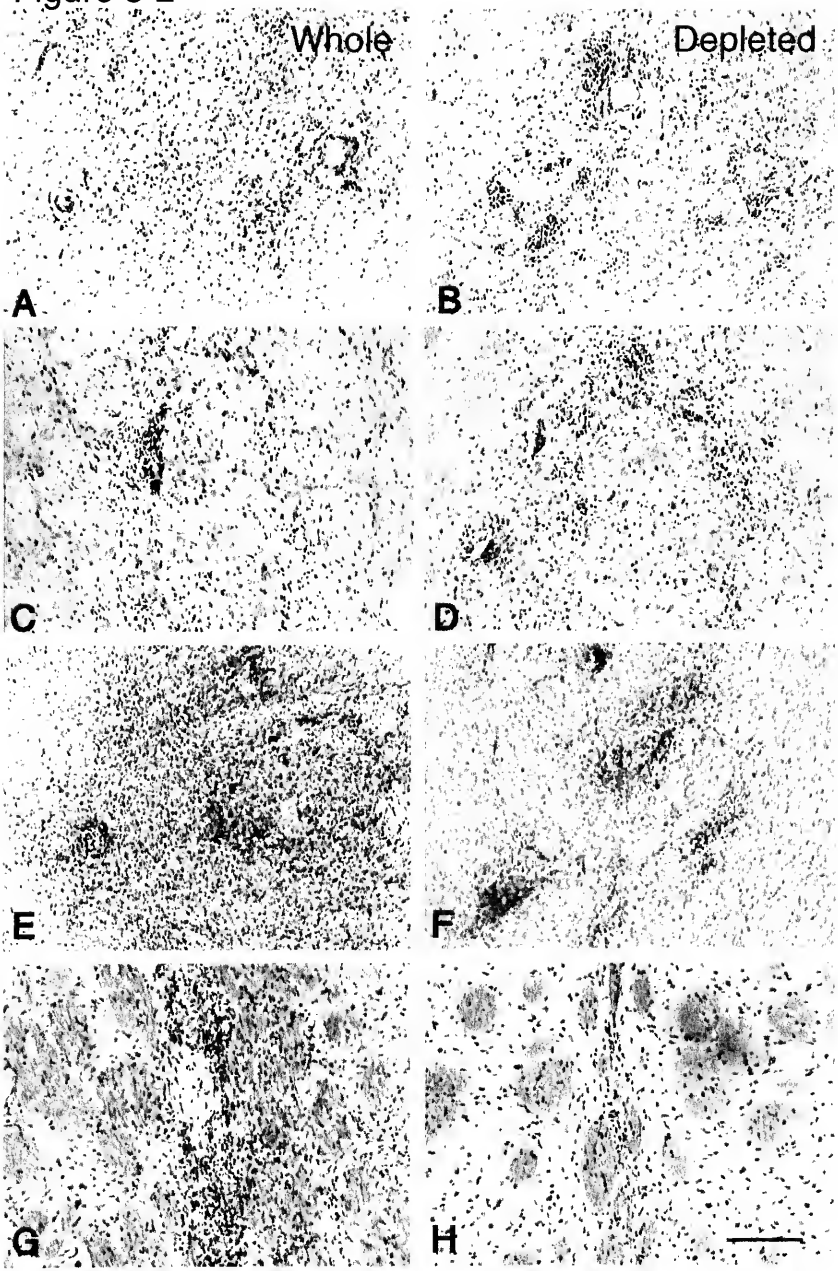


Figure 3-3. H&E stained sections of whole and depleted intraspinal allografts at 14, 21, and 30 days *pt.* **(A, B)** At 14 days *pt* all grafts in both groups (g) were large and well organized, with good integration with the host spinal cord (h). There were no signs of inflammation in any graft examined, and blood vessels were free of adherent mononuclear cells. **(C, D)** At 21 days grafts in both groups were practically identical to those at 14 days, albeit larger. Again, the grafts seemed to be surviving well with no signs of inflammation. **(E, F)** By 30 days *pt* the grafts in both groups had undergone a striking change. In several grafts in both groups there was no longer any grafted tissue evident **(E)** while in those with surviving grafts there was a robust rejection response underway **(F)**, with the remaining areas of graft inundated with inflammatory cells. Scale bar=110 μm.

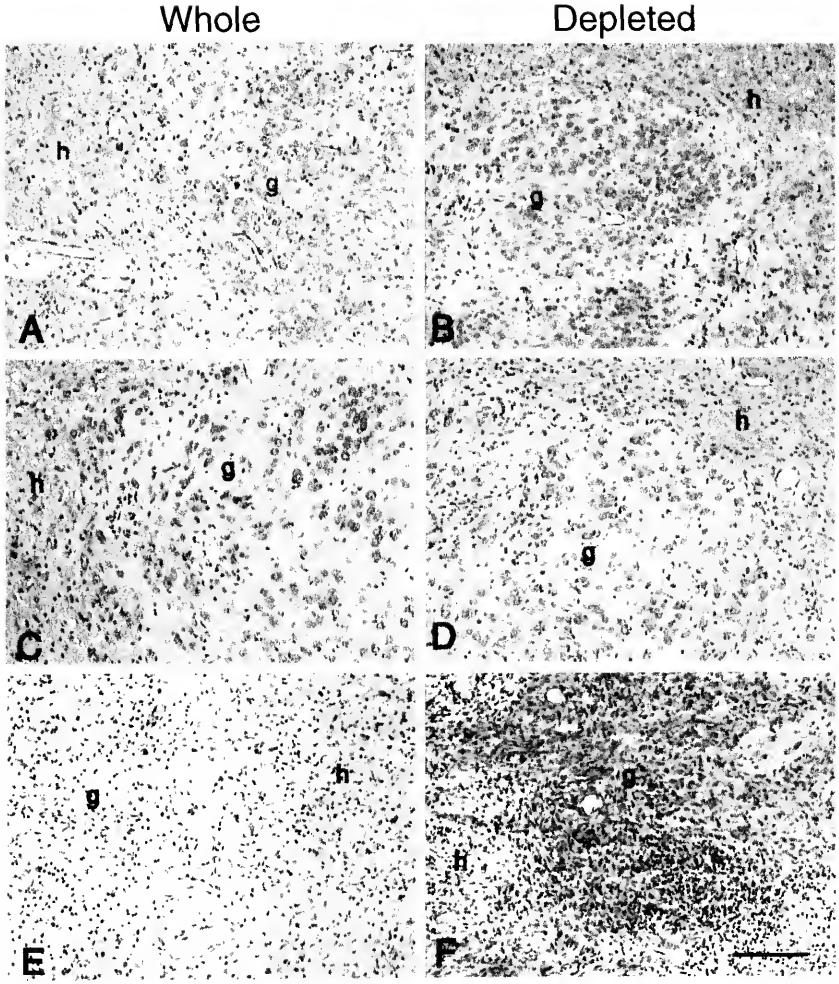


Figure 3-3

with numerous neuronal profiles. There was no sign of perivascular cuffing in any of the grafts examined in either group, although there were many macrophages within and surrounding the graft tissue. Surprisingly, there were only a few scattered OX-6-positive cells within the whole and depleted grafts at this time, although the surrounding spinal cord was filled with OX-6-positive microglia and macrophages. The first donor-specific MHC II-positive cells appeared at this time, although there were only a few round cells apparent in any graft, sometimes located in perivascular spaces. There were no differences in number or appearance of these cells between the whole and microglia-depleted grafts. W3/25 staining was still primarily limited to cells outside the grafts, with a few rounded cells within the grafts, although there was no difference in this pattern between the two groups.

By 21 days *pt,* the grafts were morphologically very similar to the 14 day grafts, although they were significantly larger (Fig. 3-3c, d, 3-4a, c). There was still no evidence of perivascular cuffing or inflammatory cell infiltrate in any graft examined in either group. Immunostaining patterns were also virtually identical to those seen at 14 days, with few MHC II or CD4-positive cells within the grafts, and a very few scattered donor-specific MHC II-positive cells (Fig. 3-4b, d). It is clear that at this time the grafts are still surviving well with no sign of immunological rejection.

At 30 days *pt,* the grafts had undergone striking change (Fig. 3-3e, f). There was no recognizable surviving graft tissue in two of the whole graft recipients and one of the depleted graft recipients, and the remaining grafts in both groups contained a dense cellular infiltrate with widespread perivascular cuffing throughout the surviving graft tissue. The surviving areas of graft were filled with OX-6-positive cells, while the

Figure 3-4. Expression of donor-derived MHC class II antigen in whole and depleted intraspinal allografts. **(A, C)** Nissl stained sections of 21 day whole (**A**) and depleted (**C**) allografts, showing large, healthy grafts free of inflammation. **(B, D)** Higher power micrographs of the approximate areas outlined in **A** and **C** in adjacent sections, stained with F17-23-2. Only a few isolated donor MHC II expressing cells are present within the grafts. **(E,G)** Nissl stained sections of 30 day whole (**E**) and depleted (**G**) allografts, showing that the grafts at this time were inundated with small, darkly stained inflammatory cells, indicative of a rejection response. **(F, H)** Higher power micrographs of the approximate areas outlined in **E** and **G** in adjacent sections, also stained with F17-23-2. Areas of graft which are undergoing rejection now contain numerous donor MHC II expressing cells, while areas of graft free of inflammation are still relatively free of donor MHC II expression (not shown). Scale bar=110 μm.

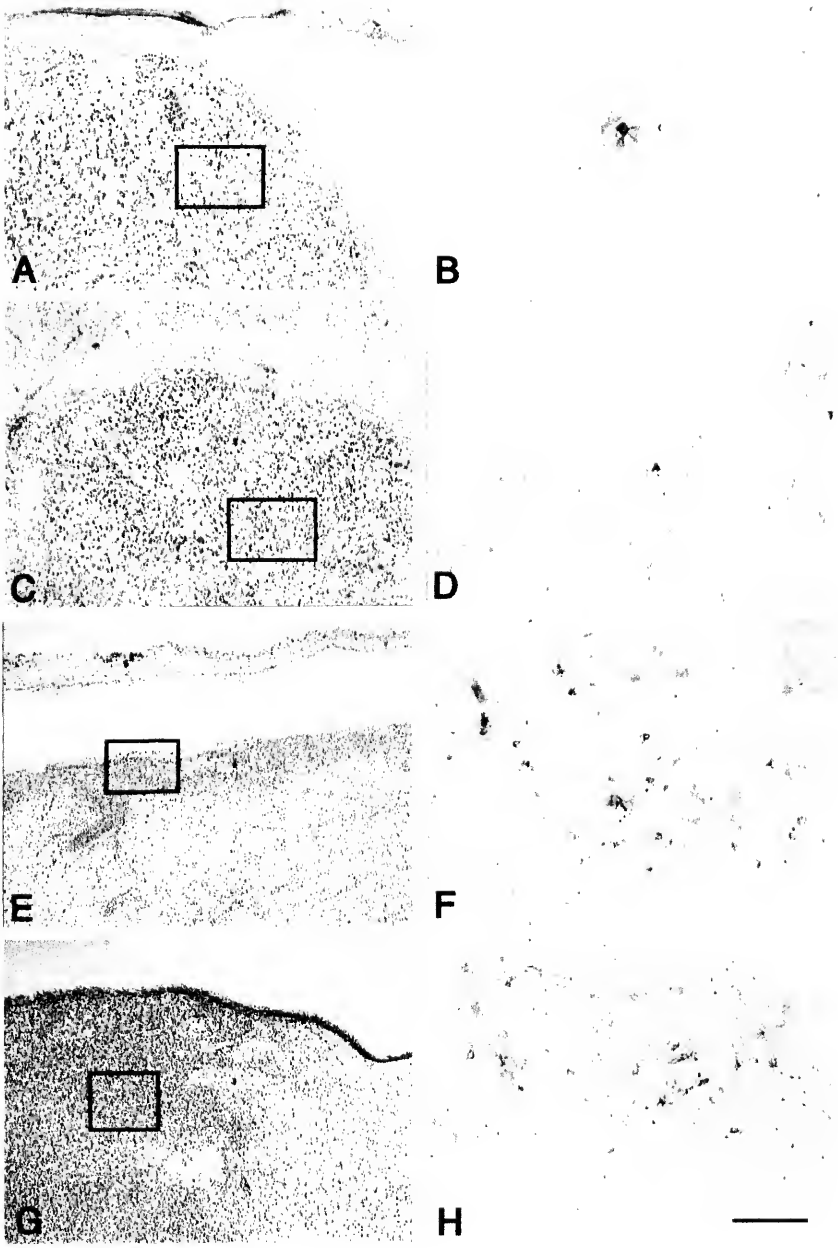


Figure 3-4

periphery of the grafts where the densest areas of mononuclear cell infiltrate were located contained numerous donor MHC II-positive cells (Fig 3-4f,h). There was no difference in the numbers or patterns of staining of donor MHC II-positive cells between whole and depleted grafts.

By 45 days *pt,* none of the whole grafted spinal cords and only one of the depleted spinal cords contained surviving graft tissue (Fig. 3-3i,j). The surviving graft was surrounded by a dense cellular infiltrate and many of the neurons within the graft appeared shrunken and unhealthy, indicating that this graft was in the final stages of rejection. All spinal cords still contained numerous OX-6 and W3/25-positive microglia and macrophages, but no CD4-positive lymphocytes were apparent in the spinal cords in which grafts had been completely rejected. There were no remaining cells expressing donor MHC II.

Antigen Presentation Assays

The results of the antigen presentation assays are presented graphically in Fig. 3-5. Donor (ACI rat)-derived splenocytes (positive control) induced proliferation of host (WF)-derived lymphocytes in a dose-specific fashion, with 4×10^4 and 4×10^5 stimulator cells inducing significant proliferation above control values ($p<0.002$ and $p<0.001$ respectively; n=4 each). Allogeneic microglia, both normal and stimulated with LPS and IFN-γ, actually seemed to suppress lymphocyte proliferation, although the suppression was not quite significant ($p<0.087$ and $p<0.097$ respectively; n=3 each). Suspensions of allogeneic E14 rat CNS tissue, whether whole or depleted of microglia, did not induce a

Figure 3-5. Graphical representation of ^{3}H-thymidine incorporation by host T-cells in response to various allogeneic stimuli, in counts per minute (CPM). Allogeneic splenocytes induced T-cell proliferation in a dose-dependent manner, with significant responses when 4 x 10^{4} and 4 x 10^{5} stimulator cells were added ($p<0.002$ and 0.001, respectively). In contrast, there was no significant proliferative response to allogeneic normal or stimulated microglia or to whole or depleted E14 CNS suspensions.

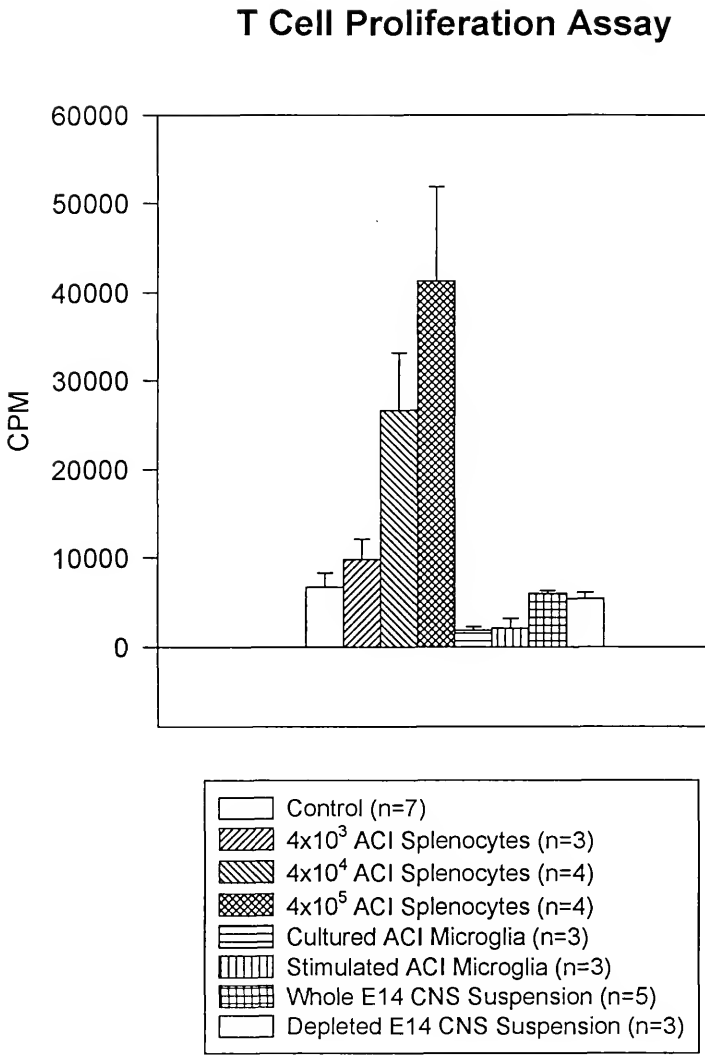

T Cell Proliferation Assay
60000
50000
40000
30000
20000
10000
0
CPM
Control (n=7)
4x10^3 ACI Splenocytes (n=3)
4x10^4 ACI Splenocytes (n=4)
4x10^5 ACI Splenocytes (n=4)
Cultured ACI Microglia (n=3)
Stimulated ACI Microglia (n=3)
Whole E14 CNS Suspension (n=5)
Depleted E14 CNS Suspension (n=3)

Figure 3-5

significant response from the responder lymphocytes (n=3 each), indicating a lack of functional antigen presenting cells within the graft tissue.

Discussion

In the present study we have attempted to elucidate the cellular components of allogeneic and xenogeneic fetal neural transplants which are responsible for the initiation of immunologic rejection. We have done this by removing those cells most likely to represent functional antigen presenting cells (APCs) within the donor tissue, i.e. microglia. We found that depletion of microglia and endothelial cells from fetal neural allo- and xenografts did not alter the pattern of rejection in any observable way, indicating that these cells are unlikely to play a significant role in the initiation of graft rejection. We also demonstrated that neither allogeneic fetal neural cell suspensions nor purified allogeneic microglia induced T-cell proliferation *in vitro*, indicating both a lack of functional APCs within the suspensions and that microglia alone do not act as APCs. Together, these findings indicate that the traditional view of the microglial cell as the resident immunocompetent cell of the CNS (Streit et al., 1988) may need to be reevaluated.

Surprisingly, we have also shown that single-cell suspensions of allogeneic fetal CNS tissue placed into the sub-acutely (7-10 day post-contusion) injured spinal cord undergo a significant delay in the onset of rejection when compared to reports of fetal spinal cord piece allografts placed into the acutely injured spinal cord, although the final outcome is unchanged. This rejection does not coincide temporally with donor MHC II expression, as has been previously shown in other allograft models (Theele et al., 1996; Lawrence et al.,

1990). Instead it occurs between one and two weeks after donor MHC II expression was first noted. This finding, along with those described in the previous paragraph, lead us to conclude that the presence of MHC-expressing cells is not the primary stimulus for allograft rejection in suspension grafts, but that other factors must be coming into play, such as indirect presentation of minor histocompatability antigens.

Xenograft Rejection

In the current study, the rejection of murine striatal xenografts followed a pattern identical to that described in previous studies (Finsen et al., 1991; Duan et al., 1995). This pattern was not altered by the removal of microglia and endothelial cells from the donor tissue prior to transplantation, for which there could be a variety of reasons. We have demonstrated that microglia and endothelial cells together make up no more than 1% of the total number of cells within the donor cell suspensions, and that we cannot detect any murine microglia within the xenografts at any time point, using species-specific markers. This duplicates results produced by Perry and Lund (1989) in which they were unable to find donor-derived microglia within murine retinae transplanted into the rat brain. A later study which followed human fetal microglia in xenografts placed into the rat brain demonstrated that these cells were few in number and that they disappeared over time (Geny et al., 1995). Taken together with the results described here, it would appear that few if any fetal microglia survive within developing xenografts, and thus their removal prior to transplantation may be unnecessary.

In addition, there is evidence that the primary stimulus for xenograft rejection is not direct presentation of foreign antigen to host T-cells by donor APCs, as is likely the case in

allograft rejection, but rather indirect presentation of minor histocompatability antigens by host APCs is the initiating event (Kaufman et al., 1995). This is supported by our observation that perivascular cuffing within striatal xenografts appears within seven days, a period of time in which there is not likely to be much graft-specific MHC expression (Takei et al., 1990). Similarly, bovine chromaffin cells transplanted into the rat CNS do not survive without immunosuppression, even after depletion of MHC expressing cells (Czech et al., 1997), further illustrating that xenogeneic cells which do not express MHC antigens are subject to rejection. Conversely, a recent study has shown that there is no immune response directed against human Schwann cell transplants placed into the adult rat brain, despite constitutive MHC II expression on the implanted cells (Hermanns et al., 1997). Taken together, these findings indicate that MHC antigens are just one among many immunogenic molecules present within xenografts, and are not themselves essential for immunologic rejection.

Allograft Rejection

The allograft model used in this study, donor ACI rats paired with recipient Wistar-Furth rats, is known to be a high responder cross. A previous spinal allograft study using these two strains demonstrated induction of donor-specific MHC II expression by 14 days *pt* which increased until it was widespread at 21 days (Theele et al., 1996). This MHC II expression coincided temporally with the onset of inflammatory cell infiltration and perivascular cuffing within the grafts as well as $CD4^+$ and $CD8^+$ T-cell infiltration, and the grafts were all destroyed by 30 days *pt.* This model clearly illustrates the three stages of rejection outlined by Lawrence and colleagues (1990): 1) the immune induction phase,

coinciding with the onset of graft MHC expression; 2) the phase of immune attack, coinciding with immune cell infiltrate and graft tissue destruction; and 3) the quiescent phase, in which inflammation recedes. It was our goal to interrupt this process at the immune induction phase, by removing the cells from the graft most likely to express MHC antigens. A similar study was performed in which MHC antigen expression was induced on allogeneic neural tissue *in vitro* by IFN-γ, following which all cells expressing MHC were removed using anti-MHC conjugated magnetic beads (Bartlett et al., 1990). Although this strategy prevented rejection of the allografts, the transplants themselves consisted only of a subset of neurons, all the glia and even some neurons having been induced to express MHC. Our donor suspensions are complete in all neuronal and macroglial elements.

We have succeeded in delaying the immune induction phase, although not in the manner we intended. Our results also demonstrate low level MHC II expression within the grafts by 14 days *pt,* but this level does not increase until between 21 and 30 days *pt.* The observation that this increase is coincident with inflammatory cell infiltration may indicate that the MHC expression is induced by the $CD4^+$ T-cells present within the graft, a likely source of pro-inflammatory cytokines such as IFN-γ. Furthermore, this delay in the onset of the phase of immune attack was independent of the removal of microglia and endothelial cells, indicating that these cells are probably not the direct cause of rejection, as was originally hypothesized. Instead, it appears that the suspension itself is the cause of the delay. Previous allograft studies have utilized either whole piece grafts which contain complete blood vessels and probably some residual meningeal tissue (Lawrence et al., 1990; Theele et al., 1996) or "slurry" suspensions which consist of large clumps and

aggregates of cells and cellular debris (Duan et al., 1995; Poltorak and Freed, 1991; Reier et al., 1992), while the present study uses only dissociated cells. This process probably results in a much lower antigenic load, filtering out the majority of vascular and meningeal elements as well as cellular debris which could contribute to the pool of alloantigen within the graft site. This may have raised the threshold of antigen necessary to initiate an immune response, or merely increased the amount of time necessary to mount a response. Interestingly, this period between 21 and 30 days is also the window of time in which new blood vessels fully mature within developing grafts (see chapters 4 and 5). It would be interesting to find out when host perivascular cells are recruited to the new vessels, providing a ready pool of non-microglial host APCs within the graft.

Another key difference between this and previous studies is the use of sub-acutely injured animals (7-10 days post-injury) as opposed to acutely injured animals (Theele et al., 1996; Theele and Reier, 1996). The sub-acute contusion model reduces the risk that the graft tissue will encounter peripheral and meningeal elements when compared to acute resection lesions, and also allows the damaged spinal cord time to repair vascular damage and reestablish the blood-brain barrier, limiting the graft's exposure to serum products and circulating lymphocytes.

Microglia as Antigen Presenting Cells

The prevailing opinion in regards to the role of the microglial cell in transplant biology, and in neuroscience in general, is that microglia represent the resident immunocompetent cells of the CNS (Streit et al., 1988; Guilian, 1995; Poltorak and Freed; 1989). This view is supported by a large body of research demonstrating *in vitro* and *in vivo*

expression of MHC I and II antigens on microglia (Suzumura et al., 1987; Matsumoto et al., 1986; Streit et al., 1989; Morioka et al., 1992; Popovitch et al., 1993; Steiniger et al., 1988) as well as their ability to present antigen to syngeneic T-cells (Frei et al., 1987). More recently, however, evidence has indicated that microglia may not be fully immunocompetent APCs after all. Ford and colleagues (1995) separated microglia from other brain-derived macrophages, i.e. perivascular cells, by flow cytometry and discovered that while perivascular cells were potent APCs, microglia were quite poor at stimulating either T-cell proliferation or IL-2 production. A recent study characterized the poor antigen-presenting ability of adult microglia as comparable to that of immature APCs from other tissues (Carson et al., 1998). Taking this idea one step farther, a recent study showed that purified microglia actually stimulate apoptosis in T-cells (Ford et al., 1996). These previous studies support the results in the current study, which demonstrate that purified allogeneic adult microglia do not function as APCs, and that fetal microglia within the cell suspensions are also incapable of antigen presentation.

Conclusions

It has been thought for some time that MHC antigens expressed by donor tissue, and by microglia in particular, have been primarily responsible for initiating graft rejection. Our results indicate that MHC expression within allo- and xenografts is probably not the primary stimulus in rejection of suspension grafts, and that donor microglia are not functional APCs and thus not responsible for immune recognition of the grafted tissue. We have also shown that spinal allograft rejection can be delayed for more than three weeks by the use of single-cell suspension grafts as opposed to the grafting of pieces of neural tissue.

These findings may have an impact on current and future clinical protocols regarding the time course of immunosuppression used in human transplants.

CHAPTER 4
COLONIZATION OF NEURAL ALLOGRAFTS BY HOST MICROGLIA: RELATIONSHIP TO NEOVASCULARIZATION

Introduction

There has been considerable interest over the last decade in the use of fetal neural tissue grafts to treat traumatic and degenerative disorders of the central nervous system (CNS) (Bjorklund, 1991; Dunnett and Richards, 1990; Gage and Buzsaki, 1989; Gash and Sladek, 1988; Reier et al., 1992). Where once neuronal loss was thought irreparable, neural transplantation has reached a point where significant recovery of function is being reported, both in animal models and in clinical studies (Cheng et al., 1996; Fiandaca, 1991; Freed et al., 1992; Freed et al., 1993; Reier et al., 1994). However, despite these successes, there are fundamental aspects of transplant biology which are not yet understood and which will need to be investigated before neural tissue transplantation can be applied clinically to the wide range of neural disorders for which it has shown potential. In the present study, we have paid particular attention to potential interactions occurring between microglial and endothelial cells, as we have hypothesized that microglia are involved in promoting graft neovascularization.

Little is known about functional roles of microglial cells in neural transplants, and the source of microglia (i.e. donor or host derived) within fetal grafts has been subject to debate (Geny et al., 1995; Perry and Lund, 1989). Most studies have focused on the

cells' capacity to act as brain macrophages and thus, as a likely source of antigen presenting cells (Duan et al., 1995; Finsen et al., 1991; Lawrence et al., 1990). More recently, the limited traditional view of microglia as a mere scavenger cell has been broadened by experimental observations demonstrating that microglia produce neurotrophic factors *in vitro* (Elkabes et al., 1996; Mallet et al., 1989; Nagata et al., 1993), and can enhance the poor regenerative capacity of the adult CNS *in vivo* (Rabchevsky and Streit, 1996). Additional support derives from experiments showing that transplants of peripheral macrophages help to overcome the inhibitory nature of the CNS towards axonal regeneration (Lazarov-Speigler et al., 1996). Although a role of macrophages in angiogenesis and neovascularization has been well described (Clark et al., 1976; Evans, 1977; Greenburg and Hunt, 1978; Mostafa et al., 1980; Polverini et al., 1977a; Polverini et al., 1977b), little attention has been paid to a possible role for microglia in transplant vascularization (for review see Horner et al., 1994).

The goal of the current investigation was to study microglia in striatal suspension grafts and to correlate their temporal appearance and morphology with the development of graft vasculature. The B_4-isolectin from *Griffonia simplicifolia* (GS I-B_4) binds specifically to microglial and endothelial cells in the developing and adult rat CNS, making it an ideal tool for visualizing both microglia and blood vessels (Ashwell et al., 1989; Peters and Goldstein, 1979; Streit, 1990; Streit and Kreutzberg, 1987). Furthermore, by depleting the grafts of donor microglia and endothelial cells prior to transplantation, we have isolated the effects of donor and host cells on neovascularization and determined the contribution of donor microglia to the overall population of microglia within the graft. Our results suggest that striatal suspension grafts are populated and vascularized entirely

by host-derived microglia and endothelial cells. We hypothesize that these invading microglia play a pivotal role in the neovascularization of the developing grafts.

Materials and Methods

Preparation of Fetal Tissue Suspension

E14 fetuses were obtained from timed-pregnant female Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.). The fetuses were delivered by Cesarean section under deep sodium pentobarbital anesthesia. Isolated fetal CNS (cortex, brainstem, and spinal cord) was stripped of meninges in a 35 mm Petri dish while immersed in 2 ml of "Solution D" (0.8% NaCl, 0.04% KCl, 0.1% Glucose, 2.2% Sucrose, 0.01% Streptomycin sulfate, 0.025% Fungizone, and 0.006% Penicillin G in dH_2O). Clean fragments were then incubated in 0.05% trypsin in "Solution D" for 20 minutes, triturated, and incubated an additional 10 min. in DNAase (5800 units/ml). An equal volume of complete media (DMEM + 10% FBS) was then added to stop the reaction and the suspension was filtered through a 130 μm Nitex filter. The filtrate was pelleted (400 x g, 10 min.), resuspended in 5 ml of complete media, and filtered through a 40 μm Nitex filter. The cells were then counted using a hemocytometer and their viability assessed using an acridine orange/ethidium bromide viability stain (live cells fluoresce green, dead cells fluoresce red). Viability was always >95%.

Depletion of Microglia and Endothelia from Fetal CNS Suspensions

GS I-B_4 was conjugated to tosylactivated Dynabeads® (Dynal, Inc.) according to the manufacturer's specifications. Optimal cell selection using Dynabeads® is obtained using a

bead:cell ratio of 40:1. Under the original assumption that microglia make up no more than 10% of the total cells in the fetal CNS suspension, $1x10^7$ cells were added to $4x10^7$ GS I-B_4-conjugated Dynabeads® in 5 ml test tubes. The tubes were then incubated at 4°C under bi-directional rotation for one hour, after which the tubes were placed in a magnetic particle concentrator which pulls bound cells to the side of the tube. The suspension was transferred to a new tube and the process repeated a second time. The freshly depleted suspension, as well as a control, undepleted (whole) suspension, was smeared on gel-subbed slides, allowed to dry, and stained with GS I B_4-HRP (Sigma L5391). By forming a ratio of GS I-B_4-positive cells in experimental suspensions and GS I-B_4-positive cells in control suspensions, we obtained a measure of depletion effectiveness.

Intrastriatal Grafting Procedure

Under ketamine/xylazine anesthesia, approximately 1 x 10^6 cells (10 µl total volume) from whole or depleted suspensions were injected stereotactically into the right striatum of adult male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) weighing approximately 250 g, using a 25 gauge Hamilton syringe. The stereotaxic coordinates were 1 mm rostral to bregma, 3 mm lateral to bregma, and 4.5 mm ventral to the dural surface. The injection was performed slowly over a period of five minutes and the needle was allowed to remain in place for several minutes afterwards to avoid reflux. Four animals were used for each timepoint (3, 7, 10, 14, 21, and 30 days), two receiving whole, non-depleted suspensions and two receiving depleted suspensions.

Tissue Processing and Lectin Histochemistry

Animals were sacrificed by transcardiac perfusion with 100 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. The brains were removed and post-fixed in 4% paraformaldehyde for at least one week prior to sectioning. 60 μm sections were cut on a vibratome and soaked in PBSC (PBS containing 0.4% Triton X-100 and 0.1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$) at 4°C for 24hrs before being incubated with GS I-B_4-HRP (10 μg/ml) in PBSC overnight at 4°C. The sections were briefly rinsed with PBS and the lectin binding sites were visualized using 3,3' -diaminobenzidine-H_2O_2. Sections were then mounted on gelatin-coated slides, allowed to air dry, dehydrated in ascending ethanols, and coverslipped with Permount.

Image Analysis

The percent area of graft covered by blood vessels was quantitated using a computerized image-analysis system and NIH Image software. The area covered by blood vessels was measured in four sections from each graft and from three noncontiguous areas of each section, and divided by the total area of graft to give a relative measure of the degree of vascularization. This method gives a better indication of overall graft vascularization than the counting of vessels, as the majority of blood vessels within the grafts are contiguous and joined by branches.

Results

Depletion of Microglia and Endothelia from Fetal CNS Suspensions

The microglial depletion procedure has been described previously (Pennell et al., 1995; Pennell and Streit, 1996). Single cell suspensions of E14 fetal CNS were obtained consistently, with a viability of 95% or higher. GS I-B_4 staining of the whole cell suspensions revealed that microglia and endothelial cells make up no more than 1% of the total cell number. Negative selection using GS I-B_4-conjugated Dynabeads® resulted in a ratio within the depleted suspensions of approximately one microglial or endothelial cell per five thousand total cells (0.02%), a depletion effectiveness of approximately 98% (Fig. 1). Student's t-test indicated statistical significance with $p<0.0001$ (n=3). The depletion procedure did not greatly affect cell viability, which remained consistently above 90% after depletion.

Morphometric Analysis of Graft Vascularization

The results from morphometric analyses of graft vascularization are summarized in Figure 3. There was no significant difference in percent area of graft covered by vessels between whole and depleted grafts at any time point. There were no vessels present at three days post-transplantation, and very few present (on average) at seven days. By ten days post-transplantation, however, the graft vascular bed covered over 8% of total graft area. Fourteen days after transplantation the blood vessels in both groups covered approximately 10% of total graft area, and by twenty-one days vascular bed coverage was at approximately 12%. There was no further increase in percent area of graft covered by vessels at thirty days post-transplantation, indicating that neovascularization was complete by twenty-one days.

Development of Microglia and Vasculature Within Striatal Suspension Grafts

Lectin staining of the grafts provided clear visualization of microglia and microvasculature (Fig. 2). Staining of microglia and vessels was stronger inside the grafts than outside, allowing clear demarcation of the graft/host border. There was no apparent difference in the number of microglial cells between the whole and depleted grafts at any time point examined (Fig. 2A-F), indicating that the relative contribution of donor-derived microglia to the overall population of microglia within the striatal suspension grafts was negligible. Similarly, there was no significant difference between the density of vasculature within whole and depleted grafts at any time point, indicating that donor endothelial cells are not essential for graft neovascularization (Fig. 3). There were no signs of immunological rejection, such as lymphocyte infiltration, at any time examined.

Little or no graft tissue was present three days after transplantation in either whole or depleted grafts. The needle track was evident, surrounded by activated microglial cells with an ameboid morphology (Fig. 5A). More ramified cells were present with increasing distance from the injection site. There was no evidence of blood vessels within the small grafts visible at this time. By seven days post-transplantation grafts were larger and evident in all animals. In both whole and depleted grafts there were numerous microglial cells, appearing hypertrophied with short, thick processes (Fig. 5B). Microglia were more numerous at the periphery of the graft, suggesting that they migrated in from the host tissue. Very few, if any, blood vessels were evident at this early time point (Fig. 4A).

By ten days the grafts were considerably larger than at seven days, and the microglial cell density within the grafts appeared decreased, leaving large areas of graft free of microglial cells (Fig. 2C, D; Fig. 4B). Most cells were still hypertrophic, similar to those

Figure 4-1. Effectiveness of depletion procedure using magnetic beads. The graph shows the percentage of GS I-B_4 -positive cells in E14 CNS cell suspensions before and after depletion. Student's t-test indicates significance at $p<0.0001$, $n=3$.

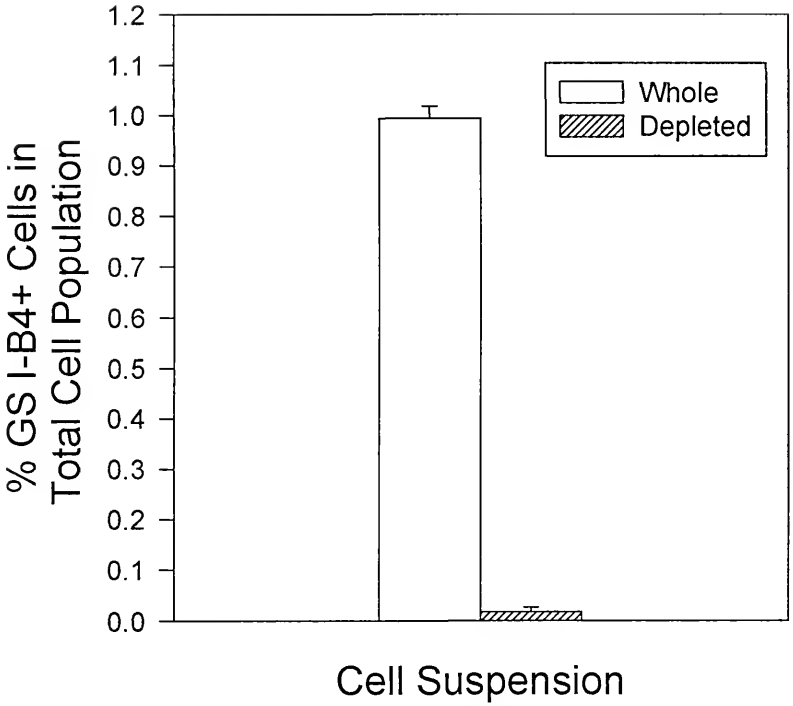

% GS I-B4+ Cells in Total Cell Population
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Whole
Depleted
Cell Suspension

Figure 4-1

seen at seven days. At this point, there was considerable vascular development, with numerous vessels growing in from the periphery of the graft (Fig. 4B). The vessels appeared very large in diameter compared to later time points, so that despite the low number of vessels they covered a large percentage of the graft. Interestingly, most microglial cells within the grafts at this time point were associated in some way with the newly formed blood vessels, either directly overlapping or wrapping their processes around them. By fourteen days post-transplantation, microglia were more numerous, maintaining their hypertrophic morphology, particularly towards the edges of the graft (Fig. 4C). Although the overall percent total area of vasculature was only slightly higher than at ten days post-transplantation, there appeared to be more branches and small diameter vessels than before. Microglia remained closely associated with blood vessels, and were frequently seen in the proximity of angiogenic sprouts from developing endothelium (Fig 5C).

By twenty-one days post-transplantation, most microglia in the graft had assumed a morphology similar to normal, resting microglia in adult rat brain (Fig. 4D). They had spread out in a characteristic fashion, each cell occupying its own distinct, non-overlapping territory. The vascularization of the grafts was complete by 21 days, and microglial cells were no longer found predominantly associated with blood capillaries, although a number continued to wrap their processes around these vessels. At thirty days after grafting there was an increase in the arborization of microglial processes (Fig. 5D), and microglia and vasculature were indistinguishable from normal adult rat brain (Fig. 4E, F).

Figure 4-2. Comparison of microglial and vascular staining in whole (**A, C, E**) and depleted **(B, D, F)** suspension grafts at 7, 10, and 21 days post-transplantation. Microglia and blood vessels are clearly visualized, and the grafts are demarcated from surrounding host tissue by their lighter background. Graft boundaries are outlined by arrows. Despite relative differences in graft size, there do not appear to be any differences in the degree of microglial colonization or extent of vascularization between whole and depleted grafts at any time point examined. GS l-B_4 -HRP; scale bar= 100μm.

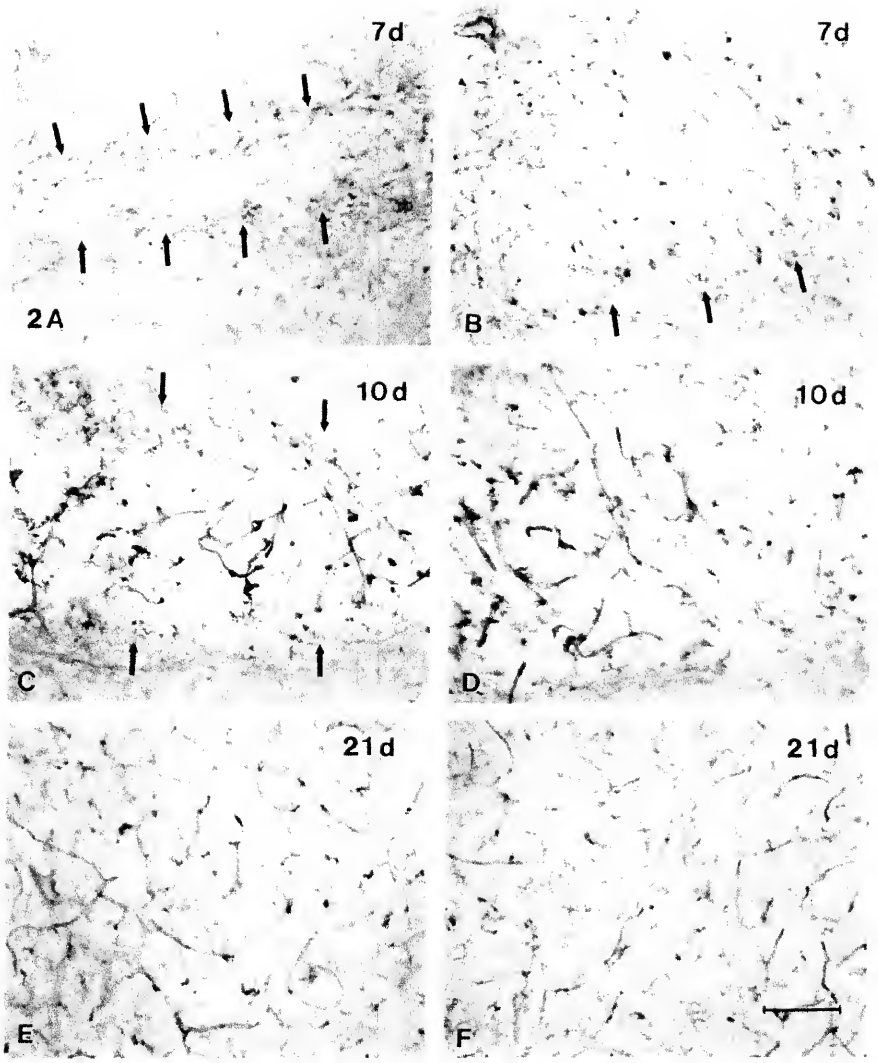


Figure 4-2

Figure 4-3. Graphical representation of graft vascularity in developing striatal suspension allografts over time. Maximum neovascular growth occurs between 7 and 10 days post-transplantation. Thereafter, little additional development takes place. Student's t-test indicates no significant difference between vascularity in grafts derived from whole or microglia-depleted CNS suspensions at any time point examined.

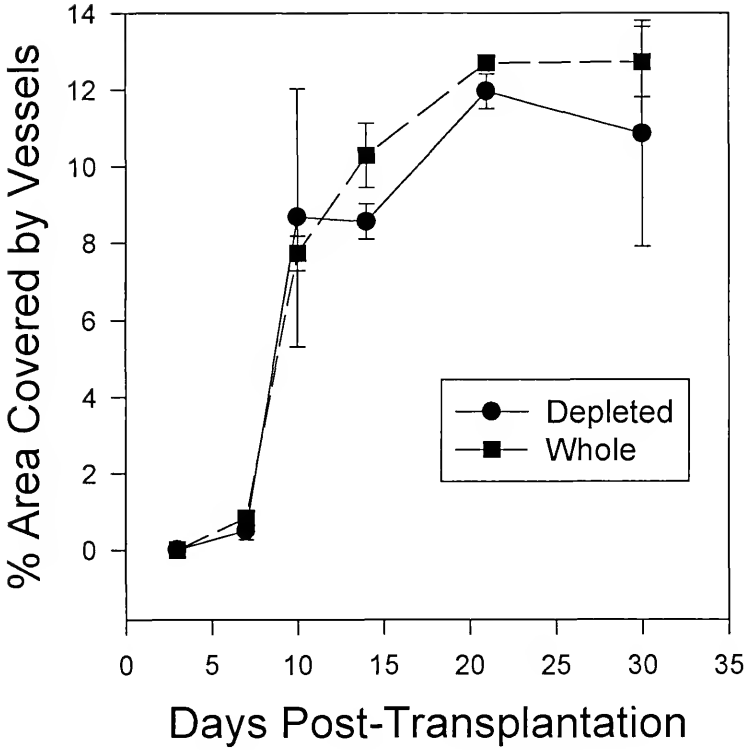

% Area Covered by Vessels
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Days Post-Transplantation
Depleted
Whole

Figure 4-3

Figure 4-4. Time course of microglial and vascular development in whole suspension allografts into the striatum. **(A)**, numerous ameboid and poorly differentiated microglial cells are present within the grafts 7 days after transplantation. There are very few blood vessels present at this time. **(B),** at 10 days there is a striking increase in vascularity within the grafts, consisting mostly of large diameter and poorly branched vessels. Most microglia are found adjacent to vessels. **(C)**, by 14 days the blood vessels are of smaller diameter and more highly branched, and microglia are more numerous. Note that areas unoccupied by microglia are also relatively free of blood vessels. **(D)**, at 21 days the graft vasculature resembles that of normal rat brain, although microglia still appear hypertrophied compared to resting cells. **(E)**, by 30 days post-transplantation, the graft microglia and vasculature are indistinguishable from that of control, normal rat brain depicted in **(F)**. GS l-B_4 -HRP; scale bar= 100μm.

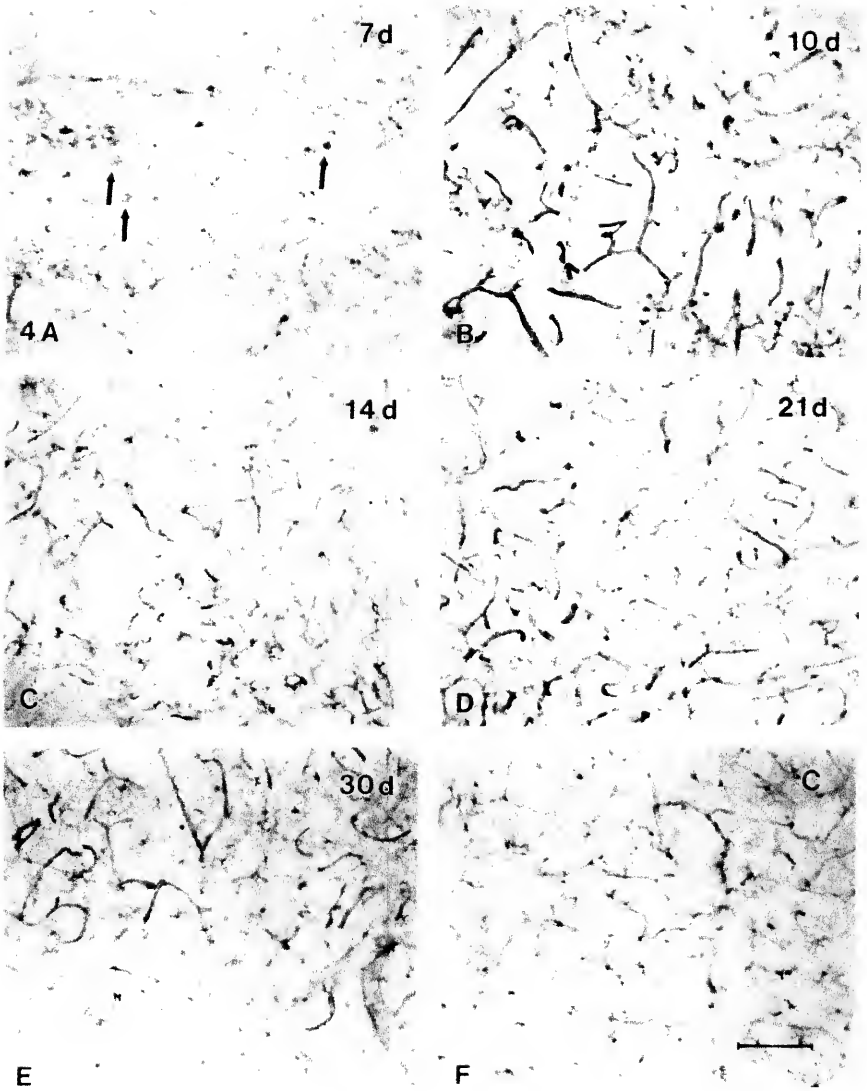


Figure 4-4

Figure 4-5. Metamorphosis of microglial cell morphology with increasing post-transplantation times. High power micrographs show GS I-B_4-stained microglia in intrastriatal suspension allografts at 3 days (**A**), 7 days (**B**), 14 days (**C**), and 30 days (**D**). **(A)**, 3 days post-transplantation microglia in and around the grafts have an ameboid morphology typical of macrophages (arrows). **(B)**, by 7 days microglia begin to put out processes (arrows), and can be seen in the vicinity of angiogenic sprouts (arrowhead). **(C)**, microglia within 14 day grafts display a more differentiated, and increasingly ramified morphology of cell processes (arrow). Most microglia are found surrounding developing blood vessels, and in this figure, angiogenic sprouts from a new vessel (arrowheads) appear to be bending towards a microglial cell. **(D)**, by 30 days microglia are fully differentiated with a small soma and long, highly branched processes. Microglial cells are no longer restricted to areas around blood vessels, although many continue to be juxtavascular (arrows), as is also seen in normal brain. Scale bar= 20μm.

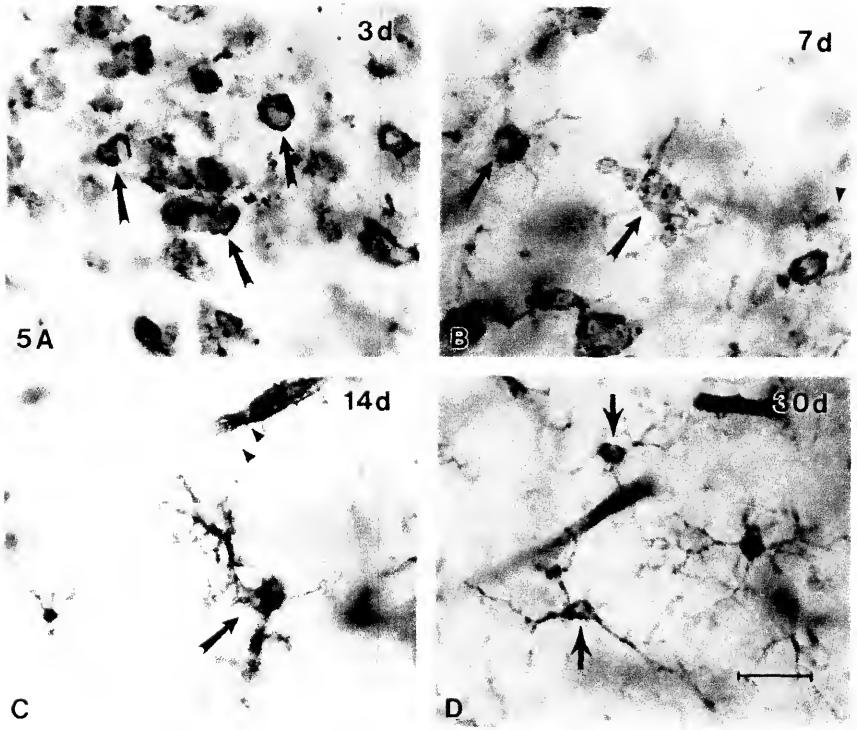


Figure 4-5

Discussion

In this study we have attempted to delineate the roles of microglial and endothelial cells in the establishment of a normal cellular microenvironment within developing fetal CNS suspension grafts. Our observations indicate that host microglia invade developing grafts at early time points, populate the grafts over a period of several weeks, and assume normal, resting morphology within the graft in a pattern indistinguishable from that of normal adult CNS parenchyma. Microglial invasion precedes the onset of neovascularization within the grafts, and the invading microglia are intimately associated with the ingrowing vessels. Most of graft neovascularization takes place between seven and ten days, after which vascular development consists of increasing ramification of larger vessels. The continuing differentiation of reactive microglial cells into resting microglia parallels the process of neovascularization within the grafts. Finally, our findings indicate that most if not all the microglia and blood vessels within the grafts are of host origin. The current study strongly suggests a role for microglial cells in the development of vasculature within fetal suspension grafts.

Microglia and Neovascularization

The idea that macrophages are involved in wound healing and inflammatory angiogenesis is not a new one. Leibovitch and Ross discovered in 1975 that macrophages were necessary for undisturbed wound repair, while other groups showed both a temporal correlation between endothelial DNA synthesis and mononuclear cell infiltration (Polverini et al., 1977a). Furthermore, macrophages isolated from wounds induced neovascularization *in vitro* and in *in vivo* assays (Clark et al., 1976; Greenburg and Hunt,

1978). It was soon discovered that macrophage conditioned medium alone promoted vascular proliferation (Polverini et al., 1977a), implicating some diffusable substance. Macrophages have also been implicated in tumor angiogenesis. Mice depleted of monocytes showed decreased tumor vascularization (Evans, 1977), and neoplastic tissue explants demonstrate angiogenic properties only when macrophages are present (Mostafa et al., 1980). It is known that activated macrophages produce a host of angiogenic cytokines, among them basic fibroblast growth factor (bFGF), tumor necrosis factor - α (TNF-α), transforming growth factor - β (TGF-β), interleukin-1 (IL-1) (Sunderkötter et al., 1994). In tissue culture, microglia have also been demonstrated to produce bFGF (Shimojo et al., 1991), TNF-α (Leibovitch and Ross, 1975), TGF-β (Leigh et al., 1994), and IL-1 (Lindholm et al., 1987).

It is reasonable to assume that when grafts reach a certain size and level of differentiation in the absence of a ready blood supply, they are placed in metabolic stress. This process has been hypothesized to be a primary stimulus for the onset of neovascularization (Horner et al., 1994). Microglial cells are uniquely sensitive to neuronal stress, and microglial activation is among the first indications of brain pathology, even when it does not result in neuronal death (Streit et al., 1988). We have demonstrated that activated microglia are present within developing grafts prior to the onset of neovascularization, and that most of this vascularization occurs between seven and ten days, a period in which the grafts undergo tremendous growth. It is possible that this period of growth places the developing graft in oxidative and metabolic stress, which the surrounding microglia react to by the secretion of pro-angiogenic cytokines. The

progressive differentiation of reactive microglia into resting microglia parallels the development of vasculature within the graft, with the completion of neovascularization corresponding to the return of all microglia to a resting state. This differentiation may reflect the lessening of metabolic stress within the graft, which would remove the stimulus for microglial activation and down-regulate the production of angiogenic cytokines. Of course, the current evidence is only suggestive of such a role for microglia in neovascularization. Future studies will test this hypothesis using *in situ* hybridization to identify the cellular source of the cytokines in question.

The Origin of Microglia and Vasculature Within Suspension Grafts

The origin of microglia and vasculature within fetal CNS grafts has been addressed in the past by several groups. Perry and Lund (1989) reported that host microglia invade solid retinal xenografts placed into the brains of neonatal rats, and concluded that they make up most if not all of the resident microglia in the transplants. Another study (Geny et al., 1995), using suspension xenografts into the adult rat brain, determined that the resident population of microglia within developing grafts is a chimera of host and donor cells, but that donor-derived cells are few and do not proliferate. Our findings support these studies by demonstrating that microglia make up a very minor percentage of E14 CNS cell suspensions and by showing that the removal of these cells does not affect the population of suspension grafts by host microglia. This finding makes sense intuitively, because although there are microglia within the developing CNS as early as E12 (Ashwell et al., 1989), the majority of microglial development within the rat CNS takes place in the last prenatal and first postnatal weeks. ameboid microglia from "pools" located throughout the developing CNS migrate into the brain and give rise to the

adult microglial population (Hurley and Streit, 1996). We have shown previously (Pennell et al., 1995) that E14 murine CNS cell suspensions maintained in culture for one week do not contain a greater percentage of microglia than do fresh suspensions, indicating the lack of proliferative potential of these early microglia and, along with the current study, demonstrating a lack of putative microglial "progenitor cells" within the transplanted tissue (Neuhaus and Fedoroff, 1994).

While it is known that the vasculature within solid CNS grafts is a chimera of anastomosing donor and host vessels (Broadwell et al., 1990; Takei et al., 1990), it has been hypothesized that the blood vessels within developing suspension grafts are entirely of host origin (Broadwell et al., 1990; Leigh et al., 1994). However, Geny and colleagues (1995) have demonstrated that the vessels within suspension xenografts also represent a chimera consisting of both donor and host endothelial cells, opening the question of whether or not donor endothelial cells are required for graft neovascularization. By depleting the grafts of donor endothelia, the present study has demonstrated that donor endothelial cells are unnecessary for proper suspension graft vascularization.

Conclusions

In every organ of the body, macrophages play a crucial part is tissue reconstruction and healing following injury, and it is becoming clear that microglial cells play the same role within CNS. Although these cells are thought by some to exacerbate injury through production of harmful substances such as nitric oxide, oxygen free radicals, and glutamate (Boje and Arora, 1992; Colton and Gilbert, 1993; Piani et al.,

1991), more and more studies are finding evidence that activated microglia produce neurotrophins and other growth factors. This study strongly supports the view that activated microglia are associated with the construction of a normal vascular environment within suspension grafts, and over time give rise to a resting microglial "network" similar to that present within the normal CNS. However, it is difficult to attribute a definitive role for microglia in vascular development based on morphological study alone, and further investigation will be required to determine the molecular mechanisms of vascular development within fetal suspension grafts.

CHAPTER 5
GLIAL AND VASCULAR DEVELOPMENT WITHIN INTRASPINAL SYNGRAFTS: PRODUCTION OF ANGIOGENIC CYTOKINES

Introduction

Transplantation of fetal neural tissue as a treatment for spinal cord injury has been under investigation for many years (for review see Reier et al., 1992). The potential for functional recovery has been sufficiently impressive in animal models (Reier et al., 1994; Anderson et al., 1995) that initial pilot studies using human fetal tissue are underway to treat patients with syringomyelia (Falci et al., 1997; D.K. Anderson, personal communication). However, there are many fundamental aspects of fetal transplantation that remain to be addressed. To date, the vast majority of published work regarding intraspinal transplantation has addressed the neuronal component within the grafts, with numerous studies of neural connectivity, neurite ingrowth and outgrowth, and functional assessment (Reier et al., 1992; Anderson et al., 1995). Investigations into functional roles of glial cells in intraspinal transplants have focused primarily on their roles in rejection in the case of microglia (Theele and Reier, 1996; Theele et al., 1996), and on their possible roles as barriers to graft/host connectivity in the case of astrocytes (Reier et al., 1983; Reier et al., 1988). This is unusual, considering the recent attention which has been paid to possible beneficial effects of purified astroglial (Wang et al., 1995; Bradbury et al., 1995; Pierret et al., 1998) and microglial (Rabchevsky and Streit, 1996; Lazarov-Speigler

et al., 1996; Prewitt et al., 1997; Franzen et al., 1998) cells placed into the brain and injured spinal cord. In addition, there has been a considerable amount of attention paid to the development of vasculature in intracerebral transplants (Lawrence et al., 1984 ; Krum and Rosenstein, 1988; Takei et al., 1990; Rostataing-Rigattiera et al., 1997; Pennell and Streit, 1997). These studies have addressed issues from metabolic support of transplant growth to vascular involvement in graft rejection. However, while there has been some work done on the development of a blood-spinal cord barrier (Horner et al., 1996a) and on the vascular density of mature transplants (Horner et al., 1996b), nothing is known about the time course or inductive mechanisms of early intraspinal transplant vascular neogenesis.

In the current study, we have focused on the relationship between glial cells (microglia and astrocytes) and developing vasculature within intraspinal syngrafts during the first month following transplantation. We have also attempted to address possible inductive mechanisms of neovascularization by showing the temporal expression of three cytokines known or suspected to play important roles in angiogenesis: vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-beta (TGF-β). Together these elements will provide a fundamental picture of the development of non-neuronal elements within intraspinal fetal transplants.

Materials and Methods

Surgery and Transplantation

Single-cell suspensions of E14 rat CNS tissue were prepared as described in Chapter 3. Briefly, whole CNS (telencephelon, brainstem, and spinal cord) were removed from E14 Lewis rat fetuses, the meninges were stripped, the clean fragments were triturated and digested with Trypsin and DNAase, and the resulting suspensions were filtered through successively smaller diameter filters to obtain single-cell suspensions with a viability >95%. Suspensions were counted and maintained at 4°C until transplantation.

Spinal surgeries were performed as described in Chapter 3. Female Lewis rats weighing approximately 200 g received lower thoracic (T11) spinal cord injuries using the NYU weight drop device, from a height of 25 cm. One week later, the lesions were exposed and approximately 1 x 10^6 cells (15 μl total volume) from the donor suspension was injected into each injury site using a 25 gauge Hamilton syringe. A total of twenty-five animals received transplants.

Tissue Processing

Under deep pentobarbital anesthesia, five animals each were sacrificed at 7, 10, 14, 21, and 30 days post-transplantation by transcardiac perfusion of 0.9% saline followed by 200 ml of Bouin's fixative (72.5% picric acid, 22.5% formalin, 5% glacial acetic acid). The spinal cords were removed and post-fixed in Bouin's fixative for 2 hours

before being embedded in paraffin for sectioning. Sections were cut on a microtome at a thickness of 12 μm and mounted on Fisher Superfrost Plus™ slides. Slides were baked at 65°C overnight and allowed to cool before being processed. Sections were deparaffinized in xylene for 10 min., rehydrated in descending ethanols, and rinsed twice in PBS. Antigen retrieval was performed by microwaving the slides in 10 mM citrate buffer (pH 6.0) for 10 min. and allowing the slides to cool to room temp. Sections were blocked for 2 hours at 4°C in PBS + 1% BSA + 1% normal goat serum. Primary antibodies were diluted in the blocking solution.

Mouse anti-bFGF (Upstate Biotechnology, Inc.), rabbit anti-VEGF (Santa Cruz Biotechnology, Inc.), rabbit anti-TGF-β (R&D Systems), and *Griffonia simplicifolia* B_4-isolectin-peroxidase (GS-1- B_4-HRP; Sigma) were used at 1:100 dilution, while mouse anti-GFAP (Sigma) was used at 1:500. The TGF-β antibody is pan-specific and recognizes TGF-β1, 2, and 3, while the VEGF antibody recognizes all four splice variants of VEGF. GS-1- B_4-HRP was used to visualize microglia/macrophages and blood vessels. Sections were incubated in primary antibodies and lectins at 4°C overnight. Controls in which primary antibodies were omitted were performed in every case. Sections were rinsed three times in PBS and incubated in either biotinylated goat anti-mouse (bFGF, GFAP) or goat anti-rabbit (TGF-β, VEGF) secondary antibody at a dilution of 1:400 for 2 hours at room temp. The sections were then rinsed three times in PBS and incubated in 1:400 avidin D-HRP (Vector) for 45 min. at room temp. Sections were again rinsed three times in PBS and the peroxidase visualized using DAB (GS-1- B_4-HRP treated sections

were visualized immediately after the lectin incubation). Some sections from each animal were stained with hematoxolin/eosin (H&E) to show general morphology.

Results

Microglia, Astrocytes, and Vasculature

By seven days post-transplantation (pt) all spinal cords had recognizable grafts when stained with H&E, although the size varied considerably from animal to animal. The grafts generally consisted of small islands of undifferentiated cells suspended within and along the edges of large cystic cavities in the lesioned spinal cord. GS-1- B_4 allows microglia/macrophages and blood vessels to be visualized in the same sections, and typically stains developing tissue, including transplants, much more strongly than adult tissue (Ashwell et al., 1991; Pennell and Streit, 1997). In every case the grafts were surrounded by numerous macrophages, as evidenced by lectin staining, and contained microglia in various stages of ramification (Fig. 5-1a). These microglia were few in number and differed from adult ramified microglia in that they were larger and had fewer processes. There were few blood vessels evident at this early time, and those present were of large diameter and extended into the grafts from the adjoining areas of host spinal cord. At this time the grafts were almost entirely free of GFAP staining, except where blood vessels were present (Fig. 5-2a). Astrocytic processes (Fig. 5-3a) always surrounded developing blood vessels.

By 10 days *pt,* the grafts were generally larger than at seven days, although there was still considerable variability. The grafts were still surrounded by macrophages, but also contained many macrophages, possibly engulfed by the growing transplants as the

small islands joined to form larger areas of graft (Fig. 5-1b). Microglia within the grafts were still larger and less ramified than resting adult microglia, and were frequently found near developing blood vessels (Fig. 5-3b). The grafts contained numerous large diameter blood vessels at this time, most still appearing to extend from the periphery of the graft (Fig. 5-1b). GFAP staining was still almost exclusively limited to the outlines of blood vessels, although there were a few isolated astrocytes that appeared to be occupying areas free of vasculature (Fig. 5-2b).

By two weeks *pt* the grafts were uniformly much larger than at ten days, frequently filling the entire center of the spinal cord and eliminating the cystic cavities evident at earlier time points. H&E stained sections showed that the grafts now contained many cells that were phenotypically neuronal, whereas earlier grafts consisted of small, undifferentiated neuro-ectodermal cells (data not shown). The grafts continued to be surrounded and populated by macrophages, and contained numerous microglia, some of which were smaller and more ramified than those seen at seven and ten days (Fig. 5-1c). Blood vessels at this time were much more numerous than at ten days, were spread evenly throughout the grafts, and appeared to be of smaller diameter than those at earlier time points. At this time the grafts contained a great many astrocytes, which were no longer associated exclusively with blood vessels (Fig. 5-2c). The astrocytes were smaller and less darkly stained than those in the surrounding host tissue.

By three weeks *pt* all grafts examined had filled the lesion cavity, smoothly integrating with the host spinal cord tissue. There were significantly fewer macrophages surrounding the grafts at this time, and very few were contained within the graft. In contrast, there were many more microglial cells within the grafts at this time, most of

which resembled normal adult ramified microglia with small soma and long, branching processes (Fig. 5-1d). The grafts were filled with evenly distributed blood vessels, but the vessels were of much smaller diameter than at earlier timepoints, and appeared more numerous (Fig. 5-1d). GFAP staining at three weeks *pt* revealed that the grafts were filled with astrocytes with a density greater than that at 14 days *pt,* but still less than that of the surrounding host tissue (Fig. 5-2d). These cells were generally smaller than those in the surrounding tissue, although the astrocytes immediately adjacent to the graft were hypertrophied, probably due to the contusion injury, at every time point examined.

At thirty days *pt* the grafts were of a comparable size to those at twenty-one days. As has been previously reported (Pennell and Streit, 1997), GS-1- B_4 staining of microglia and blood vessels was much lighter at this time point than at earlier times, probably due to downregulation of glycoproteins associated with the maturation of the graft. The microglia within the graft had differentiated further, with most of them being indistinguishable from normal resting adult microglia (Fig. 5-1e). The density and diameter of blood vessels at 30 days *pt* appeared to be relatively unchanged from 21 days. GFAP staining showed that astrocytes within the graft were morphologically indistinguishable from and approximately as numerous as the astrocytes in the surrounding host tissue (Fig. 5-2e). The graft/host interface was difficult to discern from the astrocytic staining, indicating minimal scarring and good integration of the transplant with the host spinal cord.

Cytokine Immunostaining

At seven days *pt,* bFGF staining was absent in all grafts (Fig. 5-4a), although positive staining was evident around blood vessels in the surrounding host spinal cord.

Figure 5-1. Development of microglia and blood vessels within intraspinal syngrafts. **(A)** 7 day *pt* islands of graft are surrounded by numerous macrophages. The islands contain many semi-ramified microglia but few blood vessels (arrow). **(B)** 10 days *pt* the grafts contain many macrophages and microglia. Notice the significant increase in blood vessel number at this time. **(C)** By 14 days *pt* the blood vessels are more numerous and of smaller diameter. The number of microglia within the grafts has also increased. **(D)** At 21 days *pt* the grafts are larger and the blood vessels consist primarily of small capillaries. The density of microglia is comparable to that in the surrounding host spinal cord. **(E)** By 30 days *pt,* the staining is much fainter, and although the blood vessels seem even smaller than at 21 days, the number of vessels and microglia seems mostly unchanged. GS-1-B_4-HRP. Scale bar=110 μm.

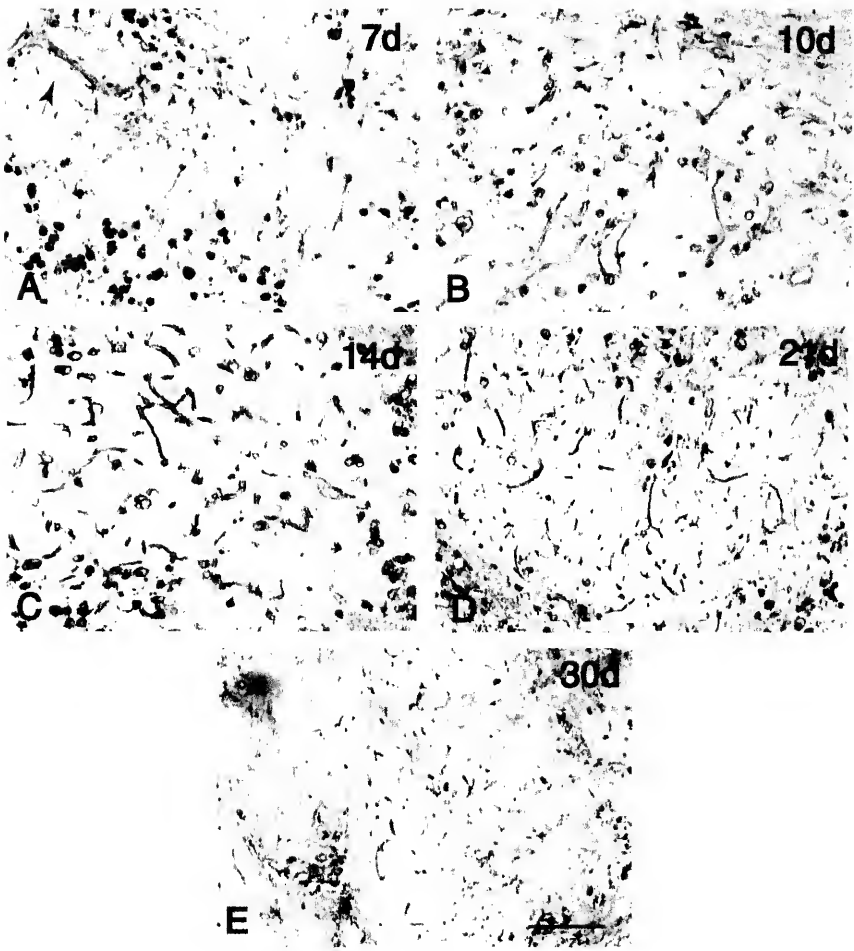


Figure 5-1

Figure 5-2. The development of astrocytes within intraspinal syngrafts. **(A)** 7 days *pt* the grafts are mostly free of astrocytes, with astrocytic processes only present surrounding blood vessels (arrows). **(B)** At 10 days *pt* the staining pattern is the same as at 7 days, allowing for increased number of blood vessels at this time point. **(C)** By 14 days *pt* astrocytes are no longer just surrounding blood vessels, but have spread out to fill the entire graft, although at much lower density than in the surrounding host tissue. **(D)** At 21 days *pt* astrocytes have increased in number. **(E)** By 30 days *pt,* the astrocytic density is comparable to the surrounding host tissue, the cells having increased in size and number over those at 21 days. GFAP. Scale bar=110 μm.

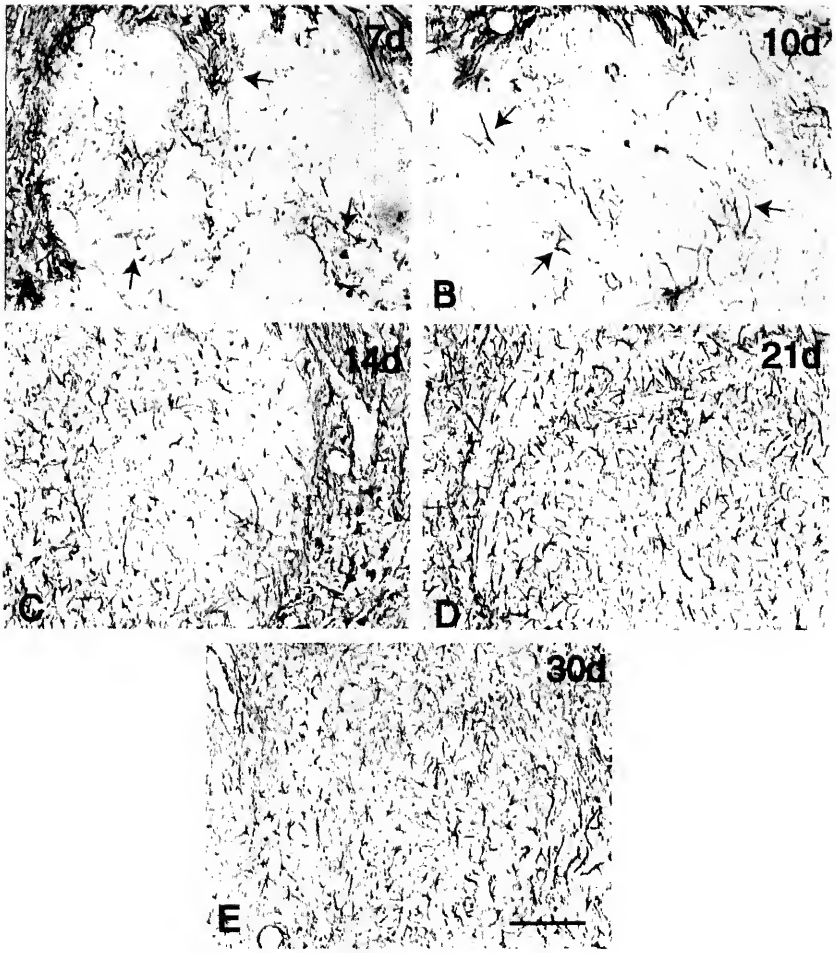


Figure 5-2

Figure 5-3. Morphological relationship between astrocytes, microglia, and developing blood vessels. **(A)** At 10 days *pt* new blood vessels are covered with astrocytic processes (arrows), leaving the non-vascularized areas of graft bare of GFAP staining. **(B)** At 14 days *pt* microglia (arrowhead) and macrophages (arrow) are frequently seen in close apposition to ingrowing vessels. GS-1-B_4-HRP. Scale bar=46 μm.

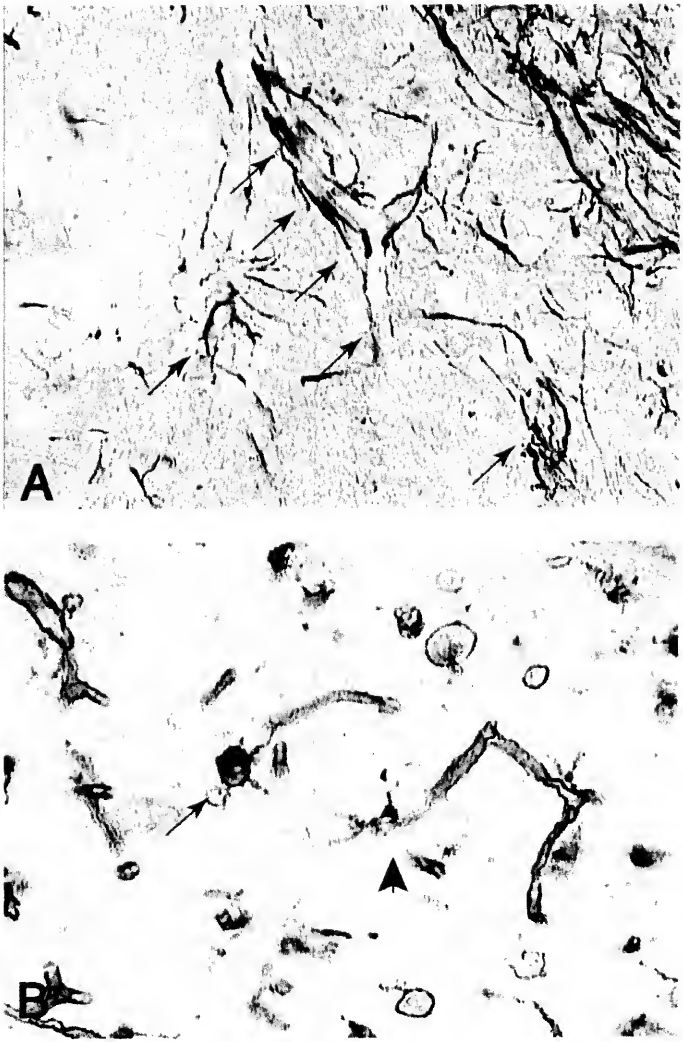


Figure 5-3

Figure 5-4. The expression of basic fibroblast growth factor (bFGF) in developing intraspinal syngrafts. **(A, B, C)** At 7, 10, and 14 days *pt,* there is little or no expression of bFGF in the grafts. **(D, E)** By 21 days *pt* and continuing at 30 days *pt,* however, there is widespread expression of bFGF on neurons. Scale bar=110 μm **(F)** Higher power micrograph shows expression of bFGF on neurons and astrocytes (arrows) at 30 days *pt.* Scale bar=27.5 μm.

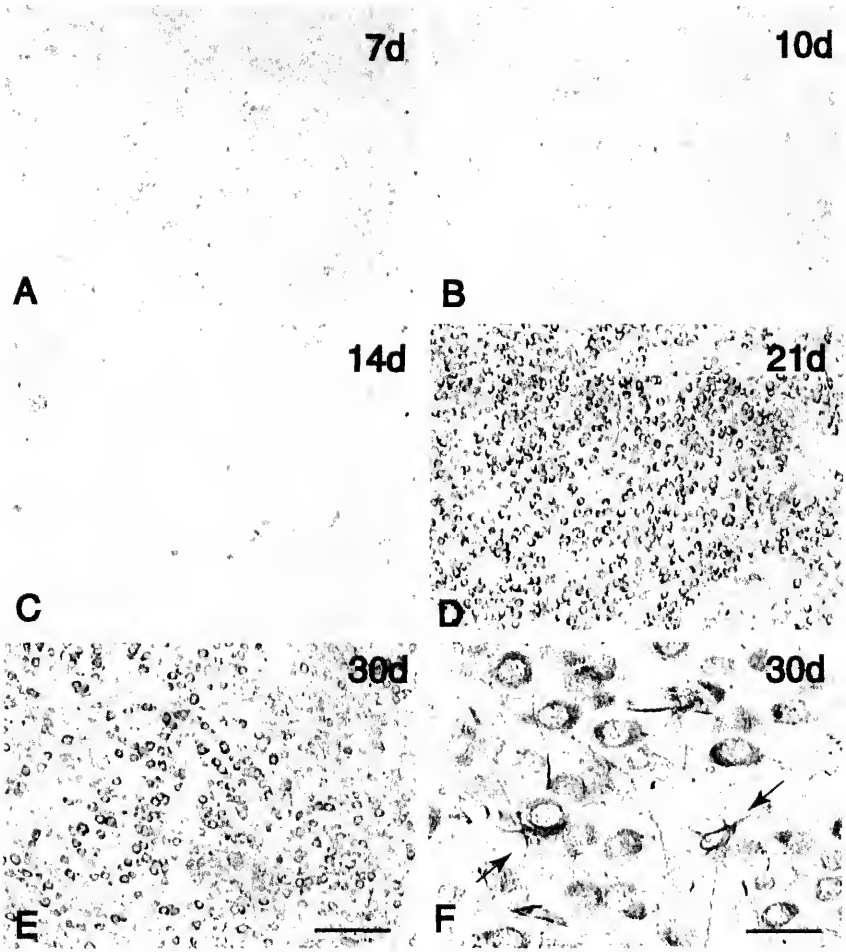


Figure 5-4

Figure 5-5. Expression of transforming growth factor beta (TGF-β) in developing intraspinal syngrafts. **(A)** TGF-β immunoreactivity is limited to blood vessels in and around 7 day *pt* grafts (arrows). **(B)** At 10 days *pt* the staining pattern is the same as at 7 days. **(C)** At 14 days *pt* the grafts are free of TGF-β immunoreactivity. **(D, E)** By 21 and 30 days *pt,* the grafted neurons all appear to be expressing TGF-β. Scale bar=110 μm.

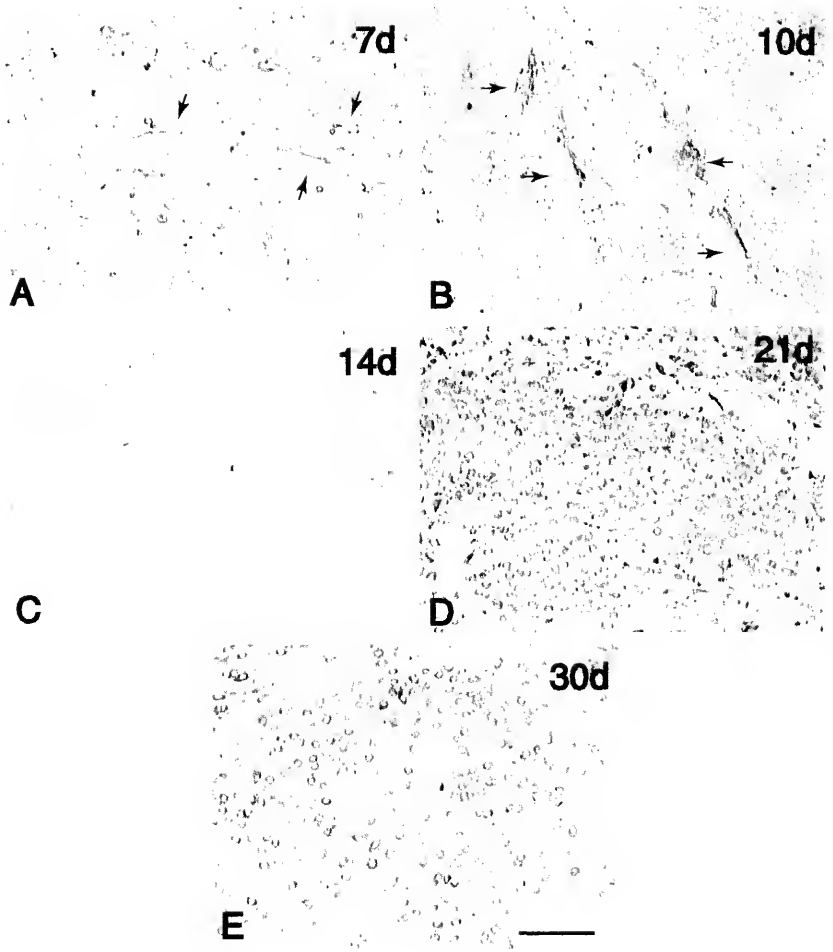


Figure 5-5

Figure 5-6. Expression of vascular endothelial growth factor (VEGF) in developing intraspinal syngrafts. **(A)** At 7 days *pt,* virtually every cell in and around the graft (g) is expressing VEGF, including the grafted cells, macrophages (arrow), and astrocytes (arrowhead). **(B)** At 10 days *pt,* the graft (g) is still expressing high levels of VEGF, so much so that cellular identification is difficult. **(C, D)** At 14 and 21 days *pt,* VEGF expression appears to be limited to neurons within the graft. **(E)** By 30 days *pt,* VEGF expression appears to be decreasing, although it is still limited to grafted neurons. Scale bar=110 μm.

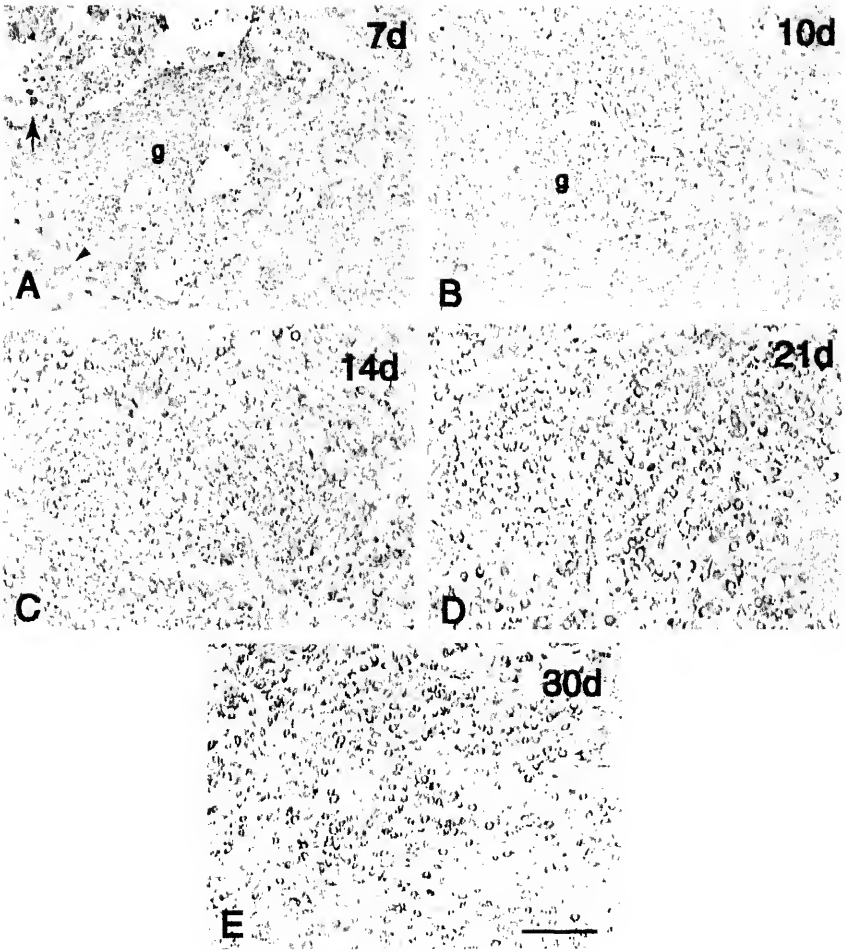


Figure 5-6

TGF-β immunoreactivity was restricted to blood vessels in the graft and in the surrounding lesion cavity (Fig. 5-5a), with widespread staining of neurons and astrocytes, but not blood vessels, in the surrounding host spinal cord. In contrast, virtually every cell in the graft and lesion area was immunoreactive for VEGF, including most if not all grafted cells, macrophages and astrocytes within and surrounding the grafts, and astrocytes throughout the host spinal cord (Fig. 5-6a).

At ten days *pt,* the patterns of bFGF and TGF-β were identical to those at seven days, with no staining and staining of new blood vessels, respectively (Figs. 5-4b, 5-5b). It is interesting to note that the patterns of TGF-β and GFAP immunoreactivity overlap at this time (Figs. 5-5b, 5-2b). Likewise, VEGF staining was very similar to that at seven days (Fig. 5-6b), with the graft staining so darkly in some cases that individual cells were difficult to pick out.

At fourteen days *pt* there was no bFGF staining apparent in four out of five transplants (Fig. 5-4c), with weak staining of neurons in the remaining graft. There was no apparent immunoreactivity for TGF-β within the grafts (Fig. 5-5c), although staining of astrocytes in the surrounding host tissue served as a positive control for the efficacy of the antibody. VEGF staining, however, was still abundant, and could now be localized to the cytoplasm of neurons within the graft (Fig. 5-6c), as well as to astrocytes immediately adjacent to the graft within the host spinal cord.

By 21 days *pt* the staining patterns had undergone a striking change. There was strong neuronal bFGF immunoreactivity in every graft examined (Fig. 5-4d). In almost

identical patterns, there was also strong TGF-β staining in neurons throughout every graft examined (Fig. 5-5d), as well as in some blood vessels. There was still strong VEGF expression (Fig 5-6d). Staining for all three cytokines remained high at 30 days *pt* (Figs. 5-4e, 5-5e, 5-6e), and was still limited to neurons within the grafts with the exception of bFGF, in which immunostaining was also localized to astrocytes (Fig. 5-4f).

Discussion

In the present study we have followed the development of glial and vascular elements within developing intraspinal syngrafts during the first month post-transplantation (*pt*), and attempted to show the cellular sources of three major cytokines/growth factors known to be involved in angiogenesis, i.e. bFGF, TGF-β, and VEGF. We have shown that neovascularization begins at approximately 7 days *pt,* is well underway at 10 days *pt,* and continues throughout the first month with progressive differentiation of vessels from large diameter to smaller diameter. Microglial infiltration of developing grafts precedes vessel ingrowth, and microglia are frequently associated with immature vessels. Astrocytes are almost exclusively found associated with blood vessels for the first ten days *pt,* after which they expand out to populate the entire graft by one month *pt.* bFGF immunoreactivity is absent within the grafts until 21 days *pt,* after which all grafted neurons and a subset of astrocytes express it. TGF-β is expressed only on blood vessels for the first two weeks *pt,* after which it is expressed by grafted neurons. VEGF, one of the most potent known angiogenic factors, is highly expressed by the grafted cells as early as 7 days *pt,* along with host macrophages and astrocytes, and

continues to be expressed by graft neurons throughout the first month *pt.* Together these findings present an interesting picture of intraspinal syngraft development, from a different viewpoint (non-neuronal) than has been previously addressed.

Glial and Vascular Development

The early migration of microglia into developing syngrafts is very similar to that which has been described in early intrastriatal allografts (Pennell and Streit, 1997). It has been fairly well established at this point that these cells are derived from host microglia/macrophages and are not primarily graft-derived cells (Perry and Lund, 1989; Geny et al., 1995; Pennell and Streit, 1997). It is interesting to note that the microglia within the graft begin to differentiate into ramified cells as early as one week *pt,* despite being suspended within a lesion cavity and being surrounded by numerous macrophages, a likely source of pro-inflammatory cytokines. It has been shown that phenotypic brain macrophages can differentiate into ramified microglia *in vivo* (Pennell and Streit, 1998), indicating that the large pool of macrophages within the lesion cavity is the likely source of the ramified microglia within the grafts at later time points.

The development of astrocytes within the grafts is also very interesting. As has been previously shown in other transplant models (Lawrence et al., 1984; Conner and Bernstein, 1987), the only astrocytic presence within the grafts during the first ten days is associated with ingrowing vessels, which are covered by astrocytic processes. Lawrence and colleagues (1984) have shown an association between streamers of new basal lamina from developing vessels and astrocytic processes at the ultrastructural level, and suggest that the astrocytes play a part in directing basal lamina assembly. Whatever their role is,

however, the absence of astrocytes with the grafts prior to blood vessel ingrowth suggests that this interaction is induced by the vasculature itself and not the other way around. The striking increase in astrocytic density within the grafts between ten days *pt,* when there is little GFAP staining, and fourteen days *pt,* where there is widespread GFAP immunoreactivity, suggests that immature precursor cells within the graft are induced to differentiate into astrocytes at this time. This makes sense since the second week *pt* corresponds temporally with the first postnatal week of development, the primary period of astrocytic differentiation in the CNS (Skoff, 1980). These early astroglia are smaller and less darkly stained than mature astrocytes, and they gradually increase in number and size until at one month *pt* they are comparable in size and density to those in the surrounding host spinal cord. Conner and Bernstein (1987) reported that graft astrocyte numbers were increased relative to the host spinal cord, but we must remember that in our case the surrounding spinal cord is injured, which probably accounts for the increased number and size of the host glia.

It has been hypothesized that transplants placed into the injured spinal cord, rather than having to rely solely on the inductive faculties of the grafted tissue, would be vascularized earlier than those placed into the uninjured CNS due to the presence of diverse angiogenic factors within the lesion cavity associated with the injury itself (Horner et al., 1994). This is supported somewhat by our data, considering the presence of some large diameter vessels seen growing in from the periphery of the grafts at one week (Fig. 5-1a). This is in contrast to previous studies in uninjured animals in which one week grafts were almost entirely free of blood vessels (Pennell and Streit, 1997; Rostataing-Rigattiera et al., 1997). The differences were far from striking, however, as

the one week grafts were still relatively free of blood vessels. In addition, the continued development of vasculature within the intraspinal grafts paralleled that previously shown in other suspension graft models (Pennell and Streit, 1997; Rostataing-Rigattiera et al., 1997), indicating that the influence of the lesion environment was minimal at best. Instead, it is probably the metabolic needs of the developing neurons within the rapidly expanding grafts which is the primary inductive stimulus, as has been hypothesized in other models (Horner et al., 1994; Pennell and Streit, 1997). Overall, it would appear that the development of glial and vascular elements within intraspinal syngrafts is not significantly different from said development in suspension grafts placed into other, uninjured areas of the CNS.

Angiogenic and Angiostatic Cytokine Production

Basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) are all cytokines/growth factors that have been shown to have potent effects regarding angiogenesis (Beck and D'Amore, 1997). It has been hypothesized that astrocytes and microglia play a role in the induction of neovascularization during graft development (Lawrence et al., 1984; Horner et al., 1994; Pennell and Streit, 1997). Thus, these particular cytokines were selected for this study because they are known to be produced by astrocytes (Ballabriga et al., 1997; Flanders et al., 1998; Stone et al., 1995) and microglia/macrophages (Shimojo et al., 1991; Lindholm et al., 1990; Sunderkötter et al., 1994).

We have shown that bFGF is not expressed within the grafts until at least two to three weeks after transplantation. This makes it unlikely to be involved in at least the

initial stages of neovascularization, although there is continued differentiation of large vessels into smaller capillaries at this time, so a role in vascular development cannot be ruled out. bFGF is potently angiogenic in *in vitro* and *in vivo* models of angiogenesis, with effects on endothelial cell proliferation, migration, and differentiation (Pötgens et al., 1995), but it's role in actual angiogenesis *in vivo* is disputed (Horner et al., 1994; Beck and D'Amore, 1997). Because bFGF also has lots of effects not related to angiogenesis, such as causing proliferation of astrocytes, smooth muscle cells, and of course fibroblasts (Pötgens et al., 1995), the upregulation of bFGF between 14 and 21 days *pt* may partially account for the increase in the number of astrocytes within the grafts. Interestingly, bFGF has been shown to induce the differentiation of neurons (Vlodavsky et al., 1991) and to greatly increase the size of neural transplants (Giacobini et al., 1991), so it is possible that it serves as a neurotrophin within the intraspinal grafts.

The expression of TGF-β within the developing grafts is quite illuminating. It is expressed only in new vessels in and around the early (7 and 10 day) transplants, in a pattern strikingly similar to the GFAP staining pattern within the grafts at these times. It is known that TGF-β is both produced by astrocytes (Flanders et al., 1998) and has significant effects upon them. TGF-β inhibits the growth of astrocytes *in vitro* (Flanders et al., 1993) and, more importantly, induces the deposition of extracellular matrix (ECM) by astrocytes, a crucial step in angiogenesis (Baghdessarian et al., 1993; Flanders et al., 1993). These findings correspond well with Lawrence and colleagues (1984) study which showed astroglial processes intimately associated with newly formed vascular basal lamina in early cortical transplants. TGF-β, in contrast to bFGF and VEGF, actually

inhibits endothelial cell proliferation, and is thought to be involved in vascular differentiation (Beck and D'Amore, 1997). This would make sense considering that the onset of TGF-β expression by grafted neurons (21 days pt) corresponds approximately with the end of neovascularization within the grafts.

Last but not least, we found that VEGF expression was widespread within the grafts at every time point examined. VEGF is generally considered the most important angiogenic cytokine, both because of its effects on endothelial cell proliferation, migration, and differentiation, and because of the specificity of its actions for vascular endothelial cells in particular (Ferrara et al., 1992; Pötgens et al., 1995; Beck and D'Amore, 1997). It is known that VEGF production by astrocytes is crucial to angiogenesis within the CNS following brain injury (Papavassiliou et al., 1997) and to vasculogenesis within the developing retina (Stone et al., 1995). VEGF production by macrophages is also very important to wound healing-related angiogenesis (Sunderkötter et al., 1994), another consideration in our grafts placed into a lesioned environment. At early time points (7 and 10 days pt) there was expression of VEGF by macrophages and astrocytes in addition to the grafted cell themselves, suggesting a combination of inflammatory and normal developmentally derived VEGF. VEGF expression after that, however, was limited to neurons within the graft and astrocytes and neurons in the surrounding host spinal cord. It is known that VEGF is expressed at high levels during development and then maintained at low levels even in the adult CNS (Breier et al., 1992), so the continued expression of VEGF by the grafts even after the apparent end of angiogenesis should not be surprising.

What is surprising is the relative lack of support for the hypothesis that glial cells are key components in graft neovascularization. Although astrocytes may be playing a role in the deposition and assembly of ECM associated with endothelial cell migration, it remains to be shown conclusively. Furthermore, while macrophages and astrocytes surrounding the grafts do express high levels of VEGF at early time points, this expression is not reflected by glial cells within the grafts and is probably more associated with the wound healing response of the injured spinal cord than it is induced by the graft. Intraspinal graft neovascularization proceeds more or less along the same time scale as in grafts not placed into lesioned environments, downplaying the significance of inflammatory angiogenesis. Instead, angiogenic and angiomodulatory cytokine production by the grafted neurons themselves appears to play the largest role in vascular neogenesis. This does not, however, rule out important roles for microglia and astrocytes in new blood vessel growth. For example, there are a number of directly and indirectly angiogenic molecules potentially produced by these cells which have not been examined, such as platelet derived growth factor (PDGF), macrophage colony stimulating factor (M-CSF), interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and countless others. Microglia have been shown to produce ECM components which both promote and inhibit angiogenesis, such as laminin and thrombospondin (Masuda-Nakagawa et al., 1993; Chamak et al., 1994). Microglia have also been shown to produce constituents of the plasminogen activator pathway (Nakajima et al., 1992) which are responsible for both for activation of latent TGF-β (Schultz-Cherry and Murphy-Ulrich, 1993) and for cleavage of membrane and matrix-bound bFGF and VEGF into soluble forms (Pötgens et al., 1995). So we see that there are still a number of

potential explanations for the intriguing morphological association between astrocytes, microglia, and developing blood vessels within neural transplants. Elucidation of these mechanisms, however, must wait for future studies.

CHAPTER 6
CONCLUSIONS

The research in this dissertation consists primarily of three separate projects which are loosely related in that they all have to do with both microglial cells and transplantation. Chapter 2 described a method for the reliable tracing of cultured microglial cells after injection into the adult rat brain. Chapter 3 attempted to test the hypothesis that microglia are responsible for graft rejection. Finally, in chapters 4 and 5, the relationship between microglia and developing graft vasculature was investigated. In this final chapter I shall attempt to summarize what was learned from these projects, propose new directions for research, and point out the common threads which tie these seemingly disparate ideas together.

The Tracing of Transplanted Microglia

There has been a great deal of recent interest in the transplantation of purified microglia/macrophages into the injured CNS (Rabchevsky and Streit, 1996; Lazarov-Speigler et al., 1996; Prewitt et al., 1997; Franzen et al., 1998). However, these studies all share the common problem of being unable to reliably trace the injected cells and differentiate them from the endogenous host microglia/macrophages. In chapter 2, Fluoro-Gold (FG) prelabeled microglia were traced for up to two weeks after injection into the

adult rat corpus callosum and lateral ventricle. They remained brightly labeled in this time, with little or no background labeling of endogenous cells. Some of the injected cells differentiated from the macrophage phenotype typical of cultured microglia into ramified cells morphologically indistinguishable from resting *in vivo* microglia. These results indicate that this method would be useful for the reliable tracing of transplanted microglia/macrophages in future studies.

Furthermore, control injections of free FG resulted in widespread labeling of neurons and perivascular cells within the injected hemisphere, but no labeling of endogenous microglia. This would indicate that resting microglia, in contrast to phagocytic microglia, are not actively pino- or phagocytic. Thus microglia represent a population of facultative macrophages within the CNS, while the role of constitutive phagocytes goes to the perivascular cells. This concept will be addressed again later in this discussion.

Single-cell Suspension Grafts, Microglial Depletion, and Rejection

Using a xenograft model , mouse to rat, and what we thought was a well characterized model of allograft rejection in the spinal cord, the ACI to Wistar-Furth cross (Theele and Reier, 1996), we attempted to test the hypothesis that microglia induce immunologic rejection by removing the microglia from the grafts prior to transplantation. Approximately 98% of microglia and endothelial cells, the primary sources of major histocompatability complex (MHC) antigens present within the prenatal CNS, were removed from single-cell suspensions of E14 mouse and ACI rat CNS prior to injection

into the intact striatum or sub-acutely injured spinal cord of Wistar-Furth rats. In the xenografted animals, all the grafts were rejected by 35 days regardless of the presence or absence of microglia. This finding was consistent with what was known about xenograft rejection, and the rejection itself followed a well defined pattern. In the allografted animals, however, we found out that our "well-characterized model" was not so well-characterized after all. We expected the non-depleted grafts to begin rejecting at about 10-14 days post-transplantation (*pt*), at the time of onset of donor MHC II expression. Instead, none of the allografts in either group showed signs of rejection in the first three weeks *pt.* After that, at 30 days *pt,* ALL of the grafts were rejecting, and were all destroyed by 45 days *pt.* What was going on here?

The answer is that we had created a new model of intraspinal transplantation. No one had previously investigated single-cell suspension grafts placed into the sub-acutely injured spinal cord, and it was unreasonable to expect graft/host interactions to be the same as they were in other models. Donor MHC II expression began at the same time as in previous models, at about 14 days *pt,* but in this case it did not precipitate an immune response from the host. The suspension grafts contained very few (~1%) microglia and endothelial cells even in the non-depleted grafts, and thus the suspensions were almost purely neuroectodermally derived. The whole-piece and slurry suspensions from former studies almost certainly contained a greater amount of vascular and menigial elements, as well as larger amounts of cellular debris that could contribute to the antigenic load of the graft. Thus, even without depleting the microglia, we had removed the vast majority of MHC expressing cells from the grafts prior to injection. In essence, we had succeeded in our goal to interrupt the immune induction stage of graft rejection by eliminating the

direct pathway of antigen presentation (see chapter 1). The question that remains, however, is why did the grafts reject at all? And why so consistently between 21 and 30 days pt? Obviously the lack of variability in this window of time indicates a discrete event taking place. However, explanations at this point remain speculative until further studies are done.

Although the direct presentation pathway is thought to represent the primary inductive mechanism for allograft rejection (VanBuskirk et al., 1994), it is clear that indirect presentation of foreign antigen can be sufficient in and of itself to induce an immune response (Steinmuller, 1983; LaRosa and Talmage, 1985). In essence, this means that donor-derived antigen presenting cells (APCs) are not always necessary to induce allograft rejection. Instead, APCs from the host can migrate into the graft, process donor (foreign) antigen, and present it to host T-cells. It had been generally thought that the microglial cell represents the CNS's endogenous APC (Frei et al., 1987; Streit et al., 1989). We know that host microglia are present within the graft as early as 7 days *pt,* presumably providing a large pool of host APCs within the graft. Why then do they not induce rejection through the indirect pathway until four weeks *pt*? The answer may be that microglia do not function as APCs at all, or at least not very effectively. We have shown that cultured microglia, even after activation with IFN-γ and LPS, do not stimulate allogeneic T-cell proliferation. This confirms previous studies which indicate that microglia are not functional APCs (Ford et al., 1995; Carson, 1998), and that it is instead the perivascular cells, i. e. macrophages within the vascular wall, that actually represent the functional APCs of the adult CNS. This, I believe, is the missing piece of the puzzle. The next logical

question is, when during the development of vasculature within grafts do host perivascular cells appear? I would hypothesize that they appear between 21 and 30 days *pt,* at the time of the onset of rejection. This would be easy to determine, using the ED-2 antibody to selectively stain perivascular cells within developing grafts, and represents perhaps the most important follow-up study to the current work. If a temporal correlation were established between perivascular cell appearance and rejection, mixed lymphocyte reactions could be carried out using FACS-sorted perivascular cells to see if they can present alloantigen to T-cells. This ability could then be compared to that of microglia. In addition, to definitively establish whether or not MHC antigens are crucial to graft rejection, transplants should be carried out in animals differing *only* at the MHC I and/or II loci, and not at both major and minor histocompatability loci, such as in the animals used in this study.

The significance of this research is twofold. First, this new model of transplantation should have an impact on our understanding of the need to immunosuppress allograft recipients. There would appear to be no real need to administer immunosuppression during the first two to three weeks after transplantation, during the patient's most vulnerable period to post-surgical infections. If immune recognition occurs between three and four weeks *pt,* this would then be the best period to target for immunosuppressive therapies. Although, since human tissue is vascularized so much slower than is rat tissue, human transplants may not be at risk of rejection for even longer periods of time. While rat tissue is completely vascularized by one month *pt,* human graft neovascularization is barely beginning by that time (Geny et al., 1994).

The second major significant addition that this research provides is in the area of microglial immunocompetence (see chapter 1 for review). We showed in chapter 2 that

brain macrophages are induced to differentiate into ramified microglia which are not actively phagocytic. We observed in chapter 3 that microglia likely do not act as functional APCs. These findings are in agreement with the recent literature that describes microglia as immature immune cells (Carson, 1998), which can actually induce apoptosis in T-cells rather than activate them (Ford et al., 1996). In light of these studies, we must conclude that microglia are not the immunocompetent cells of the CNS. That honor falls to perivascular cells, which have been conclusively shown to be functional APCs (Ford et al., 1995).

Graft Neovascularization

The vascular networks within neural transplants are vitally important both to graft survival and to immunologic rejection. In chapters 4 and 5 we followed the development of microglia and blood vessels within developing striatal and intraspinal grafts. In both cases, microglia infiltrated the grafts prior to the onset of neovascularization and were intimately associated with ingrowing vessels during the early time points *pt.* Blood vessels began to form between seven and ten days *pt* and increased in number while decreasing in diameter until completing their development by approximately 30 days *pt.* Microglial differentiation from macrophage-like morphology into ramified, adult microglia took approximately the same amount of time. This temporal correlation between microglial and vascular development led to the hypothesis that microglia play an important role in graft vascular neogenesis.

This morphological correlation in mind, we hypothesized that microglia, and possibly astrocytes, produced angiogenic factors such as the cytokines bFGF, TGF-β, and

VEGF to regulate neovascularization. TGF-β and bFGF are produced by both types of glial cells, while VEGF is known to be produced by astrocytes and peripheral macrophages. All three are generally considered to be among the primary players in angiogenesis. Thus, we examined the expression of these cytokines by immunohistochemistry during the first month *pt* in intraspinal syngrafts. What we found, however, was that bFGF was only expressed after 21 days *pt* and primarily in neurons. Its role in graft vascular development, if any, remains unclear. TGF-β was expressed by ingrowing blood vessels at early time points and then by neurons at later timepoints, perhaps as a differentiation factor. The early expression may have been a signal to surrounding astrocytes to aid in extracellular matrix (ECM) deposition, although this is only speculation. Finally, VEGF expression was widespread at every time point examined, both in the host spinal cord and in the grafted neurons. These patterns of expression would indicate that the grafted neurons are the primary inducers and regulators of neovascularization, although other roles for glial cells are not ruled out. They may be involved in ECM degradation and/or deposition, for instance. Microglia in particular are known to produce matrix metalloproteases such as MMP2 (Yamada et al., 1995) and can secrete ECM components such as laminin and thrombospondin (Masuda-Nakagawa et al., 1993; Chamak et al., 1994). Glial cells may also produce angiogenic factors other than those examined in this study, such as platelet derived growth factor (PDGF), macrophage colony stimulating factor (M-CSF), interleukin-1 (IL-1), and tumor necrosis factor-alpha (TNF-α).

These studies have been valuable first steps into the understanding of glial and vascular development in neural transplants. It will be a long time before every element

involved in graft development is understood, and our investigations have raised more questions than they have answered. For example, what signals induce the migration of microglia into developing grafts, and what induces their differentiation? What is the nature of the interaction between astrocytes and blood vessels in early graft development? What factors, if any, do astrocytes and microglia manufacture that influence angiogenesis? If microglia do not influence angiogenesis, do they play a neurotrophic role in supporting transplant growth, as has been hypothesized (Prewitt et al., 1997)? We can test these questions either by examining the expression of various molecules through immunohistochemistry or *in situ* hybridization, or by using animals in which the molecules in question have been knocked-out or inhibited. Differential display RT-PCR or subtractive hybridization could be used to identify mRNA species upregulated during early angiogenesis, which could be cloned and identified. Microglia can also be inhibited *in vivo* using microglia/macrophage inhibitory factor (MIF; Thanos and Mey, 1995). Experiments could be performed in which grafts are treated with MIF to examine effects on angiogenesis and microglial infiltration.

The studies in this dissertation have just begun to investigate potential functional roles for microglia in neural tissue transplantation. The fact that most of our hypotheses were either not supported or outright disproven by our results should not be dismaying, for these are really just first steps into a world of research previously dominated by neuronal and behavioral studies. General, descriptive studies must be performed before more specific hypotheses can be made and tested, and we did manage to carve some new ground. A new model of neural transplantation was developed and characterized, light was cast on some long misunderstood ideas about microglial immunocompetence, and some interesting

correlations can now be made between graft vascularization and immunologic rejection (see Table 6-1). These investigations should be thought of as pilot studies, starting points for many future studies. Besides, if there is anything that this author has learned over the last five years, it is that no question in science is ever really answered.

Table 6-1. Temporal relationship between graft rejection and vascular development.

Time (days) PT	*Xenograft Rejection*	**Piece Allograft Rejection*	*Suspension Allograft Rejection*	*Suspension Allograft Vascularization*
7	Onset of inflammation, perivascular cuffing	Graft healthy, no donor MHC expression	Graft healthy, no donor MHC expression	Migration of host microglia into graft; little or no vascularization at this point
10		Onset of donor-MHC II expression; first signs of inflammation		Increased numbers of large diameter blood vessels
14	Widespread perivascular cuffing	Approximately the same as 10 days	Onset of donor-MHC II expression (low levels); No signs of inflammation	Increased vascular density; Differentiation of large vessels into smaller ones begins
21	Phase of immune attack well underway	Phase of immune attack well underway	Maintained low levels of donor MHC II expression; still no signs of inflammation	Vascular density near its maximum; **Still no host perivascular cells within the grafts
30		Grafts mostly destroyed by this time	High levels of donor MHC II; phase of immune attack well underway	Graft vasculature mature; ψPerivascular cells present?
45	Graft destroyed		Grafts destroyed	

* From the literature

**Remains to be shown conclusively

ψ Still speculation

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BIOGRAPHICAL SKETCH

Nathan Adam Pennell was born on March 21, 1971, in Latrobe, Pennsylvania. An only child, Nathan was raised there by his mother and his grandparents until the age of seven when his mother and he moved to St. Petersburg, Florida. There he spent the next seven years growing up. In 1985 Nathan and his mother moved to Plantation, Florida, where Nathan attended high school. There the formerly chubby and bespectacled boy joined the junior varsity football team, lost weight, received his first pair of contact lenses, and decided the world had to call him "Nate" now. Four years later Nate was accepted to the University of North Carolina at Chapel Hill, a place that seemed like Heaven on Earth to a poor South Florida boy, and proceeded to have the time of his life. At UNC Nate was a varsity sabre fencer, making it all the way to the NCAA Championships his senior year. Nate was also a biology major, and discovered an interest in research while working as an undergraduate in Dr. Ken Lohmann's lab. In this lab Nate developed an intense dislike for all things behavioral.

In the fateful summer of 1992 Nate was working at the C.V. Whitney Marine Laboratory in Marineland, Florida. Here he was deciding that he hated anything to do with biochemistry, and was wondering if science was the way to go (although beach volleyball made up for a lot). Fortunately, Nate made a trip to the UF campus to meet with various departmental heads in the graduate school, and discovered his calling while

reading the Department of Neuroscience brochure. Nate applied, was accepted, and thus began his graduate career with no idea what he wanted to research.

After a couple of uneventful rotations, Nate heard a seminar dealing with graft rejection and was hooked. He approached Dr. Wolfgang "Jake" Streit with his interest and was told that there simply was no funding available. Here Fate smiled as Dr. Streit soon received a Paralyzed Veterans of America grant to look at graft rejection, but had no one to do the work. Not having to be asked twice, Nate took the reins and hasn't looked back since. Along the road culminating in this dissertation, Nate received a NIMH pre-doctoral fellowship and published several papers dealing with microglia and neural transplantation.

While working in spinal cord injury research, Nate also discovered an intense interest in the clinical aspects of neuroscience, and decided to attend medical school. He was accepted into the University of Florida College of Medicine, and will attend UF this Fall, with the intent of pursuing a career in academic medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Wolfgang J. Streit, Chair
Associate Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Douglas K. Anderson
C.M. and K.E. Overstreet Eminent Scholar and Professor of Neuroscience and Neurological Surgery

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Susan L. Semple-Rowland
Associate Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joel L. Schiffenbauer
Associate Professor of Molecular Genetics and Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1998

Dean, College of Medicine

Dean, Graduate School