IMMUNOSUPPRESSION WITH MONOCLONAL ANTIBODIES IN NEURAL TRANSPLANTATION

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this work is dedicated to my wife Karla

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ABSTRACT

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The ability of two different immunosuppressive monoclonal antibodies to prolong the survival and function of neural allografts and xenografts in mice and rats was studied.

YTS 191.1, a rat IgG2b monoclonal antibody against the L3T4 surface antigen found on murine helper T-cells, was injected intraperitoneally into mice for thirteen consecutive days. Mice received various grafts of central nervous system (CNS) tissue to their third ventricle or parenchyma on the third day. The mice were examined to observe the specific depletion of helper T-cells and the neural grafts were examined histologically for surviving tissue at various time points. Mice immunosuppressed with YTS 191.1 had significant prolongation of both allograft and xenograft tissue compared to control animals given saline or an antibody against the Lyt-2 surface antigen on cytotoxic T-cells. Xenografts of rat neuronal tissue containing gonadotrophin releasing hormone neurons were shown to function in the third ventricle of hypogonadal mice at thirty days.

NDS 63, a mouse IgG1 monoclonal antibody against the rat interleukin-2 receptor, found on activated T-cells, was injected intraperitoneally into rats for ten consecutive days. The rats received CNS allografts and xenografts to their third ventricle and striatum on day one. Rats immunosuppressed with NDS 63 had significant prolongation of survival of all neural grafts. Fetal human mesencephalon was shown to function for at least six months in a hemi-Parkinson model of rats immunosuppressed with NDS 63.

The benefits of monoclonal antibodies for immunosuppression was discussed.

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Chapter 1

General Introduction

The technique of neural transplantation is being investigated in numerous centres throughout the world. The last decade saw the application of knowledge gained in the laboratory to a potential neurosurgical therapy of Parkinson Disease. There are many unanswered questions about the use of central nervous system transplantation in the clinical setting. This thesis deals with the question of neural graft rejection by the host immune system and presents two methods of immunosuppression with monoclonal antibodies which prolong neural graft survival and function in rodent models.

Backlund *et al.* (1985) reported the 'first clinical trials' using autologous adrenal medullary tissue transplanted to the striatum in Parkinsonism. Later, Lindval *et al.* (1987) reported two further cases. None of the patients, however, enjoyed any long term benefits from the treatment. Madrazo *et al.* (1987a) was the first to claim 'marked improvement' in Parkinsonian patients using this technique. Further reports by Madrazo *et al.* (1987b, 1988) and by Jiao *et al.* (1988) confirmed this optimism. This led to an explosion of clinical trials with over 100 patients receiving this treatment in at least 15 different neurosurgical centres. Recent trials have included the use of human fetal mesencephalon as donor neural tissue in the United Kingdom (Hitchcock *et al.*,1988), Mexico (Madrazo *et al.*,1988), Sweden (Lindvall *et al.*,1988), as well as in Cuba and China.

This neurosurgical enthusiasm was met with caution by the scientific community. Joynt and Gash (1987) asked 'Are we ready?' and Sladek and Shoulson (1988) called for 'Patience rather than Patients'. There were numerous questions that had been raised by the surgical trials which needed investigation.

First, what was the ideal technique for transplantation? Was an open approach (Madrazo *et al.*,1987a) better than a stereotaxic approach (Backlund *et al.*,1985); was transplantation to the putamen (Lindvall *et al.*,1987) better than to the caudate (Backlund *et al.*,1985) or were multiple sites better? Should the tissue be in contact with the ventricle (Madrazo *et al.*,1987) or not (Backlund *et al.*,1985)?

Second, what was the ideal source of graft material? Were fetal dopaminergic neurons better than autologous adrenal medullary tissue? What was the ideal age of fetal tissue (Brundin *et al.*,1986)? Should the tissue be implanted as fragments or as a cell suspension (Nicholas and Arnason,1989)?

Third, how did the grafts exert their therapeutic effect? Was the adrenal medullary tissue functioning as a dopamine mini-pump or stimulating host neuronal recovery (Bohn *et al.*, 1987). Was some of the improvement due to lesioning the caudate rather than the providing a functioning neural graft (van Manen and Speelman, 1988). In 1951, Meyer reported seven patients 'regarded as much improved' following pallidofugal fibre section. Was the graft merely damaging the blood brain barrier and allowing peripheral adrenergic molecules access to the CNS (Rosenstein, 1987)?

Fourth, was the age and disability of the patient important factors in selecting candidates for surgery (Bakay and Herring,1989)? Madrazo (1988) observed a 'marked improvement in young patients but a high morbidity and mortality rate in elderly patients (>60 years)'.

Fifth, if fetal allografts were used as the source of neural tissue, would immunosuppression be required. Madrazo (1988), and Lindvall (1988) used chemical immunosuppression but Hitchcock (1988) did not!

Animal models had provided the basic knowledge of neural transplantation which had originally stimulated the interest in clinical transplantation. It seems appropriate to return to these animal models to answer some of the questions raised by clinical transplantation. At this point, a short review of neural transplantation in animal models is warranted.

Neural Transplantation in Animal Models

The reader is referred to Gash (1984) and Björklund and Stenevi (1985) for extensive reviews on this topic. The present review is a condensed version of the highlights of neural transplantation.

Dr. W. Gilman Thompson (1890) first described grafting of central nervous system (CNS) tissue to the brain. He transplanted occipital cortex from one adult dog to another and from adult cats to dogs. The grafts were examined histologically at three days and seven weeks after transplantation. Professor Thompson claimed that the grafted tissue remained viable at both time points. Although it is unlikely that the graft tissue survived (he did not show evidence of neuronal survival), he did express enthusiasm for this field of research and predicted the growing interest that followed.

In 1917, Elizabeth Hopkins Dunn at the University of Chicago was the first to demonstrate survival of transplanted CNS tissue to the brain. She used cortical tissue from developing rat pups (9-10 days) and transplanted them into cortical cavities of littermates.

She reported:

> 'The two points of chief importance in successful cerebral transplantation are first, the retention in place of the material transferred, and second, the furnishing to it of an adequate blood supply'

Although the survival rate was low (9 out of 44), the neocortical grafts had retained some degree of their original laminar organization.

Several publications followed, demonstrating that tumours survived better when transplanted to the brain than to subcutaneous sites (Ebeling,1914; Shirai,1921). Murphy and Sturm (1923) focused their studies on the immunology of transplanting tumours to the brain. Murphy and Sturm's work was of particular interest for several reasons. First, they were the first to use a canula for the intracerebral placement of the graft. Second, they suggested that the transplantation site within the brain may play a role in the transplants survival (grafts lying entirely within the brain parenchyma survived whereas grafts which came in contact with the ventricle were destroyed). Third, they showed that transplanting a piece of the host animal's spleen together with the tumour resulted in destruction of the graft. This last observation led to the later series of investigations for the possible causes of enhanced survival of tissues transplanted to the brain. The results of experiments utilizing tumours as grafts, however, must be carefully scrutinized. Tumours could potentially grow faster than the immune system could destroy them - thus appearing to avoid rejection. Tumours have been shown to modulate their surface proteins and hence present a less immunogenic stimulus to the host (Klein, Forman and Hauptfeld,1975).

Edward Le Gros Clark (1940) in Oxford, demonstrated successful fetal (E15-19) rabbit cortex survival in the brains of young rabbits. He wrote that the fetal neural grafts demonstrated, 'capability for continued growth and differentiation'.

Björklund and Stenevi (1985) suggested that these early experiments were not followed up because of the general negative impression of the feasibility of grafting neural tissue to mammalian CNS and the technical ease of transplanting into cold-blooded vertebrates. The criteria for consistent survival of CNS grafts, and the techniques to visualize them, were established in the early 1970s. Laboratories in Sweden (Olson and Malfors, 1970; Björklund and Stenevi, 1971) and at Purdue University (Das and Altman, 1971) published investigations which led to the current explosion of interest in the field of CNS transplantation.

One of the areas of interest explored was the possibility of transplanting dopaminergic neurons into Parkinsonian patients (see Chapter 8 Introduction). Perlow *et al.* (1979) and Björklund *et al.* (1980) showed that grafts from the substantia nigra could function in the host rat brain striatum. Similarly, Freed *et al.* (1981) showed that adrenal chromaffin cells could also reduce lesioned induced rotational behaviour in rats. These studies were quoted as the basis for the 'tentative clinical study' preformed by Backlund *et al.* (1985) when he transplanted adrenal medullary tissue to the striatum of two Parkinsonian patients.

Animal models have shown that transplantation of fetal mesencephalon (the site of developing dopamine neurons) is superior to adrenal medullary tissue in reversing lesion-induced behaviour in the rat (Brown and Dunnett, 1989). It seems logical that replacement of the missing nigral dopaminergic neurons in these models with neurons which normally function in the striatum would provide the best replacement therapy. Madrazo (personal communication) feels the fetal

mesencephalon transplants in Parkinson patients also function better than adrenal medullary grafts.

If fetal neuronal transplants are going to be utilized in future clinical trials, then the neurosurgeon will face two major obstacles. First, the ethical questions of using human fetal material. This lies beyond the scope of this thesis. Second, the potential of the graft recipient to reject the foreign tissue transplant. Do patients need to be immunosuppressed and if so, what is the best method?

In order to answer these questions we must have an understanding of how the immune system deals with neural grafts. At this point, a brief review of the pertinent immunology is warranted.

Transplantation Immunology

The reader is referred to Widner and Brundin (1988) for an extensive review of the immunology involved in neural transplantation. The present review is a condensed version of the highlights of transplantation immunology required for the understanding of the material included within this thesis.

It seems appropriate to begin by reminding oneself that the immune system was not designed to foil the transplant surgeon! The primary concern of the immune system is the recognition and subsequent destruction of foreign organisms (such as bacteria or viruses). One of the crucial concepts in immunology then, is the ability of an organism to distinguish self from non-self. The immune system accomplishes this by recognizing a set of glycoproteins carried on the membrane of cells. The most important of these are encoded for by the major histocompatibility gene complex (MHC) and display a high degree of polymorphism (ie. individual variation). In man, this gene complex is called the HLA and is found on chromosome 6; in mice it is referred to as H-2 and in rats it is referred to as RT1. The products of the MHC genes responsible for immune system activation can be subdivided into two main categories depending on their structure, function and tissue distribution (Klein *et al.*, 1981). MHC Class I antigens are composed of a polymorphic heavy chain non-covalently bound to β_2 microglobulin and is found on nearly all nucleated cells (most neurons being a notable exception). MHC Class II antigens are composed of two polymorphic non-covalently bound chains and are found on macrophages, dendritic cells and B cells (but can be induced to be expressed on a number of other cells).

The original concept of the MHC came about from experiments on mice acceptance or rejection of tumour allografts by Peter Gorer (1937) and George Snell (1948). The immunology of transplantation has therefore always been associated with the development of a general understanding of how the immune system functions. Mismatching for histocompatibility antigens is the *sine qua non* for graft rejection to occur (Counce *et al.*, 1956). The most rapid rejection occurs in animals given grafts which differ in the MHC Class I and II antigens. Grafts which differ in only in MHC Class I or Class II antigens can also elicit a rejection response (Klein, 1977) and grafts which differ in non-MHC antigens (called minor antigens or mH) elicit a much slower rejection.

The immune system of higher vertebrates is composed of a number of organs and several different cell types capable of distinguishing self from non-self. All cells in the immune system arise from pluripotent stem cells through two main lines of differentiation. The myeloid lineage produces phagocytes (capable of engulfing microbes or damaged cells) as well as platelets and mast cells. The lymphoid lineage produce lymphocytes (capable of the high degree of specificity involved with recognition of non-self antigens). There are two subsets of lymphocytes - T cells and B cells. T-cells differentiate initially in the thymus (T for thymus) while B cells differentiate initially in the fetal liver, spleen , adult bone marrow and in an organ unique to birds - the bursa of Fabricius (B for bursa). A third group of lymphocytes called null cells because they are neither T or B cells can be found. This third category contain cells which function non-specifically as natural killer (NK) cells and antibody dependent cellular cytotoxic (ADCC) cells described later.

Two subgroups of T-cells with different functions can be distinguished by their surface proteins. Table I shows examples of these antigens for mice:

Table I

mouse antigens	
all T cells:	Thy-1
helper T-cell:	L3T4
cytotoxic T-cell:	Lyt-2

The individual function of these different cells is best understood by following the sequence of events leading from foreign tissue recognition to its eventual destruction by the immune system. These events can be divided into two stages - the afferent limb (detection of foreign antigen and activation of the immune system) and the efferent limb (destruction of foreign tissue).

The Afferent Limb

First, the immune system must be activated. There are two suggested pathways for antigen recognition. The most common route involves antigen being taken up by an accessory cell (eg. macrophage). The antigen is then either directly associated with self MHC Class I or II molecules on its surface, or broken down and processed into smaller pieces before being associated with self MHC Class I or II molecules on antigen presenting cells (APC). In the allograft (and probably xenograft) transplant setting, however, foreign antigen is usually presented by donor APC (Lafferty *et al.*, 1983), although host APC can also serve this purpose (Sherwood *et al.*,1986). These donor APC or dendritic cells are found in most tissues (Hart and Fabre, 1981) with the notable exception of brain parenchyma. This route of activation involves the direct recognition of foreign MHC by T-cells and presumes the host T-cell mistakes foreign MHC as self MHC in association with a foreign antigen (ie. altered self). In xenograft combinations, the immunological reaction is not necessarily dominated by the MHC, although it seems likely to be a source of strong antigenic stimulus. Sachs, Winn and Russel (1971) found antibodies against mouse MHC antigens in rats.

The APC (as their name suggests) function by presenting antigens to helper T-cells. The helper T-cell binds to the complex of foreign antigen associated with MHC Class II antigens with its T-cell receptor (Unanue, 1981). This triggers helper T-cell activation and leads to the secretion of a macrophage stimulant and the formation of receptors for insulin (Helderman and Strom, 1979), transferrin (Trowbridge and Omary, 1981), interleukin-1 (Gillis and Mizel, 1981) and interleukin-2 (Robb, Munck and Smith, 1981). The cytotoxic T-cell is activated by binding foreign antigen in association with MHC Class I antigens and then

expresses the interleukin-2 receptor (IL2R). Subsequently, stimulated macrophages and other accessory cells release interleukin-1 (IL-1), which in turn stimulates the release of interleukin-2 (IL-2) from helper T-cells (Smith *et al.*, 1980). IL-2 reacts with the IL2R on activated helper and cytotoxic T-cells and promotes clonal proliferation of receptor-bearing cells. Moreover, the continued viability of activated T-cell clones is IL-2 dependent. IL-2 in turn causes the release of gamma-interferon (which activates macrophages) and B-cell growth factors (which stimulate the proliferation of antigen-activated B cells (Farrar, Johnson and Farrar, 1981; Inaba *et al.*, 1983).

The site of immune system activation depends on the type of graft and can occur peripherally in the allograft itself (Medawar, 1958) or centrally in the regional lymph nodes (Wilson and Billingham, 1967). The site of activation for primarily vascularized allografts, such as the kidney, is peripheral (Pederson and Morris, 1970) while secondarily revascularized allografts, such as the skin, are thought to take place centrally (Barker and Billingham, 1968). The lymph nodes are populated by large numbers of lymphocytes so cellular interactions are facilitated. The APC cells travel to the lymph nodes from the transplantation site by the lymphatics. The removal of regional lymph nodes near the transplantation site can slow tissue graft rejection (Dorsch *et al.*, 1983).

The Efferent Limb

The T-cell appears both necessary and sufficient for acute allograft rejection. Congenitally athymic (nude) mice fail to reject rat skin xenografts (Rygaard, 1969) and thymic grafts can restore these animals ability to reject xenografts (Rygaard, 1974). Whereas neonatally bursectomized chickens, which totally lack B-cells, are still able to reject allografts (Roitt, Brostoff and Male, 1985). The subset of T cells responsible for rejection is still debated (Mason and Morris, 1986).

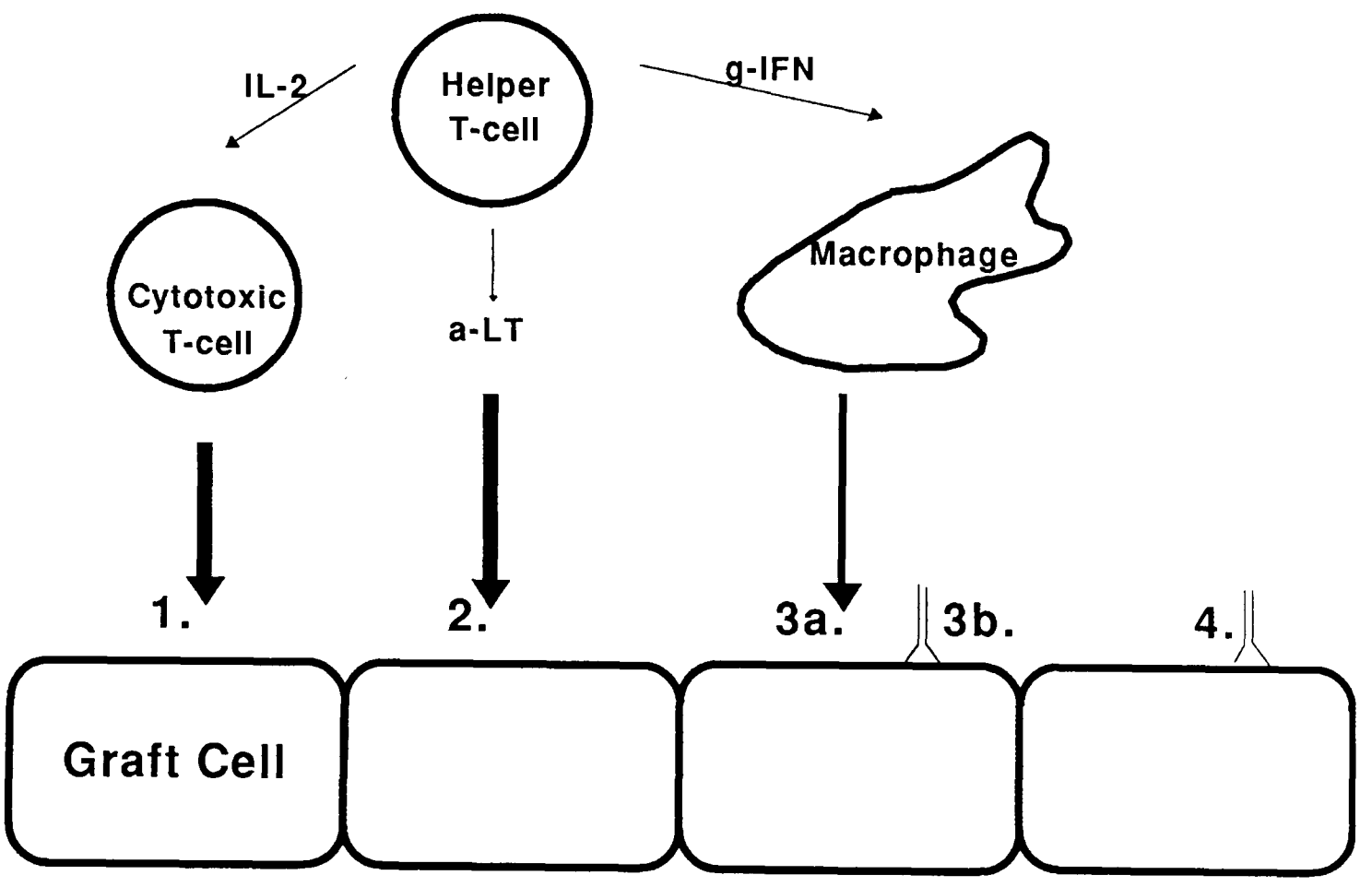
Brent *et al.* (1962) noted histologic similarities between the cell infiltrates in skin allografts and those in delayed-type hypersensitivity (DTH). The cells which mediate DTH were known to be helper T-cells, thus helper T-cells were suggested to destroy allografts in a similar manner. DTH occurs when helper T-cells recognize a previously encountered foreign antigen and release lymphokines. These lymphokines recruit and activate other cells, predominantly the macrophage, which then produce an inflammatory reaction. This inflammation results in the destruction of the foreign tissue but also results in non-specific damage to nearby cells (so called *innocent bystander* destruction). The work by Mintz and Silvers (1970), however, suggested that DTH may not be the effector mechanism. They showed specific killing of foreign melanoblasts and hair follicles in skin grafts without bystander destruction (the hallmark of DTH). The view that the helper T-cell was the effector cell in allograft rejection was supported by studies in T cell deprived mice (Loveland *et al.*, 1981) and rats (Dallman, Mason and Webb, 1982) which showed that these rodents could reject skin allografts when reconstituted with helper T-cells but not cytotoxic T-cells.

There are several lines of evidence that suggest the cytotoxic T-cell may mediate allograft rejection. Engers, Glasebrook and Sorenson (1982) have shown a cytotoxic T-cell clone (made *in vitro*) was capable of eliminating susceptible tumour cell grafts in mice. In heart allografts which differ only in MHC Class I antigens, Lowry *et al.* (1985) showed cytotoxic T-cells were essential for rejection. Mason and Morris (1986) examined the cellular composition of the infiltrate in human renal allografts. They found the cytotoxic T-cell was always more abundant than the helper T-cell. They also noted, however, that the most abundant cell during rejection was the macrophage (suggesting a DTH reaction).

There are several other possible mechanisms for graft rejection. In human renal allografts, hyperacute rejection (which takes place in <24 hours) has been shown to be due to preformed host antibodies against the foreign kidney (Kissmeyer-Nielson *et al.*, 1966). It is generally agreed, however, that antibody *per se* does not mediate allograft rejection in rodents (Mason and Morris, 1986). Another possible mechanism for allograft rejection is antibody-dependent cell-mediated cytotoxicity (ADCC). In this reaction, antibody first binds to foreign antigen. The F_c portion of the antibody then interacts with molecules of the complement pathways and a variety of effector cells (macrophages, neutrophils, eosinophils, K cells and platelets) to bring about damage to the foreign cell and the surrounding tissues. No evidence exists that ADCC plays an essential role in experimental allograft rejection (Mason and Morris, 1986) but the possibility that it may augment rejection, under certain circumstances, cannot be rule out (Carpenter, d'Aspice and Abbas, 1976).

A diagrammatic summary of the possible mechanisms for graft rejection are provided below (adapted from Widner and Brundin, 1988):

Mechanisms of Graft Rejection



1. Cytolysis by cytotoxic T-cells
2. Cytotoxic lymphokines (alpha-lymphotoxin)
3a. Macrophage non-specific killing
3b. Antibody-dependent cell-mediated cytotoxicity (ADCC)
4. Antibody mediated lysis

Figure 1

With this rudimentary explanation of how allografts are rejected, we can now understand why the brain is felt to be a 'privileged site' for transplantation.

Transplantation to the Brain

As mentioned before, Ebeling (1914) and Shirai (1921) had shown that tumours transplanted to the brain survived better than those transplanted subcutaneously. A series of investigators began to conduct experiments to ascertain why the brain was such a propitious site for transplants. Peter Medawar's classic 1948 paper showed that intracerebral skin allografts in *presensitized* rabbits (ie. rabbits which had received previous exposure to the foreign antigens) were rapidly rejected. This proved that the efferent limb of the immune system could function well against intracerebral transplants. He proposed that since intracerebral allografts did survive longer than those in peripheral sites, the afferent limb of the immune system must be hindered from functioning optimally within the brain. He suggested that the brain's lack of conventional lymphatics produced this afferent block.

Several authors have shown that the intracerebral allografts can sensitize the host animal (ie. the afferent limb does function). Scheinberg *et al.* (1966) showed that rabbits given intracerebral skin grafts were capable of mounting a secondary (or accelerated) response to subsequent peripheral skin grafts. If the immune response to the subsequent peripheral skin grafts was accelerated, the original intracerebral skin graft must have sensitized the animal. Lance (1966) showed that intracerebral thyroid allografts could systemically sensitize dogs. Geyer and Gill (1979) confirmed these results in rats and proposed that the afferent limb was slowed but not blocked.

If the brain parenchyma lacks conventional lymphatics then how are the foreign antigens on these intracerebral grafts reaching the regional lymph nodes? Prinease (1979) and Bradbury, Cserr and Westrop (1981) have described the function equivalent of lymphatics within the brain - perivascular spaces present along the larger vessels. These spaces drain into the subarachnoid space and antigen (in animal models) has been shown to cross the cribriform plate into the lymphatic vessels of the nasal mucosa and subsequently reach the cervical lymph nodes.

Another proposed impairment of the afferent limb of the immune system within the brain is its relative lack of antigen presenting cells. Hart and Fabre (1981) found an absence of classical dendritic cells within the brain parenchyma. Judith Head and Susan Griffin (1985) found potential APC lining the ependyma but described a paucity of these cells within the parenchyma. Several authors, however, have proposed alternative cells within the brain that are capable of presenting foreign antigens. The microglial cell, for example, can produce IL-1 and express MHC Class II antigens like the macrophage. Fontana, Fierz and Wekerle (1974) showed that astrocytes stimulated with gamma interferon could present antigens *in vitro*. Cerebral endothelial cells have also been proposed to function as APC. McCarron *et al.* (1986) showed that these cells could present myelin basic protein in association with MHC antigens to T-cell clones *in vitro*.

Another possible reason for the brain's reduced ability to reject grafts is the presence of the blood brain barrier (BBB). This barrier is designed to protect the brain from direct exposure to the blood. It is formed by the tight junctions, *zonula occludens*, in the cerebral microvessels (Reese and Karnovsky, 1967); a transcellular potential difference (Crone, 1986); degredative enzymes (Hardebo and

Owman, 1980); low pinocytic capacity (Wolff, 1966); and a reduced response to inflammatory mediators that normally increase permeability (Bradbury, 1984). All these factors would tend to hinder effector cell access to an intracerebral graft from the bloodstream. Wekerle *et al.* (1986), however, have shown that activated helper T-cells can cross the intact BBB at the post-capillary venule.

Kaplan and Streilein (1974) suggested that a state of immune unresponsiveness could be achieved when antigens were presented to an animal's immune system via a vascular route to the spleen (rather than a lymphatic route to the lymph nodes). They proposed that the spleen generated *enhancing antibodies* which bound the foreign antigens and paradoxically 'hid' them from cell-mediated immune destruction. Perhaps the antigens on intracerebral grafts reach the host's immune system via a preferentially blood born route.

One final suggestion for the enhanced survival of intracerebral grafts is the possible presence of local factors secreted by the brain parenchyma which may inhibit immune system function.

Whatever the mechanism, the brain does allow prolonged survival of tissue grafts. It has therefore been labelled an immunologically *privileged site* (Barker and Billingham, 1977). The apparent lack of MHC antigens in the CNS may also make it an ideal source of tissue for transplantation.

MHC Class I and II antigens are not normally found on neurons, astrocytes and oligodendrocytes (for review see Widner and Brundin, 1988). Since these antigens are the strongest stimulators of the immune system in allografts, their deficiency may reduce the host's immune response against the graft. Wong *et al.*(1984), however, showed that astrocytes and oligodendrocytes could be induced to express MHC Class I antigens by gamma-interferon *in vitro*. Several authors

have shown that neural grafts undergoing rejection do express MHC Class I (Mason *et al.*,1986) and Class I and II (Date, Kawamura and Nakashima,1988) antigens.

These combination of factors provide the transplantation of CNS tissue to the brain with a degree of privilege (ie. reduced response from the host's immune system) not afforded to other tissues transplanted to other sites. Does this mean that CNS allografts and xenografts do not require immunosuppression when transplanted to the brain? There are conflicting reports in the literature.

Interpreting the survival rate for various CNS neural transplants can be difficult since different investigators use different criteria for survival. There are, however, several consistent points. First, the survival rate varies between species and within strains. Second, the survival rate varies depending on where the transplant is placed within the host brain. Third, xenografts show very poor survival.

Xenograft survival in the rat is consistently reported to be very low. There was no survival of mouse (Brundin *et al.*,1989) or human (Brundin *et al.*,1988) mesencephalic tissue and virtually no survival of rabbit (Dusart *et al.*,1987) mesencephalic tissue transplanted to the rat striatum. Similarly, there was no survival of mouse neural tissue in the rat ventricle (Inoue *et al.*,1985) at 28 days. Higher rates of murine CNS xenograft survival have been reported in the rat by Björklund *et al.* (1982) (10/18 at 180 days) and Daniloff *et al.* (1985) (6/6 at 91 days). Survival in these last two experiments was recorded if any cell from the xenograft was found at sacrifice. Thus a xenograft which had almost been destroyed at the time of sacrifice, but still had a few surviving cells, would be considered 'surviving'. Indeed, Björklund's Nature paper reported that the 10 surviving xenografts were largely resorbed at 6 months.

There is no doubt that immunosuppression for neural xenografts is beneficial. Appropriate treatment with Cyclosporin A has been shown to raise survival rates of mouse (Brundin *et al.*,1985; Inoue *et al.*,1985) and human (Brundin *et al.*,1988) xenografts in rats to 100%.

The data for allografts is not as compelling, and certainly more confusing. In the rat, Mason *et al.* (1986) made a systematic study of the survival of neural allografts and found fully allogeneic grafts survive in the ventricle in only 25% of animals at 60 days. Low, Lewis and Bunch (1983) found 4/5 allografts survived in the parenchyma for 90 days. Once again, however, survival was recorded if the presence of any tissue (regardless how small) was found. In the mouse, neural allografts to the parenchyma appear to enjoy better survival. Widner *et al.* (1989) reported 8\8 surviving allografts at 42 days. Similarly, in the monkey, neural allografts appear to enjoy prolonged survival. Sladek *et al.* (1986); Redmond *et al.* (1986) and Freed *et al.* (1987) reported 100% survival. These apparently high figures in primate models may be artificially bolstered by short observation periods (only 69 days for Sladek's group), small number of animals (only two for Redmond's group), the reporting of small allografts as 'survived', and the possible spontaneous recovery from MTPT lesions (Eidelberg *et al.*, 1986). No examinations for infiltrating cells within the grafts were made.

The role of immunosuppression in neural allografts may not be as obvious as that for xenografts but a strong case can still be made for its advocacy. In the clinical setting, neurosurgeons will want to offer their patients the best possible chance for neural graft survival and the best possible function of those surviving grafts. If immunosuppression can increase the survival rate of long term neural grafts or increase the amount tissue surviving in any one graft, then surely it is necessary.

This thesis will show that immunosuppression with monoclonal antibodies in rodent models of neural transplantation can increase neural allograft and xenograft survival and function. In order to understand the sequence of events which led to the discovery and use of monoclonal antibodies for immunosuppression a brief history of immunosuppression is warranted.

Immunosuppression

The early attempts at immunosuppression reflected a gross attempt to kill lymphocytes by destroying all rapidly proliferating cells with total body irradiation (Murray *et al.*,1960) and antimetabolites such as azathioprine (Murray *et al.*,1962). The active metabolite of azathioprine, 6-mercaptopurine, functions by interfering with DNA synthesis. Unfortunately, its side-effect include leucopenia with increased susceptibility to infection, anæmia and thrombocytopenia as well as alopecia and hepatitis.

The addition of steroids to the immunosuppressive regime allowed the dose of azothioprine to be reduced, decreasing the incidence of toxic side-effects (Murray *et al.*,1962). Steroids produce a multitude of immunosuppressive effects including anti-inflammatory actions, sequestration of circulating lymphocytes (Germuth *et al.*, 1968), and inhibition of IL-1 production by monocytes and therefore blocking IL-1-dependent release of IL-2 (Synder and Unanue, 1982). Their side effects, however, include increased susceptibility to infection, poor wound healing, Cushingoid appearance, psychosis, hyperlipidæmia, osteoporosis and diabetes mellitus.

There was a tremendous increase in the rate of successful renal allografts in man with the advent of cyclosporin A (Calne *et al.*,1978). Although its precise actions have not been fully documented, cyclosporin A is thought to function by blocking the release of IL-2 from activated helper T-cells (Bunjes *et al.*, 1981). Unfortunately, its side-effects include nephrotoxicity and increased susceptibility to infection. Lane *et al.* (1988) has also shown that cyclosporin may directly damage the CNS.

Thus the current immunosuppressive protocols can prevent allograft rejection but their application is limited by significant side-effects. The ideal immunosuppressive regime would inhibit the mechanism of graft rejection (without serious side-effects) while not interfering with that part of the immune system responsible for protecting the host against opportunistic infections. This concept of specific or selective immunosuppression is an attractive one.

One method of achieving this selective immunosuppression is to use the exquisite specificity of antibodies to target various parts of the immune system. Serum specific for lymphocytes and resulting in lymphopenia was demonstrated by Bunting (1903). It was not until half a century later that the immunosuppressive effects of antilymphocyte serum was recorded. Inderbitzin (1956) showed that antilymphocyte serum depressed the tuberculin reaction (a type of DTH) in the guinea pig. Waksman, Arbouys, and Arnason (1961) showed antilymphocyte serum could suppress skin allograft rejection. Starzl (1967) was the first to use antilymphocyte serum clinically.

The initial problems with antilymphocyte serum included batch to batch variability and contamination with non-lymphocyte antibodies. The advent of monoclonal antibody technology (Köhler and Milstein, 1975) overcame these problems and pure and specific antibodies could be produced in abundance. The question then became - what part of the immune system is best targeted?

Referring back to Figure 1, we can see the various parts of the immune system known to be involved in allograft rejection. Three possible approaches to interfering with this system become apparent. First, removing or blocking the function of all T-cells. Second, selectively removing or blocking the function of either T-cell subset. Third, inhibiting the action of the activating lymphokines. These three mechanism will now be discussed.

Pan T cell Antibodies

OKT3 is a mouse IgG2a monoclonal antibody which reacts with the CD3 antigen of human thymocytes and mature T cells (Kung *et al.*,1979). A multicenter, randomized, prospective trial comparing OKT3 to conventional high dose steroid therapy in rejection episodes of cadaveric renal allografts showed a significantly increased rejection reversal rate and an improved 1 year grafts survival rate (Ortho Multicenter Transplant Study Group, 1985). OKT3 is felt to function by binding to T cells and inhibiting their function (Goldstein, 1987).

T cell Subset Antibodies

The recognition that the two T cell subset carried unique surface markers in mouse (Ledbetter and Herzenberg, 1979), rat (Brideau *et al.*, 1980), and human (Reinherz *et al.*, 1983), made it possible to design monoclonal antibodies against these antigens and thereby specifically target one subset of T cells. In the mouse the helper T-cell carries the L3T4 surface antigen while the cytotoxic T-cell carries the Lyt-2 surface antigen. In humans and monkeys the equivalent antigens are CD4 on helper T-cells and CD8 on cytotoxic T-cells.

Monoclonal antibodies against subsets of T cells have shown marked immunosuppressive effects in animal models. OKT4, an anti-CD4 antibody, prolonged monkey renal (Cosimi *et al.*,1981) and skin (Jonker *et al.*,1983) allograft survival. YTS 191.1, an anti-L3T4 monoclonal antibody, prolonged skin (Cobbold *et al.* ,1984) and cardiac (Madsen, Wood and Morris, 1987) allografts in mice. Cobbold's work deserves a detailed examination since this was the antibody used for immunosuppression of mice in Chapters 3, 4 and 5 of this thesis.

Cobbold injected thymectomized and normal mice with the YTS 191.1 and the YTS 196.4 (anti-Lyt-2) monoclonal antibodies. In both cases, the anti-Lyt-2 antibody did not result in prolongation of skin allografts, while the anti-L3T4 antibody led to greatly increased graft survival. They suggested that the YTS 191.1 antibody worked by depleting their target helper T-cells, since no residual antibody could be detected during the immunosuppressive effect (ie. none was available to block cell function). Cobbold, Thierfelder and Waldmann (1983), have also shown that the antibody isotype was crucial for the immunosuppressive effect. They showed that two rat IgG2b anti-mouse Thy-1 antibodies were potently immunosuppressive *in vivo*, compared to a panel of other antibodies with the same specificity but different isotypes. The other antibodies were binding to the same target (they had identical specificities) but their different F_c portions could not bring about target destruction. Presumably antibody targeted cells are either removed from circulation in the reticular endothelial system or destroyed by complement or antibody-dependent cell-mediated cytotoxicity (Waldmann, 1989). Other authors have shown that cell depletion is not essential for immunosuppression in some models (Qin *et al*, 1988).

Activated T cell antibodies

Monoclonal antibodies against lymphocyte activation markers would be specific for activated cells. Therefore, only activated T-cells responding against transplanted tissue would be inhibited. The value of this approach has been confirmed both experimentally (Billing and Chatterjee, 1983; Kirkman *et al.*, 1985; Kupiec-Weglinski *et al.*, 1986) and clinically (Soulillou *et al.*, 1987).

A panel of monoclonal antibodies (NDS 61 to NDS 66) against the rat interleukin-2 receptor (IL2R) were produced at the John Radcliffe Hospital in Oxford by George Tellides. He showed that the mouse IgG1 antibody, NDS 63, inhibited the binding of interleukin-2 (IL2) on activated rat lymphocytes. This antibody can also prolong cardiac and renal allografts (Tellides, Dallman and Morris, 1989). These authors suggest that the antibody exerts its effect by inhibiting T-cell function rather than depleting these cells. Evidence in support of this included (i) the lack of correlation between antibody isotype and its immunosuppressive capabilities and (ii) the presence of target cells within the allografts.

If the NDS 63 antibody had prolonged rat cardiac and renal allografts, it seemed likely that it could prolong neural graft survival as well. The antibody was therefore used for immunosuppressing rats throughout Chapters 6, 7 and 8.

Aims of this Thesis

The four main aims of this work are as follows:

1. To determine if anti-L3T4 monoclonal antibody treatment can prolong neural allograft and xenograft survival in the mouse.

2. To determine if anti-IL2R monoclonal antibody treatment can prolong neural allograft and xenograft survival in the rat.

3. To determine if neural graft function can also be prolonged with these two treatments.

4. To determine if there are any detrimental effects of these two treatments.

Chapter 2

Materials and Methods

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2.1 Animals

The inbred strains of mice (AKR, Balb/c, C3H/He, C57BL/6, DBA/2, and 129) and rats (AO, DA, LOU, PVG, and Wistar) were obtained from Harlan Olac Limited (Bicester, United Kingdom) and then housed in the Department of Human Anatomy Animal House (University of Oxford, United Kingdom). The *hpg* mice were obtained from the colony continuously bred in our department. (LOU X $DA)_{F1}$ hybrids rats and (Balb/c X $DBA/2)_{F1}$ hybrid mice were bred in our animal house. All animals were fed *ad libitum* and housed in a fourteen hour light / ten hour dark cycle. The details of each strain used will be elaborated as required in the following experimental chapters.

2.2 Preparation of anti-L3T4 MAb

The preparation of the anti-L3T4 MAb was separated into the following three stages: production, purification and characterization.

2.2.1 Production of anti-L3T4 MAb

The tissue culture medium was prepared from Iscove's Medium (Gibco, Paisley, United Kingdom) supplemented with:

L-Glutamine (BDH Limited, Atherstone, U.K.)		2 mM
Penicillin	(Gibco)	100 µg/mL
Streptomycin	(Gibco)	100 µg/mL
Kanamycin	(Gibco)	100 µg/mL
Fetal calf serum	(Gibco)	5 %

The hybridoma cell line, YTS 191.1 (Cobbold et al., 1984), which produced the anti-L3T4 MAb was stored in 1 ml aliquots of freezing medium in liquid

nitrogen. Tissue culture medium was supplemented with 10% dimethyl sulphoxide (BDH) to produce the freezing medium. The cells were thawed, placed in 20 ml of sterile tissue culture medium at 4 °C and spun at 220g for 7 minutes. The pellet was then resuspended in 10 ml tissue culture medium at room temperature.

The number of viable cells was determined by adding FDA, fluorescein-di(methylaminodiacetic acid) (BDH), to a sample of cell suspension and counting the number a fluorescing cells in a hæmocytometer with an ultraviolet light.

The cell suspension was diluted with tissue culture medium to give a final concentration of approximately 10^5 cells/ml. The cells were then transferred to tissue culture flasks, 'gassed' with 5% CO_2 and allowed to incubate at 37 °C.

The cells began to divide and as the metabolic products of the increasing cell population accumulated, the resultant reduction of the pH showed as a change in colour of the tissue culture medium. The old medium, now containing a large cell population, was split in two and diluted to the original cell concentration with fresh medium. This process could be repeated until the desired number of cells had been grown.

While grown in tissue culture, the hybridoma cells continued to secrete antibody. The tissue culture supernatant (TCS) was therefore full of the anti-L3T4 monoclonal antibody. The concentration of anti-L3T4 MAb in TCS was approximately 32 µg/ml (see Appendix). This TCS was used in the Pilot Study to determine if anti-L3T4 MAb could prolong neural xenografts in mice (see Chapter 3). TCS has been used successfully to prolong cardiac allografts in mice (Madsen, Wood and Morris, 1987).

In order to produce large quantities of purified anti-L3T4 MAb required for this work, hybridoma cells were grown as intraperitoneal tumours. Three month old $(LOU\ X\ DA)_{F1}$ hybrid rats were injected intraperitoneally with 0.25 ml pristane (2,6,10,14-tetramethylpentadecane, Koch Light Ltd., Haverill, U.K.) in each abdominal quadrant to induce an inflammatory reaction. Ten days later, the hybridoma cells were spun at 220g and the pellet resuspended in medium to a concentration of 2×10^7 viable cells/ml. The cell suspension was then injected intraperitoneally into the pristane primed rats (1 ml in each quadrant) through a large bore needle. The hybridoma cells were allowed to grow as intraperitoneal tumours producing monoclonal antibody-rich ascitic fluid. When the abdominal cavity was distended the rats were sacrificed by cervical dislocation and the ascites harvested.

The ascites was collected by Pasteur pipette and each 5 mL anticoagulated with 200 µL 0.1M EDTA (ethylenediaminetetra-acetic acid, BDH) and then spun at 400g for 15 minutes. The clear ascitic supernatant was then drawn off by passing a Pasteur pipette through the top layer of Pristane. The ascites was then frozen at -20 °C to await purification.

2.2.2 Purification of anti-L3T4 MAb

The monoclonal antibodies were purified from ascites by sodium sulphate precipitation according to the method of Mason and Williams (1980) and then by ion exchange chromatography.

2.2.2.1 Sodium Sulphate Precipitation

The ascites was spun at 10000g for 20 minutes at 4 °C using a Beckman JA-20 rotor. Any remaining pristane was removed and the final volume was measured. 29.5% ammonium sulphate (BDH) was slowly added with constant stirring. The solution was incubated at 37 °C for 30 minutes and then spun at 10000g for 15 minutes at 25 °C. The pellet was dissolved in milli-Q water to half the initial volume. This was then dialysed for two days against 5 L of 25 mM tris (hydroxy-methyl) methylamine (BDH), 70 mM NaCl (BDH) at pH 7.4.

2.2.2.2 DEAE-Sephacel Anion-Exchange Column

The column was prepared using a 50 mL syringe clamped vertically with a small amount of glass wool, dampened with buffer (25 mM Tris / 70 mM NaCl at pH 7.4), packed into the bottom. Diethylaminoethyl (DEAE) Sephacel (Pharmacia, Milton Keynes, U.K.) suspension was shaken and poured into the syringe to the 50 mL level. The rubber end of the plunger was removed and a 19 gauge needle inserted through it. This was then inverted and inserted into the top of the syringe creating a seal. A reservoir of buffer was connected by tube to the needle and an outlet tube connected to the bottom of the syringe. 500 mL of buffer was allowed to run through, and when the pH and conductance of the outflow matched that of the buffer, the column was equilibrated.

The outlet tube was then connected to a LKB 2112 Redirac fraction collector set to collect 5 mL samples. The input tube was then placed in the dialysed ascites solution and when all the ascites had been drawn into the column it was replaced into the buffer. The immunoglobulin was bound to the column while the other components of the ascites remained in solution. The optical density (O.D.) of each fraction was measured with a Pye Unicam SP6-500 UV Spectrophotometer at 280 nm against a blank of buffer alone. The protein content of each fraction could then be calculated using Beer's Law:

$$E_{280} = k\ [protein]$$

where Extinction = O.D.

k for IgG at 280 = 1.4

The O.D. was plotted against the fraction tube number (see Figure 2.1) and when the readings returned to base levels the input tube was placed in a buffer of 25 mM Tris / 80 mM NaCl in pH 7.4. The increased sodium ion concentration bound to the column forcing the immunoglobulin into solution and hence out of the column were its presence in the collected fractions was revealed by a rise in the O.D. as shown in Figure 2.1. When the O.D. had again returned towards baseline levels, the column was washed with a solution of 25 mM Tris/ 90 mM NaCl in pH 7.4. This forced more protein off the column and into solution resulting in a third peak of protein in the collected fractions. Samples from the fractions were tested for purity by SDS-PAGE electrophoresis.

2.2.2.3 SDS-PAGE Electrophoresis

The following reagents were used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

A[1] 30% acrylamide + 0.8% N,N,-methylamine-bis-acrylamide (Sigma Chemical Company Ltd., Poole, U.K.)

B 0.75 M Tris-HCl pH 8.8

C 1.00 M Tris-HCl pH 6.8

D 10% SDS (Lauryl sulphate) (Sigma)

E 1% fresh ammonium persulphate (Sigma)

DTT: 31 mg dithiothreitol (Sigma) in 5 mL sample buffer

IAA: 123 mg iodoacetamide (Sigma) in 1 mL water

Running Buffer: 60.6 g Tris + 288.0 g Glycine in 2 L distilled water at pH 8.3 and diluted x10 for use

Sample Buffer: 4.4 g Tris + 96.96 g Urea (Sigma) + 4.6 g SDS in 600 mL distilled water

Coomassie Blue Stain: 1 g Coomassie (Sigma) + 73.6 mL acetic acid+ 364 mL methanol + 364 mL d.H_2O

Destain: 150 mL acetic acid + 100 mL methanol + 1750 mL d.H_2O

[1]dangerous neurotoxin (kept stored at 4 °C)

The gels were prepared according to the following formulae:

	10%	3%
A	30 mL	2.4 mL
B	45 mL	-
C	-	3.0 mL
D	0.9 mL	0.24 mL
E	4.5 mL	1.2 mL
distilled water	9.6 mL	17.13 mL
TEMED	60 µL	45 µL

(TEMED: N,N,N',N'-tetramethylethylenediamine (Sigma))

The separation gel (10%) was poured between two glass plates to form a 1.5 mm slab and then overlayed with distilled water. After the gel had set the stacking gel (3%) was poured on top and allowed to set with well holes in place. The complete electrophoresis unit was assembled and placed in Running Buffer. Equal volumes of DTT were added to the samples and boiled for two minutes to break the disulfide bonds. Sample volumes were taken to give approximately 10 µg protein per well. 20 µL Bromophenol blue (Sigma) was added to visualize the samples and then 10 µL IAA was added to block the disulfide bonds from reforming and boiled for two minutes. A standard with known molecular weights[2] (Pharmacia) was added to the first well and then each sample was added to separate wells. The gel was run with a current of 35 mA until the bromophenol blue marker reached 1 cm from

[2]bovine serum albumen 67 kilodalton (kD); soybean trypsin inhibitor 20.1 kD; α-lactalbumen 14.4 kD

the bottom. The gel was then removed and stained for protein in the Coomassie blue stain for one hour. The gel was then destained overnight and photographed.

An example of a gel can be seen in Figure 2.2. This gel corresponds to the samples taken from the DEAE-Sephacel anion exchange column purification of anti-L3T4 MAb seen in Figure 2.1. Samples A and B show contamination with molecules of many different molecular weights, thus the first peak of protein through the column was discarded. Samples C, D and E show strong bands at approximately 50,000 MW and 25,000 MW corresponding to the heavy and light chains of the IgG. There was a slight contamination with a protein of approximately 20,000 MW which may have been a light chain fragment. The samples between C and E were pooled and concentrated to 1 mg/mL with an Amicon filter and used for all experiments which required anti-L3T4 MAb.

2.2.3 Characterization of anti-L3T4 MAb

The purified anti-L3T4 MAb was then checked for activity by using it as the primary antibody in an indirect immunohistochemical staining of a mouse spleen (see section 2.7.1 for staining method). Spleens were obtained from C3H/He mice, frozen (see seection 2.6) and sectioned on a cryostat. The spleens, which contained numerous L3T4-positive cells, showed strong staining with all batches of anti-L3T4 MAb tested. The negative controls, omitting the primary antibody, showed no staining of the sleen cells.

2.3 Preparation of anti-Lyt-2 MAb

The production of this monoclonal antibody from the YTS 169.4 cell line (Cobbold et al. 1984) followed the method outlined above with the following modification. The ammonium sulphate cut ascites was dialysed into 25 mM Tris / 50 mM NaCl at pH 7.4 and then the ascites was eluted off the DEAE-Sephacel Column with a constantly increasing salt gradient (upto 25 mM Tris / 100 mM NaCl at pH 7.4). The resultant protein collection from the DEAE-Sephacel Column can been seen in Figure 2.3 and the purity of selected samples run on an electrophoresis gel is shown in Figure 2.4. Sample A showed heavy contamination and therefore the first protein peak off the column was discarded. Samples C, D and E showed strong bands corresponding to the heavy and light immunoglobulin chains. There was a slight contamination of a heavier protein probably from heavy and light chain dimers. The samples between C and E were pooled, concentrated to 1 mg/mL with an Amicon filter, and used for all experiments which required anti-Lyt-2 MAb.

2.4 Preparation of anti-IL2R MAb

This monoclonal antibody from the NDS 63 cell line (Tellides 1988) was prepared by the method outlined above, with several modifications, and was then tested for its activity.

2.4.1 Production of anti-IL2R MAb

Spleen feeder cells were prepared by sacrificing a BALB/c mouse and aseptically removing its spleen into 10 mL RPMI-1640 (Gibco). A single cell suspension was produced by teasing the spleen apart with watchmakers forceps. This suspension was then irradiated the next day with 2000 rads to prevent replication. The hybridoma cells were grown in RPMI-1640 with spleen feeders (2 mL/ 10 mL) and supplemented with the following:

L-Glutamine	(BDH)	2 mM
Penicillin	(Gibco)	100 µg/mL
Steptomycin	(Gibco)	100 µg/mL
Kanamycin	(Gibco)	100 µg/mL
Heat inactivated FCS	(Gibco)	10%

Three month old (BALB/c x $DBA/2)_{F1}$ hybrid mice were injected intraperitoneally with 0.5 mL pristane and then fourteen days later received 1 x 10^6 viable hybridoma cells to their peritoneal cavity. Acites was harvested as above.

The ascites was precipitated with 18% sodium sulphate (BDH), spun at 10,000g for 25 minutes at 25 °C, and resuspended in milli-Q water at 33% the original volume. This was then dialysed for 48 hours against 25 mM Tris at pH 7.4 and run on a DEAE-Sephacel Column. The immunoglobulin was eluted off the column with a constantly increasing salt gradient (upto 25 mM Tris / 200 mM NaCl at pH 7.4). The resultant protein collection from the DEAE-Sephacel Column can been seen in Figure 2.5 and the purity of selected samples run on an electrophoresis gel is shown in Figure 2.6. Samples A, B and C were very pure with no bands

other than those of the heavy and light immunoglobulin chains. The samples between A and C were pooled and used for all experiments which required anti-IL2R MAb.

2.4.2 Assessing the activity of anti-IL2R MAb

The binding capacity of the purified anti-IL2R MAb was compared to a known sample of anti-IL2R MAb in an indirect fluorescent binding assay. The reagents used were as follows:

NRS: normal rat serum (Serotec, Kidlington, U.K.)

DAB: Dulbecco's A+B, 1 litre of distilled water +
10 tablets (BR1 4a, Oxoid Ltd., Basingstoke, U.K.) +
5 mL Dulbecco mineral salts (Oxoid Ltd.)

Wash Solution: DAB + 10% fetal calf serum (Gibco)

RPMI-Complete: RPMI-1640 + supplements (see page 39)

Formalin: wash solution + 2% Formalin (BDH)

GAM-FITC: Goat-anti-mouse IgG conjugated to
fluoroscein isothiocyanate (Sigma)

Con A: Concanavalin A (Sigma)

LOU rats were sacrificed and their cervical and mesenteric lymph nodes removed aseptically into ice cold wash solution. A single cell suspension was produced by passage of the lymph node through a wire mesh. Viable cells were counted by FDA staining (see page 31). Cells were resuspended in 10 mL RPMI-Complete at 2.5 x 10^5 cells/mL and Con A was added at 10 µg/mL and incubated at 37 °C in 5% CO_2 for 72 hours. Blast cells were then spun at 200g for 10 minutes and resuspended in wash solution.

5 x 10^5 viable cells in 50 µL were incubated with 50 µL of various monoclonal antibodies in LP3 tubes (Luckham Ltd., Burgess Hill, U.K.) at 4 °C for 60 minutes. The antibodies tested were known anti-IL2R MAb; unknown anti-IL2R MAb; a positive control, MRC OX-1 (Woollet *et al.*, 1985) which binds rat leucocyte common antigen; and a negative control, MRC OX-21, (Hsuing *et al.*, 1982). The cells were washed with 1 mL wash solution, spun at 200g for 5 minutes and the pellet resuspended. This was repeated to remove all unbound antibody from the blast cells. 50 µL of 5% GAM-FITC blocked with 10% normal rat serum was added and incubated at 4 °C for 60 minutes. The cells were then washed twice as above and fixed in formalin and stored in the dark at 4 °C.

Flow cytometric analysis was preformed by Dr. Margaret Dallman at the John Radcliffe Hospital on a Cytofluorograf 50 L with a 2150 Data Handler (Ortho Diagnostic Systems Inc., Massachucetes, U.S.A.). 10,000 cells were acquired within the region of interest (R1 of HP2) which was gated to exclude dead cells and debris. The gains setting was standardized with FluoroTrol (Ortho Diagnostic Systems Inc.) as a reference. The background fluorescence was determined with the negative control antibody. The flow cytometer then measured the fluorescence of the blast cells and hence indirectly how much primary antibody had bound to the blast cells. All batches of anti-IL2R MAb showed good binding activity.

2.5 Transplantation Techniques

2.5.1 Donor Tissue

The precise details of the source of neural grafts will be outlined in each experimental chapter. The majority of experiments, however, used less than 24 hour old (day 1) mouse or rat pups as the source of neural tissue. Animals were decapitated with a razor blade and their brains removed aseptically into iced saline. The meninges were dissected off under a Zeiss bench dissecting microscope. The appropriate piece of neural tissue was then drawn into a sterile SGE 10 μL glass syringe (Scientific Glass Instruments PTY Ltd., Australia) ready for transplantation.

2.5.2 Host Animal

Once again the precise details of the recipient animals will be described in the appropriate experimental chapter. All experiments involved transplanting into adult mice or rats.

Mice receiving neural transplants were anaesthetized with an intraperitoneal injection of a 1:10 dilution of Hypnorm[3] 0.1 mL/g (Janssen Pharmaceutical Ltd., Grove, U.K.). They were then fixed in a modified Baltimore stereotaxic apparatus and the scalp incised. The stereotaxic coordinates were determined and a small hole drilled in the skull with a dental drill above the transplantation site. The SGE syringe containing the transplant tissue was lowered through the brain to the desired implantation site. The graft was then slowly expelled at 1 μL/minute. When the

[3]Hypnorm = fentanyl citrate 0.315 mg/mL + fluanisone 10 mg/mL

graft was completely expelled, the canula was left *in situ* for 5 minutes and then slowly withdrawn pausing for 30 seconds after each millimetre. This slow process of graft placement reduced the tendency of the graft to be extruded back up the canula tract. Hæmostasis was effected, the scalp sutured and the animal left to recover in a warm chamber overnight.

Rats receiving neural transplants were anæsthetized with Valium[4] 0.025 mL/100g IP (Roche Products Ltd., Welwyn Garden City, U.K.) and a 1:5 dilution of Hypnorm 0.3 mL/100g IP in a separate syringe. Anæsthetized animals were placed in a stereotaxic instrument with the incisor bar 3.3 mm below the interaural line and their scalps incised. The stereotaxic coordinates were taken from Paxinos and Watson (1982). The transplantation procedure was the same as for mice.

[4]Valium = 10 mg diazepam in 2 mL benzyl alcohol

2.6 Sacrifice of Animals

The following reagents were used:

Heparinized saline: 20 units/mL Heparin (BDH) in 0.9% NaCl

Phosphate Buffer: A 0.2M $Na_2PO_4.2H_2O$ (BDH)

B 0.2M $NaH_2PO_4.2H_2O$ (BDH)

0.1M = 36 mL A + 14 mL B + 50 mL d.H_2O

Zamboni's Fixative: 1.8% paraformaldehyde (BDH) + 7.5% saturated Picric acid (BDH) in 0.1M phosphate buffer at pH 7.3

The vast majority of animals were sacrificed with the intention of performing indirect immunohistochemistry on their brains. Animals were deeply anæsthetized with Hypnorm and placed supine on a dissecting board. Their inferior vena cava was clamped, the right atrium cut and the left ventricle cannulated and perfused with heparinized saline until the thymus gland became white and its various lobes separated. When the thymus was flushed of all erythrocytes (and hence white in colour) it was likely that the brain's blood vessels had also been cleared.

Aluminum foil boats were prepared and filled with Tissue-Tek OCT embedding medium (Miles Laboratories Inc., Elkhart, U.S.A.). Animals were decapitated and their brains dissected out and placed in the boats. The boat was then lowered into liquid nitrogen cooled iso-Pentane (2-methylbutane, BDH) to freeze the tissue. Animals in the Dose Response Study (see section 3.3) had their spleens and cervical lymph nodes frozen in a similar manner. The blocks were then removed and stored at -20 °C until sectioning.

The *hpg* mice examined for Gonadotrophin Releasing Hormone staining, were perfused for one minute with heparinized saline and then for ten minutes with Zamboni's fixative. Their brains were removed and placed in the fixative until sectioning.

The rats which were examined for tyrosine hydroxylase staining were perfused with heparinized saline for one minute and then with 500 mL 0.1M phosphate buffer pH 7.4 with 2% paraformaldehyde (BDH) and 0.1% glutaraldehyde (BDH). Their brains were removed and stored in phosphate buffer until sectioning.

2.7 Histology

2.7.1 Indirect Immunohistochemistry

The protocol for the localization of antigens in tissue sections was adapted from a method by Barclay (1981). The reagents used were as follows:

PBS: phosphate buffered saline

BSA: bovine serum albumin (Sigma)

NRS: normal rat serum (Serotec)

NMS: normal mouse serum (Serotec)

RAM-HRP: peroxidase conjugated rabbit anti-mouse antibody (Dako Ltd., High Wycombe, U.K.)

RAR-HRP: peroxidase conjugated rabbit anti-rat antibody (Dako Ltd.)

Diaminobenzidine tetrahydrochloride (Sigma)

Sodium chloride (NaCl analar) (BDH)

Tris (hydroxy-methyl) methylamine (BDH)

12 µm sections were cut on a Leitz Kryostat 1720 and air dried for one hour on clean glass slides. Sections were either stained immediately or stored for less than 48 hours at 4 °C in plastic bags with silica gel (BDH). Stored sections were allowed to warm to room temperature for one hour before opening to prevent any condensation on the sections. A thin circle of petroleum jelly was painted around the sections to contain the various antibodies. The sections were fixed in absolute alcohol at 4 °C for 10 minutes and then washed three times in PBS for 10 minutes each. The sections were placed in 1% Hydrogen Peroxide (BDH) in PBS at room temperature for 10 minutes and washed as before. This removed any endogenous peroxidase in the section. A primary antibody was used to detect the desired antigen and then a secondary antibody (conjugated to horse radish peroxidase) which only recognizes the primary antibody was added. Thus the desired antigen was bound to the peroxidase via the primary and secondary antibodies and its presence could be revealed with an oxidase sensitive chromogen. Since many molecules of secondary antibody would bind to the primary, the signal was amplified. Care was taken to insure the secondary did not bind directly to the section causing nonspecific staining.

The primary antibodies used will be described as they are encountered in the experimental chapters. In each case, the primary was placed on the section (50 µL for mouse and 100 µL for rat brain) for one hour at 4 °C. The slides were placed in a humidity chamber to prevent the sections drying out. The sections were washed three times in PBS for 20 minutes each and then the secondary was applied as above for one hour at 4 °C. The secondary antibodies were either RAR-HRP used for mouse brains or RAM-HRP for rat brains. Mouse serum was added to the RAR-HRP to block any secondary which may have nonspecifically bound mouse

antigens. Similarly, rat serum was added to the RAM-HRP. The secondaries were prepared as follows:

2.5 µL RAR-HRP + 30 µL 10% BSA + 10 µL NMS + 7.5 µL 2M NaCl

5 µL RAM-HRP + 60 µL 10% BSA + 20 µL NRS + 15 µL 2M NaCl

The sections were washed three times in PBS for 10 minutes each. The enzyme substrate was prepared by dissolving 150 mg diaminobenzidine in 300 mL (0.14M NaCl + 0.025M Tris + 0.02% sodium azide at pH 7.6) and then activated with 100 µL hydrogen peroxide. The sections were placed in this solution for 10 minutes at room temperature and then washed three times in PBS for 5 minutes each.

Sections were then counterstained for 30 minutes in 0.5% toluidine blue (BDH) made up in 0.5% acetic acid (BDH) and then dehydrated through 70%, 95% and 100% ethanol to xylene (BDH) and mounted using DPX mountant (BDH).

2.7.2 GnRH Staining

The following antibodies were used:

Primary: anti-GnRH serum (Benoit) 1:2000 in 0.1M PO_4 buffer with 0.3% Triton X-100 (BDH)

Secondary: peroxidase conjugated sheep-anti-rabbit (Serotec) 1:200 in PO_4 buffer

The *hpg* mouse brains were removed from the Zamboni's fixative, embedded in paraffin wax (BDH), and 60 μm sections cut with a vibrotome. Deparaffined sections were placed in PBS, washed with 2% hydrogen peroxide in PBS for 5 minutes and then washed twice with PBS. Sections were incubated with the primary antibody for 48 hours at 4 °C and then washed in PBS three times. Sections were incubated with secondary for one hour at room temperature and then washed as above. The sections were revealed with diaminobenzidine, counterstained, dehydrated and mounted as described in the previous section.

2.7.3 Tyrosine Hydroxylase Staining

Rat brains were sectioned on a vibrating microtome (Bio-rad, USA) and prepared for tyrosine hydroxylase (TH) staining by a method adapted from Freund *et al.* (1985). The following reagents were used:

PBS: phosphate buffered saline

NGS: normal goat serum (Serotec)

anti-TH serum (van den Pol et al. 1984)

goat-anti-rabbit IgG (Miles Laboratories, U.K.)

Rabbit peroxidase-anti-peroxidase complex (Miles)

3,4-diaminobenzidine (BDH)

1% OsO_4 pH 7.4 (BDH)

Seventy micron thick coronal sections were obtained from the caudate-putamen and the mesencephalon at the level of the substantia nigra. The sections were rinsed three times in PBS and then preincubated in normal goat serum (Serotec) for 30 minutes. The sections were then incubated for 48 hours at 4 °C in anti-TH serum (kindly given by Dr. D. Clarke, Oxford, U.K.) diluted to 1:1600 in PBS. After rinsing, the sections were incubated in goat-anti-rabbit IgG (1:40 in PBS) for 12 hours at 4 °C, and then incubated with rabbit peroxidase complex (1:100 in PBS) for 3 hours at room temperature under constant agitation, according to the peroxidase-anti-peroxidase method (Sternberger *et al.* 1970). Diaminobenzidine was utilized as the chromogen in the visualization reaction. Finally, the sections were treated with 1% OsO_4 (pH 7.4) to enhance the staining. The sections were dehydrated through ethanol and xylene and mounted.

2.7.4 Hæmatoxylin and Eosin Staining

Sections were stained according to the method outlined in *Carleton's Histologic Technique* (fourth edition, 1967). Sections were fixed for ten minutes in absolute alcohol at 4 °C and then washed in tap water. Sections were placed in 50% Harris's hæmatoxylin 'Gurr' (BDH) for 20 seconds, washed in tap water, placed in 1% aqueous Eosin 'Gurr' (BDH) for 40 seconds and washed in tap water. Sections were dehydrated and mounted as described in section 2.7.1.

2.8 Microscopy and Photography

All black and white photomicrographs were taken on an Olympus PM-10AD Photomicrographic system using Technical Pan 100 ASA film.

Figure 2.1 **Protein Content of Anion-Exchange Column Fractions**

The protein content of the fractions collected off the anion-exchange column were determined by measuring their optical density at 280 nm. Samples of the fractions (**a-h**) were tested for their purity by SDS-PAGE.

Figure 2.2 **SDS-PAGE Electrophoresis**

Samples from the anion-exchange column (**a-h**) were tested against a standard (**s**) with known molecular weights. The second protein peak (**c-e**) was eventually used as the source of anti-L3T4 MAb.

FIGURE 2.1

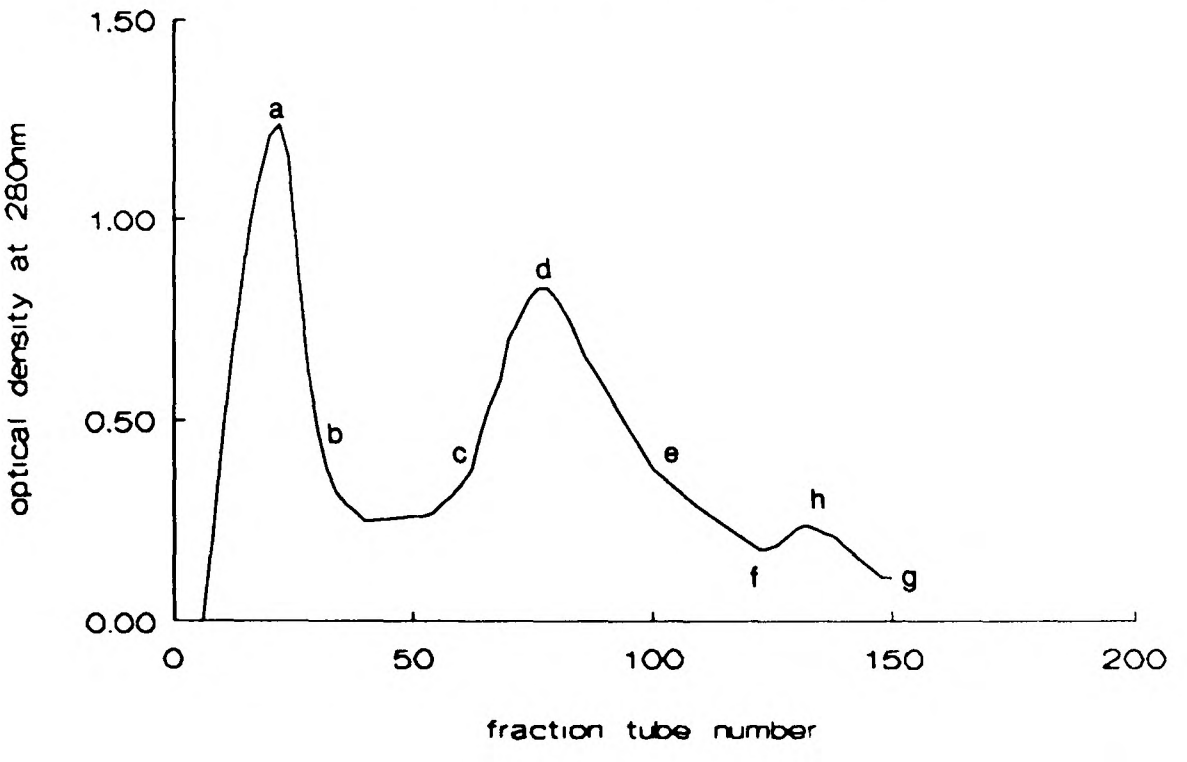


FIGURE 2.2

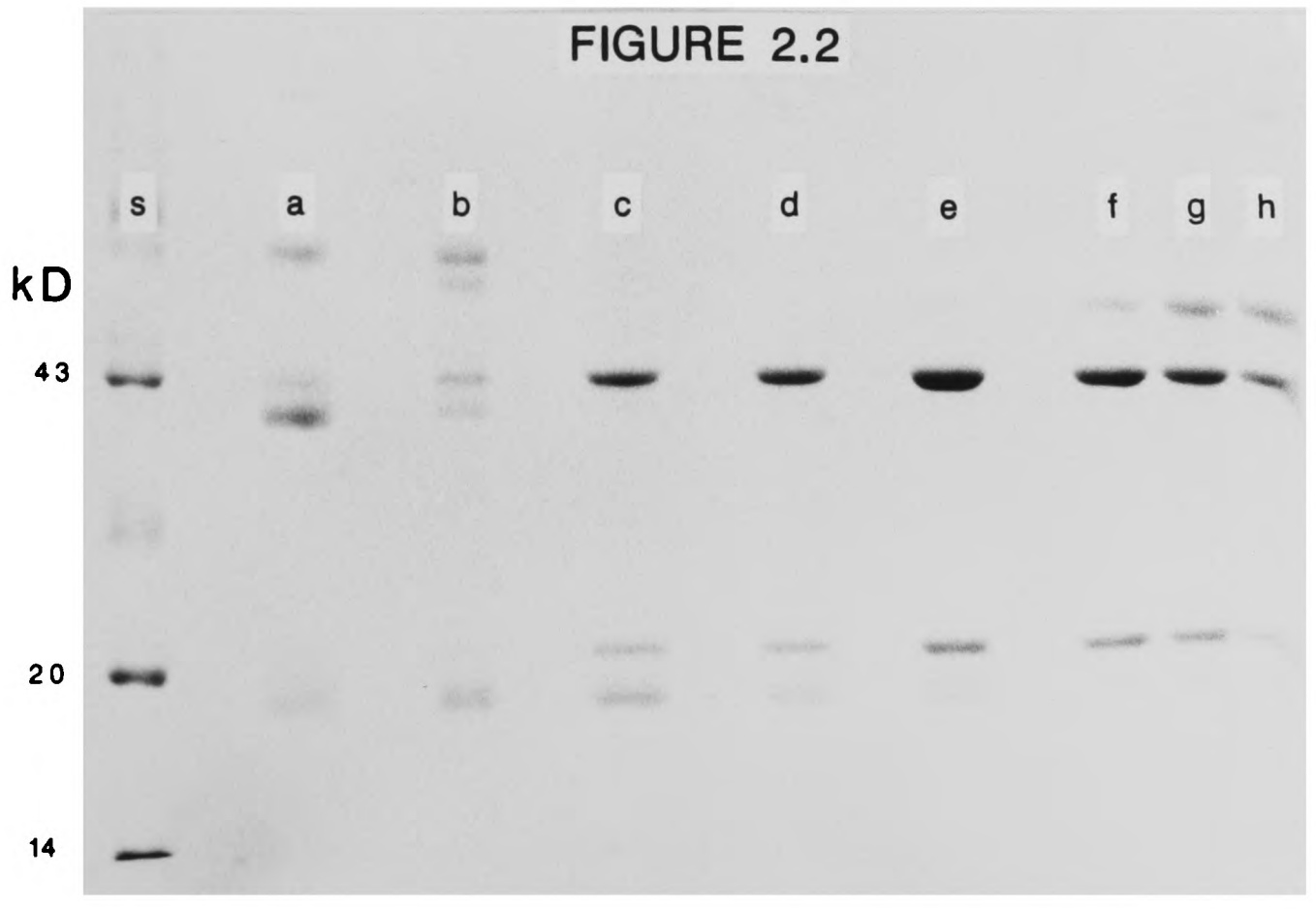


Figure 2.3 **Protein Content of Anion-Exchange Column Fractions**

The protein content of the fractions collected off the anion-exchange column were determined by measuring their optical density at 280 nm. Samples of the fractions (**a-f**) were tested for their purity by SDS-PAGE.

Figure 2.4 **SDS-PAGE Electrophoresis**

Samples from the anion-exchange column (**a-f**) were tested against a standard (**s**) with known molecular weights. The second protein peak (**c-e**) was eventually used as the source of anti-Lyt-2 MAb.

FIGURE 2.3

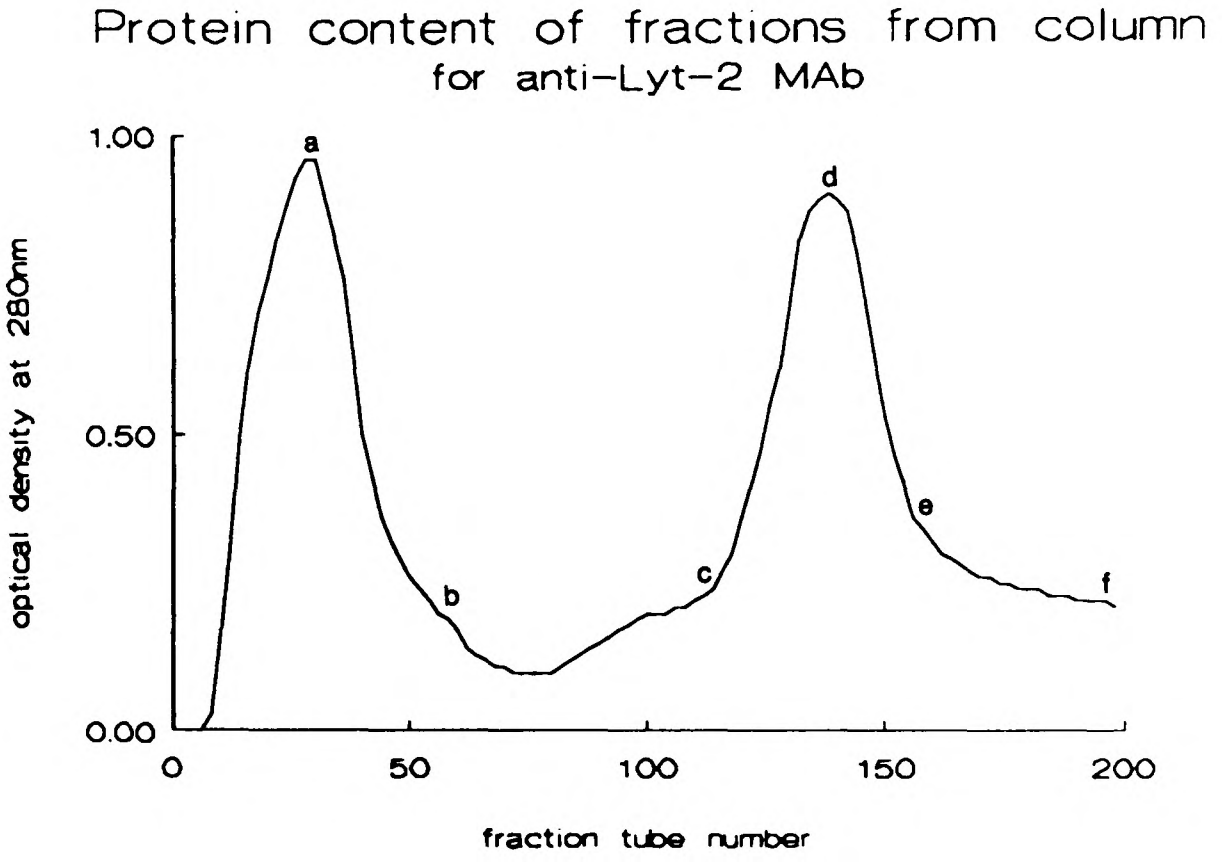


FIGURE 2.4

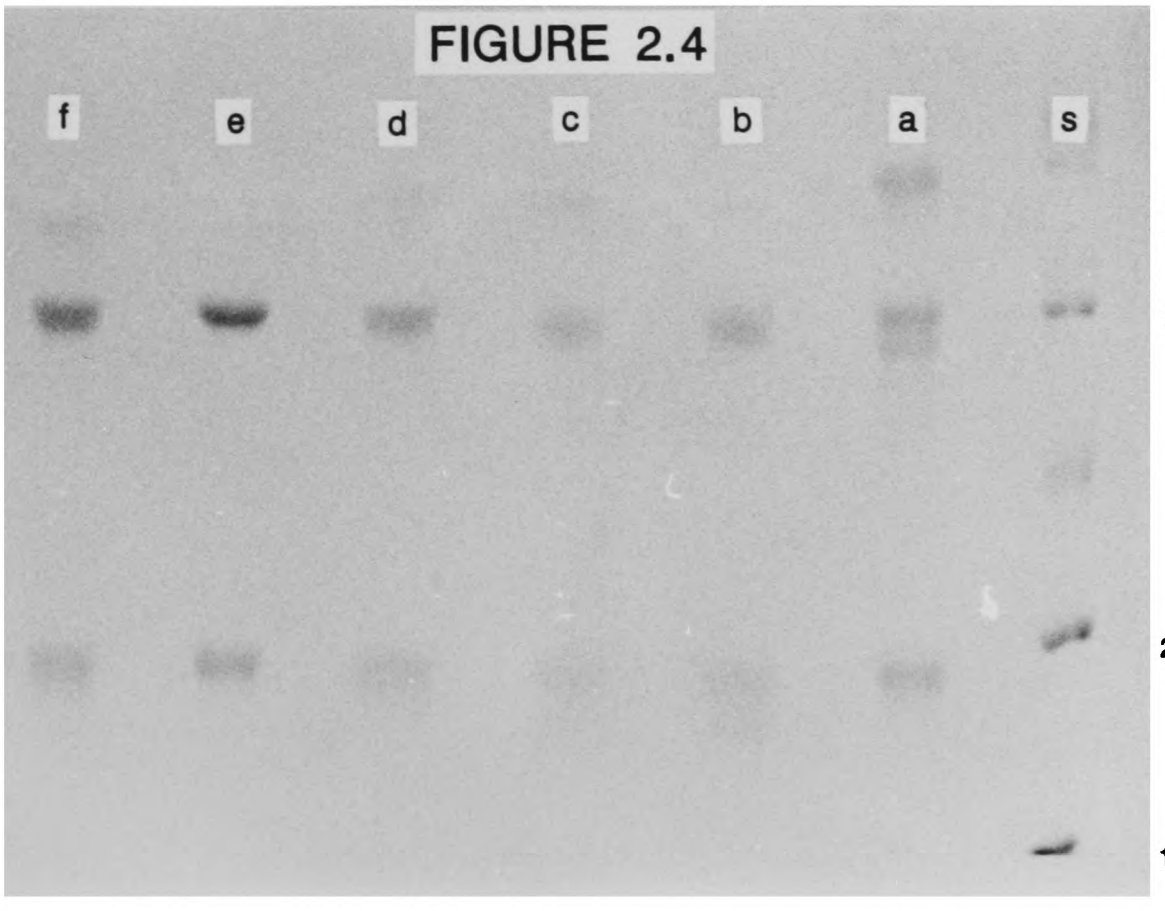


Figure 2.5 **Protein Content of Anion-Exchange Column Fractions**

The protein content of the fractions collected off the anion-exchange column were determined by measuring their optical density at 280 nm. Samples of the fractions (**a-f**) were tested for their purity by SDS-PAGE.

Figure 2.6 **SDS-PAGE Electrophoresis**

Samples from the anion-exchange column (**a-f**) were tested against a standard (**s**) with known molecular weights. The first protein eak (**a-c**) was eventually used as the source of anti-IL2R MAb.

FIGURE 2.5

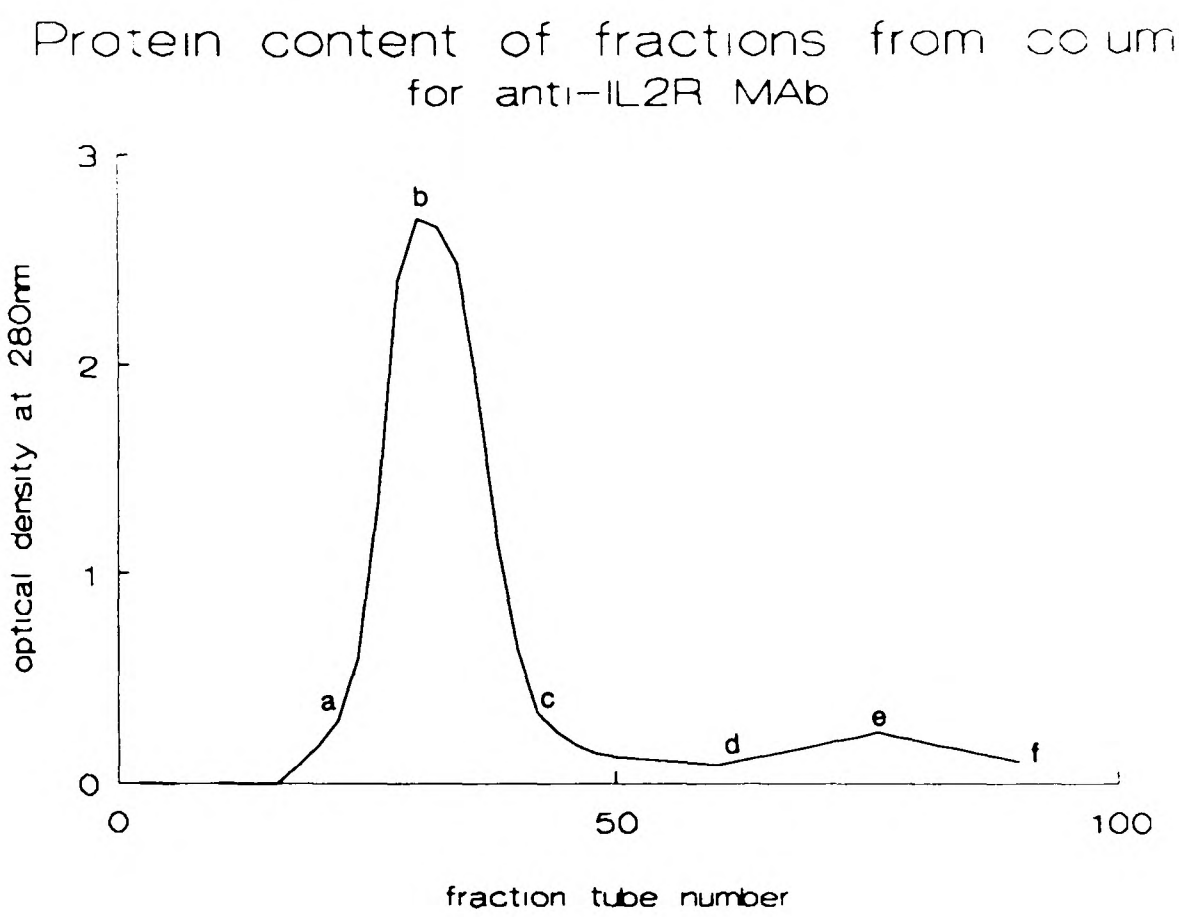
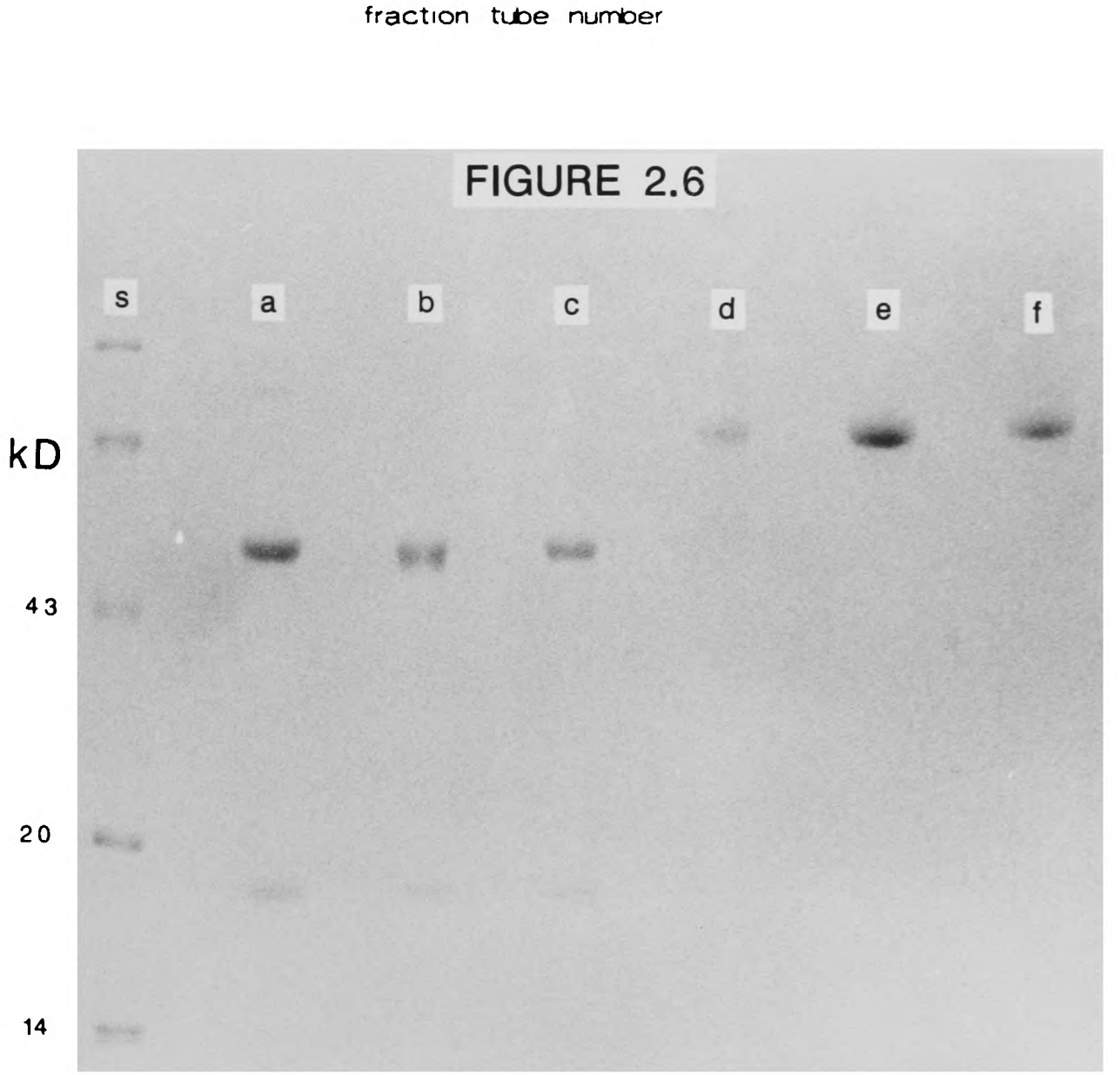


FIGURE 2.6



Chapter 3

anti-L3T4 MAb:

Pilot and Dose Response Studies

Table of Contents

3.1 Introduction

The theoretical advantages of immunosuppression with monoclonal antibodies were outlined in the Introductory Chapter. The purpose of this chapter was to determine if anti-L3T4 MAb treatment could prolong neural graft survival in mice.

The model chosen to test the immunosuppressive capabilities of anti-L3T4 MAb was rat neural xenografts transplanted into the third ventricle of mice. In his 1988 review on xenograft transplantation, Auchincloss points out that xenografts provide a greater stimulus to rejection than allografts. Lund *et al.* (1987), and work described later in this thesis, show that the tissues transplanted to the ventricles are rejected more vigorously than similar tissue transplanted to the brain parenchyma. Thus, in order to provide the greatest stimulus to rejection, and hence the greatest test for immunosuppression, this xenograft model of transplantation to the ventricle was chosen.

The third ventricle was selected since it was hoped that eventually this immunosuppressive technique could be applied in a model which could assess the physiological functioning of the graft - the *hpg* mouse model described in Chapter 5.

3.2 Pilot Study

3.2.1 Experimental Procedure

Sixty adult (older than 12 weeks), male C3H/He mice were divided into four groups of fifteen. Group 1 received thirteen consecutive daily intraperitoneal injections of 1 mL of TCS from the cell culture producing anti-L3T4 MAb. Group 2 received a similar injection protocol with TCS from the cell culture producing anti-Lyt-2 MAb. Group 3 received daily intraperitoneal injection of 1 mL saline (0.9% NaCl) for the same period. Group 4 received the same injection protocol with a total of 2 mL TCS, 1 mL from both cell cultures (anti-L3T4 MAb and anti-Lyt-2 MAb).

All animals were operated on the third day of injections. 4 μL of day 1 PVG rat cortex was stereotaxically transplanted (see 2.5) into their third ventricle using the following coordinates:

anterior: 1 mm posterior to Bregma

lateral : 0 mm from midline

vertical: 5 mm below dura

All animals were sacrificed 21 days after transplantation and their brains processed for immunohistochemistry (see section 2.7.1). Serial coronal sections through the third ventricle were stained with the following primary antibodies:

Antibody	Specificity	Reference
MRC OX-7	Thy-1.1 (rat brain)	Mason & Williams 1980
YTS 191.1	L3T4 (mouse T_h-cells)	Cobbold *et al.* 1984
YTS 169.4	Lyt-2 (mouse T_c-cells)	Cobbold *et al.* 1984
M142	mouse MHC class 1	Kennett 1980

3.2.2 Results

The transplantation technique involved lowering a canula vertically through the superior sagittal sinus into the third ventricle below (see section 2.5). This operation carried with it an overall mortality rate of 28%. Two animals from Group 1, three from Group 2, three from Group 3, and nine from Group 4 died postoperatively.

The mortality rate in Group 4 (60%) was significantly higher ($p< 0.05$ by Chi-squared Test) than any of the other groups (all ≤20%). Within Group 4, three animals died within 48 hours of operation (comparable to other operative mortality) but six died later. Most of these suffered from otitis media premorbidly. Perhaps the depletion of both L3T4-positive and Lyt-2-positive cells had left the animal's immune system unable to combat bacterial infections. The animals in Group 4 were excluded from all further studies since no immunosuppressive regime which caused a three-fold increase in death rate could be justified. Future studies with this combination could be preformed in a specific pathogen free unit.

All deaths in the remaining three groups occurred within 48 hours of operation and all animals which died (except for one anæsthetic overdose) showed evidence of devastating neurological damage such as paralysis or mydriosis. This damage was probably caused by perioperative cerebral hæmorrhage and may have

been avoided by using stereotaxic equipment capable of rotation in the coronal plane and thus avoiding the sagittal sinus during cannula placement. Unfortunately, this was not available.

Another potential cause of death could have been conduction hydrocephalus following blockage of the cerebral aqueduct by the graft in the third ventricle. This seemed unlikely since no animals had enlarged ventricles at *post mortem* and one surviving animal in Group 2 had dilated ventricles at sacrifice. Another potential cause of death could be acute elevation in intracerebral pressure caused by the addition of the graft volume to the intracranial space. This also seems unlikely since similar graft volumes to the parenchyma have very low mortality rates (see section 4.3.2).

3.2.2.1 Control Staining

Four lots of five serial sections were taken through the third ventricle to cover as much of the xenograft as possible. The first section in each lot was not stained with a primary antibody but was treated with secondary and revealed with diaminobenzidine. None of these sections showed any non-specific staining (not shown).

3.2.2.2 Thy-1.1 Staining

The second section in each lot was stained with a direct peroxidase conjugate of MRC OX-7 (Charlton, Barclay and Williams, 1983). Therefore, each animal in the study had four sections through its xenograft stained for rat brain tissue.

Figure 3.1 (1a-c) shows representative photomicrographs of Thy-1.1 stained sections from one animal in each group. The xenograft from Group 1 is strongly positive whereas the section from Group 2 and Group 3 are obviously negative.

The sections were scored as positive (+) or negative (-) depending on whether there was staining for the Thy-1.1 antigen. Table IIIa shows the scoring for the three groups of animals.

Table IIIa

animal	Group 1	Group 2	Group 3
1	++++	----	+---
2	----	----	----
3	-++-	----	----
4	+--+	----	----
5	++++	-+-+	----
6	++++	----	----
7	----	----	----
8	----	----	----
9	+++-	----	--+-
10	+--+	----	----
11	++--	----	----
12	++++		----
13	--+-		

Each animal was grouped as either positive or negative depending on whether or not it had positive staining for Thy-1.1 in any of its sections. Group 1 had more than 75% (10 out of 13) of animals with Thy-1.1 positive staining

xenografts while Group 2 had less than 10% (1 out of 11) and Group 3 had only 17% (2 out of 12). The result were significantly different ($p< 0.001$) by the chi-squared test. The difference was even more dramatic since the one positive animals in Group 2 and the two in Group 3 only had the smallest vestiges of remaining xenograft.

3.2.2.3 L3T4 Staining

The third section in each lot (four per animal) was stained for L3T4-positive cells. The third ventricle was visualized with a Zeiss microscope under high power (X 250) and the number of positive cells in and around the xenograft was counted. The diameter of the field of view was calibrated with a stage graticule (Graticules Ltd., Tonbridge, U.K.) at 0.69 mm and the cross-sectional area was therefore calculated to be 0.37 mm^2.

Figure 3.1 (2a-c) shows representative photomicrographs of L3T4 stained sections from one animal in each group. The xenograft from Group 1 has very few L3T4-positive cells whereas the sections from Group 2 and Group 3 are filled with positive cells.

The number of L3T4-positive cells per high power view in each sections was counted and displayed in Table IIIb.

Table IIIb

animal	Group 1	Group 2	Group 3
1	20,10,15,30	*, *, *, *	*, *, *, *
2	*, *, *, *	*, *, *, *	*, *, *, *
3	*,50,45, *	*, *, *, *	*, *, *, *
4	35, *, *, *	*, *, *, *	*, *, *, *
5	30,20,10, 5	*, *, *, *	20,10, 5,15
6	5,20,15, 5	*, *, *, *	*, *, *, *
7	*, *, *, *	*, *, *, *	*, *, *, *
8	*, *, *, *	*, *, *, *	*, *, *, *
9	15,40,20,75	25,30,15,10	*, *, *, *
10	*, *,80,45	*, *, *, *	35,25 10 20
11	35,70,60,85	35,15, 5,20	*, *, *, *
12	15, 5, 5,10		*, *, *, *
13	*, *, *, *		

* = cells confluent (statistics)

There was a significant difference between the means of the three groups ($p<0.01$) by analysis of variance. Individual T-Tests were preformed between pairings of each of the groups. Group 1 had fewer L3T4-positive cells in their xenografts than either Group 2 ($p<0.001$) or Group 3 ($p<0.001$). There was no significant difference between Group 2 and Group 3.

In xenografts undergoing active rejection, the grafts were often confluent with L3T4-positive cells - making counting them difficult. In order to illustrate the difference between xenografts with few L3T4-positive cells within them and those filled with these cells, the xenografts were assigned to one of two groups - those

with less than 50 cells per high power view (h.p.v.) and those with more than 50 cells/h.p.v. The percentage of grafts in each of the three experimental groups with either more or less than 50 L3T4-positive cells is shown diagrammatically in Figure 3.2.

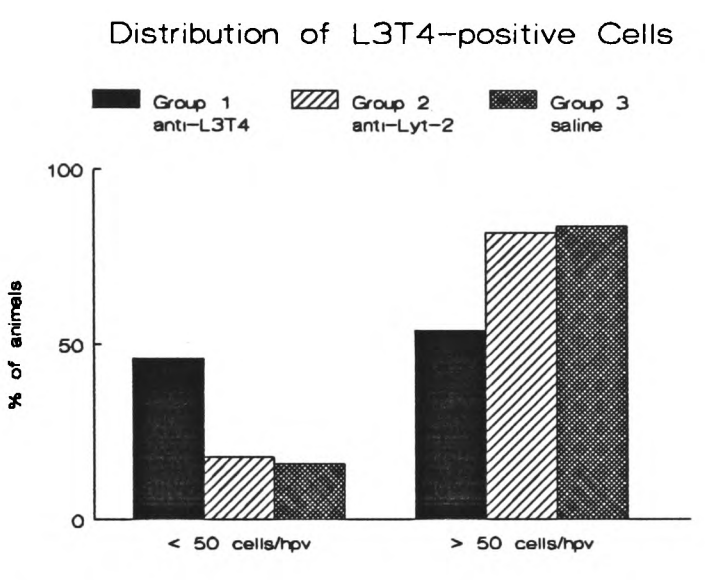


Figure 3.2

Half of the xenografts from Group 1 had fewer than 50 L3T4-positive cells per high power field, whereas atleast 80% of grafts in Group 2 and Group 3 had more than 50 positive cells in the same area.

3.2.2.4 Lyt-2 Staining

The fourth section in each lot (four per animal) was stained for Lyt-2-positive cells. Figure 3.1 (3a-c) shows photomicrographs of representative sections from one animal in each group. Positive staining cells were counted and the means of each group calculated as above. There was a significant difference between the means ($p<0.01$) by analysis of variance. Individual T-Tests were

performed between pairings of each of the groups. Group 2 had significantly less Lyt-2-positive cells in their xenografts than either Group 1 ($p<0.05$) or Group 3 ($p<0.001$). Group 1 also had significantly less positive cells than Group 3 ($p<0.001$). The percentage of grafts in each group with either more or less than 50 Lyt-2-positive cells is shown diagrammatically in Figure 3.3.

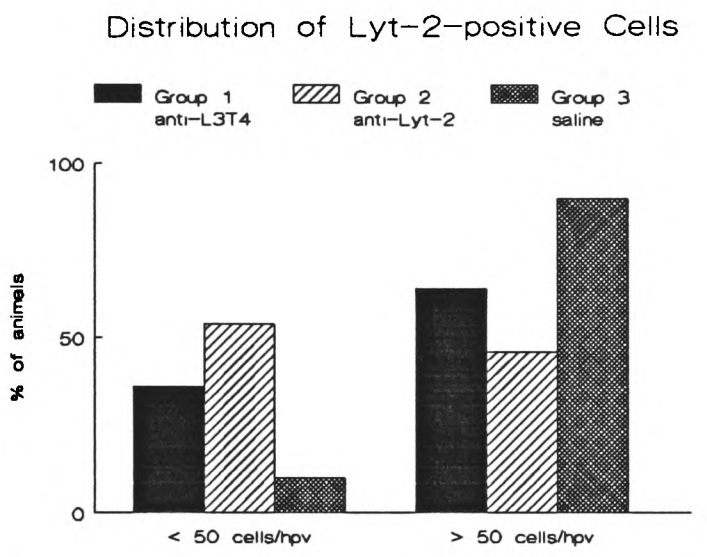


Figure 3.3

3.2.3 Discussion

The use of rat xenografts in this model had two advantages. First, it allowed species specific markers to distinguish graft from host brain. Barclay and Hyden (1978) showed the Thy-1.1 antigen is widely found on the surface membranes of central and peripheral neurons. In the mouse, the glycoprotein exists in two allotypic forms, Thy-1.1 and Thy-1.2, differing by one amino acid at residue 89 (Williams and Gagnon 1982). Charlton, Barclay and Williams (1983) demonstrated the use of monoclonal antibodies against the different allotypic forms of the Thy-1 marker to distinguish AKR mouse brain grafts (Thy-1.1) from the brains of recipient *hpg* mice (Thy-1.2). All rat strains studied by this group have been Thy-1.1-positive, thus rat neural tissue transplanted into Thy-1.2-positive mouse brains can be selectively stained with a direct peroxidase conjugate of the MRC OX-7 antibody (which binds the Thy-1.1 antigen).

The antibody, however, must be directly conjugated since the MRC OX-7 antibody itself is a mouse IgG1 antibody. The secondary antibody (Rabbit-anti-mouse) required to indirectly bind the peroxidase to an unconjugated form of MRC OX-7 would bind all mouse immunoglobulin - including the immunoglobulin found throughout the surrounding host mouse brain! The primary antibody would stain the rat brain graft but it would be obscured by the background staining of the surrounding mouse brain. Directly conjugating the peroxidase to the primary antibody removes this problem.

The second advantage of this model is that the rat xenograft is a very strong stimulus for rejection and hence provides a rigorous test for any immunosuppressive regime. Provided the species are not vastly disparate, xenografts provide a greater stimulus to rejection than allografts. In a model which measures graft survival and

not graft function, it is difficult to quantify how well a graft has survived. Graft histology can be measured by various scales and differentiated with statistics but this is subject to observer bias and arbitrary scales. It is easier to determine whether a graft has survived or not. This makes observations less subjective and the statistics simpler. Using a xenograft model increases the likelihood that control animals will not have surviving grafts. Thus if experimental animals do have surviving grafts, the treatment must be having a dramatic effect.

Xenograft survival was inferred by positive Thy-1.1 staining. The presence of the Thy-1.1 surface glycoprotein does not prove the graft was functioning but its absence was indicative of a non-functioning or destroyed graft. In order to demonstrate graft function, the *hpg* mouse model was used in later experiments (see Chapter 5). It then became clear that positive Thy-1.1 staining did indeed correlate with rat xenograft function.

Figure 3.1 shows the dramatic difference between xenograft survival in animals treated with anti-L3T4 MAb and those given anti-Lyt-2 MAb or saline. 75% of animals given anti-L3T4 MAb had surviving grafts whereas only one animal given anti-Lyt-2 MAb and two animals given saline had any hint of Thy-1.1 staining. It was clear that the anti-L3T4 MAb was a very good immunosuppressive agent.

Sections of the xenografts were stained and analyzed to see if the anti-L3T4 MAb and the anti-Lyt-2 MAb had depleted $L3T4^+$ and $Lyt\text{-}2^+$ cells respectively. Figure 3.2 demonstrates that xenografts from animals treated with anti-L3T4 MAb had significantly fewer L3T4-positive cells than xenografts in animals given anti-Lyt-2 or saline. This is not surprising since the anti-L3T4 MAb, YTS 191.1, is designed specifically to deplete L3T4-positive cells.

Figure 3.3 shows the percentage of xenografts in each group with more or less than 50 L3T4-positive cells per high power view. A graft which is not being rejected would be more likely to have fewer T-cells within it. Once the host animal's immune system had begun to reject the graft, however, the graft would likely be flooded with cells of the immune system. In this model, the number of T-cells in a given graft probably fell into two categories (rather than one gradually increasing gradient) depending on whether the graft is being rejected or not.

Figure 3.3 attempts to delineate these two categories by including all grafts with less than 50 cells per high power view as one category (little or no rejection) and all grafts with more then 50 cells per high power view as another category (rejection). Table IIIb shows that there were very few grafts which had between 50-100 cells/h.p.v. It appeared that once a graft had 50 cells/h.p.v., it was soon flooded with cells - a sure sign of graft destruction.

Table IIIa and IIIb shows a strong correlation between grafts with few L3T4-positive cells and grafts with Thy-1.1-positive staining. In Group 1, all sections which had less than 50 cells/h.p.v. had Thy-1.1-positive staining, whereas no sections with more than 50 cells/h.p.v. had any Thy-1.1 staining. In Group 2 and Group 3, there were sixteen sections with less than 50 cells/h.p.v. but no Thy-1.1 staining. Once the graft had been destroyed, there was no longer any stimulus for cells of the immune system to remain and these sections represented the dwindling number of residual cells after a graft had been destroyed. In order to avoid the confusion between a graft with few cells because it has not been rejected and a graft with few cells because it has long since been rejected, a new category was added in future experiments to include grafts which had been rejected. Thus

Figures in future experiments will included three groups: less than 50 cells/h.p.v., more than 50 cells/h.p.v., and no graft.

Figure 3.4 demonstrates that xenografts from animals treated with anti-Lyt-2 MAb had significantly fewer Lyt-2-positive cells than xenografts in animals given anti-L3T4 or saline. This was not surprising since the anti-Lyt-2 MAb treatment, with YTS 169.4, was designed specifically to deplete Lyt-2-positive cells. What was interesting was that the xenografts in Group 2 were rejected despite having fewer Lyt-2-positive cells than those in Group 1. In Group 2, the remaining cytotoxic T-cells, activated by the abundant helper T-cells, were able to effect graft rejection. In Group 1, the more abundant cytotoxic T-cells were unable (or slower) to reject the xenograft. Perhaps the helper T-cells, known to be able to directly destroy foreign tissue with alpha-lymphotoxin (see Figure 1), significantly aided in the destruction of xenografts in Group 2.

Animals in Group 1, depleted of L3T4-positive cells, also had fewer Lyt-2-positive cells in their xenografts than animals given saline. This was consistent with the theory outlined in the Introductory Chapter that helper T-cells (L3T4-positive) are required for the proliferation of cytotoxic T-cells (Lyt-2-positive).

In summary, the anti-L3T4 MAb treatment showed excellent promise as an effective immunosuppressive agent. There were, however, two immediate problems with the pilot experiment. First, there was not 100% xenograft survival in the anti-L3T4 MAb treated group. Indeed, the xenografts were filled with cytotoxic T-cells (section 3.2.2.3). It appeared that the xenograft rejection had been slowed but not ablated in this group. Perhaps this problem could be overcome by using higher

concentrations of anti-L3T4 MAb. This was the genesis of the Dose Response Experiment outlined in the following section.

Second, the method of scoring xenograft survival (ie. any Thy-1.1 staining in the graft) had shown survival in three control animals. This was misleading since there was only a hint of staining in these sections and the grafts were not really surviving but were just not yet completely destroyed. In order to overcome this problem, the xenografts were allowed to remain *in situ* for four weeks instead of three, by which time it was hoped that the last vestiges of Thy-1 staining would have been destroyed in all the control xenografts.

3.3 Dose Response Study

3.3.1 Experimental Procedure

In the Pilot Study, the monoclonal antibodies were given as intraperitoneal injections of 1 mL tissue culture supernatant (TCS). The concentration of anti-L3T4 and anti-Lyt-2 in their respective TCS was 32 μg/mL and 40 μg/mL (see Appendix). Therefore the animals in Group 1 received 32 μg anti-L3T4 MAb each day for thirteen days.

In order to standardize the dosage for each animal, the purified monoclonal antibodies (see section 2.2) were given according to weight. Three dosages were used:

A: 2 μg/g body weight/day (≈ 50 μg/animal/day)

B: 4 μg/g body weight/day (≈ 100 μg/animal/day)

C: 8 μg/g body weight/day (≈ 200 μg/animal/day)

280 adult (older than 12 weeks) male C3H/He mice were divided into seven groups of forty. Each animal received thirteen consecutive daily intraperitoneal injections of antibody (Groups 1 and 2) or saline (Group 3) as follows:

Group 1A: anti-L3T4 MAb 2 μg/g/day

Group 1B: anti-L3T4 MAb 4 μg/g/day

Group 1C: anti-L3T4 MAb 8 μg/g/day

Group 2A: anti-Lyt-2 MAb 2 μg/g/day

Group 2B: anti-Lyt-2 MAb 4 μg/g/day

Group 2C: anti-Lyt-2 MAb 8 μg/g/day

Group 3 : saline 0.02 mL/g/day

All antibodies were diluted in sterile phosphate buffered saline such that a typical 25g mouse received a 0.5 mL injection (ie. 100 µg/mL for Group 1A). The effect of the monoclonal antibody treatment on the animals was assessed by quantifying mortality rates, following weight gain and observing renal histology.

All animals received neural xenografts to their third ventricle from day 1, male, PVG rat pup cortex on the third day of injections as described in the Pilot Study. Animals were sacrificed at 4, 8, and 12 weeks after transplantation and their brains processed for immunohistochemistry.

Serial coronal sections through the third ventricle were stained with the following primary antibodies:

Antibody	Specificity	Reference
MRC OX-7	Thy-1.1 (rat brain)	Mason & Williams 1980
30H12	Thy-1.2 (mouse brain,T-cells)	Ledbetter & Herzenberg 1979
YTS 191.1	L3T4 (mouse T_h-cells)	Cobbold *et al.* 1984
YTS 169.4	Lyt-2 (mouse T_c-cells)	Cobbold *et al.* 1984
F4/80	macrophages	Austin & Gorden 1981
M142	mouse MHC class 1	Kennett 1980

In order to determine the extent of T-cell depletion by the monoclonal antibody treatment, the animals' spleens and cervical lymph nodes were removed at sacrifice and prepared for immunohistochemistry (see section 2.6).

6 μm cryostat sections of spleen and lymph node were air dried onto glass slides for one hour. Sections were fixed in acetone (BDH) at room temperature for ten minutes, air dried and then washed three times in PBS for ten minutes each. The primary antibodies used for staining were as follows:

30H12	Thy-1.2 (all mouse T-cells)	Ledbetter & Herzenberg 1979
YTS 191.1	L3T4 (mouse T_h-cells)	Cobbold *et al.* 1984
YTS 169.4	Lyt-2 (mouse T_c-cells)	Cobbold *et al.* 1984

The sections were incubated with primary and then secondary antibody (rabbit-anti-rat IgG conjugated to peroxidase), revealed and mounted as described in section 2.7.1. The area of staining in spleen and lymph node sections were quantified using a Kontron Image Processing System (Watford, U.K.).

3.3.2 Results

3.3.2.1 Animal Survival

The Pilot Study showed that transplantation to the third ventricle carries with it a significant operative mortality. It is important to determine if the immunosuppressive treatment increased the risk of mortality. Animal deaths were divided into two groups i) perioperative: up to 48 hours postoperative, and ii) nonoperative: after 48 hours.

Group 3, which received no immunosuppression, had a 20% perioperative mortality and a 5% nonoperative mortality. Only Group 2C had more deaths but comparison by chi-square analysis with Group 3 showed no significant difference.

All animals were weighed to see if the monoclonal antibody treatment had any effect on weight gain. Figure 3.4 shows mean weight gain for animals given saline (Group 3) or the maximum amounts of monoclonal antibodies (Group 1C and 2C).

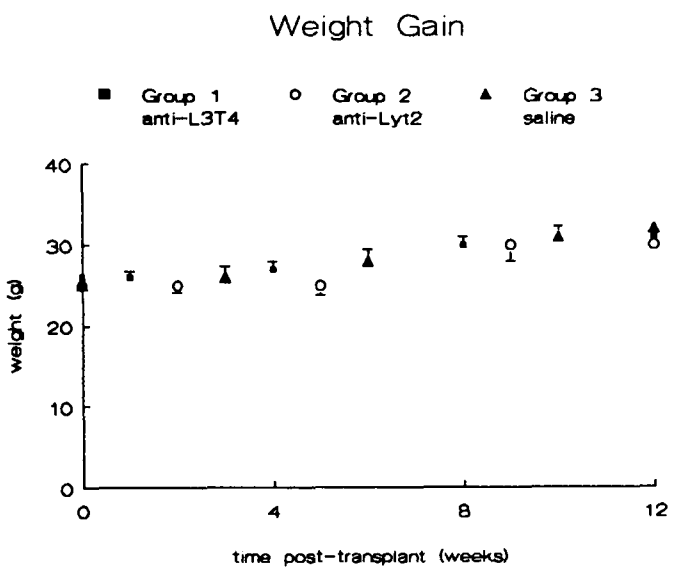


Figure 3.4

Analysis of variance showed no significant difference between any of the groups mean weight at any time point.

The kidneys from representative animals in each group were frozen, sectioned and stained with hæmatoxylin and eosin (see section 2.7.4). Sections were examined microscopically for any signs of renal damage. Kidneys from all groups were healthy and showed no signs of glomerulonephritis or nonspecific damage from immune complex deposition. Figure 3.5 shows the renal histology from animals treated with the maximum dose of anti-L3T4 MAb, anti-Lyt-2 MAb and from an animal given saline.

3.3.2.2 Xenograft Survival

Four lots of seven serial sections were taken through the third ventricle to cover as much of the xenograft as possible. The first section in each lot was processed for immunohistochemistry without applying a primary antibody. None of these control sections showed any staining. The second section in each lot (four per animal) was stained for rat brain tissue with a direct conjugate of the MRC OX-7 antibody. No animal given anti-Lyt-2 MAb or saline showed any positive staining. Figure 3.6 shows representative photomicrographs of the xenografts four weeks post-transplant in animals which received different dosages of anti-L3T4 MAb. Animals were categorized as either positive (+) or negative (-) depending on whether or not there was Thy-1.1 staining in any of four sections of xenograft. The results are shown in Table IIIc.

Table IIIc

	Group 1A	**Group 1B**	**Group 1C**
4 weeks	+++++	+++++	+++++
	+++--	+++++	+++++
8 weeks	-----	+++++	++++-
	-----	-----	-----
12 weeks	-----	++---	++---
	-----	-----	-----

There was no significant difference between the groups at four weeks using the chi-squared test. Group 1B and 1C, however, were clearly better than Group 1A at eight and twelve weeks. Group 1C appeared no better than Group 1B at any time points. It was decided to use the dosage from group 1B (4 μg/g animal/day) for all future experiments using anti-L3T4 MAb. This dosage would maximize neural graft survival and conserve antibody use.

The third section in each lot (four per animal) was stained for Thy-1.2. The Thy-1.2 antigen is found on all mouse T-cells (Schlesinger and Yron 1969) and on mouse brain (Moore *et al.* 1971). Positive cells were counted as in the Pilot Study (see section 3.2.2.2) and xenograft sections were allocated to one of three groups: xenografts with less than 50 cells/h.p.v; xenografts with more than 50 cells/h.p.v.; or no surviving xenograft. Figure 3.7 shows the results diagrammatically for Group 1B, 2B and 3.

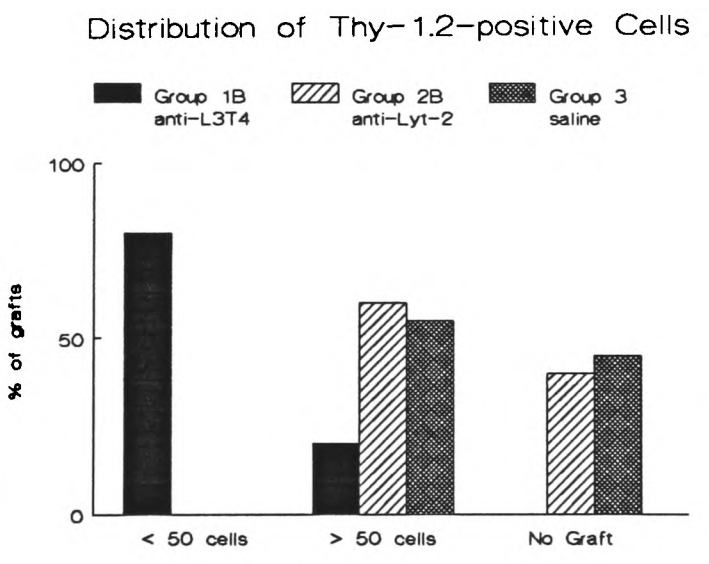


Figure 3.7

The fourth and fifth section in each lot (four per animal) were stained for L3T4-positive and Lyt-2-positive cells respectively (see Figure 3.8 for representative photomicrographs). Similar analyses were performed and the results are shown diagrammatically below in Figure 3.10 and Figure 3.11.

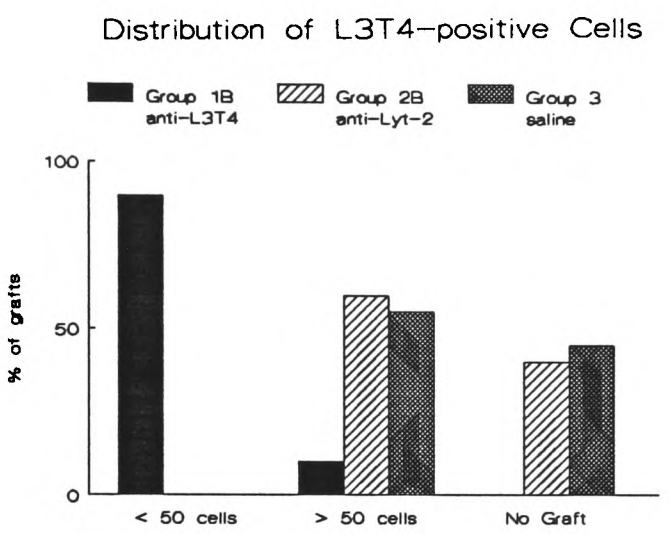


Figure 3.10

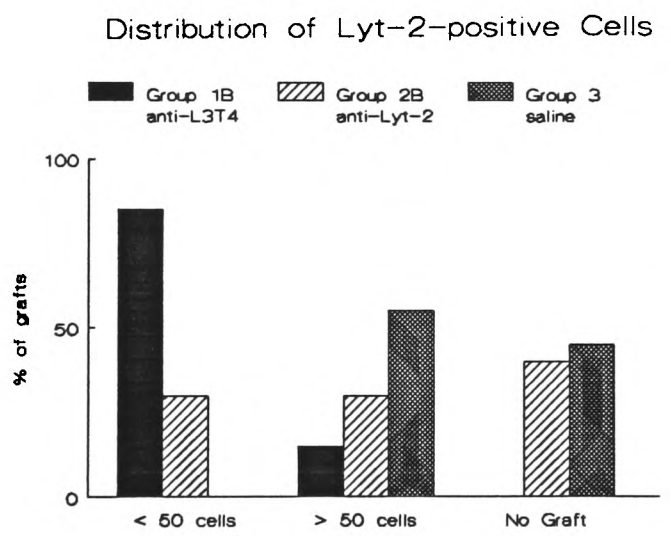


Figure 3.11

Figure 3.9 shows sections stained for macrophages and for mouse MHC Class 1 from representative animals in Group 1B, 2B, and 3. The host mouse brain in the animals treated with anti-L3T4 shows fewer macrophages within it and less induction of MHC Class 1 antigen compared to the other groups.

3.3.2.3 T-cell Depletion

The spleens and lymph nodes from animals in Groups 1B, 2B, and 3 (at 30 days post-transplant) were stained for L3T4-positive cells and displayed in Figure 3.13. It is clear that the animal treated with anti-L3T4 MAb (Group 1B) had very little staining for L3T4-positive cells compared to the animal treated with anti-Lyt-2 MAb (Group 2B) or given saline (Group 3).

The area of strong staining was measured using an image analyzer and calculated as a percentage of the total area of the section. These percentage areas were then compared to the normal percentage areas in untreated animals and displayed in Figure 3.14.

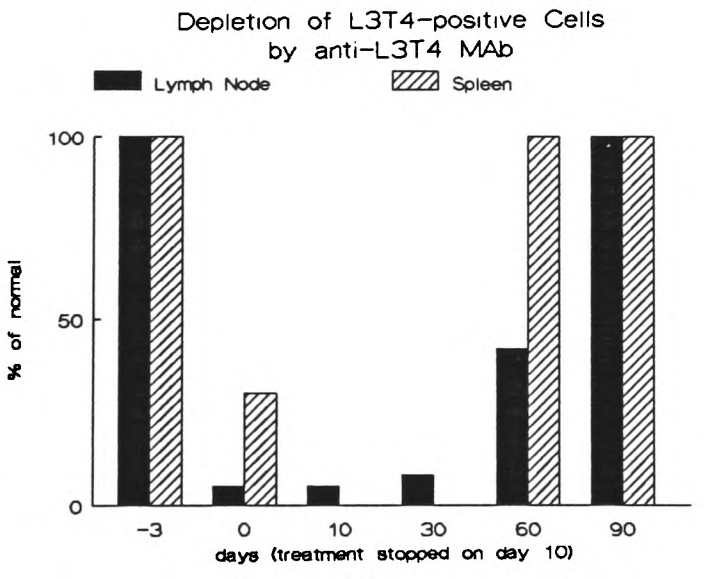


Figure 3.14

T-cells tend to occupy specific areas of the spleen and lymph node. In the spleen, the periarteriolar lymphoid sheath has a T-cell area around the central arteriole while B-cells are found beyond this zone (Roitt, Brostoff and Male, 1985). In the spleen, the T-cells are found in the paracortex while B-cells occupy the cortex (Roitt, Brostoff and Male, 1985). As T-cells were removed by antibody treatment, these T-cells areas (and hence the area of strong staining) tended to get smaller. The premise of this method was that the area of strong staining for a specific cell type was proportional to the number of those cells in the section. This may be true only for large numbers of cells. At low cell numbers, the density of cells in the T-cell zone was too low to show staining above background levels. There were T-cells present (they could be seen at high power) but there was no discrete area of strong staining. Thus, the method can not detect very low levels of T-cells.

In order to precisely quantify T-cell numbers, the cell populations of spleen and lymph nodes could be analyzed by a fluorescence activated cell sorter. Unfortunately, this was not available. The method outlined does give an excellent qualitative assessment of T-cell depletion over time.

The depletion of Lyt-2-positive cells is shown in Figure 3.15.

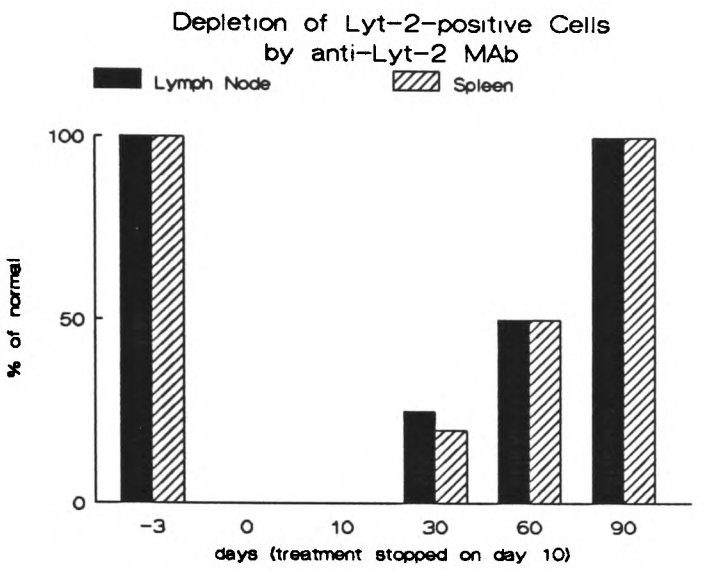


Figure 3.15

The method used did not show a difference in T-cell depletion between the various dosages of antibody used.

Another premise of this method was that the absence of specific staining for T-cells meant they were not present. In fact, the T-cell could still have been present - but just without its identifying surface antigen. For example, if helper T-cells lose their L3T4 surface antigen, then staining for L3T4-positive cells would show no cells. It could be argued that without their L3T4 surface antigen, however, helper T-cells are not functional and thus one is quantifying functional cells with this method.

An answer to this question of 'cell depletion or antigenic modulation' came by staining for a different antigen present on the cell. Figure 3.16 shows the area of staining for the Thy-1 antigen was reduced with anti-L3T4 MAb treatment.

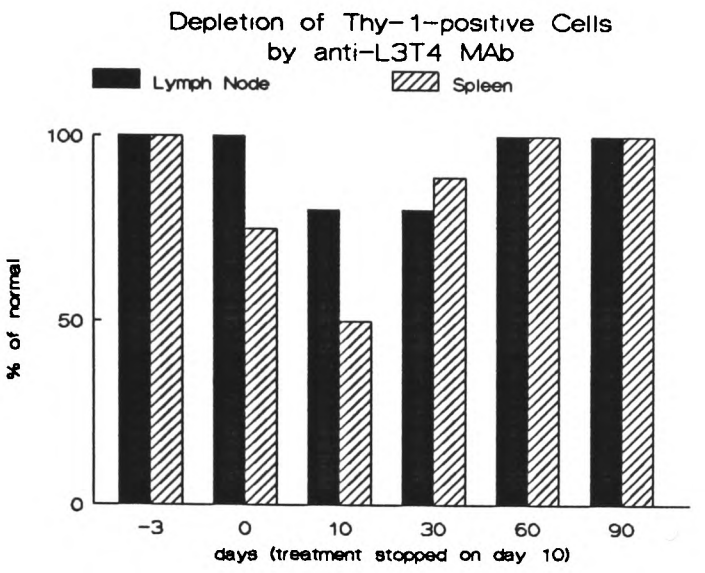


Figure 3.16

It was very unlikely that the anti-L3T4 MAb could modulate the Thy-1 antigen, thus reduced staining did correlate with cell reduction. The area of Thy-1-positive staining was not reduced to zero since both cytotoxic and helper T-cells have the Thy-1 antigen.

3.3.3 Discussion

The main purpose of this chapter was to determine if anti-L3T4 MAb could be used as an effective immunosuppressive agent for mice receiving neural grafts. It is clear that it could.

It was important to determine if the anti-L3T4 MAb treatment had any deleterious effects on the mice. There would be no point in using this technique if all the animals died, albeit with their neural grafts intact (as had happened with Group 4 in the Pilot Study). In order to address this question, the mice were examined for three indicators of health: death rate, weight gain and renal histology. Animals given the maximum dose of anti-L3T4 or anti-Lyt-2 MAb showed no

significant difference in death rate or weight gain compared to the control animals given saline.

Dixon (1969) has suggested that glomerulonephritis in humans can be due to the deposition of antigen-antibody complexes in the glomerular filter which then bind complement and attract neutrophils. This occurs when there is moderate antigen excess to antibody since small aggregates are trapped in the glomerular filter. Poststeptococcal and membranous glomerulonephritis may be examples of such immune complex renal disease. When there is equivalent or excess antibody to antigen, the large aggregates formed are taken up by the reticuloendothelial system.

The anti-L3T4 MAb was designed to form complexes with L3T4-positive cells and be cleared by the reticuloendothelial system. There was the possibility that small aggregates could have been trapped in the glomerular filter and triggered glomerular inflammation. The signs of glomerulonephritis range from tubular casts with proteinuria and hæmaturia to swollen, hypercellular glomeruli with anuria (Anderson 1971). The renal sections examined showed no signs of glomerulonephritis.

The monoclonal antibody treatment showed no deleterious effects on the mice. This may be one of its greatest assets since the current forms of chemical immunosuppression in mice (eg. cyclosporin) have a notoriously high mortality rate.

It was clear that the anti-L3T4 MAb treatment was not harmful, but was it effective? The photomicrograph of xenograft survival at thirty days (Figure 3.6) showed that it was indeed very effective at prolonging neural xenograft survival. Table IIIc showed that 100% of xenografts could be expected to survive thirty days and 50% survive sixty days post-transplant.

The next question addressed was were the surviving xenografts 'healthy'? A healthy graft was considered one which did not have any signs of ongoing rejection. The signs of rejection assessed were i) graft infiltration with T-cells and macrophages ii) induction of MHC Class 1 antigen on host brain. The animals treated with anti-L3T4 MAb showed significantly fewer Thy-1.2, L3T4, Lyt-2, and appeared to have fewer F4/80-positive cells in their xenografts than either control group.

Wong *et al.* (1984) reported that:

> '...cells in the brain express undetectable levels of the molecules encoded by the genes of the major histo-compatibility complex...'

She continued to demonstrate enhanced expression of these antigens on brain cells when exposed to a T-cell lymphokine, gamma-interferon. Gamma-interferon is produced by activated T-lymphocytes (Steeg 1982). Several authors have used the level of MHC class 1 (Mason *et al.*, 1986) and class 1 and 2 (Date, Kawamura, and Nakashima, 1988) expression on donor neural grafts as an indication of the host immune system activation. It seems likely that the gamma-interferon produced would also induce MHC Class I antigen expression on the host brain. Sloan (personal communication) has corroborated this in his doctoral thesis on rat neural allografts. The animals treated with anti-L3T4 MAb showed less induction

of MHC class I antigens around their grafts. At thirty days post-transplant, then, all surviving xenografts appeared 'healthy'.

This last statement raised two questions. How was xenograft survival prolonged and why was the effect not permanent? The treatment was designed to deplete the crucial cell of acute transplant rejection - the helper T-cell. The results from section 3.3.2.2 show that the anti-L3T4 MAb treatment was indeed effective at depleting L3T4-positive cells. The helper T-cell carries the L3T4 surface antigen and was therefore depleted. While there was sufficient depletion of the helper T-cell, there was good xenograft survival. Figure 3.14 shows the secondary lymphoid organs were repopulating with L3T4-positive cells at around 30 to 60 days - the same time the grafts were beginning to be destroyed.

It was hoped that the new helper T-cells repopulating the secondary lymphoid organs would be tolerant to the xenograft and thus the graft would survive permanently (Waldmann 1989). This was not the case. The possible reasons for this are complex and quickly transgress from known to theoretical immunology.

The simplest reason for the failure of longterm tolerance is that either not all the helper T-cells were depleted or other cells (L3T4-negative) were capable of xenograft rejection. The method of detecting T-cell population in lymph node and spleen was not sensitive to low numbers as outlined in section 3.3.2.2. There may very well have been a small population of remaining cells. Work by Marrack and Kappler (1975) and Janeway (1975) have suggested that a small subpopulation of T-cells in man, capable of helper/inducer functions are T4-negative. If similar cells exist in the mouse, they would not have been depleted by the anti-L3T4 MAb treatment.

In conclusion, the anti-L3T4 MAb treatment proved to be successful at prolonging xenograft survival in the mouse. Perhaps, if a neural graft with a less potent stimulus to rejection was used, this treatment could induce very longterm graft survival or even tolerance. This was the origin of the next series of experiments: *Neural Allografts in Mice* and is discussed in the following chapter.

Figure 3.1 Histology of Third Ventricular Xenografts

1 a-c **Thy-1.1 Staining**

Xenografts from animals treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). Only the anti-L3T4 MAb treated animal had positive Thy-1.1 staining (seen as an even brown staining within the ventricle). *x116*

2 a-c **L3T4 Staining**

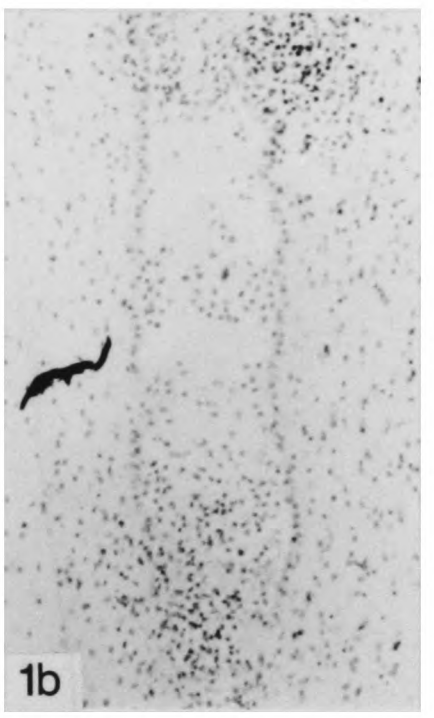
Xenografts from animals treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The xenografts in **b** and **c** were filled with L3T4-positive cells (which stained as black cells larger than the background nuclei). The xenograft in **a** had few of these cells. *x116*

3 a-c **Lyt-2 Staining**

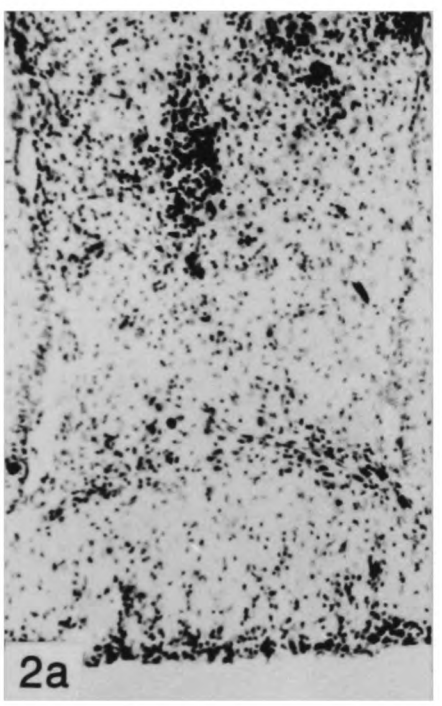
Xenografts from animals treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The xenografts in **b** and **c** were filled with Lyt-2-positive cells whereas the xenograft in **a** had few of these cells. *x116*

FIGURE 3.1


1a


1b


1c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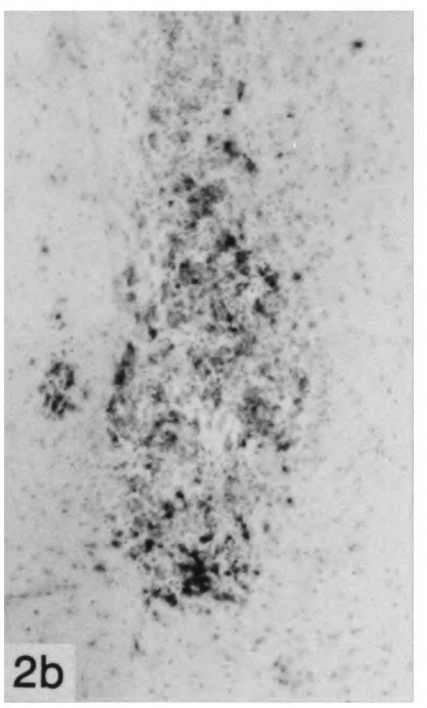
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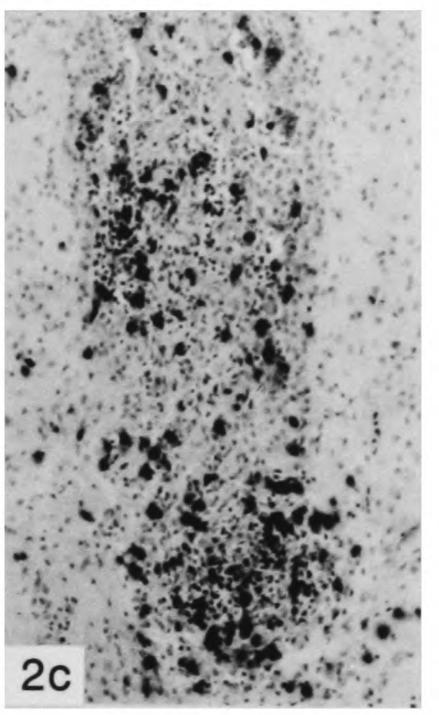
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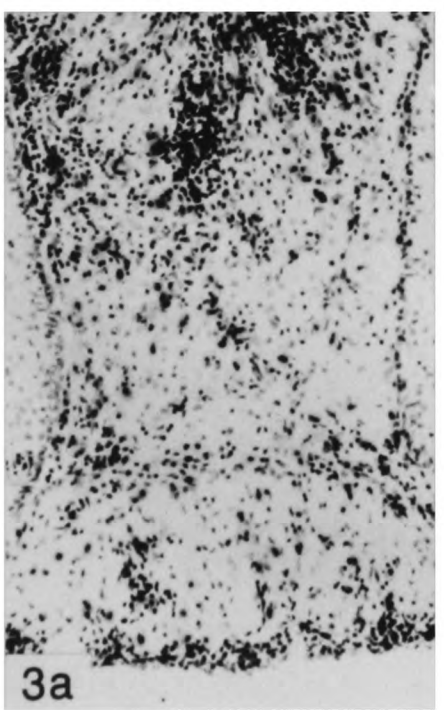
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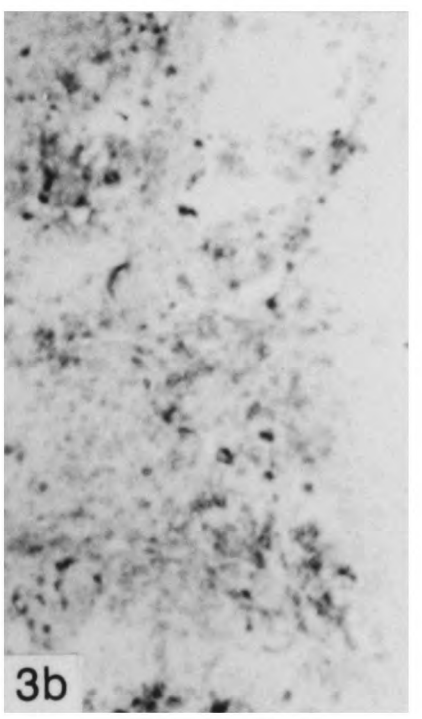
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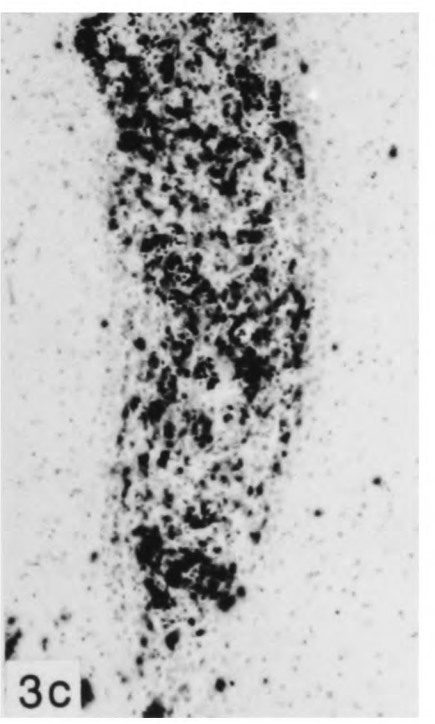
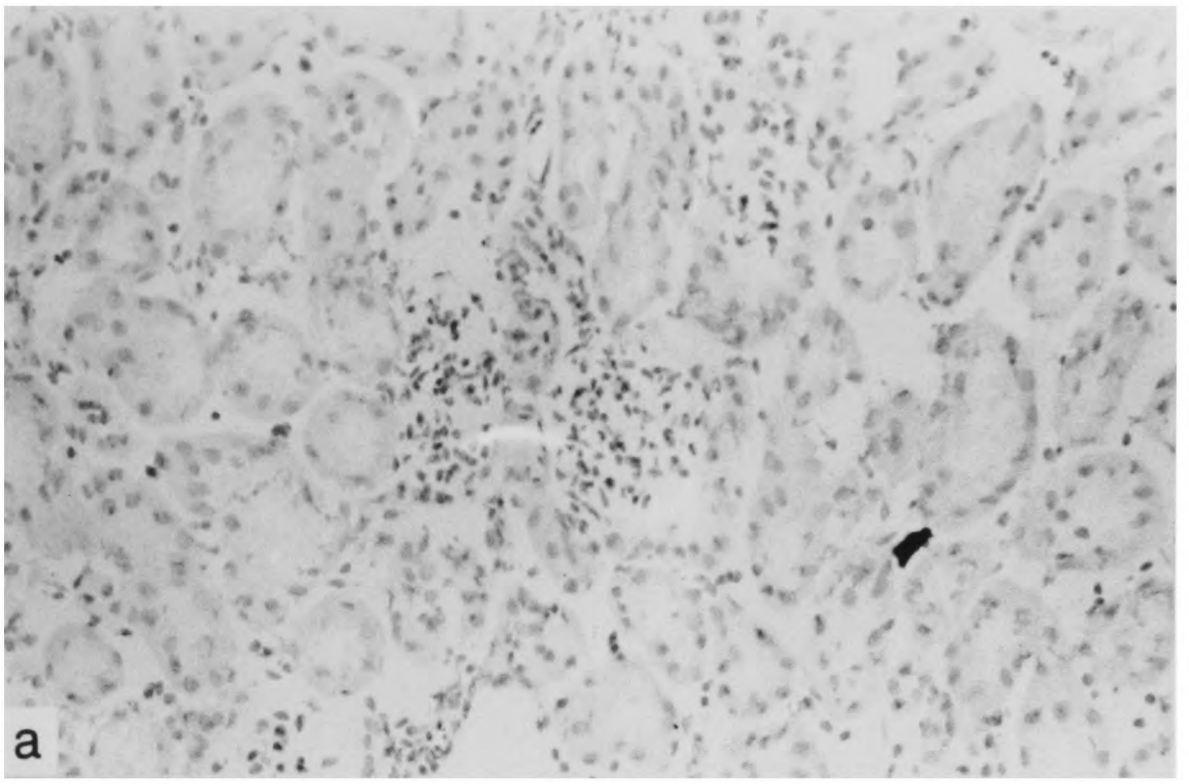
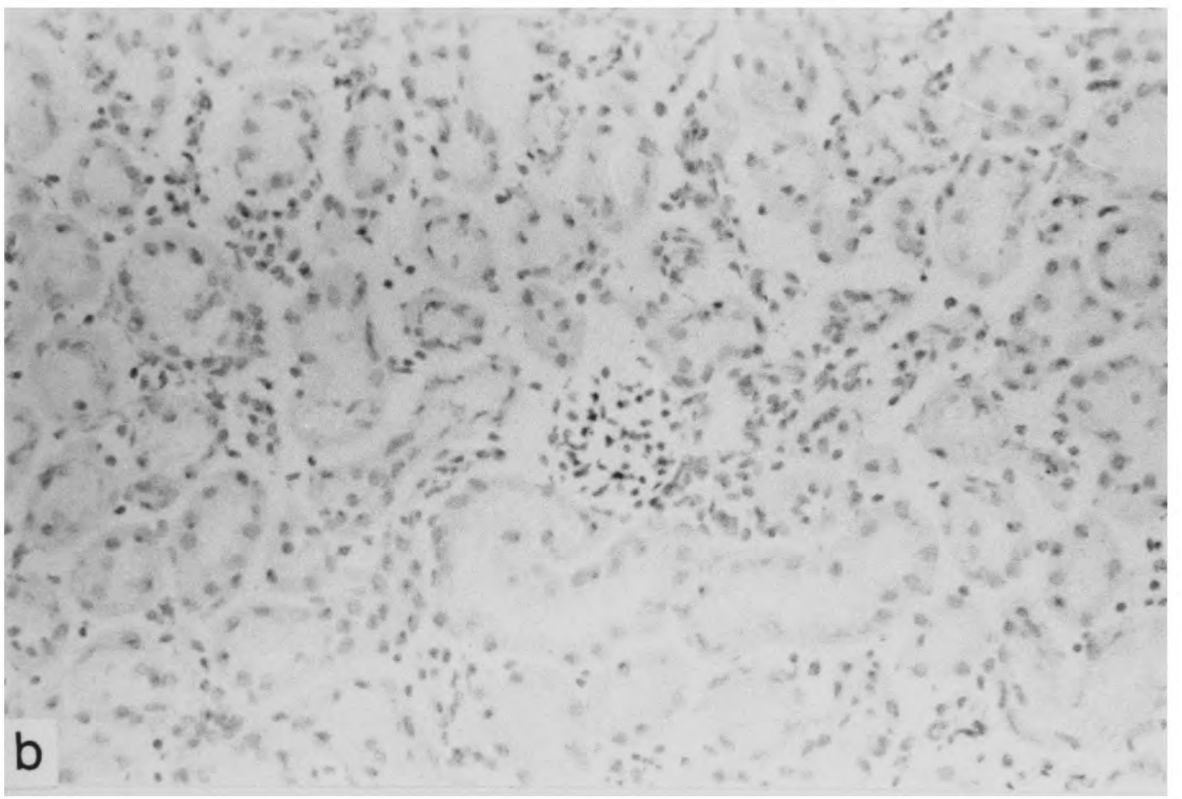

3c

Figure 3.5 **Renal Histology**

Sections through the kidney of an animal treated with the maximum dose of anti-L3T4 MAb (**a**), the maximum dose of anti-Lyt-2 MAb (**b**), and given saline (**c**). No signs of glomerulonephritis or non-specific damage were present. *x232*

FIGURE 3.5





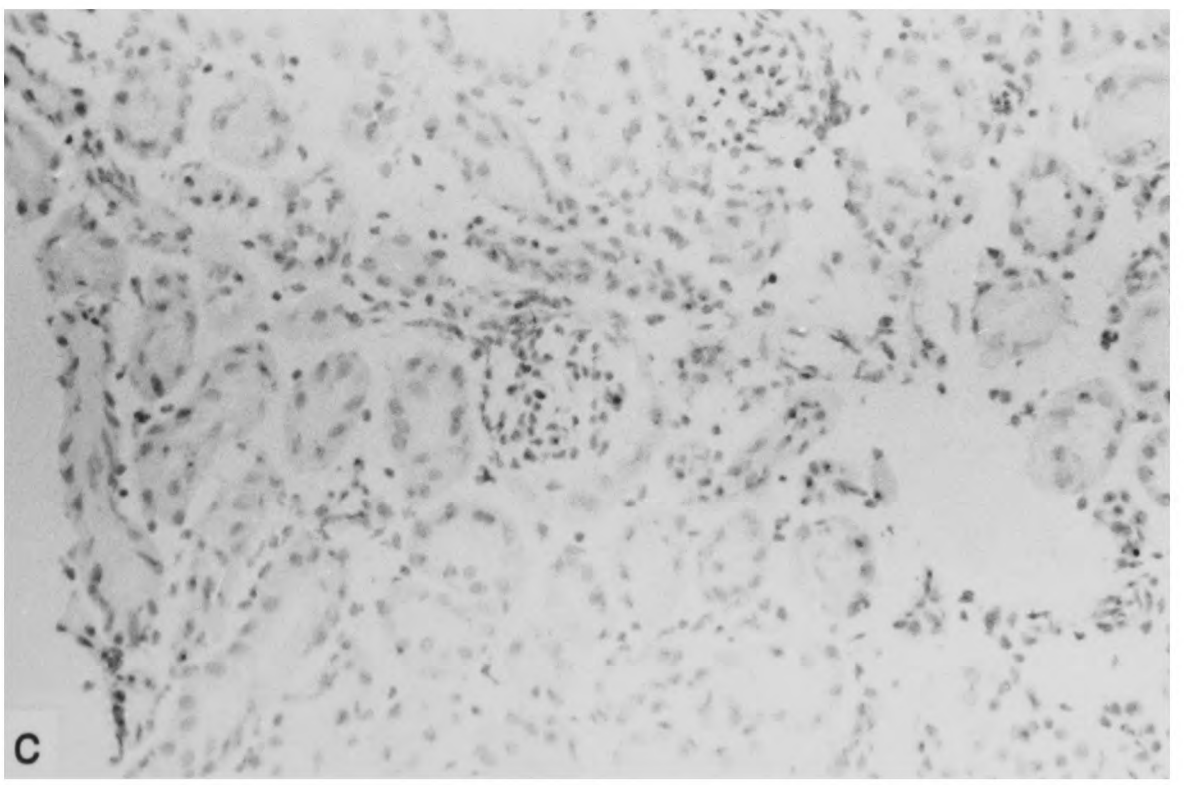
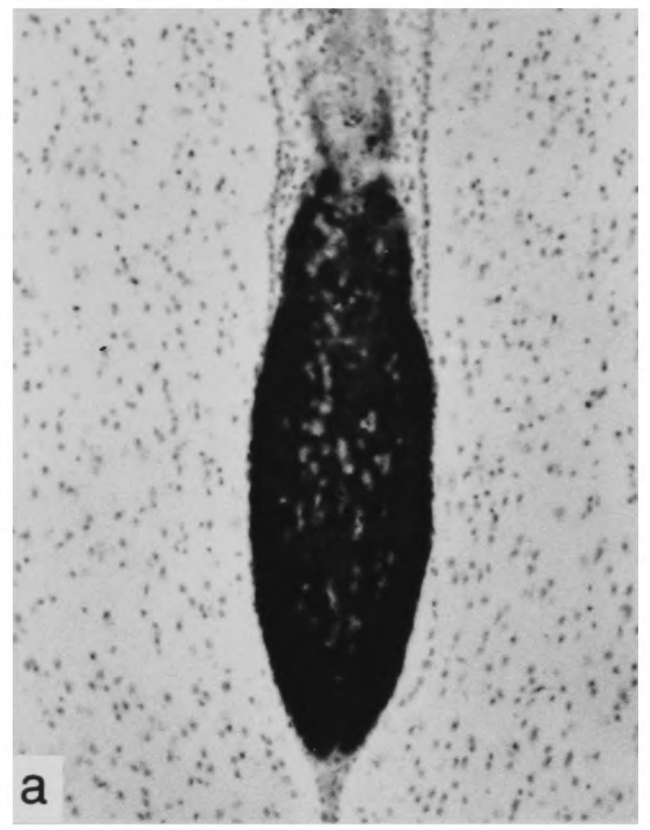
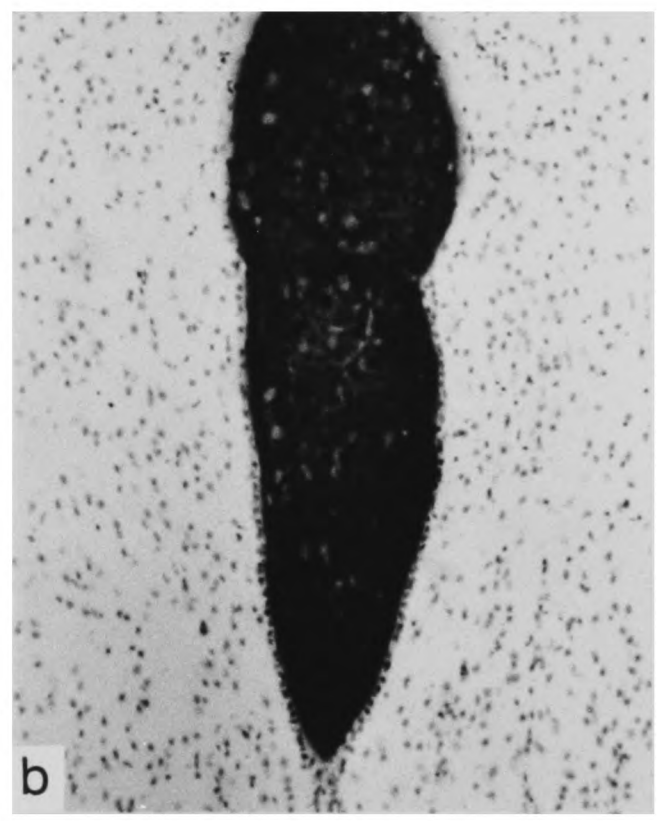


Figure 3.6 **Thy-1.1 Staining of Ventricular Xenografts in Mice Treated with anti-L3T4 MAb**

Animals given 2 μg/g/d of anti-L3T4 MAb (**a**), 4 μg/g/d (**b**), and 8 μg/g/d (**c**) all showed positive staining for the Thy-1.1 antigen on the xenograft. *x116*

FIGURE 3.6





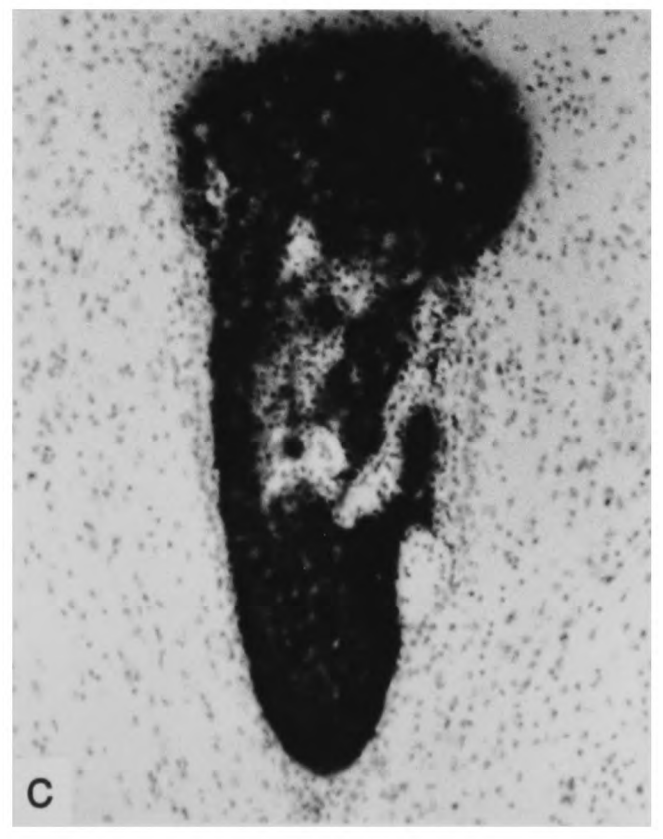


Figure 3.8 Histology of Third Ventricular Xenografts

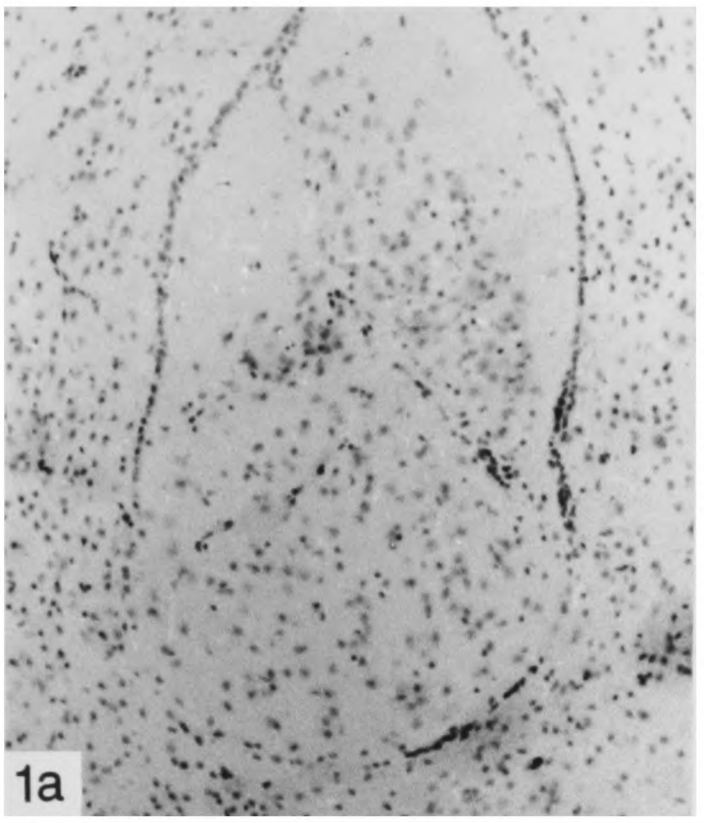
1 a-c **L3T4 Staining**

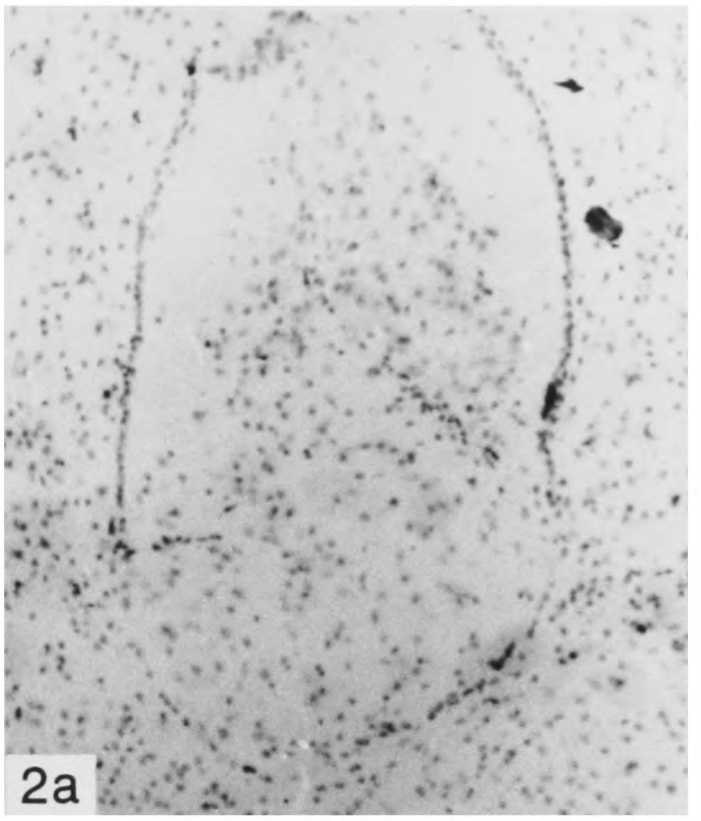
Xenografts from mice treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The xenografts in **b** and **c** were filled with L3T4-positive cells whereas the xenograft in **a** had very few of these cells. *x116*

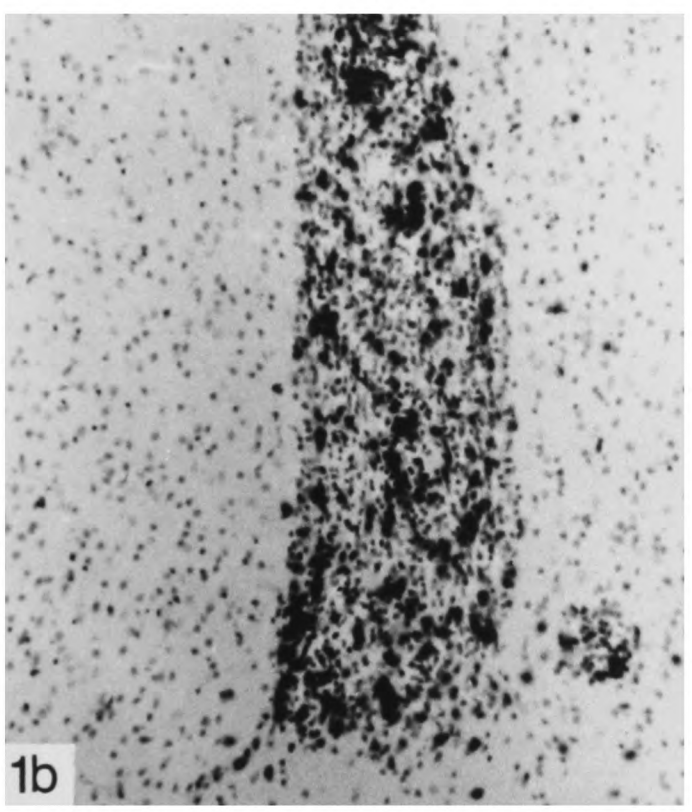
2 a-c **Lyt-2 Staining**

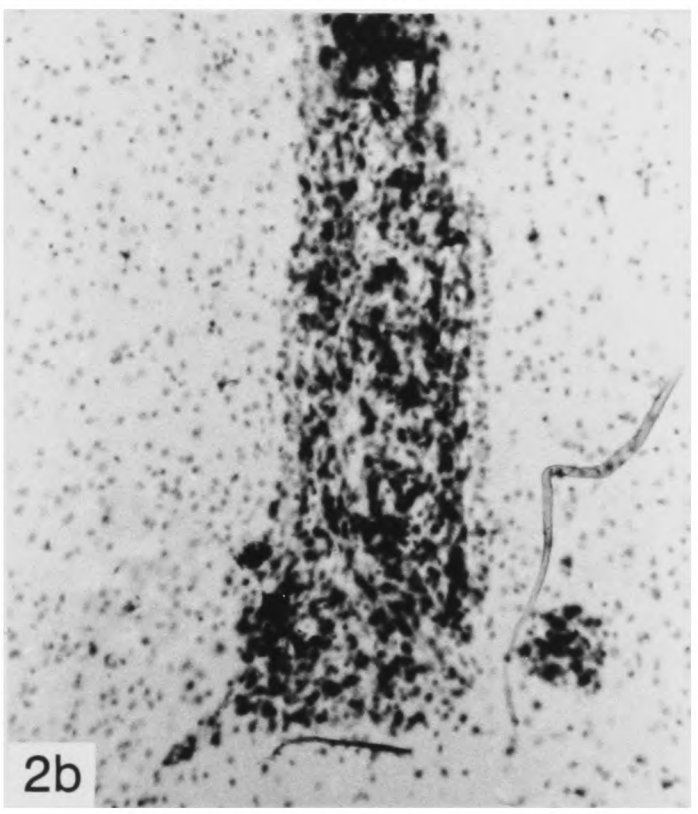
Xenografts from mice treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The xenografts in **b** and **c** were filled with Lyt-2-positive cells whereas the xenograft in **a** had very few of these cells. *x116*

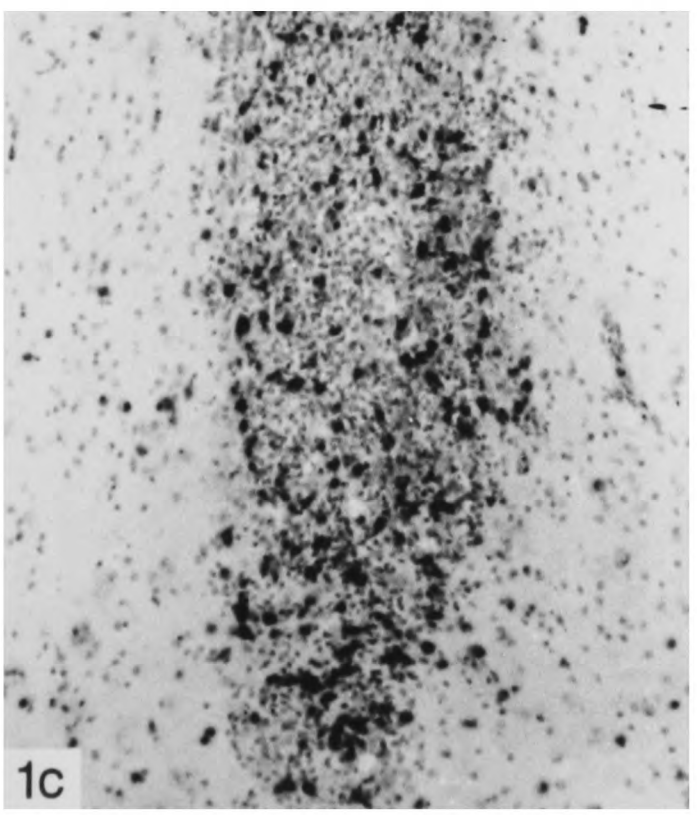
FIGURE 3.8











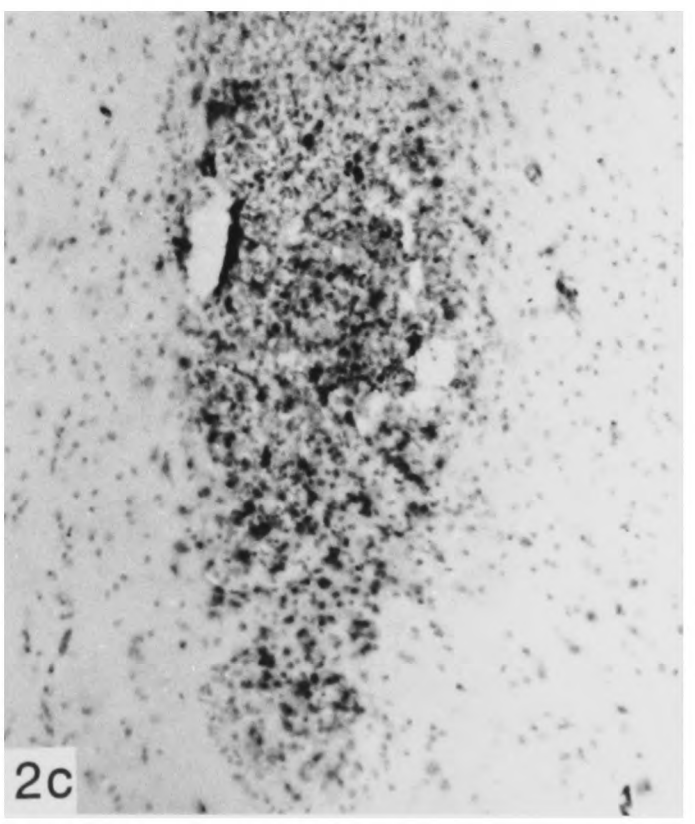


Figure 3.9 Histology of Ventricular Xenografts

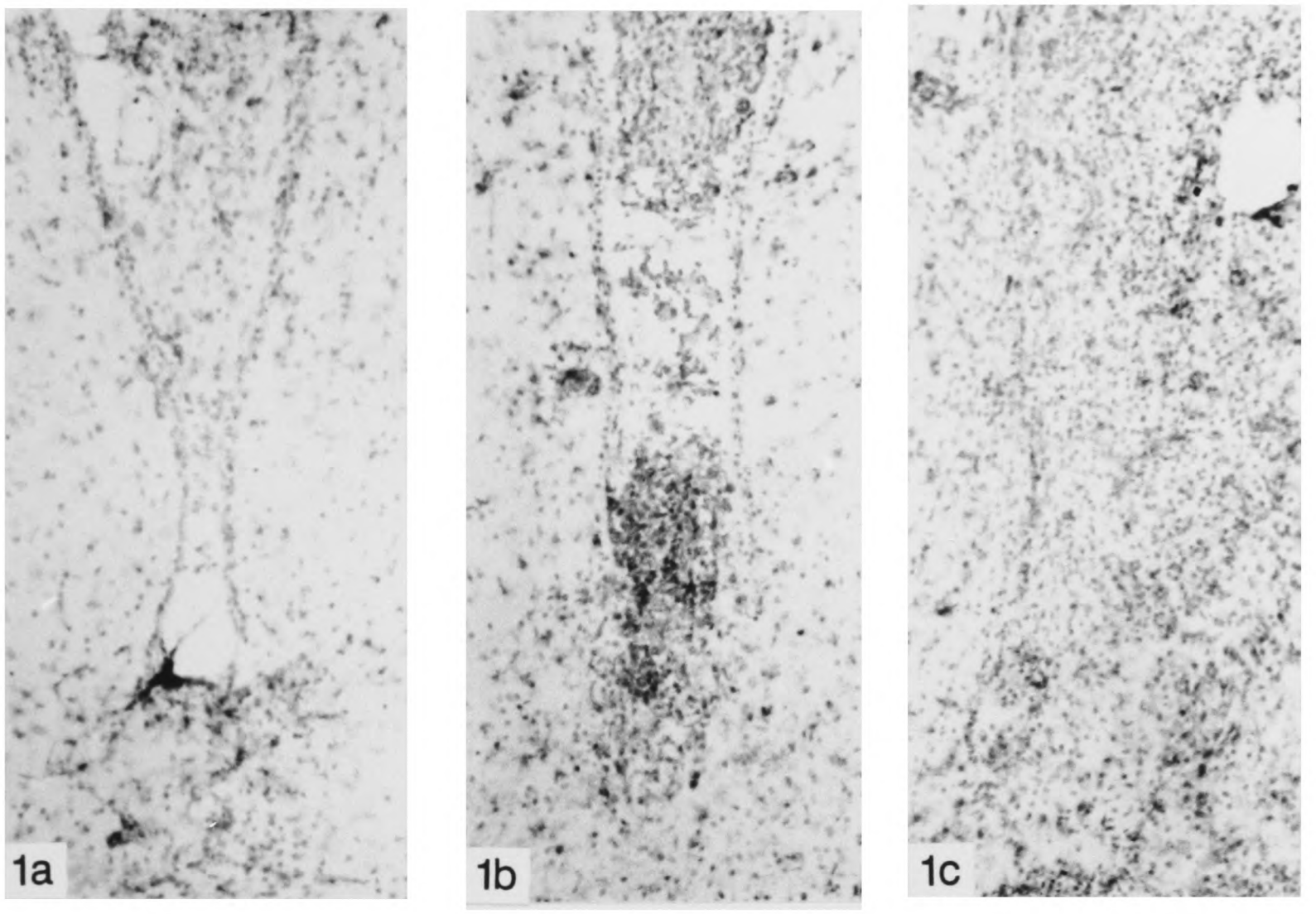
1 a-c **F4/80 (Macrophage) Staining**

Xenografts from mice treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The xenograft in **a** had less macrophages than those in **b** or **c**. *x116*

2 a-c **Mouse MHC Class I Staining**

Xenografts from mice treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The host brain in **a** had less induction of MHC Class I antigens than those in **b** or **c**. *x43*

FIGURE 3.9



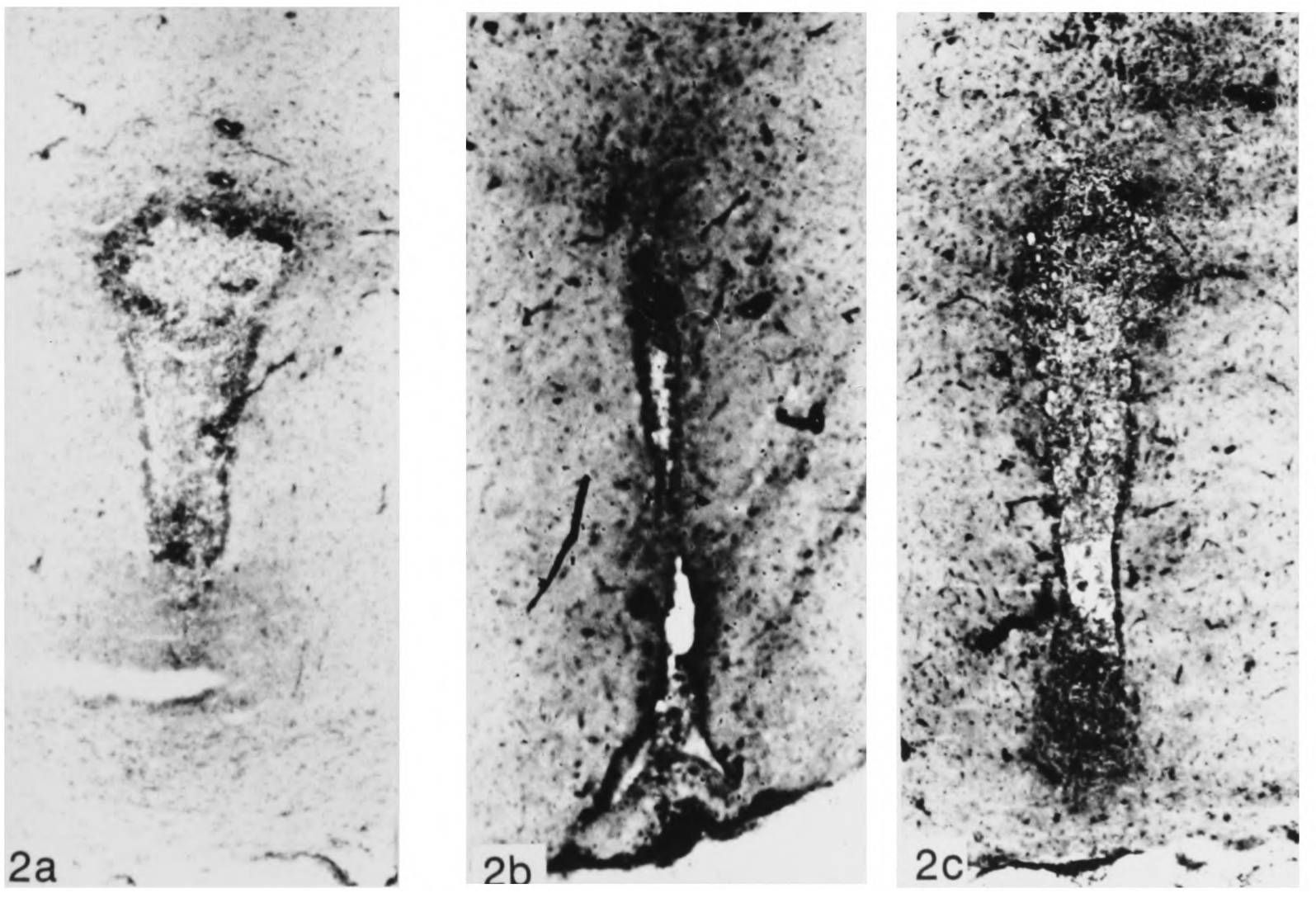


Figure 3.13 L3T4 Cell Population in Lymph Node and Spleen

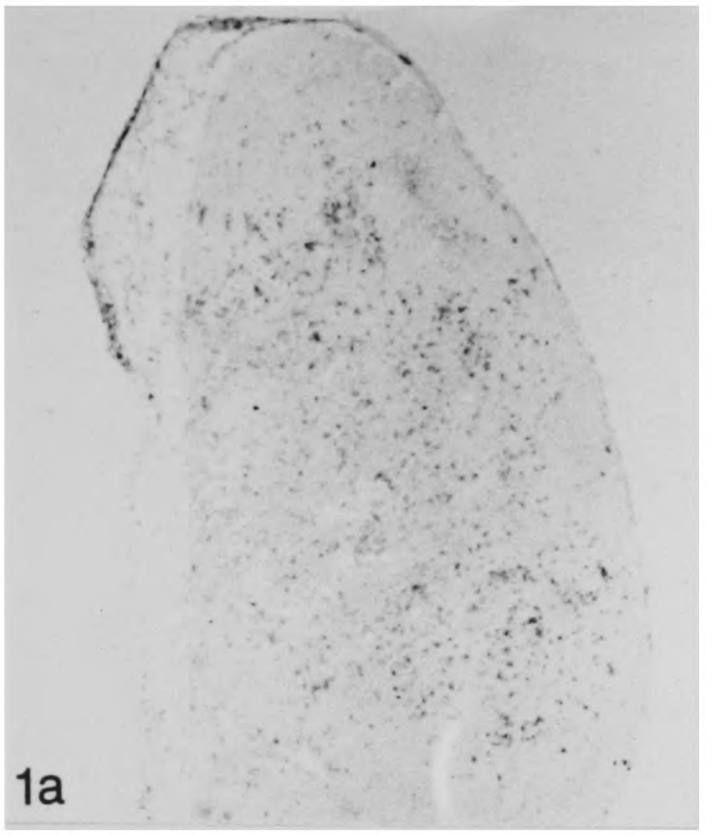
1 a-c Lymph Node

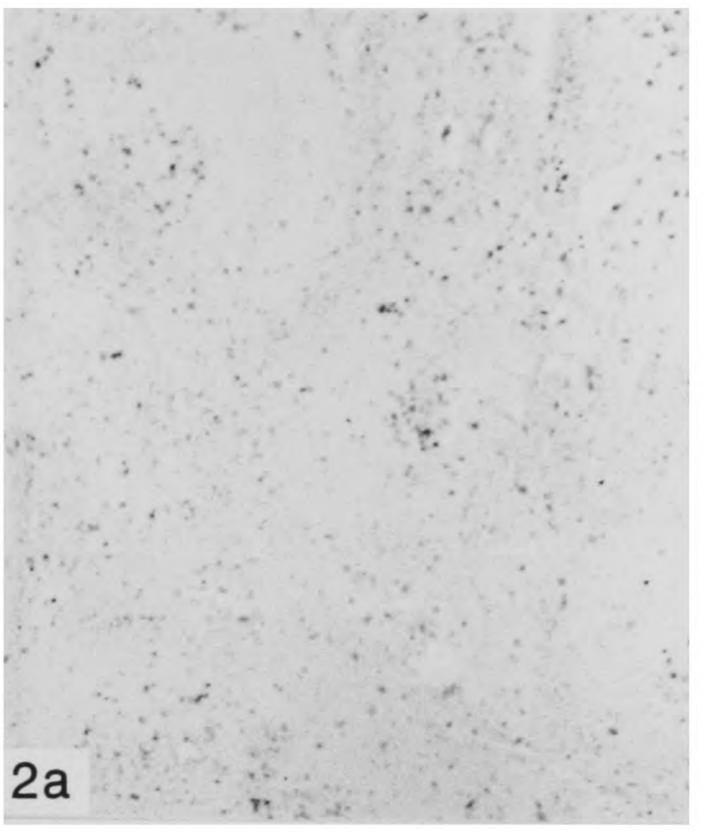
Cervical lymph nodes from mice treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The paracortex in **b** and **c** were filled with L3T4-positive cells whereas **a** had been depleted of these cells. *x36*

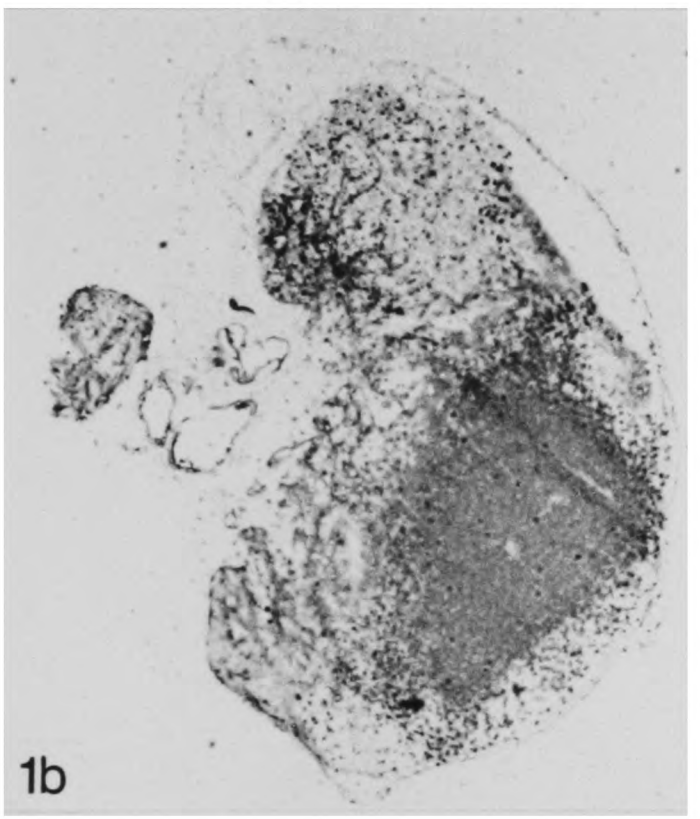
2 a-c Spleen

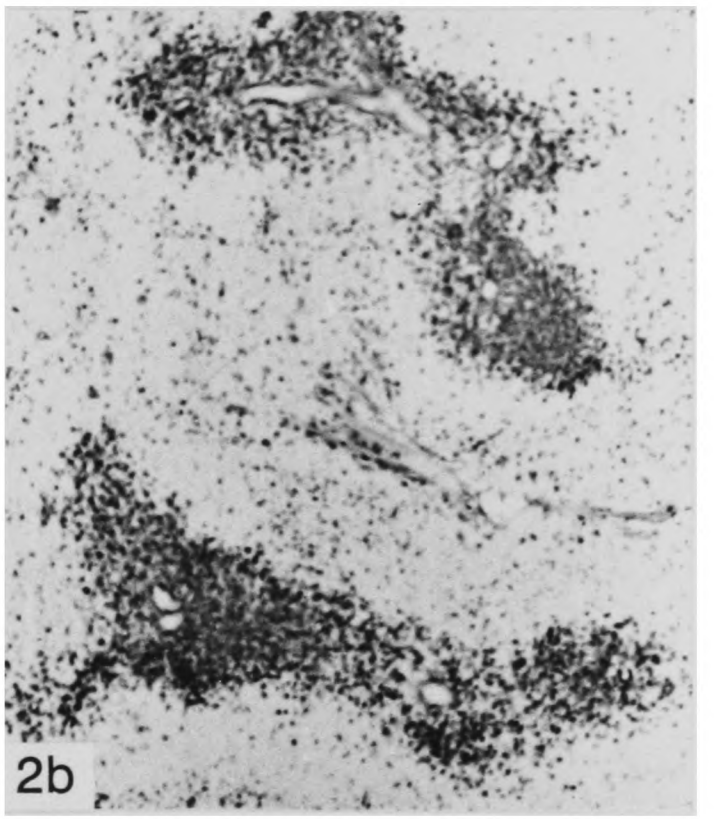
Spleens from mice treated with anti-L3T4 MAb (**a**), anti-Lyt-2 MAb (**b**), and saline (**c**). The periarteriolar lymphoid sheaths were filled with L3T4-positive cells in **b** and **c**, whereas **a** had been depleted of these cells. *x43*

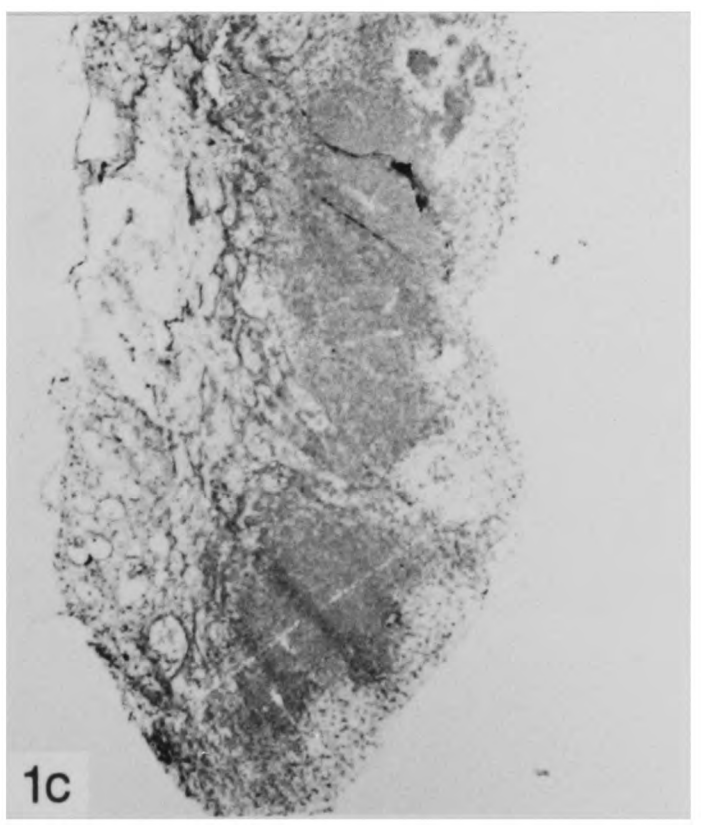
FIGURE 3.13

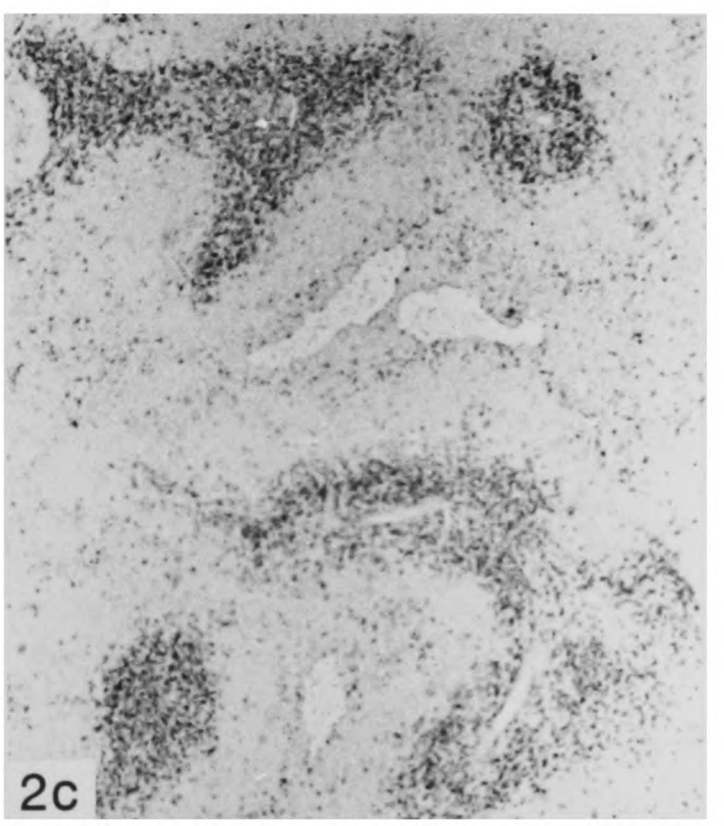












Chapter 4

Neural Allografts in Mice

Table of Contents

4.1 Introduction

This chapter explores two aspects of neural transplantation: the source of graft tissue and the site of transplantation.

The previous Chapter showed that xenografts were eventually rejected from the third ventricle of mice, even if they were immunosuppressed with anti-L3T4 MAb. Could allografts have survived in the previous model of neural transplantation when xenograft had been rejected? This Chapter, then, deals with the transplantation of neural allografts.

Allografts can vary in their stimulus to rejection. Mason *et al.* (1986) showed that the survival of rat neural allografts was reduced when the genetic disparity between the donor and host tissue was increased. Nicholas *et al.* (1987) showed the same was true with mouse neural allografts. This work examined the ability of anti-L3T4 MAb to suppress the rejection response to a range of allogeneic tissue.

Murphy and Sturm (1923) reported that xenogeneic tumours survived transplantation into the rat frontal cortex but were destroyed when they came in contact with the ventricles. More recent studies by Lund *et al.* (1987) confirmed this finding. This Chapter, then, also addresses the question: do allografts survive transplantation to the brain parenchyma better than to the ventricles?

4.2 Experimental Procedure

Three combinations of transplants were chosen to represent the spectrum of the rejection responses seen when transplanting neural tissue between different strains of mice. The first were syngeneic neural grafts which should have elicited no rejection response. The second were allogeneic neural grafts which differed only in minor histocompatibility antigens and should have elicited a rejection response. The third were allogeneic neural grafts which differed in the major and minor histocompatibility antigens and should have elicited a strong rejection response.

Table IVa shows the three strains of mice used in these experiments.

Table IVa

Histocompatibility Loci of Mouse Strains

strain	H-1	**H-2**	H-3	H-4	H-7	H-8	H-9	H-12	H-13
C57Bl/6	c	**b**	a	a	a	a	-	a	b
129	b	**b**	b	b	a	b	a,b	b	b
AKR	b	**k**	-	a	a	-	a,b	-	a

(not a or b designated with "-")

The three pairings used were as follows:

Syngeneic:	day 1	C57Bl/6	→	adult	C57Bl/6
Allogeneic (minor):	day 1	129	→	adult	C57Bl/6
Allogeneic (maj+min):	day 1	AKR	→	adult	C57Bl/6

All neural grafts (4 μL) were transplanted into the third ventricle of adult (older than 12 weeks), female, C57Bl/6 mice. All groups had 60 animals and were subdivided into three: Group 1 were immunosuppressed with anti-L3T4 MAb, Group 2 were given anti-Lyt-2 MAb and Group 3 were given saline as in Chapter 3. Half were sacrificed at four weeks post-transplant and the rest at twelve weeks.

Reference to Table IVa shows that in the Allogeneic (minor) group, the donor neural graft differed from its host in many of the minor histocompatibility antigens but was identical for the major histocompatibility antigens coded by the H-2 gene complex. In the Allogeneic (maj+min) group, the donor tissue differed in both major and minor histocompatibility antigens.

The AKR mouse strain also had the advantage of expressing the Thy-1.1 antigen on its neural tissue and could thus be distinguished from the Thy-1.2-positive brain of the C57Bl/6 host. There was no marker available which could distinguish the brains of donor C57Bl/6 or 129 from the host C57Bl/6. This was why the third ventricle was chosen as the site for all the neural grafts in this experiment. Any neural tissue found within the ventricle was likely to have been of donor origin.

Sloan (personal communication) had suggested that, in the rat, solid tissue neural allografts to the brain parenchyma were rejected much slower than those grafted to the ventricles. It seemed likely that this would be the case with mice as well. Transplanting to the third ventricle, then, presented the problem of possible increased rejection response. In order to avoid this, a second experiment was designed to follow allograft survival in the brain parenchyma.

Three groups of 24, adult (older than 12 weeks), female, C57Bl/6 mice received 4 μL neural allografts to their brain parenchyma from day 1, male, AKR mice. Group 1 was immunosuppressed with anti-L3T4 MAb, Group 2 received anti-Lyt-2 MAb, and Group 3 received saline as above. Half the animals were sacrificed at 12 weeks and the rest at 24 weeks. Only AKR donors were used so that donor allografts could be distinguished from host parenchyma with a marker for the Thy-1.1 antigen.

With the incisor bar set 2 mm below the interaural line, the stereotaxic coordinates used to place the allografts deep within the putamen were as follows:

Anterior: 3.8 mm (from interaural line)
Lateral: 2.5 mm (from midline)
Deep: 3.0 mm (from dural surface)

None of the animals died perioperatively. After sacrifice, all animals had their brains processed for immunohistochemistry. Serial sections through the allografts were stained with the following primary antibodies:

Antibody	**Specificity**	**Reference**
MRC OX-7	Thy-1.1 (AKR mouse brain)	Mason & Williams 1980
30H12	Thy-1.2 (mouse brain,T-cells)	Ledbetter & Herzenberg 1979
YTS 191.1	L3T4 (mouse T_h-cells)	Cobbold *et al.* 1984
YTS 169.4	Lyt-2 (mouse T_c-cells)	Cobbold *et al.* 1984
F4/80	macrophages	Austin & Gorden 1981
M142	mouse MHC class 1	Kennett 1980

4.3 Results

4.3.1 Transplantation into the Third Ventricle

All syngeneic transplants examined at four and twelve weeks had survived. Figure 4.1 shows photomicrographs of representative syngeneic grafts (stained for Thy-1.2) in the ventricles of immunocompetant mice at four and twelve weeks post-transplant. Both showed excellent tissue survival although there were areas of focal graft necrosis. These focal areas of graft necrosis were found in all groups of syngeneic grafts.

In the animals which received allografts, graft survival was scored as positive if there was any staining for neural tissue in the ventricle (Thy-1.2 for grafts from 129 donors and Thy-1.1 for grafts from AKR donors). Figure 4.2 shows the percentage of surviving allografts and syngeneic grafts in immunocompetant animals (Group 3).

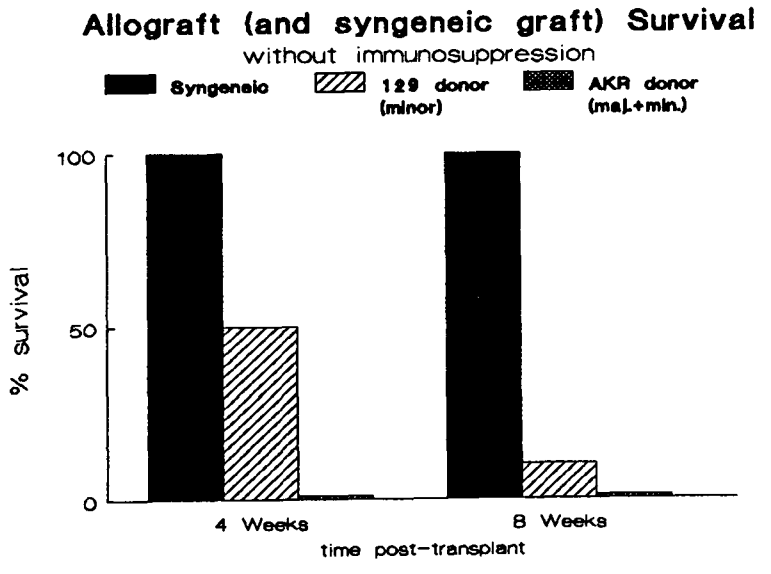


Figure 4.2

It was clear that the survival of allografts from 129 donors was better than those from AKR donors. None of the AKR allografts had survived even for four weeks. This was in keeping with current immunological theory that the antigens coded by the H-2 locus are the strongest stimulus for rejection. The AKR donors differed from the host C57Bl/6 at its H-2 locus, whereas the 129 donors did not.

The 129 allografts were also rejected, but at a slower rate. 50% of 129 allografts were rejected by four weeks and 90% by twelve weeks. The remaining grafts at twelve weeks were undergoing active rejection - they stained poorly and were filled with infiltrate. The 129 allografts differed from the host C57Bl/6 at many loci other than the H-2 locus. These minor histocompatibility antigen differences were enough to result in the ultimate destruction of all these grafts.

Figure 4.3 shows the percentage of surviving allografts in animals immunosuppressed with anti-L3T4 MAb (Group 1).

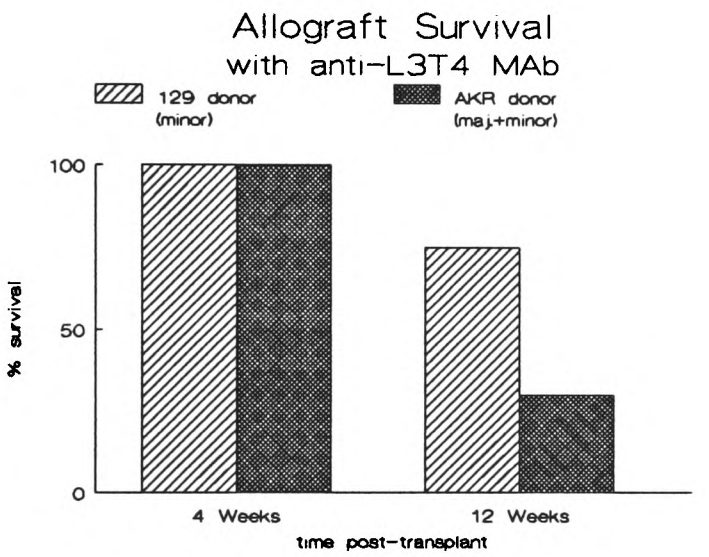


Figure 4.3

The allografts from both 129 and AKR donors showed 100% survival at four weeks. This was expected since xenografts had shown 100% survival at four weeks in Chapter 3. At twelve weeks, however, the allografts had begun to be rejected. Once again the rate of rejection followed the same pattern - AKR allografts were rejected faster than those from 129. Chapter 3 (Table IIIc) showed that xenografts were rejected even faster than AKR allografts.

4.3.2 Transplantation into the Brain Parenchyma

Figure 4.4 shows the survival of AKR allografts transplanted into the brain parenchyma of C57Bl/6 hosts.

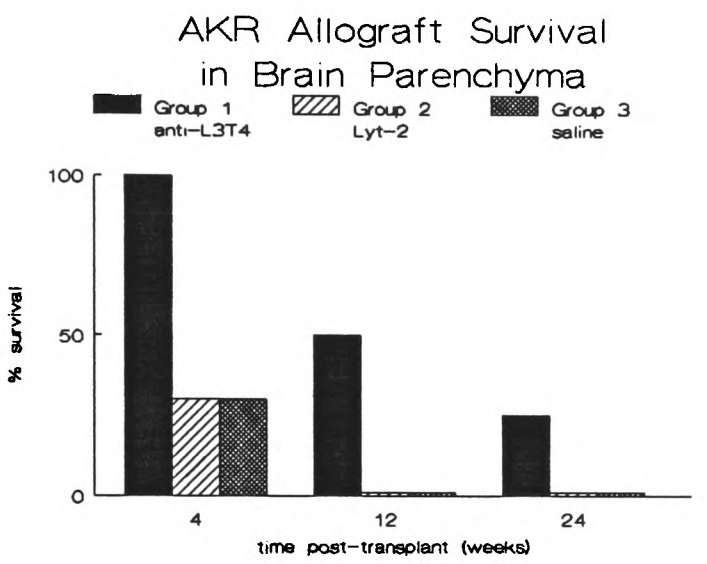


Figure 4.4

The AKR allograft survival in the host brain parenchyma was significantly better in animals immunosuppressed with anti-L3T4 MAb than in animals treated with anti-Lyt-2 MAb or saline at twelve and twenty-four weeks.

In animals immunosuppressed with anti-L3T4 MAb, the results of AKR allograft survival in the third ventricle and parenchyma were compared. Survival in the third ventricle had been 30% at twelve weeks but all of these grafts were being actively rejected and it was predicted that none of them would have survived much longer. Survival in the parenchyma had been 50% at twelve weeks and 25% at twenty-four weeks.

When the AKR allografts in the parenchyma were examined at twenty-four weeks, none of the surviving grafts (3 out of 12) showed any signs of being rejected. This was very surprising. In all previous experiments using the third

ventricle as the site of transplantation, the percentage of grafts surviving showed a steady decline over time. Even animals immunosuppressed with anti-L3T4 MAb eventually rejected their grafts. Figure 4.5 shows photomicrographs of serial sections from a representative allograft which had survived twenty-four weeks, stained for various antigens.

The allograft had even staining for the Thy-1.1 surface antigen (donor AKR brain) with no large patches of necrosis. There was very little staining for the Thy-1.2 surface antigen (host T-cells) within the allograft. There was a slight staining for mouse MHC Class 1 antigens and a few scattered F4/80-positive cells. This pattern was seen in all three surviving allografts.

In contrast, all the surviving allografts at twelve weeks in the third ventricle (3 out of 10) had showed clear evidence of being actively rejected (filled with infiltrate and strongly expressive MHC Class I antigens). Figure 4.5 shows photomicrographs of serial sections from a representative allograft stained for the same antigens. There was very poor staining for the Thy-1.1 antigen in the small allograft. The remaining tissue was strongly expressing MHC Class 1 antigens and filled with Thy-1.2-positive T-cells and F4/80-positive macrophages.

It seemed that allografts to the third ventricle were doomed to rejection once the host animals helper T-cells repopulated after the anti-L3T4 MAb treatment. On the other hand, a small percentage (≈25%) of allografts to the brain parenchyma showed extremely prolonged survival after anti-L3T4 MAb treatment. This prompted a closer analysis of the allografts to the parenchyma at earlier times post-transplant. Could the grafts which were going to be rejected be differentiated from those which were not?

Table IVb shows a subjective scoring of the staining for various antigens in all the allografts to the parenchyma examined twelve weeks post-transplant. No staining was scored as "-", some staining as "+", and strong staining as "++".

Table IVb

Animal	Thy1.1	Thy1.2	F4/80	Class1
1.	-	++	++	++
2.	-	++	++	++
3.	-	++	++	++
4.	-	++	++	++
5.	-	++	++	++
6.	+	++	++	++
7.	+	++	++	++
8.	++	+	+	+
9.	++	-	-	+
10.	++	-	-	+

The animals were arranged to show the different patterns of staining clearly. The first five allografts showed no staining for the Thy-1.1 antigen and were labelled as 'rejected' for Figure 4.4. The last five allografts did show some Thy-1.1 staining and were labelled as 'surviving' for Figure 4.4. Within this last group, however, two animals had no signs of rejection, two had clear signs of rejection, and one was ambiguous. Therefore, out of the original grouping two out of ten animals showed no signs of rejection. This rate of rejection-free, allograft survival correlated well with that seen at twenty-four weeks.

4.4 Discussion

Two major aspects of neural transplantation were dealt with in this chapter: the source of graft tissue (genetic discordance from host) and the site of transplantation.

A variety of neural grafts were used for transplantation in order to develop a complete spectrum of rejection responses. Combined with the results from Chapter 3, the neural grafts could be ranked in order of stimulus for rejection as follows:

strong rejection	xenogeneic
↑	allogeneic (major and minor differences)
↑	allogeneic (minor differences only)
no rejection	syngeneic

This order was consistent with the work of many authors. Mason *et al.* (1986) showed that the survival of rat neural grafts was reduced when the genetic disparity between the donor and host tissue was increased. Nicholas *et al.* (1987) showed the same was true with mouse neural allografts.

The syngeneic neural grafts showed 100% survival after 30 days in immunocompetent mice (Group 3). This was consistent with the work of Mason *et al.* (1986), who had shown complete survival of syngeneic fetal preoptic area grafts (5 out of 5) to the third ventricle of rats and Date, Kawamura and Nakashima (1988), who showed complete survival (9 out of 9) of syngeneic fetal brain stem neural grafts to adult mouse cerebellum. The survival of syngeneic grafts in Group

1 (immunosuppression with anti-L3T4 MAb) and Group 2 (given anti-Lyt-2 MAb) was also 100%.

All syngeneic grafts elicited weak expression of MHC Class 1 antigens on the surrounding host brain. This has been documented in syngeneic neural grafts by Mason *et al.* (1986) and in syngeneic cardiac grafts by Milton and Fabre (1985). The cause of this weak expression of MHC Class 1 is unknown but may be due to the IFN-α released by macrophages recruited to phagocytose the necrotic tissue inevitably produced by transplantation. This should not be confused with the strong expression of MHC Class 1 antigen expression seen with allogeneic and xenogeneic grafts which is probably due to the IFN-g released by activated T-cells.

This weak expression of MHC antigens did not seemed to cause any harmful effects to the syngeneic grafts. T-lymphocytes must recognize 'foreign' antigens in association with its own MHC antigens in order to be activated. By definition, the syngeneic grafts lacked any 'foreign' antigens.

The allografts which differed only in their minor histocompatibility (mH) antigens (129 → C57Bl/6) were actively rejected in immunocompetant mice (Group 3). Immunosuppression with anti-L3T4 MAb prolonged the survival of these grafts in the ventricle to 75% at 12 weeks (see Figure 4.3) but all allografts eventually showed signs of active rejection. These minor or non-MHC antigens are largely undefined gene products (Loveland and Simpson, 1986). Inbreeding has produced strains of mice which differ by only one mH antigen. Skin grafts between these strains are rejected at a much slower rate than between strains which differ at the MHC. When strains differ by many of these mH antigens, however, the rejection rate may match those of discordant MHC (Loveland and Simpson, 1986).

The allografts which differed in their MHC and mH antigens (AKR → C57Bl/6) were rapidly rejected in immunocompetant mice (Group 3). Immunosuppression with anti-L3T4 MAb prolonged ventricular allograft survival to 30% at 12 weeks (see Figure 4.3) but all allografts eventually showed signs of active rejection.

The site of graft transplantation proved to have a dramatic effect on the survival of the allografts. Transplantation to the brain parenchyma, instead of the ventricle, in immunosuppressed mice (anti-L3T4 MAb) increased allograft survival (AKR → C57Bl/6) from 30% to 50% at 12 weeks and from an estimated value of 0% to 25% at 24 weeks. More importantly, a subpopulation of parenchymal allografts had readily visible signs of rejection - even at 24 weeks.

The survival of neural grafts in various areas of the brain has been investigated by many authors. Beginning with Murphy and Sturm's (1923) report that mouse sarcoma xenografts survived in the frontal lobe of rats, but...

> 'In practically every instance in which the implant failed to grow, examination showed that it had come into contact with the ventricle.'

The fact that neural grafts survive better in brain parenchyma than in the ventricles was predicted by Head and Griffin (1985), and confirmed by Lund *et al.* (1987). There are several reasons why intraparenchymal neural grafts may illicit a smaller rejection response than intraventricular grafts.

First, the Introductory Chapter outlined why the brain's immunologic privilege is thought to be due to its relatively poor ability at activating the immune system (ie. the afferent arm of the immune response). A natural extension of this premise would be that, within the brain, grafts in the parenchyma are even more restricted from activating the immune system than those in the ventricle.

Parenchymal tissue lack conventional lymphatics, thus graft antigens are not readily transported away from the transplant. The ventricles, however, have a high cerebral spinal fluid (CSF) flow away form any intraventricular transplant towards the choroid plexus.

Second, the distribution of potential antigen presenting cells also favours immune activation from the intraventricular site. These cells characteristically express abundant Ia, or class II, histocompatibility antigens necessary for effective presentation of antigen to host T-cells (Unanue, 1981). Hart and Fabre (1981) reported an abundance of Ia^+ cells in the choroid plexus while Head and Griffen (1985) found the grey matter of the brain was almost devoid of these cells.

Third, as outlined in the Introductory Chapter, the integrity of the blood brain barrier (BBB) may play a role in the brain's immunologic privilege. Broadwell *et al.* (1989) demonstrated that allografts of CNS tissue do exhibit a BBB when grafted to the parenchyma. Rosenstein (1987) argued against this theory but his work may have inadvertently proved that CNS transplants near the ventricles (or meninges) lack a BBB. Grafts within the ventricles are directly exposed to non-fenestrated vessels in the median eminence, area postrema, subfornical organ and organovasculosum laminum terminalus. These sites may provide a pathway whereby the immune system may be activated in the early post-grafting period.

Allografts are used extensively by the transplant surgeon to treat organ failure in man. There are several problems, however, that the neurosurgeon will face when using central nervous system allografts for transplantation.

First, Stenevi, Bjorklund and Svendgaard (1976) have shown that the age of the donor tissue is crucial for graft survival. Brudin *et al.*, (1986) showed that cell suspensions of human fetal tissue older than 9-10 weeks gestation may not survive

grafting. There are many ethical questions raised by the use of human fetal tissue which are beyond the scope of this thesis. At the present time, the Canadian government has banned government funded research on any tissue from an elective abortion; the United States government has banned NIH participation in human fetal transplants; and a moratorium has been placed on clinical experiments in the United Kingdom.

Second, the source of fetal tissue is too scarce to allow neurosurgeons the luxury of tissue type matching donor tissue to prospective recipients. The allografts would most likely be discordant for the major and many minor histocompatibility antigens. This work has shown that such allografts provide a strong stimulus for rejection.

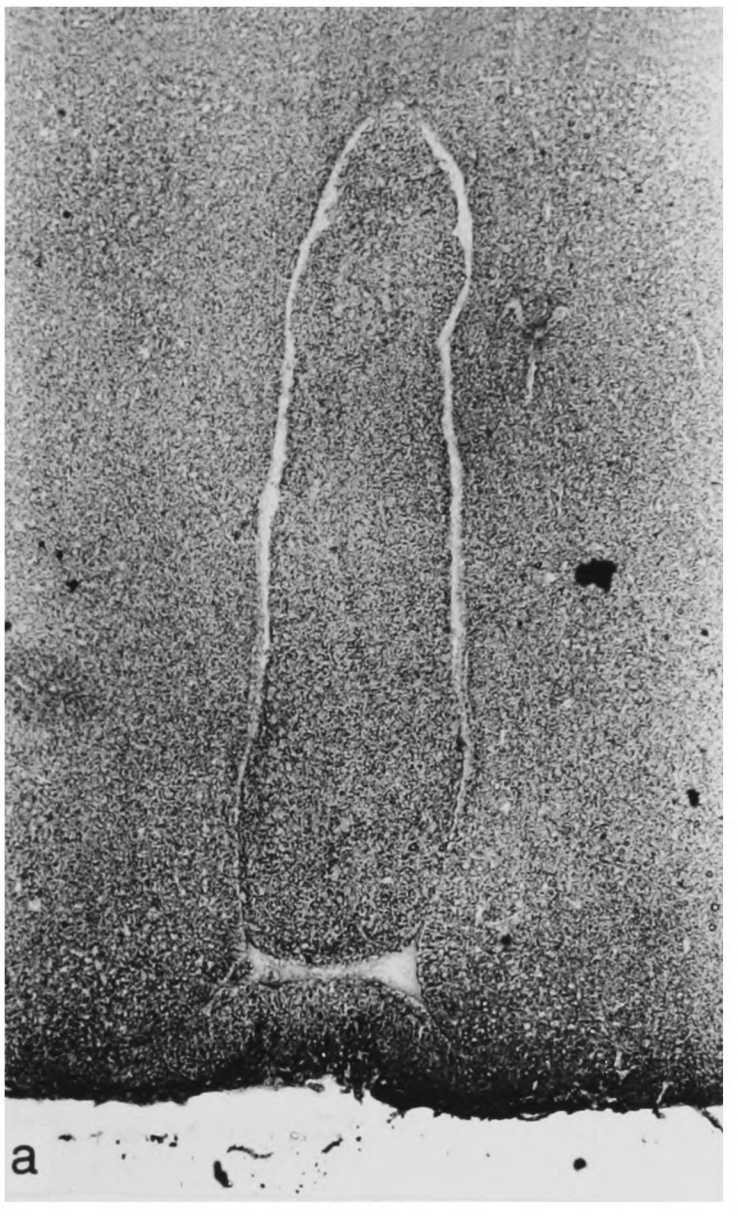
In order to avoid the ethical problems of harvesting human fetal tissue, neurosurgeons may have to turn to other potential sources of tissue. The most abundant alternative is xenogeneic tissue. This could provide a ready supply of specific cell populations at the desired age of maturation. The second potential source would be genetically transformed cells. This exciting new technique is early in the experimental stages but preliminary reports at the IIIrd International Symposium on Neural Transplantation were encouraging (Dr. Olson's group in Stockholm; Dr. Gage's group in San Diego; and Dr. Freed's group in Washington).

Genetically modified cells will provide the neurosurgeon with an excellent source of neural grafts but its use may lie well in the future. Xenogeneic grafts are more immediately available but their use will require appropriate immunosuppression. The next Chapter explores the possibility of utilizing neural xenografts with an animal model.

Figure 4.1 **Syngeneic Grafts in the Third Ventricle**

Coronal sections through the third ventricle were stained for Thy-1.2. The syngeneic neural grafts at four weeks (**a**) and twelve weeks (**b**) filled the ventricle. *x116*

FIGURE 4.1



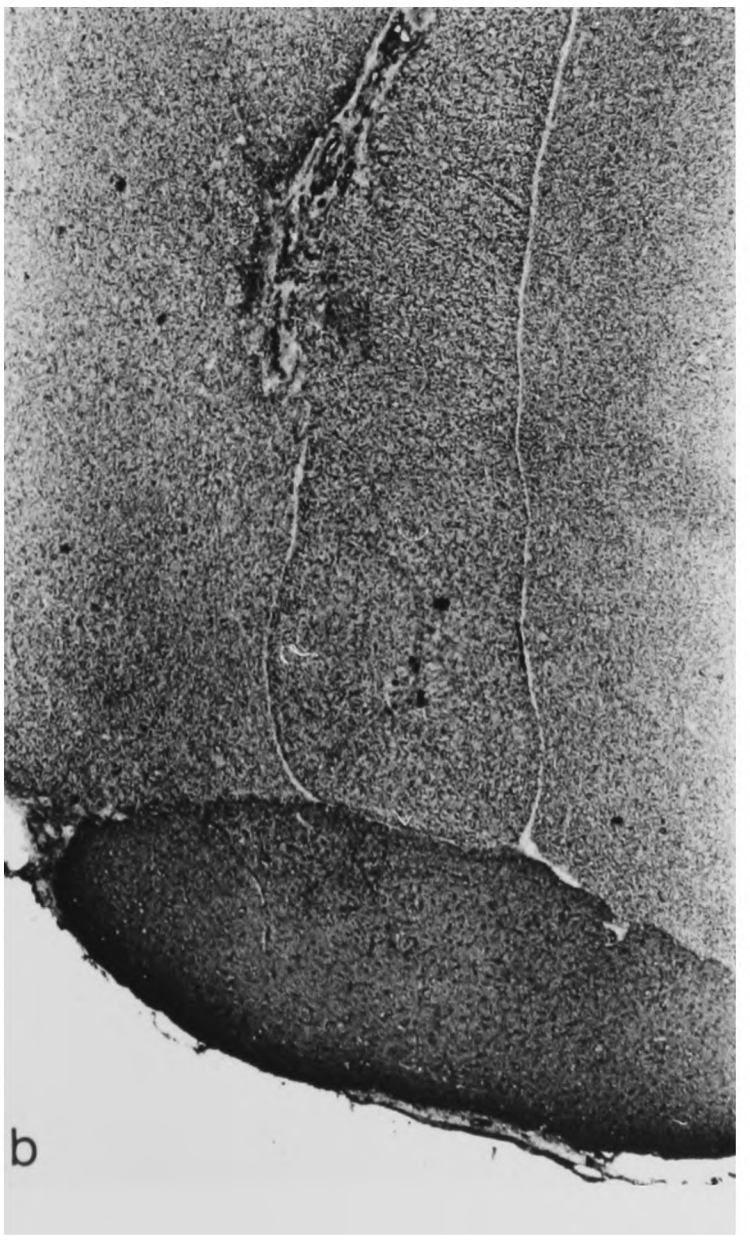


Figure 4.5 Histology of Parenchymal and Ventricular Allografts

1 a,b Thy-1.1 Staining

AKR allograft in C57Bl/6 host parenchyma (**a**) at 12 weeks showed good Thy-1.1 staining whereas the ventricular allograft (**b**) at 4 weeks had very little. *x43*

2 a,b Thy-1.2 Staining

Parenchymal allograft (**a**), clearly outlined within host brain, had few infiltrating cells whereas ventricular allograft (**b**) had many. *x43*

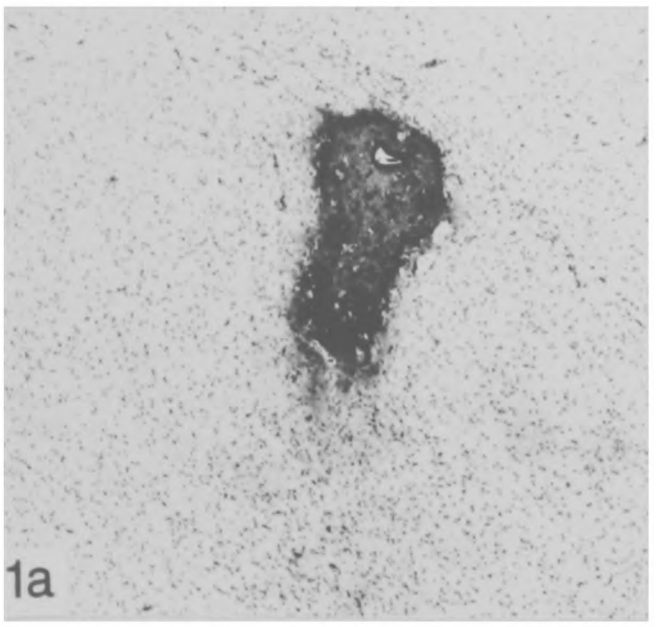
3 a,b MHC CLass I Staining

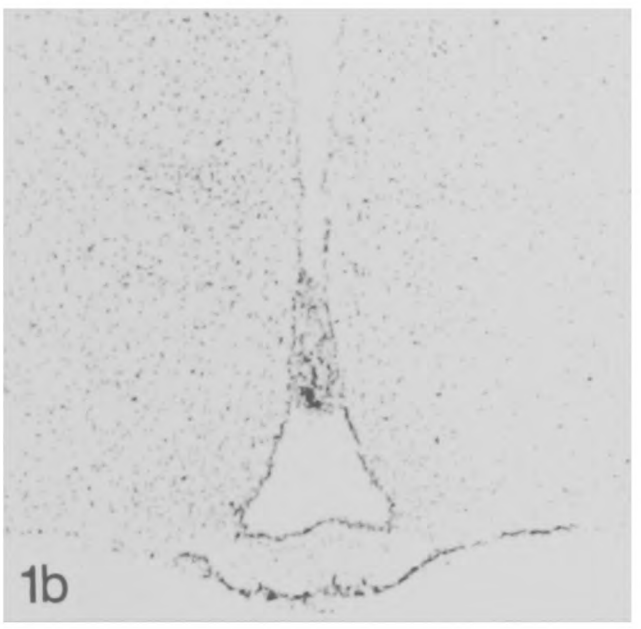
Parenchymal allograft (**a**) had little Class I induction whereas the ventricular allograft (**b**) was strongly staining for these antigens. *x43*

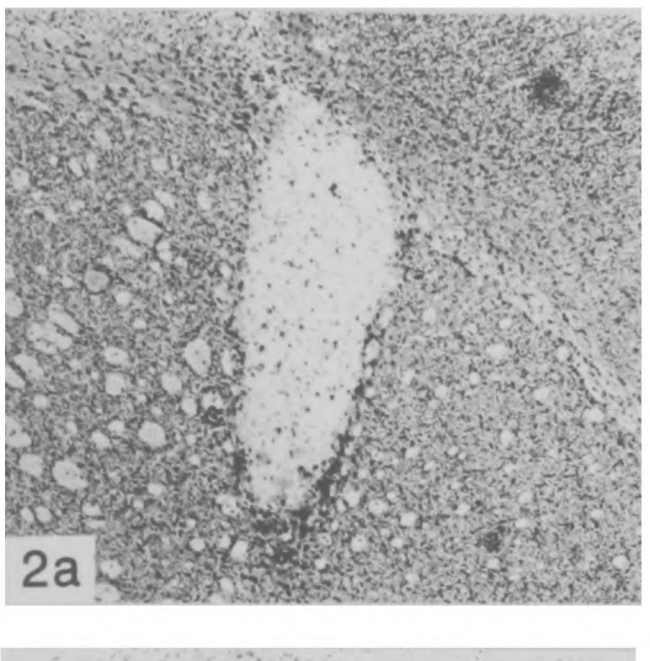
4 a,b F4\80 (Macrophage) Staining

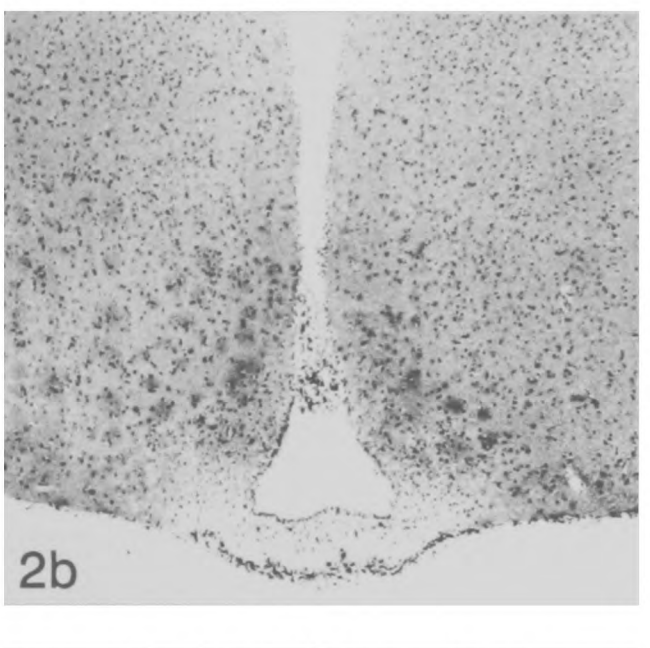
Parenchymal allograft (**a**) had little staining for macrophages whereas the small ventricular allograft (**b**) appeared filled with them. *x43*

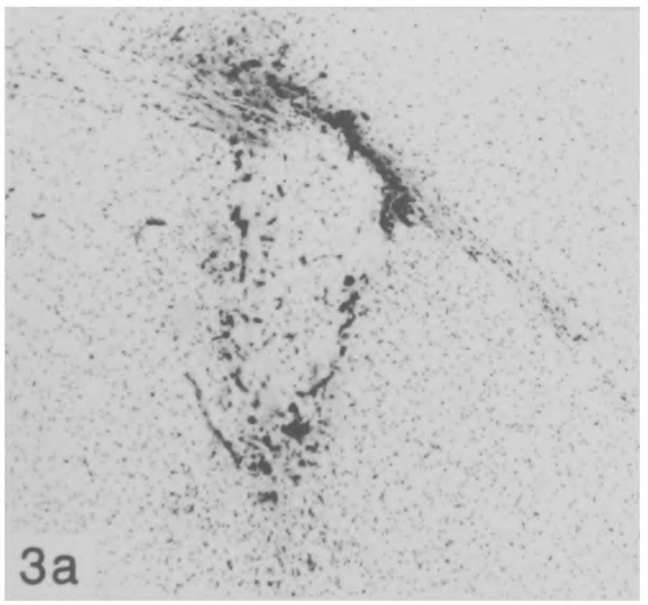
FIGURE 4.5

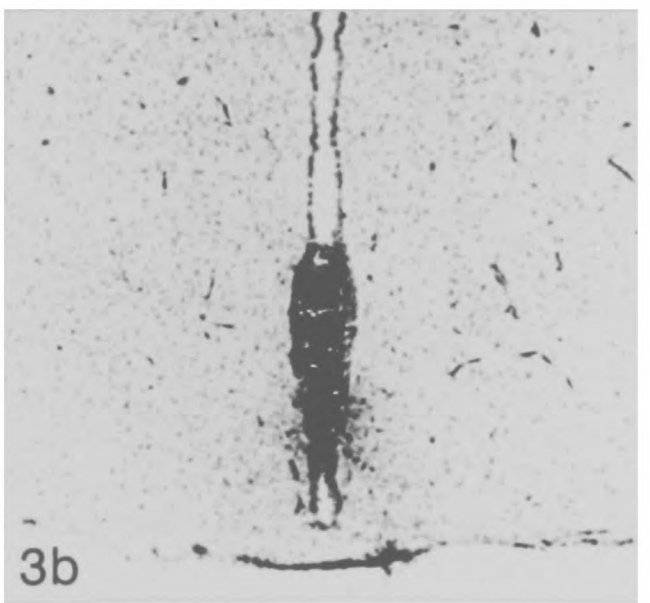


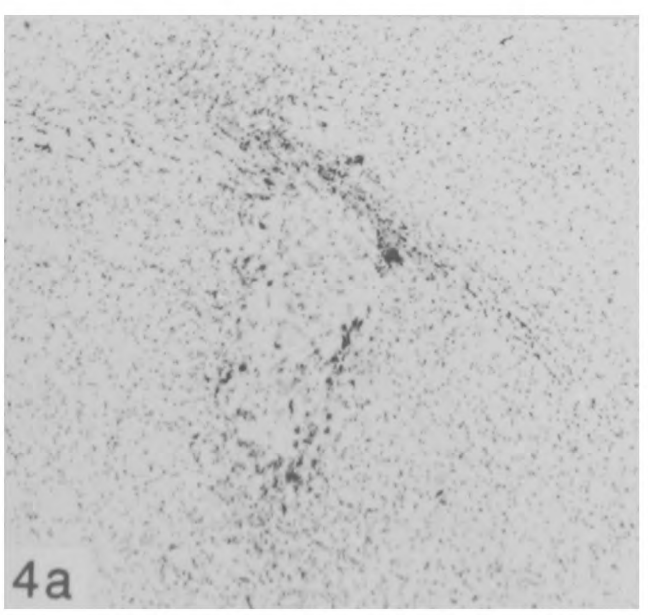


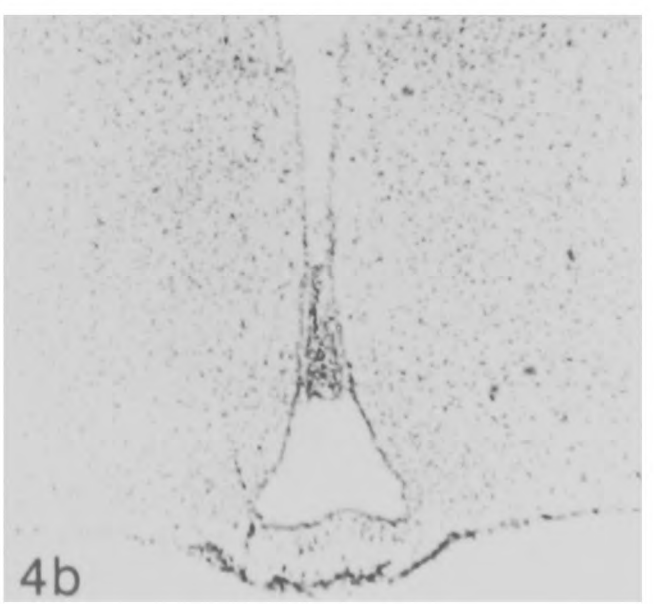












Chapter 5

Functional Neural Xenografts in Mice

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5.1 Introduction

The results from the previous two chapters showed that neural allografts (Chapter 4) and xenografts (Chapter 3) could survive in mice immunosuppressed with anti-L3T4 MAb. It is important to determine if these neural grafts are functional. This chapter investigates whether neural xenografts can function in mice immunosuppressed with anti-L3T4 MAb.

The model used was the hypogonadal (*hpg*) mouse. The *hpg* mouse was discovered in a breeding colony at the Medical Research Council Radiobiology Unit, Harwell, U.K. There is a severe deficiency of hypothalamic Gonadotrophin Releasing Hormone (GnRH) with a consequent depletion in pituitary Luteinizing Hormone (LH) and Follicular Stimulating Hormone (FSH) synthesis and secretion (Cattanach *et al.*,1977). Without LH and FSH, the gonads in both sexes fail to grow post-natally.

Recombinant DNA techniques have demonstrated that there is probably only one copy of the GnRH gene in mammals and that the gene consists of 4 exons which when transcribed and translated produce a peptide containing the decapeptide GnRH and an additional 56 amino acid sequence, the gonadotrophin releasing hormone associated peptide or GAP (Adelman *et al.*, 1986). Structural analysis of the GnRH gene in *hpg* mice has shown that there is a deletion removing 2 exons coding for a large part of the GAP moiety (Mason, Hayflick, Zoeller *et al.*, 1986). This severely truncated gene still contains the codons for GnRH but no one has ever been able to identify immunostainable GnRH in the brains of *hpg* mice (Lyon, Cattanach, and Charlton, 1981; Charlton 1986). Mason, Hayflick, Zoeller *et al.*

(1986) showed that aberrant messenger RNA may indeed be formed in the GnRH neurons of *hpg* mice which presumably cannot be translated effectively.

The *hpg* mouse provides an excellent model to test the survival and functioning of rat neural xenografts. The xenograft's survival can be demonstrated with immunohistochemistry against the Thy-1.1 antigen and its function observed by its effect on the mutant phenotype (ie. gonadal growth).

Merchenthaler *et al.* (1984) showed that the majority of GnRH neurons in the rat were found in the preoptic area (POA) projecting to the median eminence. Using fetal POA grafts from normal littermates, Krieger *et al.* (1982) showed that the mutant phenotype of the male *hpg* could be altered by transplanting the grafts to the third ventricle. 7 out of 8 grafted *hpg* mice showed significant increases in testicular weight two months post-transplant. Charlton *et al.* (1987) showed that the age of the POA donor tissue was crucial. They found a 69% response rate with fetal day 16-18 grafts, a 77% rate with day 1 grafts, a 22% rate with day 5 grafts and no response from day 10 grafts.

This chapter attempts to repeat this work using day 1 PVG rat preoptic area xenografts as the source of GnRH neurons.

5.2 Experimental Procedure

Forty-eight adult (older than 12 weeks) male *hpg* mice were divided into four groups of twelve. Groups 1 and 4 were immunosuppressed with anti-L3T4 MAb (4 μg/g/day i.p. for 13 days) as described in Chapter 3, Group 2 received anti-Lyt-2 MAb, and Group 3 received saline. Groups 1, 2, and 3 received 6 μL of day 1 PVG rat preoptic area, transplanted to their third ventricle as described in Chapter 2. Group 4 had 6 μL of day 1 PVG rat cortex transplanted to their third ventricle.

A diagrammatic summary of the four groups is shown in Table Va.

Table Va

	Treatment	Transplant
Group 1	anti-L3T4 MAb	POA
Group 2	anti-Lyt-2 MAb	POA
Group 3	saline	POA
Group 4	anti-L3T4 MAb	cortex

Thirty days post-transplant, all animals were bled and the serum analyzed for follicle stimulating hormone (FSH). The testes were removed, weighed, and processed for histology as described in Chapter 2. Half of the animals which showed enlarged testicular weight (3 animals in Group 1) were processed for GnRH staining of their brains by perfusion fixation and the remaining animals were all processed for immunohistochemistry of their brains as described in Chapter 2.

5.3 Results

Throughout the Results and Discussion sections, the animals in Group 1 were split into 'responders' (Group 1R) and 'non-responders' (Group 1N) depending on whether there was any testicular enlargement (see section 5.3.4).

5.3.1 Xenograft Survival

Lyon, Cattanach and Charlton (1981) and later Charlton (1986) showed that the brain tissue of *hpg* mice did not stain for GnRH-positive neurons. Charlton, Barclay and Williams (1983) showed the brain tissue of *hpg* mice did not stain for the Thy-1.1 antigen with a direct peroxidase conjugate of the MRC OX-7 antibody (Mason and Williams, 1980). The rat preoptic area neural xenografts, however, do stain positive for both these markers. Thus any positive staining for either of these markers represented surviving xenograft within the *hpg* brain.

Figure 5.1 shows photomicrographs of sections through the xenograft from a representative animal in each group, which were stained for the Thy-1.1 antigen with a direct peroxidase conjugate of the MRC OX-7 antibody. The xenografts from Group 1 and Group 4 showed excellent Thy-1.1. staining. All xenografts examined for Thy-1.1 in Groups 1 and 4 had strong positive staining while none of the xenografts in Groups 2 or 3 were positive. The three xenografts in Group 1 which were examined for GnRH (see section 5.3.2), showed positive staining.

In summary, all animals in Groups 1 and 4 had surviving xenografts while none of the animals in Groups 2 or 3 had any. This was expected from the results of Chapter 3, which showed 100% xenograft survival over 30 days in mice immunosuppressed with anti-L3T4 MAb.

5.3.2 GnRH Neuron Survival

It was important to established that, within the surviving xenografts in Group 1, there were GnRH neurons. Three animals which had shown enlarged testes were processed for GnRH staining as described in Chapter 2. Figure 5.2 shows GnRH-positive staining in the xenograft of an animal from Group 1. All three animals examined, showed positive GnRH staining. The axons from GnRH-positive neurons were always directed toward the median eminence.

If the GnRH neurons had survived and released GnRH into the portal system, then the pituitary would have been stimulated to release FSH and LH. Enough serum was recovered from each animal (100 μL) to assay the content of FSH.

5.3.3 Serum FSH Levels

Serum from each animal was frozen and sent to Dr. P O'Shaughnessy at the Royal Veterinary College, London[1]. The animals in Group 1 were separated into 'responders' and 'non-responders', as outlined in the beginning of the results section, and the results shown in Figure 5.3.

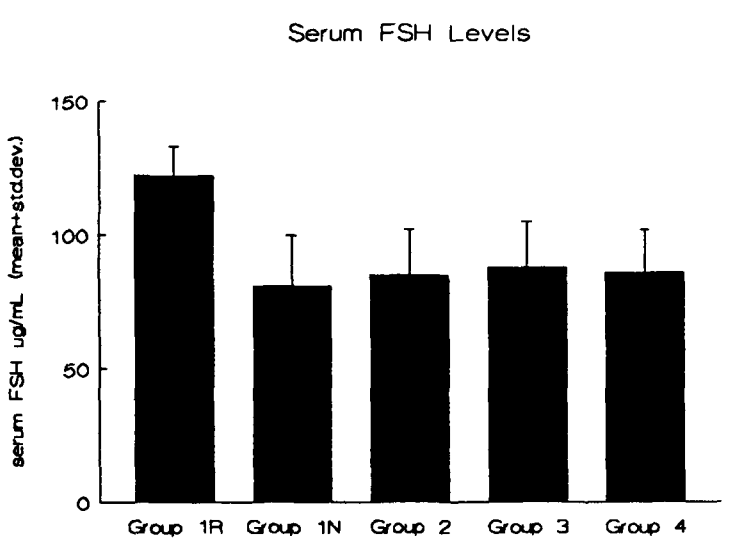


Figure 5.3

There was a significant difference ($P<0.01$) between the groups by analysis of variance. The 'responders' in Group 1 had increased levels of serum FSH. If enough FSH had been released into the blood by the pituitary, then the testes would have been stimulated to mature and enlarge.

[1]Royal College Street, London, U.K., NW1 OTU

5.3.4 Testicular Response

The testes were removed from each animal and weighed. The combined testicular weight for each animal was arranged in order and displayed in Table Vb.

Table Vb

Group1	Group2	Group3	Group4
55.4	8.8	8.0	8.8
30.9	8.0	7.0	8.6
30.8	7.1	6.7	7.8
30.6	6.2	5.8	6.4
25.8	5.8	5.6	5.5
24.0	4.9	5.5	5.5
8.2	4.4	4.9	5.0
5.8	3.9	4.2	4.8
5.4	3.4	4.2	3.9
4.0	3.0	3.9	3.8

The animals in Group 1 with a combined testicular weight of more then 10 mg were labelled as 'responders' (Group 1R) and those with less, as 'non-responders' (Group 1N). The mean combined testicular weights for each group are shown diagrammatically in Figure 5.4.

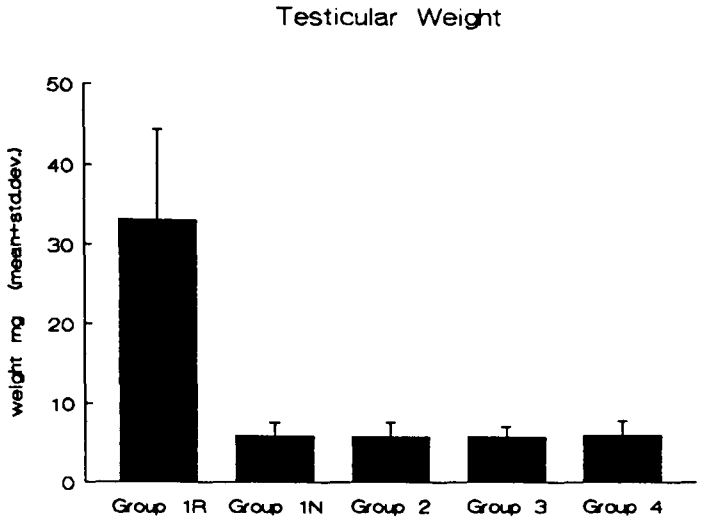


Figure 5.4

The mean combined testicular weight for each group was compared by analysis of variance. The 'responders' had a significantly ($p<0.01$) increased testicular weight.

The testes were then examined histologically to determine if they had matured as well as enlarged. Figure 5.5 shows photomicrographs of representative testicular sections from a 'responder' and a control animal. The 'responder' showed dramatically enlarged seminiferous tubules with all stages of spermatogenesis.

5.4 Discussion

This chapter showed that rat neural xenografts could survive and function in mice immunosuppressed with anti-L3T4 MAb.

GnRH neurons in the xenografts survived transplantation to the third ventricle of *hpg* mice (see Figure 5.2). Thy-1.1 neural tissue could be seen leaving the xenografts superiorly and laterally but the GnRH fibres were always directed inferiorly towards the median eminence. The same specific innervation of the median eminence has been described with POA allografts by Silverman *et al.* (1985), and by Charlton, Parry and Jones (1985). It appears that the signals for axonal guidance are still present in the adult *hpg* mouse and that rat GnRH neurons can follow them!

The GnRH, released into the portal system of the median eminence, was able to stimulate the pituitary to secrete FSH (see Figure 5.3). Presumably LH was also released, although this was not measured, since GnRH is thought to control the release of both these gonadotrophins (Dalkin *et al.*, 1989).

The increased levels of gonadotrophins were able to stimulate the testes to grow (see Figure 5.4) and mature (see Figure 5.5). The response rate in transplanted *hpg* mice, immunosuppressed with anti-L3T4 MAb was 60% (6 out of 10) with a mean testicular weight of 33.1 ± 4.6 mg at one month. Using POA neural grafts from normal littermates of the *hpg* recipients, Charlton *et al.* (1987) showed a response rate of 77% (21 out of 27) with a mean testicular weight of 65.2 ± 14.6 mg by 40 days post-transplant.

There were several reasons why the xenografts may not have produced as large testes as the allografts. First, there may have been fewer GnRH cells in the

xenografts. The rat pup preoptic area is larger than the mouse and thus the 6 L grafts may have included all the mouse pup's POA but only part of the rat pup's. This could be avoided in future studies by using the smaller fetal rat brain or by using purified GnRH neuronal suspensions. Second, the rat GnRH neurons may not have functioned as well as the murine GnRH neurons in the host mouse brain. This seemed unlikely since the rat neurons appeared to follow the murine axonal guidance signals -preferentially sending their axons to the median eminence. Third, the xenografts may not have survived as well as the allografts. Although all xenografts survived the duration of the experiment, there was infiltration by cells of the immune system in most (not shown). With only one time of sacrifice, it is difficult to tell if the testes had been larger but had then decreased due to the reduced GnRH stimulus from a xenograft undergoing rejection. Finally, the anti-L3T4 MAb, itself, may have had an inhibitory effect on restoration of neuroendocrine function. This could be ruled out by repeating the allograft studies with the anti-L3T4 MAb.

The anti-L3T4 MAb treatment in the mouse enables xenografts to survive and function in the third ventricle for at least one month but the results from Chapter 3 suggested that these grafts would not have survived much longer. In an effort to develop a model that might provide consistent longterm neural xenograft survival and function, I moved to a different method of immunosuppression.

The Introductory Chapter outlined the pathway of immune system activation and the proposed mechanics of transplant rejection. The secretion and function of the interleukin-2 molecule appeared to play a crucial role in both. Could blocking this molecule, with a monoclonal antibody against its receptor, provide effective immunosuppression? The pilot study used to test this model is described in the next Chapter.

Figure 5.1 **Thy-1.1 Staining in Ventricular Xenografts**

Thy-1.1-positive xenografts could been seen in the third ventricle of *hpg* mice treated with anti-L3T4 MAb and given rat preoptic area (**a**) or cortex (**d**) but not in those animals treated with anti-Lyt-2 MAb (**c**) or saline (**b**). *x43*

FIGURE 5.1

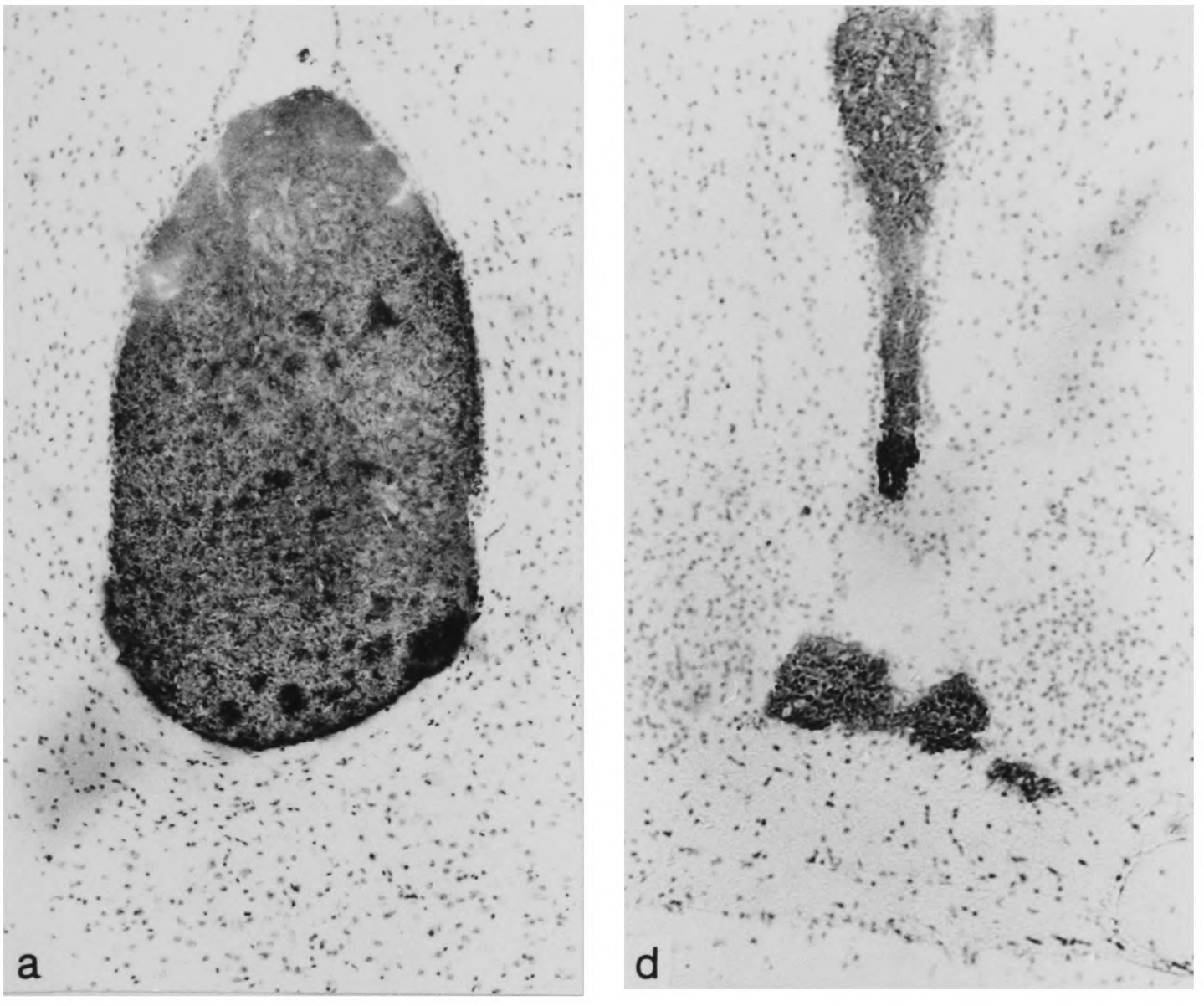


Figure 5.2 **GnRH staining in *hpg* mice given rat preoptic area xenografts**

The coronal section at the level of the median eminence showed positive staining cell bodies (large arrow) with axons projecting toward the infundibular process of the median eminence. *x232*

Figure 5.5 **Testicular Sections**

Testicular sections from *hpg* mice with rat preoptic area xenografts and treated with anti-L3T4 MAb (**a**) and anti-lyt-2 MAb (**b**). **a** showed significantly enlarged seminiferous tubules with all stages of spermatogenesis present. *x232*

FIGURE 5.2

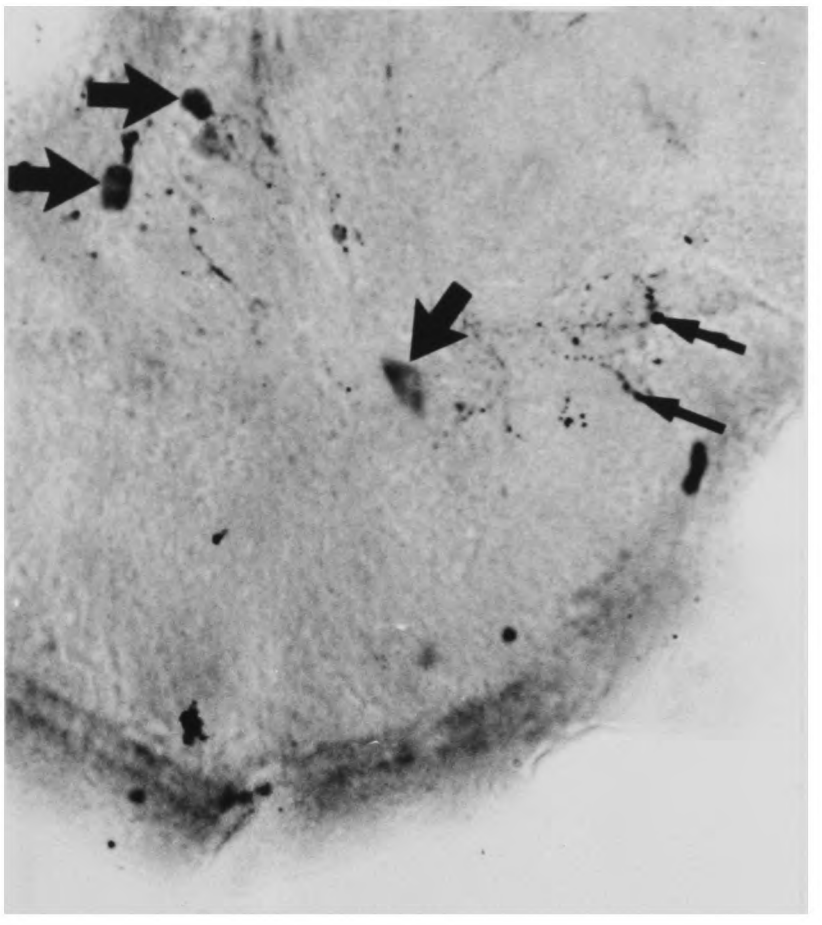
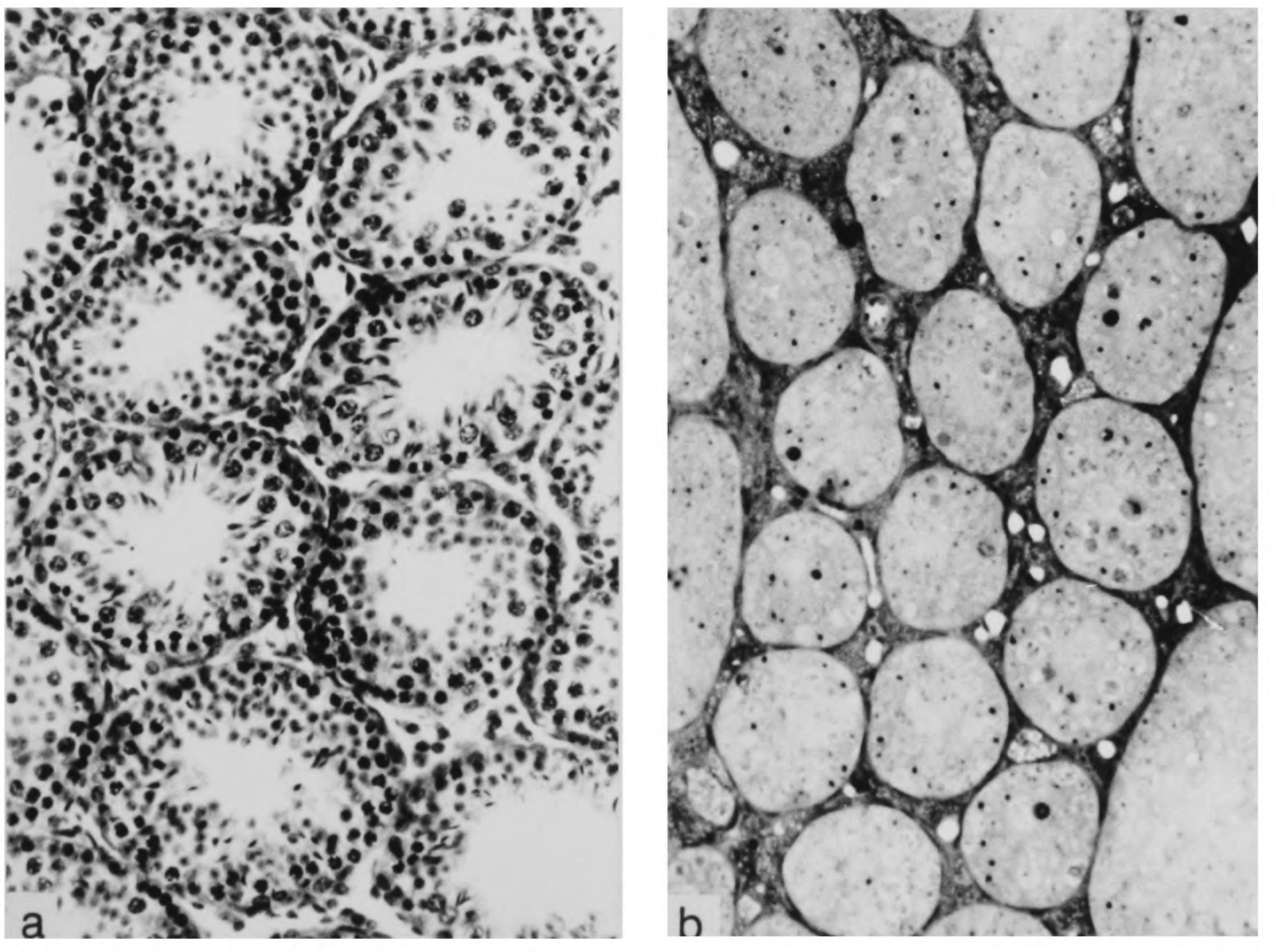


FIGURE 5.5



Chapter 6

anti-IL2R MAb:

Pilot and Dose Response Studies

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6.1 Introduction

The Introductory Chapter outlined the work that has been done using anti-IL2R MAb to prolong allografts in mice (Kirkman *et al.*, 1985) and rats (reviewed by Kupiec-Weglinski *et al.*, 1988). This chapter examines if treatment with NDS 63, an anti-IL2R MAb, could prolong neural graft survival in rats.

The model chosen for this work was mouse cortex transplanted into rat brain parenchyma. Using a xenograft had two advantages. First, it allowed species-specific markers to detect mouse neuronal tissue presence, and hence survival, within the host rat brain. Second, it provided a strong stimulus for rejection and hence a good test of the immunosuppressive capabilities of the anti-IL2R MAb treatment.

The site of transplantation chosen was the rat brain parenchyma - specifically the striatum. This site was chosen because it was hoped that ultimately xenograft function would be tested in the rat 'Parkinsonian' model (see Chapter 8). In this model, grafts are transplanted to the striatum.

Tellides, Dallman and Morris (1988) had shown that NDS 63, an anti-IL2R MAb, at 300 μg/kg/day for 10 days could prolonged cardiac allografts in a rat model with a weak immune response (eg. LEW-RT1^{l} → DA-RT1^{av1}). They also demonstrated that 2700 μg/kg/day for 10 days was required to prolong renal allografts in the same model. In the Pilot Study presented below, the lower dose was used. At that time, I relied on the generous donation of NDS 63 by Dr. Dallman. Enough antibody was received to test the model only at this low dosage. It was hoped, however that if cardiac allografts were prolonged then neural

xenografts might also be prolonged because of the relative immunological privilege of the CNS site.

By the time the Dose Response Study had begun, a large supply of NDS 63 had been prepared and purified (see Chapter 2). The various dosages of anti-IL2R MAb used were chosen to cover as large a range as possible. Unfortunately, insufficient antibody was available to test the 2700 µg/kg/day dosage in the large number of animals required for this model for neural transplantation. A compromise of 300 µg/kg/day, 750 µg/kg/day and 1500 µg/kg/day was eventually chosen for the experimental groups.

The control used for the anti-IL2R MAb treatment raised a problem. Ideally, the control group should have received an anti-interleukin-2 receptor monoclonal antibody which did not block the receptor (ie. bound to an epitope far from the active site). This became commercially available (NDS 66, Serotec) half way through my thesis, but was prohibitively expensive. This work, therefore, was able to show whether or not the anti-IL2R MAb treatment could prolong neural grafts in rats but relied on the work of others (see Introductory Chapter) to suggest how it exerted its effect.

6.2 Pilot Study

6.2.1 Experimental Procedure

Twenty adult (older than 12 weeks) male PVG rats were divided into two groups of ten. Group 1 received 300 μg/kg/day anti-IL2R MAb as intraperitoneal (i.p.) injections for ten consecutive days beginning on the operative day. Group 2 received 1 mL saline i.p. for ten days. All animals had 5 μL day 1 C3H/He mouse cortex xenograft stereotaxically transplanted to their striatum with the following coordinates (Paxinos and Watson, 1982):

anterior:	bregma
lateral:	2.5 mm from midline
deep:	4.5 mm below dura

The animals were all sacrificed 60 days post-transplant and their brains processed for immunohistochemistry. Serial sections through the xenograft were stained with the following panel of primary antibodies:

Antibody	**Specificity**	**Reference**
MRC OX-7	Thy-1.1 (rat brain)	Mason & Williams 1980
30H12	Thy-1.2 (mouse brain,T-cells)	Ledbetter & Herzenberg 1979
MRC OX-27	rat RT1^c MHC Class I	Jefferies *et al.* 1985b
MRC OX-6	rat MHC Class II	McMaster & Williams 1979

6.2.2 Results

Ten out of ten rats given anti-IL2R MAb had indirect evidence of surviving mouse xenograft at 60 days. Figure 6.1 shows photomicrographs of sections through the xenograft from representative animals in Group 1 and Group 2 stained for Thy-1.1, Thy-1.2, and a negative control secondary antibody only.

The Thy-1.1 antibody was a direct peroxidase conjugate and gave a clear picture of rat neural tissue. The animal in Group 1 had an obvious space in its striatum which did not stain for Thy-1.1. This was the same location as the Thy-1.2-positive mouse neural xenograft. The animal in Group 2 did not have a similar hole in its Thy-1.1-positive striatum - only a scar where the xenograft had been. This suggested that the mouse xenograft in the control animal was no longer occupying any space within the striatum.

The Thy-1.2 antibody was not a direct peroxidase conjugate and required a secondary antibody (rabbit-anti-rat immunoglobulin conjugated to peroxidase) to reveal its presence in an indirect immunohistochemical procedure. Unfortunately, this did not produce a clear picture of mouse neural tissue. The secondary antibody bound to the primary (specific staining of mouse brain) but also to residual rat immunoglobulin (non-specific background staining) present in the rat brain. It was hoped that flushing the cerebral vasculature with heparinized saline prior to sacrifice would have removed any immunoglobulin from the rat's brain but this was not the case.

Figure 6.1 shows intense Thy-1.2 staining in the animal from Group 1 but both animals had a halo of staining around the graft site. The two sections stained with secondary antibody alone, showed that this halo of staining was due to non-

specific staining by the secondary antibody - presumably it had bound to extravasated rat immunoglobulin around the graft site.

Figure 6.1 also shows photomicrographs of sections through the xenograft from representative animals in Group 1 and Group 2 stained for MRC OX-27 and MRC OX-6.

MRC OX-27 is a polymorphic mouse anti-rat monoclonal antibody that is specific for PVG-RT1^c (PVG) MHC Class I. As discussed in Chapter 3 (section 3.3.3), expression of host Class I antigens is a good marker for the level of host immune system activation. Figure 6.1 shows that the level of host Class I expression is much less in the animal from Group 1 than in the control from Group 2.

Similarly, the xenografts were examined for host MHC Class II expression. MRC OX-6 is a non-polymorphic mouse anti-rat monoclonal antibody that binds all forms of rat MHC Class II. Mason *et al.* (1986) had shown that allografts undergoing rejection in the rat brain were filled with MHC Class II-positive cells. MRC OX-6 staining was therefore a good marker for active graft destruction.

Figure 6.1 shows little MHC Class II expression in and around the xenograft from the animal in Group 1, but very strong staining in the control animal.

6.2.3 Discussion

The results suggested that anti-IL2R MAb treatment could prolong neural xenografts in rats. There were, however, several problems raised by this Pilot Study.

First, the Thy-1.2 staining of mouse neural xenograft was obscured by non-specific background staining. The stronger Thy-1.2 staining (coupled with the lack of Thy-1.1 staining) in the transplantation site of experimental animals compared to controls was highly suggestive of surviving xenograft in these animals. But was it conclusive? This lead to the second problem, the results were qualitative not quantitative. What was needed was an antibody which would bind mouse neural tissue without any background staining of rat brain. In order to solve this problem, I began the long process of preparing a direct peroxidase conjugate of the 30H12 antibody. At the same time, however, Serotec (Kidlington, U.K.) came out with a new antibody - F7D5.

This mouse-anti-mouse Thy-1.2 monoclonal antibody was raised in AKR/J mice immunized against the Thy-1.2 antigen from CBA mice. It was designed to be used with complement to deplete T-lymphocytes in mice. If this antibody could recognize the Thy-1.2 on murine T-cells, there was no reason why it could not recognize the Thy-1.2 on murine brain tissue. Since the antibody was a mouse IgG2b, the secondary antibody used to reveal it could be a rabbit-anti-mouse immunoglobulin linked to peroxidase. This secondary would bind only mouse immunoglobulin and thus illiminate the problem of non-specific staining of the host rat brain. If xenograft survival could be clearly shown with the F7D5 antibody,

then the results could be quantified and the immunosuppressive technique analyzed statistically.

The third problem with the Pilot Study was the antibody used to stain infiltrating cells, MRC OX-6. This antibody binds the rat MHC Class II antigen. It was hoped that this antibody would serve two purposes: it would stain Class II-positive infiltrating cells within the xenograft and would show any Class II induction on the host rat brain. The majority of Class II-positive infiltrating cells are macrophages (Mason *et al.*, 1986). They represent only a fraction of the possible cell population present in xenograft rejection. In order to characterize graft infiltration better, xenografts were stained with a panel of antibodies against various infiltrating cells in all future experiments.

In conclusion, this immunosuppressive technique showed enough promise to be tested in a more detailed study. The above modifications were incorporated into the following Dose Response Experiment.

6.3 Dose Response Study

6.3.1 Experimental Procedure

Seventy-two adult (older than 12 weeks) male PVG rats were divided into four groups of eighteen. Each animal received ten consecutive days of intraperitoneal injections of saline or various amounts of anti-IL2R MAb according to Table VIa beginning on the day of operation.

Table VIa

Group 1	300 µg/kg/day IL2R MAb
Group 2	750 µg/kg/day IL2R MAb
Group 3	1500 µg/kg/day IL2R MAb
Group 4	saline 1 mL/day

All animals had 5 µL day 1 C3H/He mouse cortex xenograft stereotaxically transplanted to their striatum as in the Pilot Study. Six animals from each group were sacrificed at 60 days, 90 days and 120 days post-transplant. Their brains processed for immunohistochemistry and serial sections through the xenograft were stained with the following panel of primary antibodies:

Antibody	Specificity	Reference
MRC OX-7	Thy-1.1 (rat brain)	Mason & Williams 1980
F7D5	Thy-1.2 (mouse brain)	Lake & Clarke 1979
MRC OX-19	rat pan T-cell	Dallman *et al.* 1984
MRC OX-52	rat pan T-cell	Robinson *et al.* 1986b
NDS 63	rat IL2R	Tellides 1988
MRC OX-27	rat $RT1^c$ MHC Class I	Jefferies *et al.* 1985
MRC OX-42	microglia	Robinson *et al.* 1986a
MRC OX-21	human C3b INA	Hsiung *et al.* 1982

The MRC OX-21 is a mouse IgG1 MAb specific for the human C3b inactivator. It was used as a negative control primary antibody on one section from each xenograft. None of the sections stained with this antibody showed any staining (not shown).

In order to assess the health of the recipient animals, each was weighed weekly and, after sacrifice, their kidneys were examined histologically for signs of immune complex glomerulonephritis.

6.3.2 Results

6.3.2.1 Health of Transplant Recipients

It was important to determine if the anti-IL2R MAb treatment had any deleterious effects on the rats. In order to address this question, the rats were examined for three indicators of health: death rate, weight gain and renal histology. There were no perioperative or postoperative deaths in any of the groups. There was no significant difference between the mean weight of any group at any time point by analysis of variance. At sacrifice the kidneys were frozen, sectioned and stained with hæmatoxylin and eosin. There were no signs of glomerulonephritis in any animal. Renal sections from representative animals in each group are shown in Figure 6.2.

6.3.2.2 Xenograft Survival

Sections through the xenograft were stained for the Thy-1.2 surface antigen with the new F7D5 antibody. Figure 6.3 shows photomicrographs of such sections from representative animals in each group. Xenografts were labelled as 'surviving' if there was F7D5-positive staining in any of the sections. The results are shown in Table VIb.

Table VIb

	Group 1	Group 2	Group 3	Group 4
60 Days	6/6	6/6	6/6	2/6
90 Days	3/6	6/6	6/6	0/6
120 Days	3/6	6/6	6/6	0/6

There were several points raised by these results. First, two out of the six control animals in Group 4 had positive staining for xenograft tissue at 60 days. These xenografts, however, were small and filled with infiltrating cells. Second, 50% of the animals in Group 1 rejected their xenografts by 90 days. The dosage of anti-IL2R MAb in this group must have been too low. Third, was no difference in survival rate of xenografts in Groups 2 and 3 (100% at 120 days). The xenografts in these two groups would have to be examined for other factors to determine if the higher dose of anti-IL2R MAb in Group 3 was necessary.

6.3.2.3 Infiltrating Cells

Sections through the xenograft were stained for all T-cells with a cocktail of the MRC OX-19 and MRC OX-52 antibodies and for the IL2R on activated T-cells with the NDS 63 MAb. Figure 6.3 shows photomicrographs of such sections from a representative animal from Group 2 and 4. As expected, the xenograft in the control animal (Group 4) were filled with T-cells. The xenograft in the animal immunosuppressed with anti-IL2R MAb also showed a number of infiltrating cells. This was expected since the anti-IL2R MAb treatment was designed to block the activation of T-lymphocytes not deplete them as the anti-L3T4 MAb had done in Chapters 3, 4 and 5.

6.3.2.4 Host Brain Response

Sections through the host rat brain at the site of transplantation were stained for MHC Class I antigens with MRC OX-27 and for microglia with MRC OX-42. Figure 6.3 shows photomicrographs of such sections from representative animals in each group. All groups showed induction of MHC Class

I antigens around the transplantation site although Groups 2 and 3 had less than Groups 1 and 4. MHC Class 1-positive host blood vessels could also be seen throughout surviving xenografts. All groups also showed a glial response around the xenograft.

6.3.3 Discussion

The anti-IL2R MAb treatment did not have any obvious deleterious effects on the rats. Their weights and renal histology were no different than those of control animals.

The treatment did significantly prolong xenograft survival from 0% at 90 days in controls to 100% at 120 days in experimental groups 2 and 3 (see Table VIb). Three experimental groups were chosen to find the optimal dosage of anti-IL2R MAb required to prolong xenograft survival. 300 µg/kg/day (Group 1) appeared to be too low since 50% of xenografts were rejected by 90 days. There were no differences in any of the parameters of xenograft survival examined between those animals given 750 µg/kg/day (Group 2) and those given 1500 µg/kg/day (Group 3). All future experiments with anti-IL2R MAb used 750 µg/kg/day for 10 days.

This dosage of anti-IL2R MAb had enabled murine xenografts to survive in the rat brain parenchyma for 120 days with very little sign of rejection. The host brain had been triggered to express MHC Class I antigens and had formed a glial response but this may always occur with transplantation - even with syngeneic grafts (Mason *et al.*, 1986). There was no reason to believe that these xenografts would not have gone on to survive much longer.

One advantage of using xenografts in this model of neural transplantation was that the vascular supply of the graft could be determined using species-specific markers. The host rat brain provided a large blood supply to the xenograft in all cases (host-specific MHC Class I positive staining blood vessels could be seen throughout the xenograft). Baker (1989) had shown that the major vascular supply of neural allografts was also of host origin. A directly conjugate antibody to a cell marker on mouse endothelial cells would be required to demonstrate if the donor xenograft retained any of its own vascular supply.

This chapter showed that the IL2R MAb treatment was very effective at prolonging neural graft survival in the rat. The next step was to ensure that these grafts were functioning. Two models which showed the function of neural grafts in rats were tentatively chosen: vasopressin neuronal grafts into the Brattleboro rat and dopamine neuronal grafts into the 'hemi-Parkinsonian' rat.

The next chapter explores the survival of neural allografts in the third ventricle of rats in preparation for use in the Brattleboro model. The subsequent chapter explores the 'hemi-Parkinsonian' model.

Figure 6.1 Histology of Murine Xenografts in Rat Brain Parenchyma

1 a,b Thy-1.1 Staining

Positive staining host brain outlined the negative staining xenograft in rats treated with anti-IL2R MAb (**a**) and saline (**b**). *x43*

2 a,b Thy-1.2 Staining

Xenograft in rat treated with anti-IL2R MAb (**a**) had positive Thy-1.2 staining. The xenograft in the rat treated with saline also appeared to have positive Thy-1.2 staining but the control sections stained with secondary antibody alone (see below) showed that this staining is non-specific. *x43*

3 a,b Control Sections with Secondary Antibody Alone

The staining in **2b** was shown to be due mostly to non-specific staining whereas that in **2a** was due mostly to specific staining. *x43*

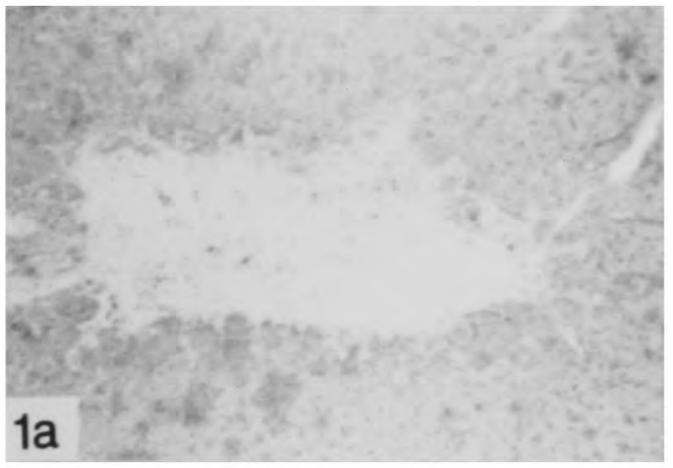
4 a,b MRC OX-27 (Rat RT1^c MHC Class I) Staining

The xenograft in the rat treated with anti-IL2R MAb (**a**) had little rat Class I induction around the transplantation site whereas the control animal (**b**) had strong staining for these antigens. *x43*

5 a,b MRC OX-6 (Rat MHC Class II) Staining

The xenograft in the rat treated with anti-IL2R MAB (**a**) had few Class II-positive cells within it whereas the xenograft in the control animal was filled with these cells. *x43*

FIGURE 6.1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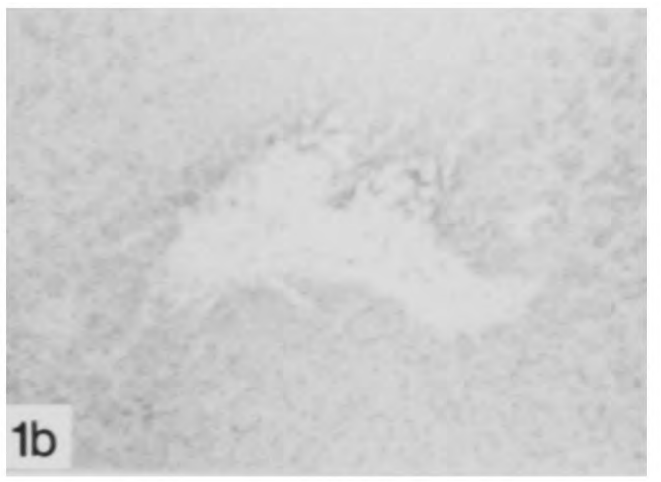
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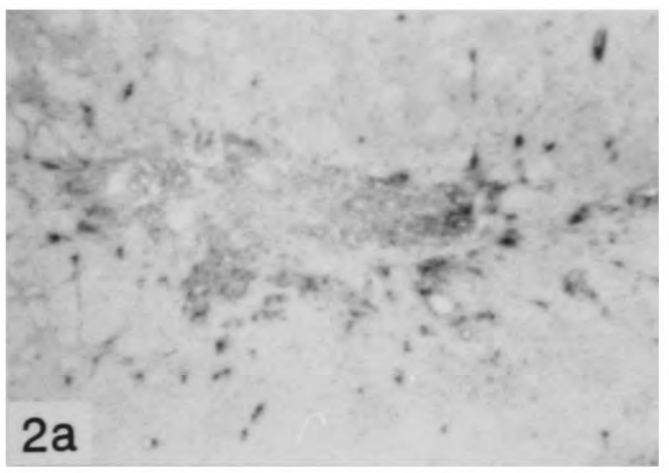
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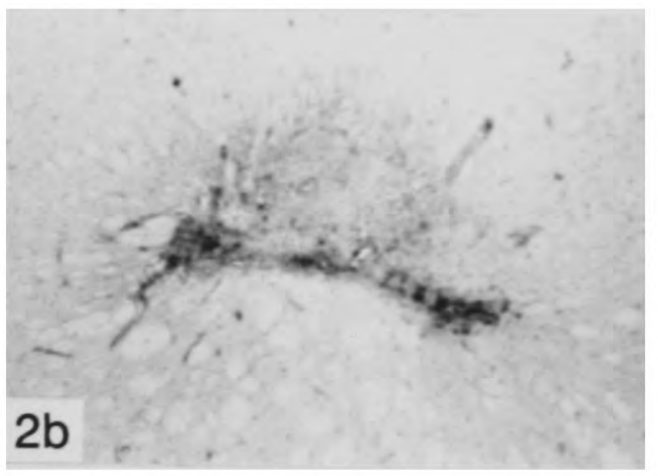
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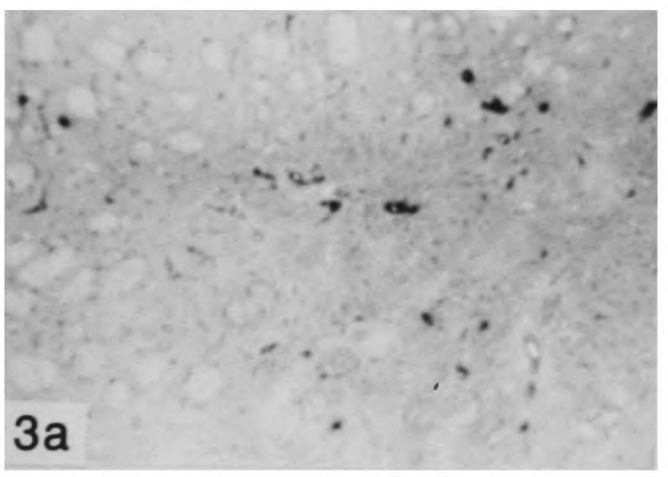
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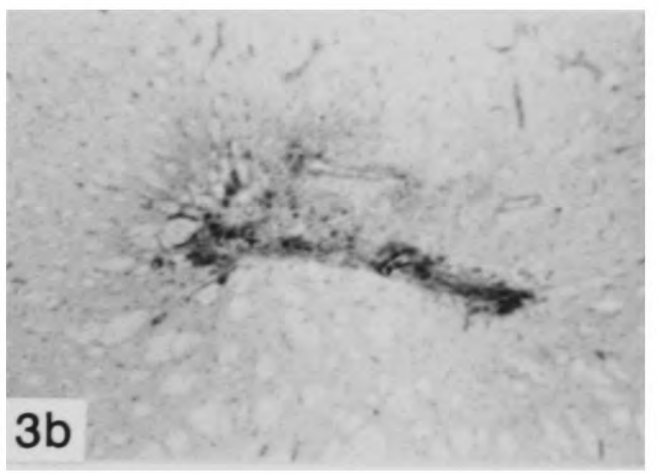
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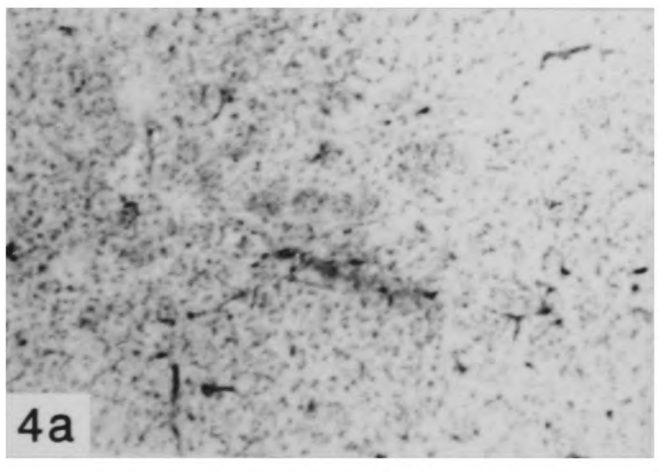
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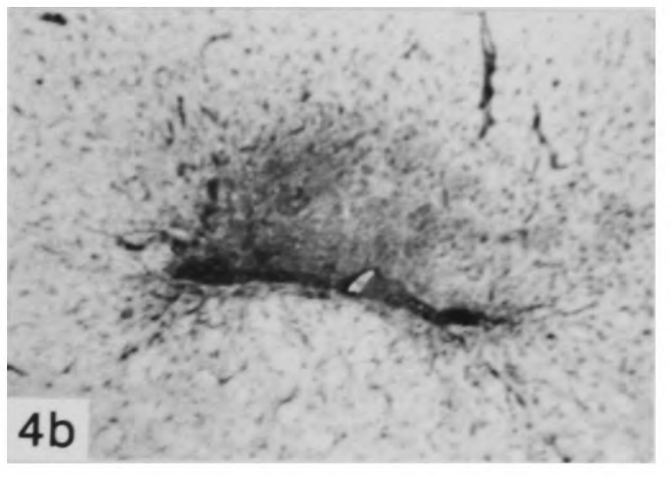
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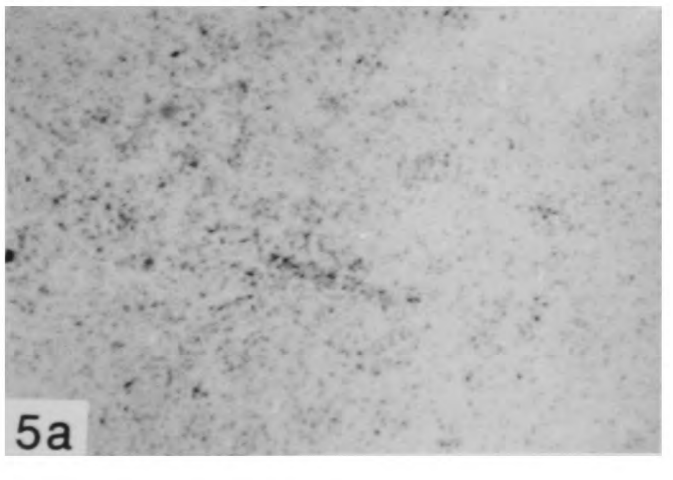
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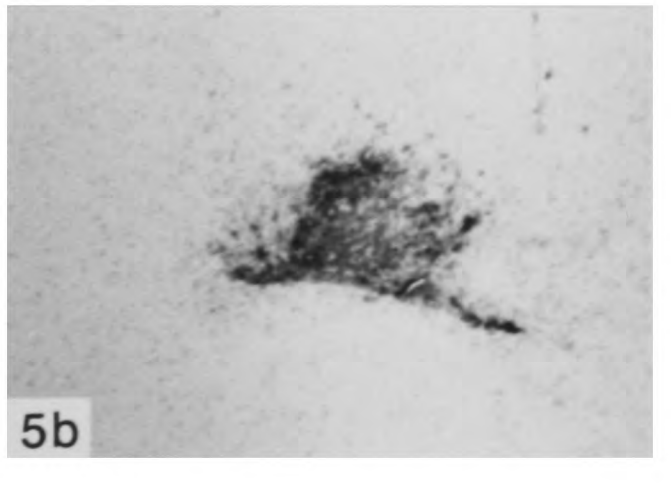
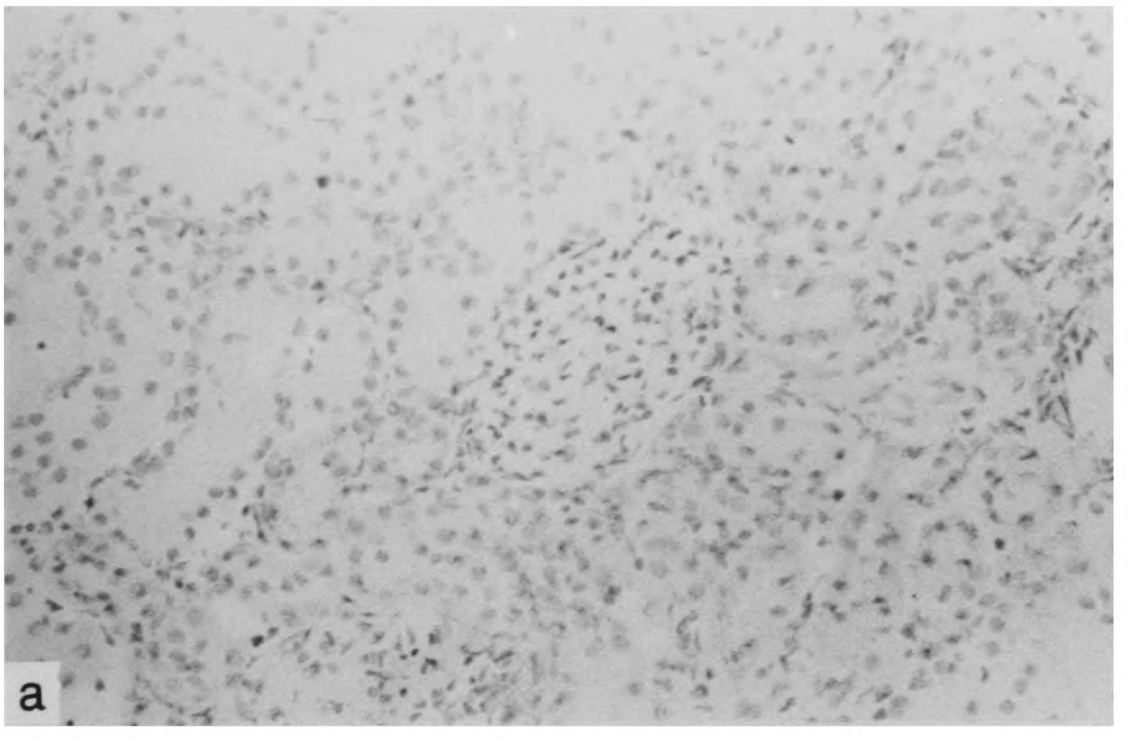

5b

Figure 6.2 **Renal Histology**

Sections from the kidney of a rat treated with anti-IL2R MAb (**a**) and one given saline (**b**). There were no signs of glomerulonephritis or non-specific damage in the anti-IL2R MAb treated animal. *x232*

FIGURE 6.2



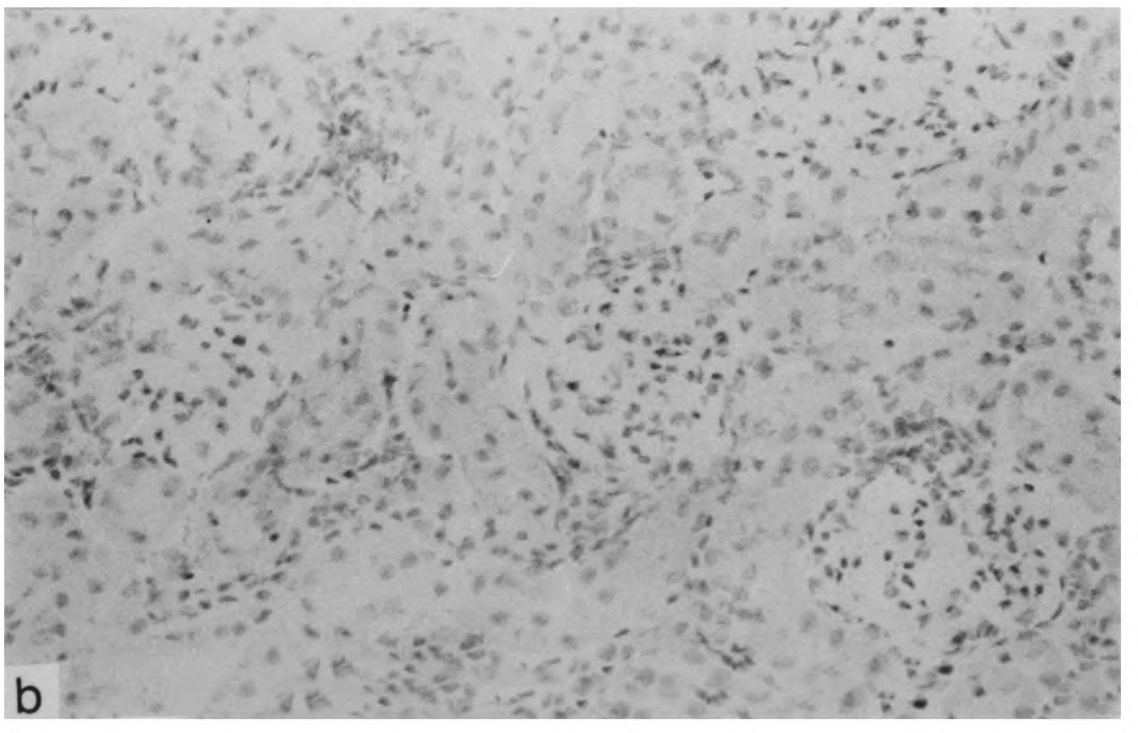


Figure 6.3 Histology of murine xenografts in rat brain parenchyma

1 a,b F7D5 (Thy-1.2) Staining

The xenograft in the rat treated with anti-IL2R MAb (**a**) showed strong staining for Thy-1.2 whereas the xenograft in the rat given saline (**b**) had very weak staining. *x43*

2 a,b MRC OX-19 + MRC OX-52 (pan T-cell) Staining

The rat treated with anti-IL2R MAb (**a**) had few T-cells within its xenograft whereas the rat given saline (**b**) had numerous T-cells within its xenograft. *x43*

3 a,b IL2R Staining

The rat treated with anti-IL2R MAb (**a**) had few IL2R-positive cells within its xenograft whereas the rat given saline (**b**) had a large number of these cells within its xenograft. *x43*

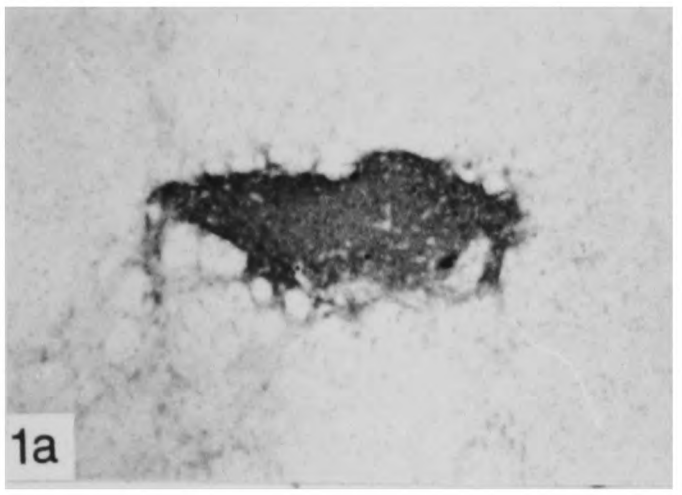
4 a,b MRC OX-27 (Rat RT1^c MHC Class I) Staining

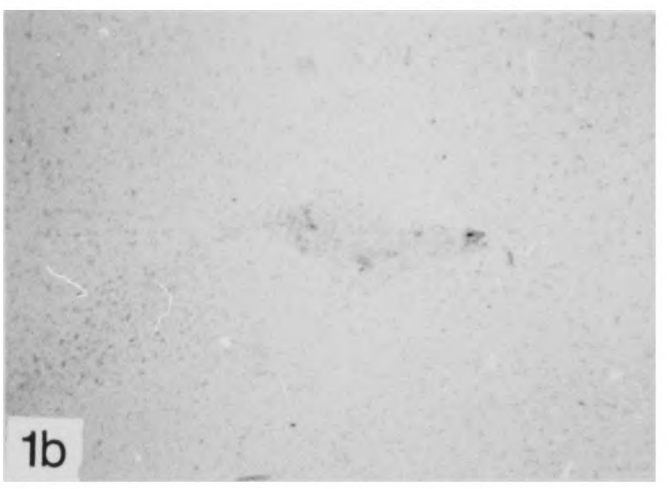
The rat treated with anti-IL2R MAb (**a**) had little induction of MHC Class I antigens around its transplantation site whereas the rat given saline (**b**) had strong Class I. *x43*

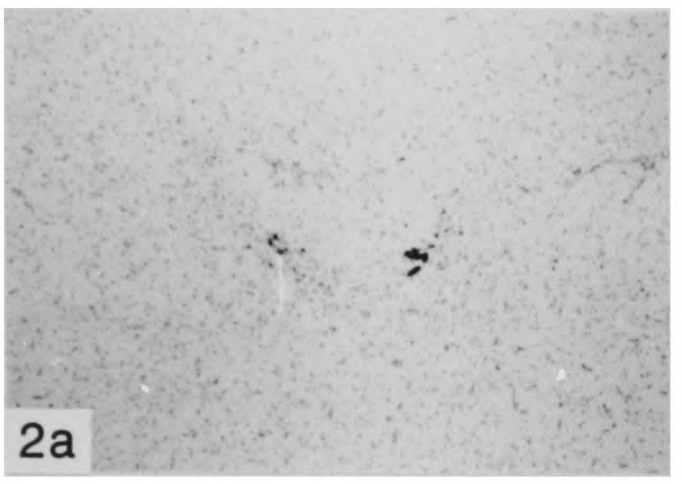
5 a,b MRC OX-42 (Microglia) Staining

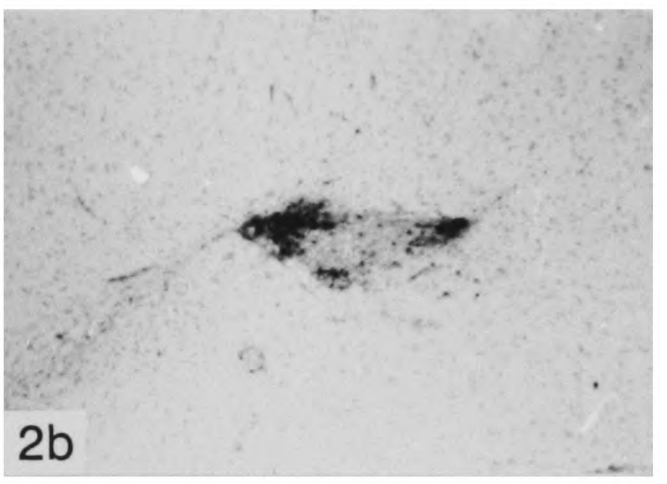
The rat treated with anti-IL2R MAb (**a**) had a smaller glial reaction than the rat given saline (**b**). *x43*

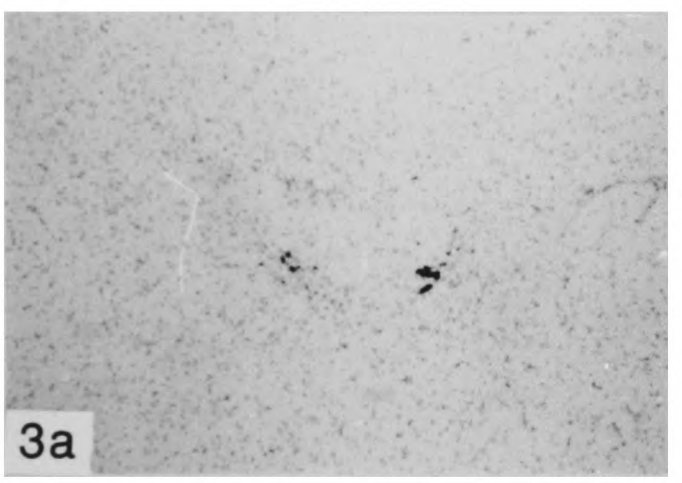
FIGURE 6.3

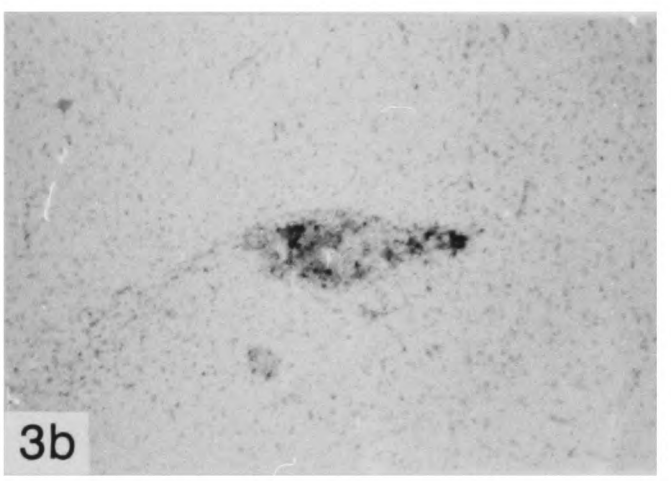


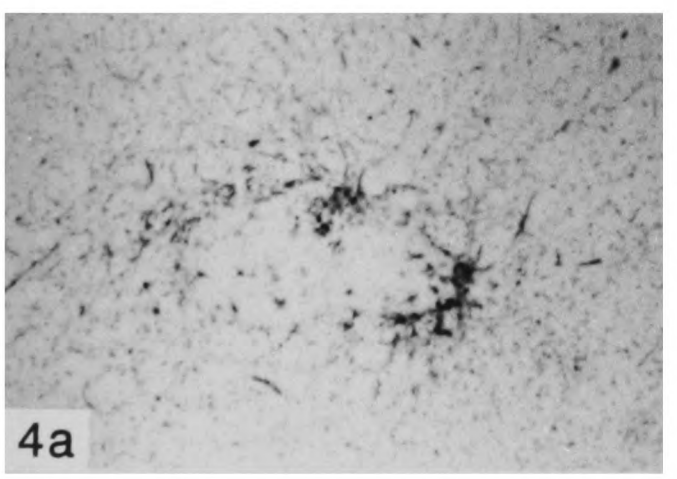


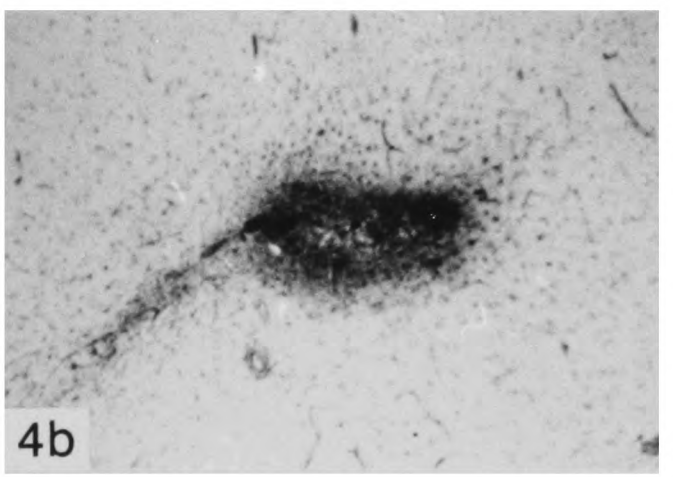


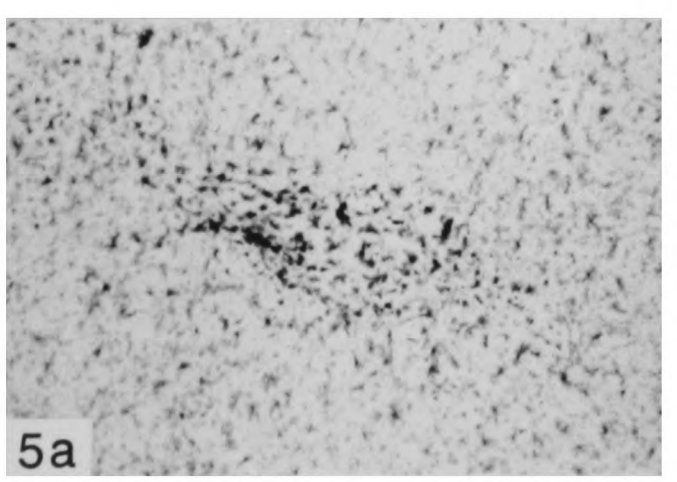


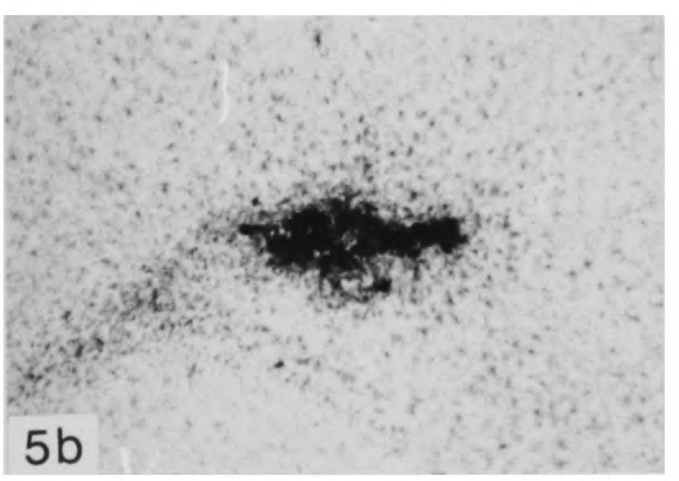












Chapter 7

Neural Allografts in Rats

Table of Contents

7.1 Introduction

This chapter investigates the viability of neural allografts transplanted into the third ventricle of rats immunosuppressed with anti-IL2R MAb.

Day 1 PVG rat cortex was transplanted into the third ventricle of AO rats and then assessed at 45 and 90 days for viability. These strains were chosen because they differed in both major and minor histocompatibility antigens and thus provided a strong stimulus for rejection. Sloan (1989) had shown that day 1 PVG cortex transplanted into the third ventricle of adult AO rats were actively rejected by 80% of animals by 30 days and by 100% of animal by 60 days.

If there was good survival of the allograft in the AO rat treated with anti-IL2R MAb, the experiment would be repeated using the Brattleboro rat as the recipient. The Brattleboro rat has a deficiency of vasopressin neurons which results in polyuria (Valtin, 1962). Gash and Sladek (1980) showed that transplanted vasopressin neurons could survive in the adult third ventricle and that the vasopressin released could alleviate the polyuria. The experimenters could thus follow urine output as a measure of the neural graft function.

The third ventricle was chosen as the site of transplantation in the AO rat in preparation for utilizing the Brattleboro model.

7.2 Experimental Procedure

Forty adult (older than 12 weeks) female AO rats were divided into two groups of twenty. Group 1 was immunosuppressed with 750 μg/kg/day for 10 days, while Group 2 received saline. All animals received 10 μL day 1 PVG rat cortex stereotaxically transplanted into their third ventricle with the following coordinates (Paxinos and Watson, 1982):

anterior:	8.2 mm from interaural line
lateral:	0.0 mm from midline
deep:	6.0 mm from dura

Half the animals from both groups were sacrificed at 45 days and the rest at 90 days post-transplant. Their brains were processed for immunohistochemistry and stained with the following panel of primary antibodies:

Antibody	Specificity	Reference
MRC OX-7	Thy-1.1 (rat brain)	Mason & Williams 1980
MRC OX-27	rat RT1^c MHC Class I	Jefferies *et al.* 1985
MRC OX-18	all rat MHC Class I	Fukumoto *et al.* 1982
MRC OX-19	rat pan T-cell	Dallman *et al.* 1984
MRC OX-52	rat pan T-cell	Robinson *et al.* 1986b
NDS 63	rat IL2R	Tellides 1988
MRC OX-42	microglia	Robinson *et al.* 1986a
MRC OX-21	human C3b INA	Hsiung *et al.* 1982

MRC OX-21 is a mouse IgG1 MAb specific for human C3b inactivator. It was used as a negative control primary antibody on one section from each allograft. None of the sections showed any staining with this antibody.

7.3 Results

7.3.1 Allograft Survival

Sections through the third ventricle were stained for rat neural tissue with MRC OX-7 antibody, for donor MHC Class I antigens with the polymorphic MRC OX-27 antibody, and for all rat MHC Class I antigens with the monomorphic MRC OX-18 antibody. Photomicrographs of representative sections are shown in Figure 7.1. The animals in Group 1 showed even and strong staining for the Thy-1.1 antigen at both 45 and 90 days post-transplant. The control animals showed staining at 45 days but very little at 90 days. Each animal was scored as positive or negative depending on whether or not there was any Thy-1.1-positive staining in the third ventricle. The results are shown below in Figure 7.2.

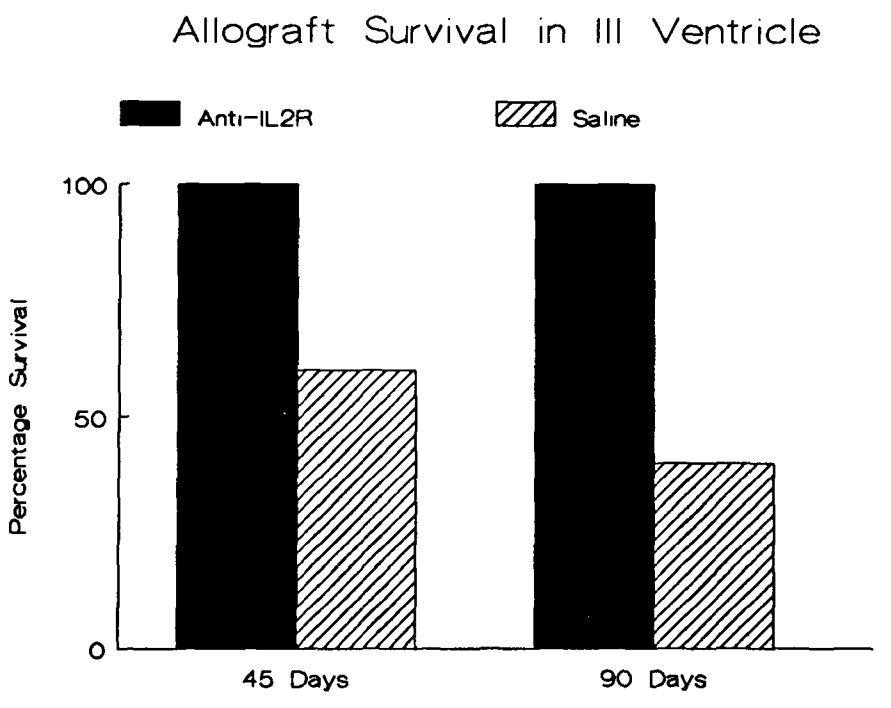


Figure 7.2

These results highlighted two points. First, four out of ten control animals had allografts with some Thy-1.1-positive staining even 90 days post-transplant. Chapter 4 had shown that, the C57Bl/6 mouse had rejected all fully allogeneic grafts by 28 days. The AO rat appeared to less effective at rejecting fully allogeneic neural grafts than the C57Bl/6 mouse. The immune system of the mouse has been shown to be more effective than that of the rat at rejecting other allografts such as cardiac and renal grafts (personal communication with Drs. Wood and Dallman). Second, the anti-IL2R MAb treatment prolonged allograft survival to 100% at 90 days compared to 40% in controls. This was a significant difference ($p<0.01$ by chi-squared test).

Figure 7.1 also shows the expression of donor MHC Class I antigens (MRC OX-27) on the allografts. Expression of these antigens on rat (Mason *et al.*, 1986) or mouse (Date, Kawamura, and Nakashima, 1988) neural grafts has been correlated with graft rejection. What was remaining of the allografts in the control animal were all strongly expressing MHC Class I antigens. The allograft in the animal treated with anti-IL2R MAb was not expressing these antigens. There were also no donor Class I-positive blood vessels in this allograft. Expression of host and donor MHC Class I antigens (MRC OX-18) shows positive staining blood vessels throughout the host brain and the allograft. All these vessels were of host origin.

Only two out of ten allografts in animals given anti-IL2R MAb showed any staining for donor MHC Class I antigens at 45 days post-transplant. By ninety days post-transplant, however, all allografts were expressing these antigens.

7.3.2 Infiltrating Cells

Sections through the third ventricle were stained for all T-cells with a cocktail of the MRC OX-19 and MRC OX-52 antibodies, and for the IL2R on activated T-cells with the NDS 63 MAb. Figure 7.3 shows photomicrographs of such sections from representative animals in both groups. The control animal showed large numbers of T-cells within the third ventricle. The anti-IL2R MAb treated animal also had a number of these cells within the graft.

At ninety days, the remaining allografts in the control group (3/10) were all filled with infiltrating cells. The allografts in the animals which had received anti-IL2R MAb all had some infiltrate but none of them showed the large numbers of cells seen in the control animals.

7.3.3 Host Brain Response

Sections through the third ventricle were stained for microglia with the MRC OX-42 antibody. Figure 7.3 shows photomicrographs of such sections from representative animals in both groups. Both the control and anti-IL2R MAb treated animals showed a glial response, although, the control animals appeared to have a stronger response.

7.4 Discussion

The results showed that neural allograft survival in the third ventricle was prolonged in rats treated with anti-IL2R MAb.

At 45 and 90 days, the allografts in animals treated with anti-IL2R MAb always had a population of leucocytes within them. A large proportion of these leucocytes were T-cells and a small percentage of these were expressing the IL2-receptor (found on activated T-cells). No allograft in this group, however, showed the dramatic clonal expansion and graft infiltration of T-cells associated with graft rejection seen in all the control animals. The presence of T-cells within the allografts of treated animals did not seem to lead to inevitable graft rejection, at least over 90 days. Tellides, Dallman, and Morris (1988) also showed numerous IL2R-positive cells in permanently accepted rat cardiac allografts.

What was attracting the T-cells to the allografts? Why were they not undergoing clonal expansion? The local damage caused by transplantation will trigger an inflammatory response in the host brain. Leucocytes, particularly polymorphonuclear cells and to a lesser extent macrophages, migrate to the site of inflammation by chemotaxis. The increased capillary permeability enable T-cells to transverse the endothelium and enter the graft. Once helper T-cells recognize foreign antigen, they are transformed into blast cells. These activated cells express the interleukin-2 receptor (amongst other activation markers) and release a series of lymphokines including interleukin-2. At this point, there should be no difference between control and treated animals.

Interleukin-2 causes clonal expansion of activated cells. The T-cells in animals treated with anti-IL2R MAb are blocked from responding to the interleukin-2 released by blast cells. The clonal expansion is thus prevented in the treated

animals. This explains why there were activated T-cells (expressing IL2R) seen in the allografts of experimental animals but only the controls showed the large clonal expansion which accompanies rejection.

Both the control and experimental animals showed host MHC Class I antigen induction and a glial response around the transplantation site (although in both cases the controls showed a larger response). These host brain responses may be triggered by the inflammation which inevitably accompanies transplantation (Mason *et al.*, 1986) and by IFN-gamma released by activated T-cells (Steeg, 1982). The experimental animals showed none (8/10) or very little (2/10) donor MHC Class I induction at 45 days but all (10/10) allografts expressed these antigens by ninety days. Cytotoxic T-cells require MHC Class I expression on target cells in order to destroy them. The delay in expression of these antigens on the allografts in the experimental animals suggests that the majority of allografts in these animals were not susceptible to rejection until after 45 days.

In conclusion, anti-IL2R MAb treatment allowed allografts to survive in the third ventricle of rats for at least 90 days. In order to demonstrate the functional capacity of neural allografts, the Brattleboro rat was used.

7.5 The Brattleboro Rat

The experiments with this model are dealt with in this section (rather than in a separate chapter) because they met with uniform failure.

7.5.1 Introduction

The Brattleboro rat is deficient for vasopressin neurons and thus suffers from diabetes insipidus (Valtin *et al.*, 1962). Gash and Sladek (1980) showed that fetal hypothalamic vasopressin neurons transplanted into the third ventricle of adult Brattleboro rats could alleviate the polyuria. This work attempted to repeat these results using allografts and anti-IL2R MAb for immunosuppression.

7.5.2 Experimental Procedure

Fifteen adult (two year old) male Brattleboro rats were divided into three groups of five. The animals were donated by Dr. J.F. Morris from the Department of Human Anatomy, Oxford, U.K. The animals were treated and received transplants to their third ventricle as outlined in Table VIIa.

Table VIIa

Group	Treatment	Transplant
Group 1	anti-IL2R MAb	SOA
Group 2	anti-IL2R MAb	cortex
Group 3	saline	SOA

The neural grafts for Groups 1 and 3 were taken from the supra optic area (SOA), a source of vasopressin neurons, of two PVG day 15 fetuses and combined into one 8 μL allograft. Those for Group 2, were taken from the cortex of day 1

PVG rat pups. All allografts were transplanted as in section 7.2 and the animals daily urine output measured for 90 days until sacrifice. The brains from animals in Group 2 and 3 were prepared for immunohistochemistry. The brains from animals in Group 1 were intended to be stained for vasopressin neurons but were eventually processed for immunohistochemistry as well.

7.5.3 Results

The mean daily urine output for each group was calculated and displayed below in Figure 7.4.

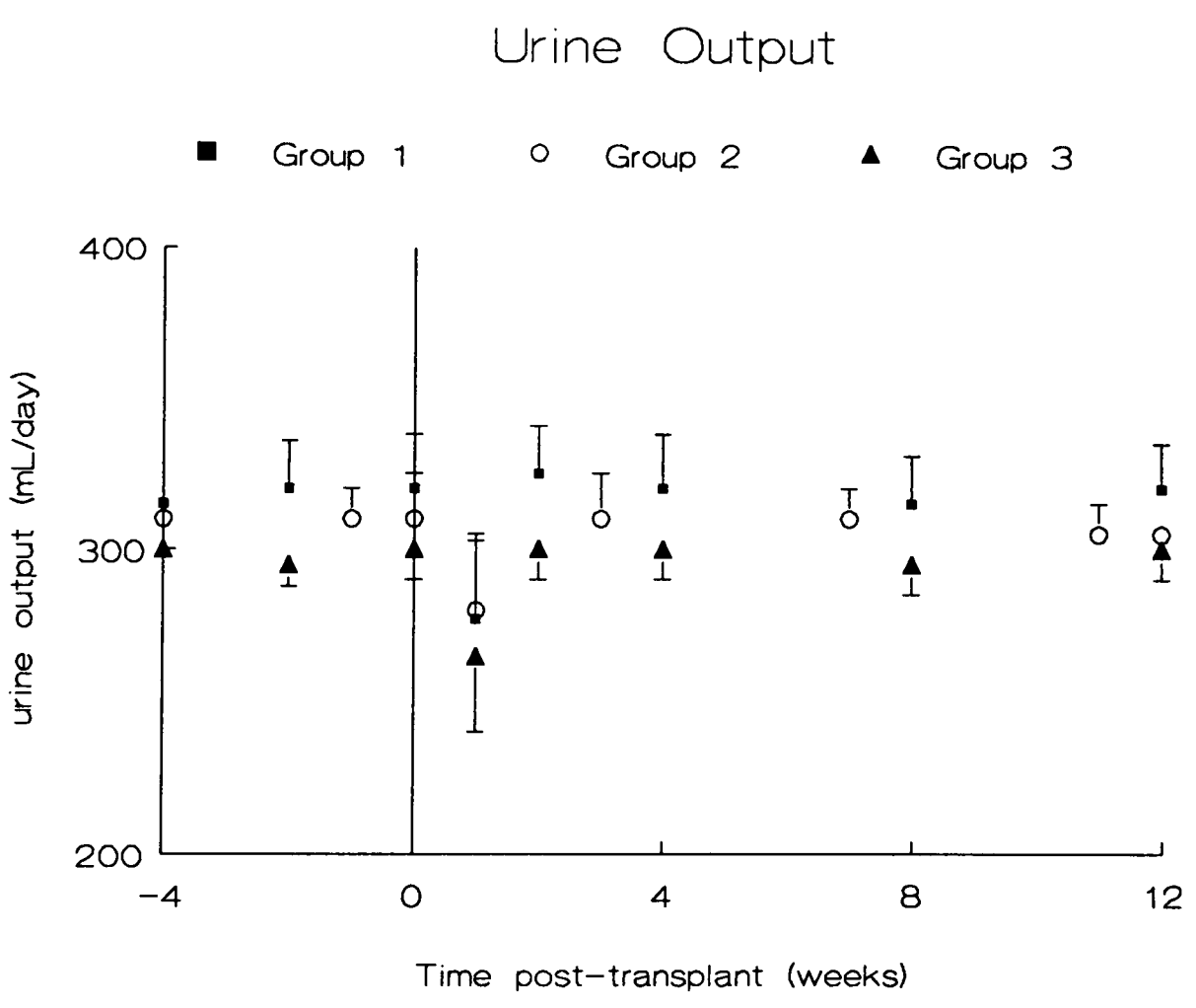


Figure 7.4

There was a slight reduction in urine output immediately post-operative in all groups. This was probably caused by the reduced water intake which occurred while the animals were recovering from the anæsthetic and trauma of surgery. All groups had returned to their preoperative levels by two weeks. After, this point, there was no significant reduction in urine output in any of the groups. The allografts had not demonstrated any function.

Sections through the third ventricle were stained for rat neural tissue with the MRC OX-7 antibody. The animals in Groups 2 and 3 were sacrificed first and none showed any allograft survival in the third ventricle. If the allografts in Group 2 had not survived then it was unlikely that those in Group 1 would have survived. Instead of processing the brains of the animals in Group 1 to look for vasopressin neurons, the brains were processed to look for any allograft tissue. None of the animals in Group 1 showed any surviving allografts.

7.5.4 Discussion

The Brattleboro rat had been chosen in an attempt to demonstrate the functional capacity of transplanted vasopressin neurons. The results, however, failed to show any donor neuronal survival (and hence no function). One reason the allografts may have been rejected was that Brattleboro rats of this age have significant renal disease with hydronephrosis and proteinuria (Henderson *et al.*, 1982). The anti-IL2R MAb may never have reached therapeutic levels because it was excreted in the urine. The older rats had been used because they had been donated by Dr. Morris and the younger animals available from Harlen Olac (Bicester, U.K.) were very expensive. This model could be tried in younger Brattleboro rats which have polyuria but have not yet developed proteinuria.

At the same time as these disappointing results were obtained, experiments with another functional model for neural grafts in rats, the hemi-Parkinsonian model, had shown very promising results. Because of the failure of the preliminary studies and the expense involved in any future work, the Brattleboro model was never tried again. All my attention moved to the hemi-Parkinsonian model which will be discussed in the next chapter.

Figure 7.1 Histology of Ventricular Allografts in Rats

1 a,b Thy-1.1 Staining

The third ventricle of the rat treated with anti-IL2R MAb (**a**) was filled with Thy-1.1-positive staining allograft whereas the rat given saline had no Thy-1.1 staining within its ventricle. *x116*

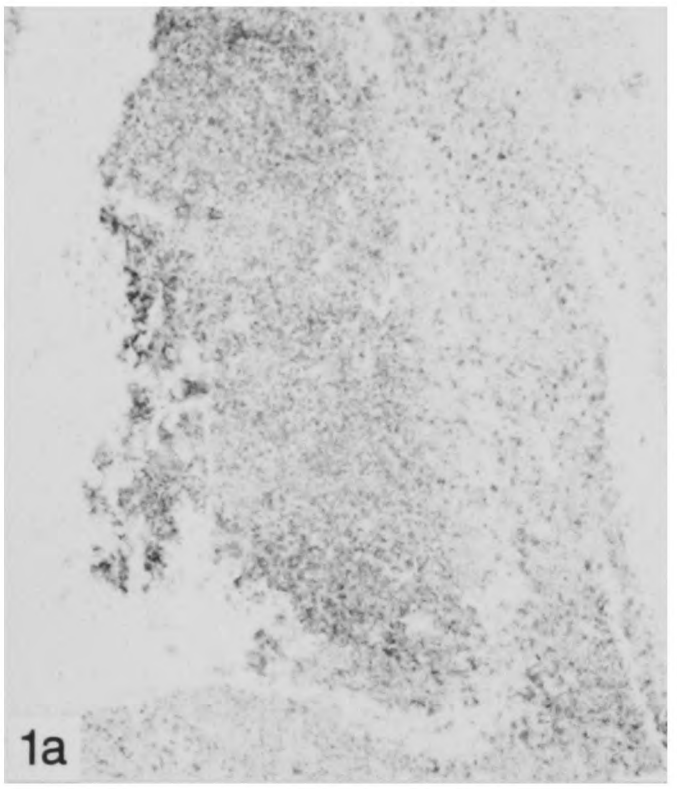
2 a,b MRC OX-27 (Donor MHC Class I) Staining

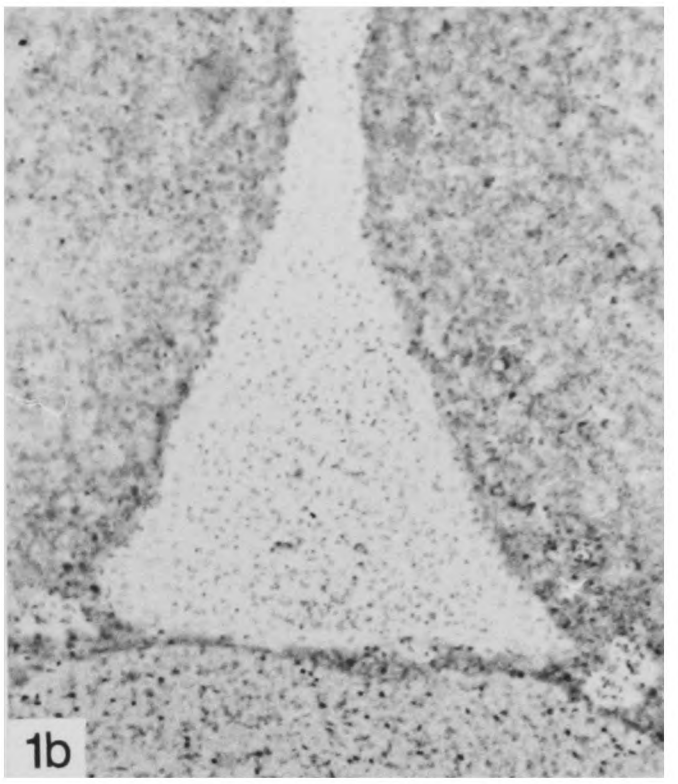
The xenograft in the rat treated with anti-IL2R MAb (**a**) had little induction of MHC Class I antigens whereas the xenograft in the animal given saline (**b**) had strong staining for these antigens. *x116*

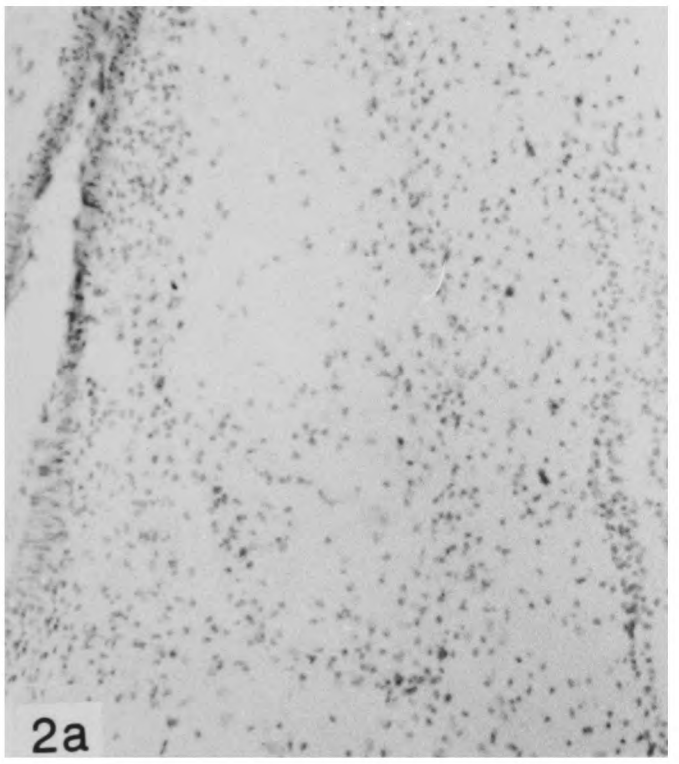
3 a,b MRC OX-18 (Donor and Host MHC Class I) Staining

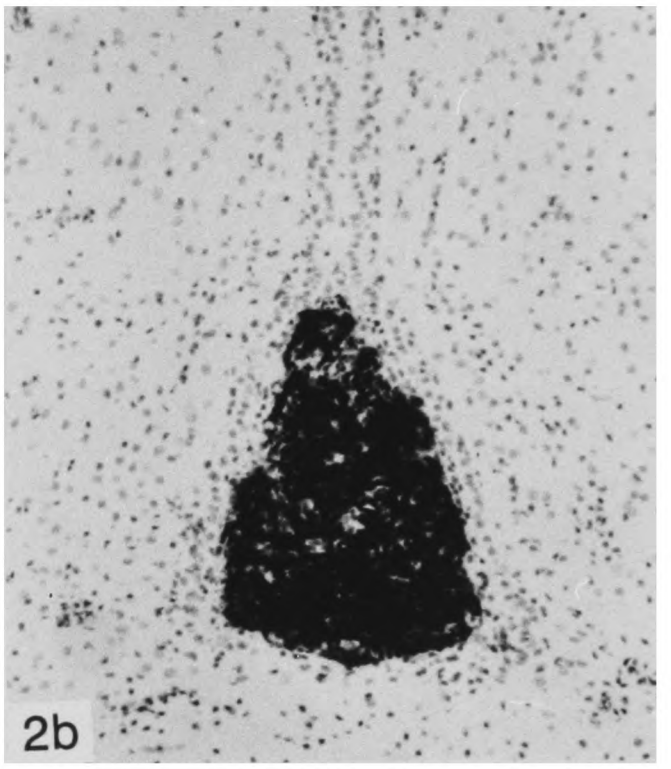
The rat treated with anti-IL2R MAb (**a**) had little induction of MHC Class I antigens around the transplantation site whereas the animal given saline (**b**) had strong staining for these antigens. *x116*

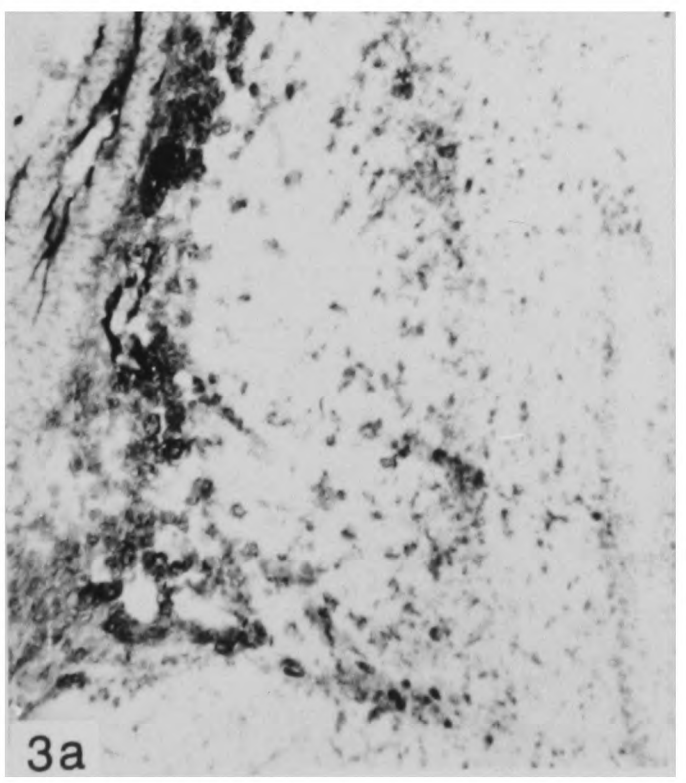
FIGURE 7.1











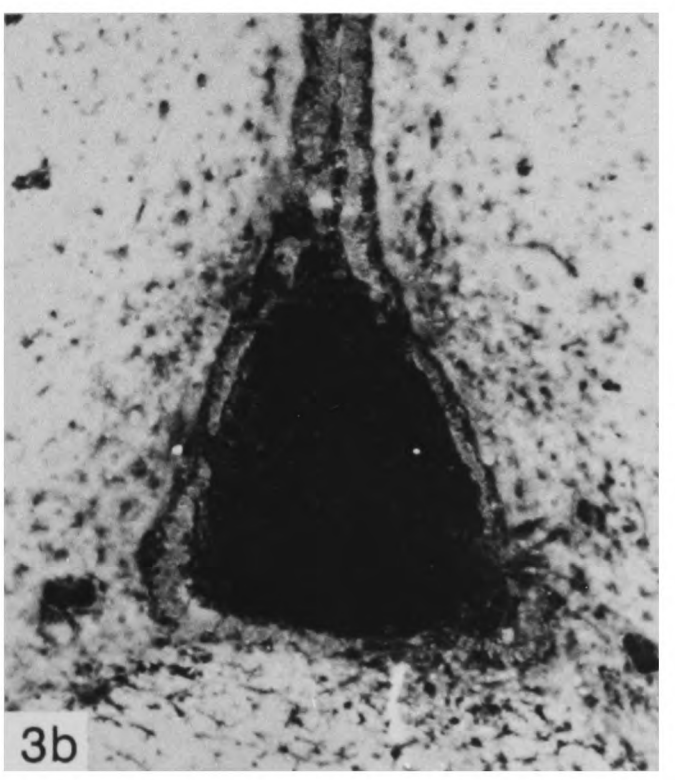


Figure 7.3 Histology of Ventricular Allografts in Rats

1 a,b MRC OX-19 + MRC OX-52 (pan T-cell) Staining

The rat treated with anti-IL2R MAb (**a**) had few T-cells within its xenograft whereas the rat given saline (**b**) had numerous T-cells within its xenograft. *x116*

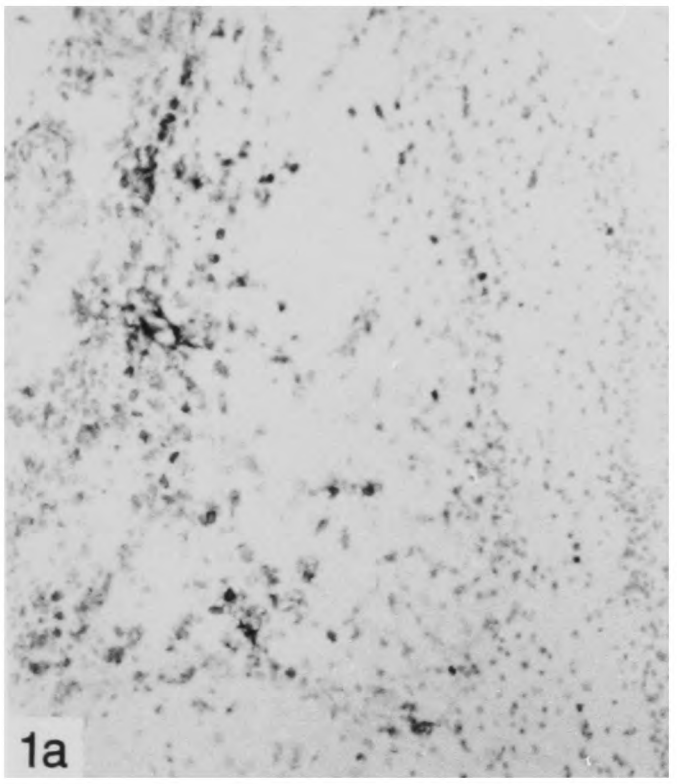
2 a,b IL2R Staining

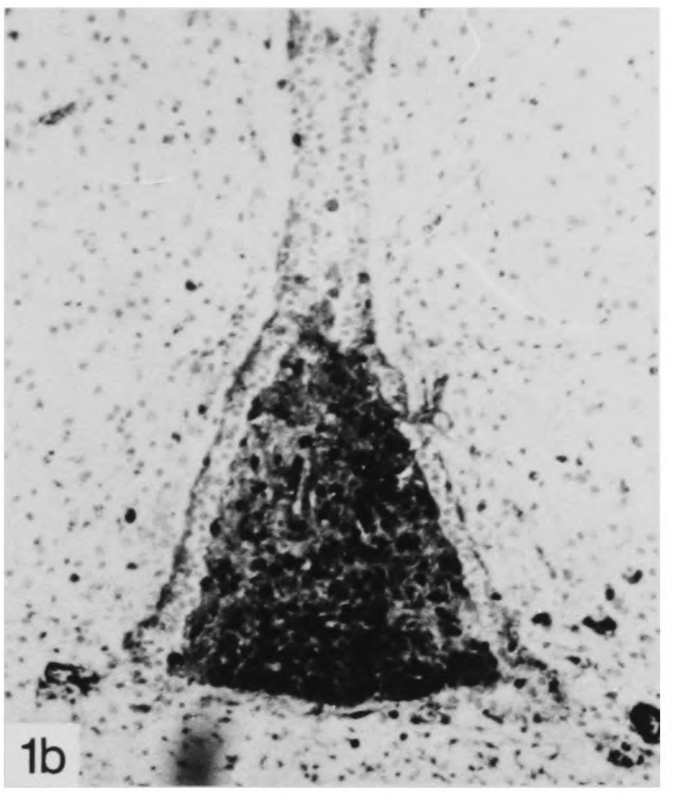
The rat treated with anti-IL2R MAb (**a**) had few IL2R-positive cells within its xenograft whereas the rat given saline (**b**) had many of these cells. *x116*

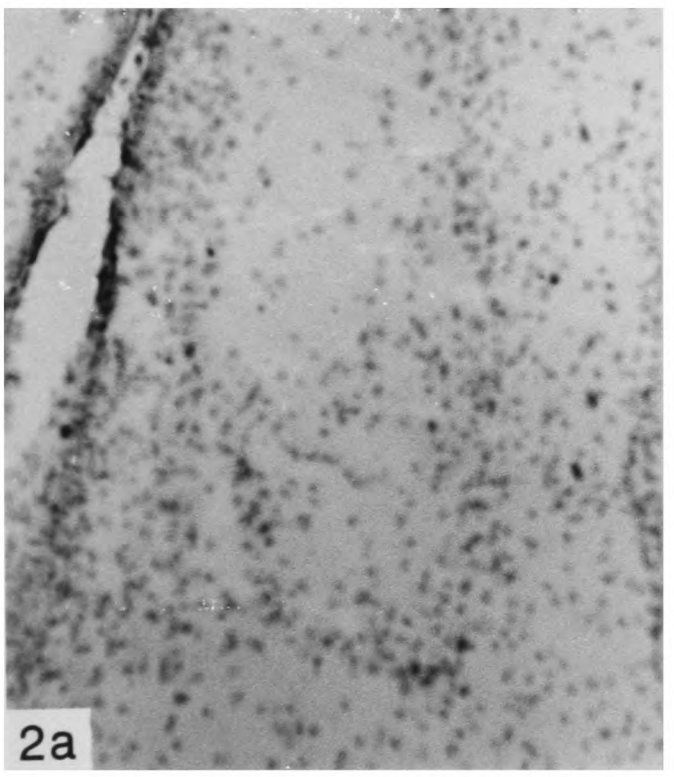
3 a,b MRC OX-42 (Microglia) Staining

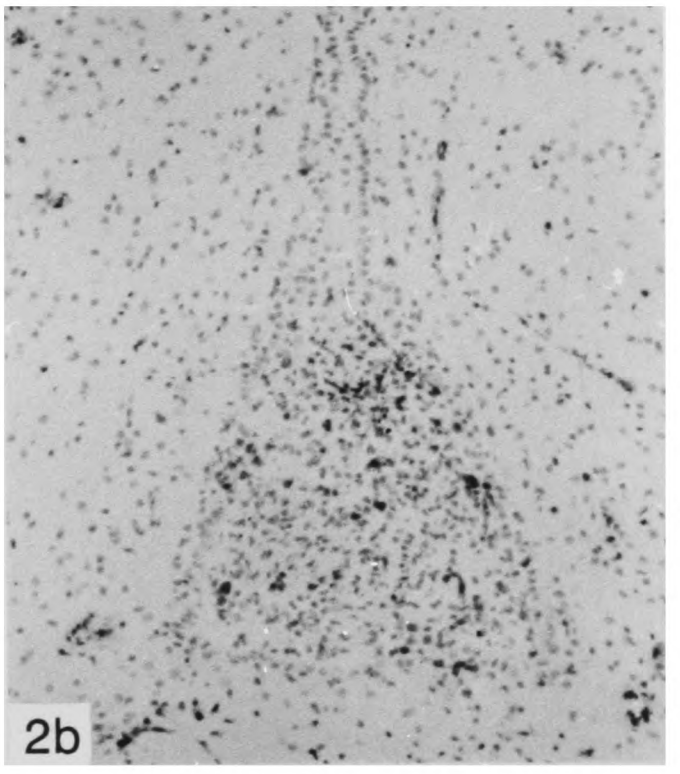
The rat treated with anti-IL2R MAb (**a**) had a smaller glial reaction than the animal given saline (**b**). *x116*

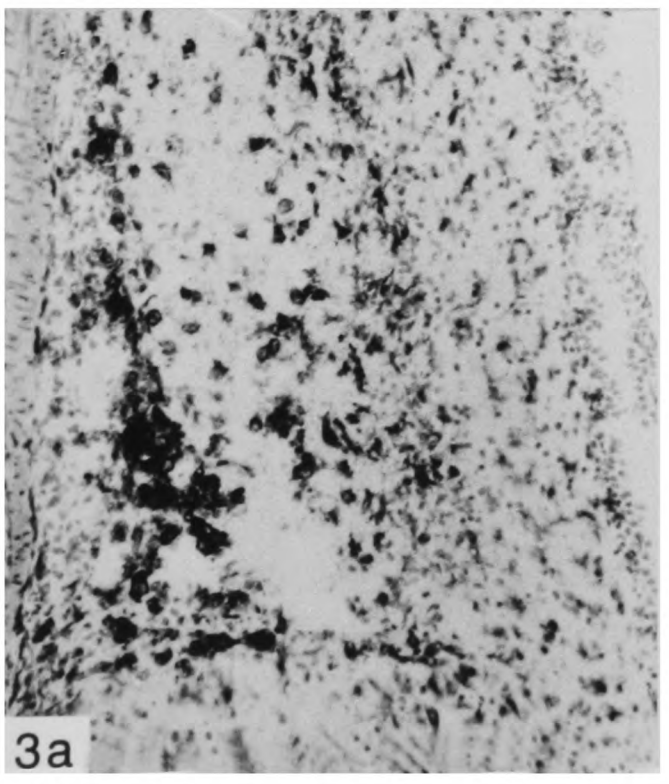
FIGURE 7.3

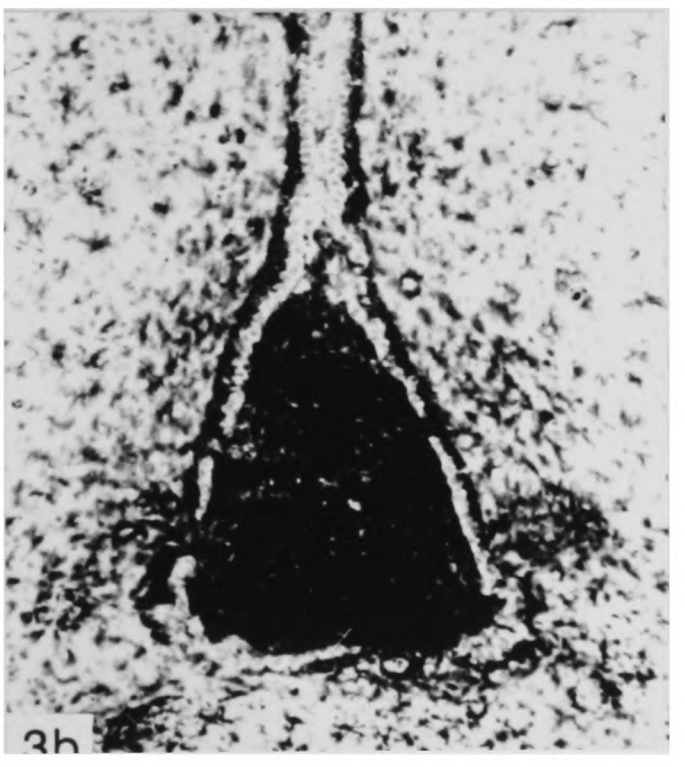












Chapter 8

Functional Neural Xenografts in Rats

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8.1 Introduction

This chapter investigates the ability of anti-IL2R MAb treatment to prolong human neural xenograft survival and function in the hemi-Parkinsonian rat model.

In 1959, Carlsson first proposed a dopamine deficiency as the cause of Parkinson's Disease (PD). A year later, Ehringer and Hornykiewicz (1960) showed that Parkinsonian patients did have reduced dopamine levels in their striata. The current literature (review Agid *et al.*, 1987) suggests that the clinical symptoms of PD are primarily due to degeneration of dopamine neurons in the midbrain. The nigrostriatal neurons which contain dopamine (Andén *et al.*, 1964) undergo idiopathic degeneration resulting in a constellation of symptoms including tremor, rigidity, akinesia and postural abnormalities (review Barbeau, 1986). Patients with hemi-Parkinsonism (one side of the brain effected more than the other) also demonstrate a circling behaviour (Trabucchi *et al.*, 1979; Bracha *et al.*, 1987). The hemi-parkinsonian rat model attempts to artificially recreate this loss of dopamine in the nigrostriatal system.

Ungerstedt (1968) showed that 6-hydroxydopamine (6-OHDA) injected into the mesostriatal dopamine system of the rat caused a dramatic depletion of dopamine in the caudate-putamen. Bilateral 6-OHDA lesions resulted in akinetic rats (Ungerstedt 1971) which resembled the motor impairment seen in Parkinson Disease. Unfortunately, these rats also developed aphagia and adipsia with a concomitant high mortality rate. Unilaterally lesioned rats, however, were able to feed and drink and demonstrated a postural curve and spontaneous rotation towards the side of lesioning. Quantifying this rotational behaviour has been used extensively as an animal model of nigrostriatal function (Anden, 1975; Ungerstedt

1976). These animals also demonstrate a contralateral sensorimotor deficit (Marshall et al., 1974) but this study will focus on the rotational behaviour.

This rotational asymmetry can be augmented by drugs which stimulate dopamine release (amphetamine) and by dopaminergic drugs (apomorphine). Ungerstedt and Arbuthnott (1970) showed that amphetamine increased the animals rotation towards the lesioned side. Amphetamine releases dopamine from intact neurons (Zetterström *et al.*, 1983) and thus amplifies the difference between the normal and lesioned sides resulting in an increased rotation towards the lesioned side. Ungerstedt (1971) also showed that apomorphine caused the animals to rotate contralaterally - away from the lesioned side. He suggested that the lesioned striatum had developed super-sensitive receptors and responded to the direct dopamine agonist more than the normal side. Several authors have suggested that the apomorphine-induced response does not correlate with graft function as well as the amphetamine-induced response (reviewed by Dunnett *et al.* 1988). This study attempts to assess the function of mesencephalic grafts and therefore only deal with amphetamine-induced rotation.

Björklund and Stenevi (1979) and Björklund *et al.* (1980) showed that fetal mesencephalon, the source of developing dopamine neurons (Olson and Sieger, 1972), could reverse the rotational asymmetry when transplanted into the lesioned striatum. This model thus provided a measure of transplanted dopaminergic neuron function. Björklund *et al.* (1982) showed that transplanted murine fetal dopaminergic cells were also capable of functioning in this model. Finally, Brundin *et al.* (1986) showed that human fetal dopamine neurons could survive and function in this model provided the rats were immunosuppressed with cyclosporin A.

This chapter utilizes this model for transplanted human dopamine neuron survival and function in rats immunosuppressed with anti-IL2R MAb.

8.2 Materials and Methods

8.2.1 Animals and Lesion Surgery

Five adult female (older than 6 months at grafting) PVG rats and Twenty-two adult (as above) Wistar rats were given two stereotaxic injections of 6-hydoxydopamine (6-OHDA) in the right ascending mesostriatal dopamine pathway by Dr D. Clarke at the Department of Pharmacology, Oxford, U.K. The 6-OHDA (Sigma Chemical Company, Ltd., Poole, U.K.) at 3 µg/mL, HBr salt, was dissolved in 0.2 mg/mL ascorbate-saline. The two injections were given with the following coordinates:

2.5 µL 6-OHDA		and	2.0 µL 6-OHDA
anterior:	-4.4 mm from Bregma	anterior:	-4.0 mm
lateral:	1.2 mm from midline	lateral:	0.8 mm
vertical:	7.8 mm from dura	vertical:	8.0 mm
incisor bar:	-2.4 mm from interaural	incisor:	+3.4 mm

These two 6-OHDA injections produced an ipsilateral dopamine denervation in the caudate-putamen (Schmidt *et al.*, 1982) and the nucleus accumbens and olfactory tubercle (Dunnett *et al.*, 1984). The extent of dopamine denervation in the striatum could be measured by assessing metamphetamine-induced unilateral rotation.

8.2.2 Metamphetamine-induced Rotation

Two to four months after lesioning, the rats were given 5 mg/kg of metamphetamine (McArthy Medical), intraperitoneally, and their rotational behaviour was monitored in automated "rotometer bowls" for one hour (Ungerstedt and Arbuthnott, 1970). All rats used for grafting exhibited a greater than 6 turns/minute predominance towards the lesioned side. This rate of turning has been shown to correspond to a permanent reduction in neostriatal dopamine of more than 97% (Tallman, Thomas and Gallager, 1978). Four animals fell below this score, presumable due to insufficient lesioning or spontaneous recovery, and were re-lesioned and re-tested. The animals were then tested for their rotation at various time points after the xenograft was transplanted to their striatum.

8.2.3 Donor Tissue

The human fetal xenografts were obtained by Dr. D. Clarke from standard abortions at the Churchill Hospital, Oxford, U.K. The work was done with the approval of the Ethics Committee of the Oxford Area Health Authority and in compliance with the standards of the British Medical Association.

All fetuses were 7-9 weeks gestational age as judged by crown-rump length and date of last menstruation. The central nervous system was recovered and the region of the mesencephalon, containing the developing dopamine cell groups, dissected out. A piece of hindbrain was also taken for control grafts. Cell suspensions were made according to the method of Björklund *et al.*, 1983.

8.2.4 Cell Suspension

The following reagents were used:

Basic Medium:	0.6% D-glucose in sterile 0.9% saline
Trypsin:	0.1% Trypsin (Sigma type II) in basic medium

The mesencephalon pieces were transferred to a glass micro test tube containing 200 µL of the trypsin solution and incubated for 20 minutes at 37 °C. The trypsin was then washed off with four changes of basic medium and the final volume reduced to approximately 30 µL. The tissue pieces were then dissociated into a suspension of single neurons by pipetting through the fire-polished opening of a Pasteur pipette twenty times. The suspension was then ready for transplantation.

8.2.5 Transplantation

The rats received two stereotaxic transplants of cell suspension (3 µL each) to their striatum using the following coordinates (incisor bar 2.3 mm below the interaural line):

anterior:	1.0 mm from Bregma	and	anterior:	1.0 mm
lateral:	2.8 mm		lateral:	2.8 mm
vertical:	5.0 mm from dura		vertical:	4.1 mm

The cell suspension was injected at 1 µL/min both sites with a 5 minute pause after each graft placement.

8.3 Experimental Procedure

Four experimental groups of animals were arranged as shown below in Table VIIIa.

Table VIIIa

Group	Number	Treatment	Transplant
Group 1	10	anti-IL2R	mesencephalon
Group 2	4	anti-IL2R	hindbrain
Group 3	5	cyclo A	mesencephalon
Group 4	8	none	mesencephalon

Groups 1 and 2 received 750 µg/kg/day anti-IL2R MAb (i.p.) for 10 days beginning on the day of transplantation. Group 3 received 10 mg/kg (i.p.) of Cyclosporin A (Sandoz, dissolved to 10 mg/mL) starting on the day of transplantation and continuing for the length of the study. Group 4 received 1 mL saline (i.p.) for 10 days.

Two animals from Group 4 were sacrificed at 4 weeks and another two at 6 weeks for histological examination of their xenografts. The remaining animals were rotated monthly for upto 24 weeks post-transplant to follow the function of the xenograft. Five animals from Group 1 and four from Group 4 were left for very longterm follow-up by Dr. Clark. At sacrifice, three animals from Group 1 were processed for tyrosine hydroxylase to observe dopamine neurons within the xenograft. The remaining animals were prepared for immunohistochemistry and serial sections through the xenograft stained with the following panel of primary antibodies:

Antibody	Specificity	Reference
B7	human Thy-1 (brain)	Ritter[1]
PA2.6	human MHC Class I	Fuggle[2]
NDS 21	human MHC Class II	Fuggle[2]
MRC OX-19	rat pan T-cell	Dallman *et al.* 1984
MRC OX-52	rat pan T-cell	Robinson *et al.* 1896b
NDS 63	rat IL2R	Tellides 1988
MRC OX-18	rat MHC Class I	Fukumoto *et al.* 1983
MRC OX-6	rat MHC Class II	McMaster & Williams 1979
MRC OX-42	microglia	Robinson *et al.* 1986a
MRC OX-26	rat transferrin receptor	Jefferies *et al.* 1985
MRC OX-21	negative control	Hsiung *et al.* 1982

MRC OX-21 is a mouse IgG1 MAb specific for the human C3b inactivator. It was used as a negative control primary antibody on one section from each xenograft. One section from each xenograft was also processed without any primary. None of these control sections had any staining.

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[1] The B7 antibody was a gift from Dr. Ritter at the Hammersmith Hospital, London.

[2] The PA2.6 and NDS 21 antibodies were gifts from Dr. Fuggle at the John Radcliffe Hospital, Oxford.

8.4 Results

8.4.1 Animal Health

After the transplant surgery, none of the anti-IL2R MAb treated animals died before sacrifice. Four of the five animals treated with cyclosporin A, however, died during the course of the experiment and the fifth animal was not healthy enough to withstand the stress of metamphetamine-induced rotation.

8.4.2 Rotational Behaviour

Figure 8.1 shows the change in uni-directional rotation for the Groups 1, 2 and 4.

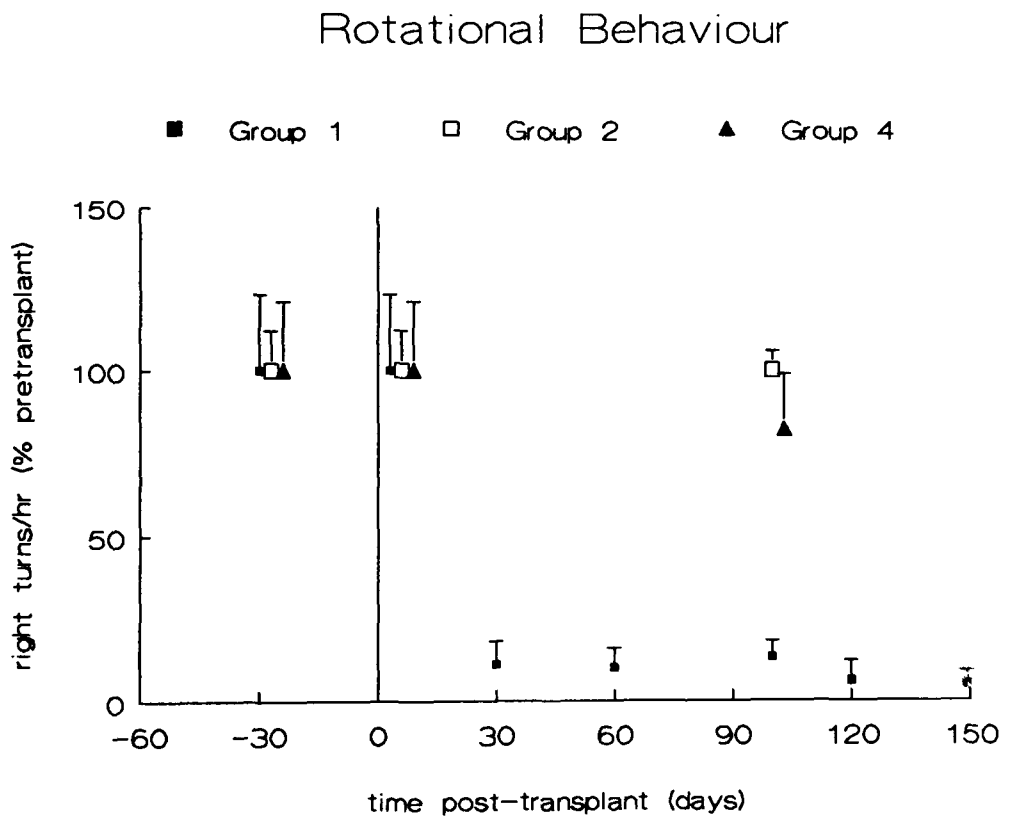


Figure 8.1

All animals in Group 1 showed a greater than 80% reduction in amphetamine-induced rotation by thirty days post-transplant. The mean rotational score was significantly reduced ($p < 0.01$, Students's t-test) from the pre-grafting level. The rotational scores remained reduced for the length of the experiment (six months). There was no significant difference in the scores at any of the post-transplantation time points by analysis of variance. Groups 2 and 4 showed no significant reduction in rotational behaviour after transplantation (individual Student's t-test).

8.4.3 Xenograft Histology

8.4.3.1 Xenograft Survival

Serial sections through the xenograft were stained for human Thy-1 with the B7 antibody. Figure 8.2 shows photomicrographs of such sections from representative animals in Groups 1, 2, and 4. The animals in Groups 1 and 2 have xenografts staining strongly for the Thy-1 expressed on human neural tissue. Both animals examined in Group 1, had excellent xenograft survival. None of the animals in Group 4 examined, had surviving Thy-1-positive staining tissue at 4 weeks (0/2) or 6 weeks (0/2).

8.4.3.2 Dopamine Neurone Survival

Sections through the xenograft in three animals from Group 1 were stained for tyrosine hydroxylase (TH). TH is the rate limiting enzyme in the synthesis of dopamine and its presence in the xenograft is highly suggestive of surviving dopaminergic neurons from the human mesencephalon. Figure 8.3 shows a photomicrograph of such a section. The xenograft was filled with cells staining strongly for tyrosine hydroxylase. All xenografts examined in Group 1 (3 out of 3) had excellent TH staining.

8.4.3.3 Xenograft MHC Expression

Sections through the xenograft in two animals from Group 1 were stained for MHC Class I and Class II expression with the PA2.6 and NDS 21 antibodies respectively. Figure 8.4 shows photomicrographs of such sections from a representative animal. The PA2.6 antibody appeared to cross-react with rat MHC Class I because there was staining of rat blood vessels (which express MHC Class I) throughout the brain. This was also seen in control sections of rat brain stained with this antibody (not shown). The xenograft itself expressed little or no MHC Class I or Class II antigens.

8.4.3.4 Infiltrating Cells

Sections through the xenograft in two animals from Group 1 were stained for all T-cells with a cocktail of the MRC OX-19 and MRC OX-52 antibodies; and for activated T-cells with NDS 63. Figure 8.4 shows photomicrographs of such sections from a representative animal in Group 1. Six month post-transplantation, there were no T-cells, within or around the xenograft. The positive control, staining sections of rat spleen, was strongly positive for the MRC OX-19 and MRC OX-52 antibodies (not shown).

8.4.3.5 Host Brain Response

Sections through the transplantation site in two animals from Group 1 were stained for rat MHC Class I and Class II expression with the MRC OX-18 and MRC OX-6 antibodies respectively and for microglia with the MRC OX-42 antibody. Figure 8.5 shows photomicrographs of such sections from a representative animal in Group 1. MHC Class I antigen expression appeared to be confined to either blood vessels or microglia. There was no generalized expression on the adjacent host brain parenchyma. Around the periphery of the xenograft, there were a very few cells which expressed MHC Class II. These may have been reactive astrocytes or microglia. There was an obvious glial response with strong staining around and within the xenograft.

8.4.3.6 Xenograft Vascularization

Sections through the xenograft in two animals from Group 1 were stained for the rat transferrin receptor found on rat cerebral vessels with the MRC OX-26 antibody. Figure 8.5 shows photomicrographs of a representative sections from an animal in Group 1. There was sparse, weak staining for rat cerebral vessels within the xenograft.

8.5 Discussion

The results showed that human neural xenografts could survive and function in rats immunosuppressed with anti-IL2R MAb for at least 24 weeks.

The benefit of anti-IL2R MAb immunosuppression over cyclosporin A treatment was twofold. First, the animals were much healthier. Four out of five cyclosporin A treated animals died during the study and the remaining one was not thriving. This may have been a strain-specific poor tolerance to the drug - Wistar rats were also noted by Professor Björklund's group in Sweden to be prone to poor health while on cyclosporin (personal communication with Dr. D. Clarke). The side effects of cyclosporin, however, have been well documented in the clinical situation. The nephrotoxicity of this drug (Tilney *et al.*, 1983) often precludes or terminates its use by the transplant surgeon (European Multicenter Trial Group, 1982a and 1982b). Lane *et al.* (1989) has also shown that cyclosporin has a deleterious effect on the central nervous system.

The second advantage of anti-IL2R MAb treatment is its short course of treatment, and hence ease of its administration. Animals were only treated for ten consecutive days after transplant. This short course of immunosuppression may be the reason why animals treated with anti-IL2R MAb remain healthy for the

length of the experiment. Perhaps the immune system was suppressed only long enough for xenograft tolerance to occur but then the animal regained its immune competence and was able to defend against infection. Dallman (personal communication) has also found no increased mortality in rats immunosuppressed with IL2R MAb for cardiac and renal allografts. The cyclosporin A group required daily injections for the entire length of the experiment (Brundin *et al.*, 1985) and were thus an easy target for opportunistic infections.

The rotational data provided two interesting points. First, the functional effect of the xenograft must have been due to the dopamine neurons. Plunkett *et al.* (1989) showed functional recovery in a model of the hemi-Parkinson rat following intracerebral transplantation of inflammatory cells. Several authors (Bankiewicz *et al.* (1989); Plunkett *et al.* (1989)) have suggested that fetal tissue transplants, with their accompanying trophic factors, may not replace the lost function of host brain but may act by stimulating regeneration of the host brain's own function. In the animal model discussed in this chapter, transplantation of fetal hindbrain (Group 2) had no effect on rotational behaviour. Only transplantation of fetal mesencephalon, with its developing dopamine neurons, resulted in reduced rotation.

Second, the rotational scores were reduced very quickly after transplantation. Figure 8.1 showed that the xenografts had dramatically reduced rotation by 30 days post-transplant. This was in contradistinction to the results of Brundin *et al.* (1986) which did not show reduced rotation in five rats given human xenografts until 12.5 weeks post-transplant. A second paper by Brundin *et al.* (1988) also found initial reduction in rotation at 13 weeks, although this was the first time point reported. The results from this chapter were more in keeping with the rapid reduction in

rotation seen with murine xenografts (Björklund, Schmidt and Stenevi, 1980; Dunnett *et al.*, 1983; Brundin *et al.*, 1985). The function of the human xenografts were either hindered in cyclosporin A treated animals or aided in anti-IL2R MAb treated animals. The latter seems unlikely. Perhaps the xenografts in the cyclosporin A treated animals were subjected to a low-grade rejection response and the resultant MHC Class I antigen induction may have hinder intercellular interactions. Unfortunately, the cyclosporin A treated animals in this study were not rotated because of their precarious health. Further studies will be required to determine if this rapid restoration of function was connected with the anti-IL2R MAb treatment or just a manifestation of variability in the techniques of fetal harvest and transplantation or host species response.

The histology of the xenografts were remarkable. Six months after ceasing immunosuppression, the xenografts were 'healthy' (strong Thy-1 expression with no MHC antigen induction), filled with dopamine neurons and showed no signs of rejection (no infiltrating cells). It appeared the xenografts would have continued to function for a much longer period. Indeed, one subgroup of animals (N=5) have been left to see just how long these xenografts will continue to function. Unfortunately, these animals could not be rechallenged with another neural xenograft from the same source to see if the animals were truly tolerant to the xenograft. A second human fetus would carry different MHC antigens (and any fragment of the first fetus may not survive frozen until it was time to rechallenge). This question could be answered by using murine xenografts in further experiments.

The results on xenograft vascularization confirmed that the host provided a substantial part of the vascular supply of the xenograft. It did not rule out the

possibility, however, that the xenograft did retain a small portion of its own vasculature.

In summary, this chapter proved to be the successful culmination of my work. The original goal of this thesis, outlined in the Introductory Chapter, was to develop a method of immunosuppression which would enable neural xenografts to survive and function for long periods without the any deleterious side-effects in an animal model. The anti-IL2R MAb treatment certainly fulfilled this goal.

Suggestions for further experiments with this technique of immunosuppression and the potential benefit of this therapy for the neurosurgeon are discussed in the final chapter.

Figure 8.2 **Thy-1 Staining in Human Neural Xenograft in Rat Striatum**

Section through the rat striatum showed strong staining for human Thy-1 with the B7 antibody. A positive-staining axon could be seen extending into the host brain (arrow). *x116*

Figure 8.3 **Tyrosine Hydroxylase Staining of Human Xenograft**

The section through the xenograft within the rat striatum showed numerous positive-staining cell bodies (white arrow) and axons. *x232*

FIGURE 8.2

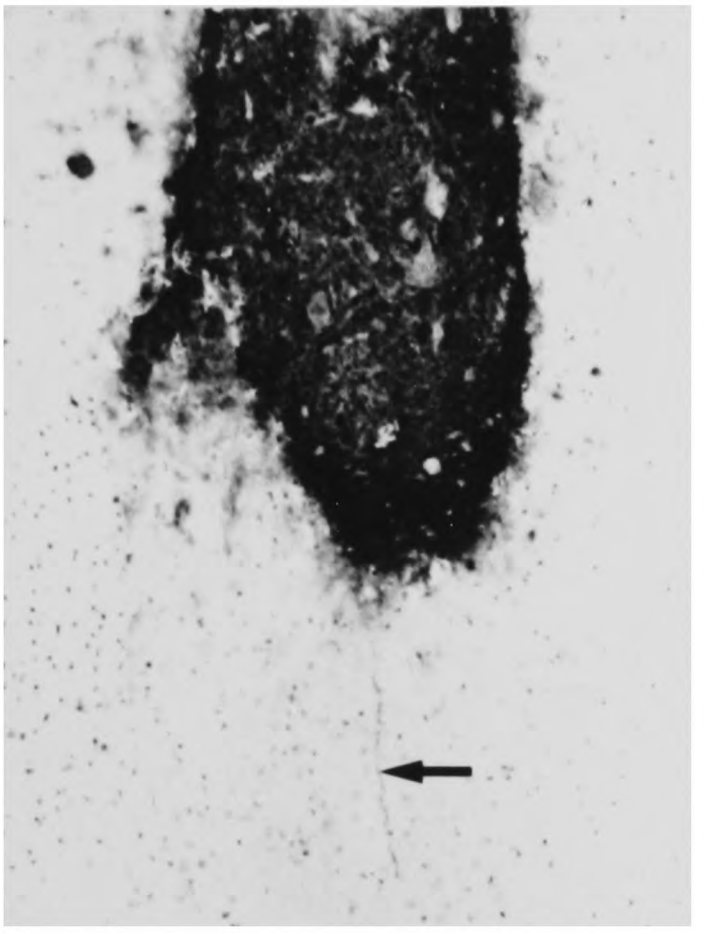


FIGURE 8.3

Figure 8.4 Histology of Human Neural Xenografts in Rat Striatum

a Human MHC Class I Staining

This antibody (PA2.6) appeared to cross react with rat MHC Class I antigens since blood vessels throughout the host brain were stained. The xenograft showed slight expression of these antigens. *x116*

b Human MHC Class II Staining

There appeared to be very little staining with this antibody (NDS 21). *x116*

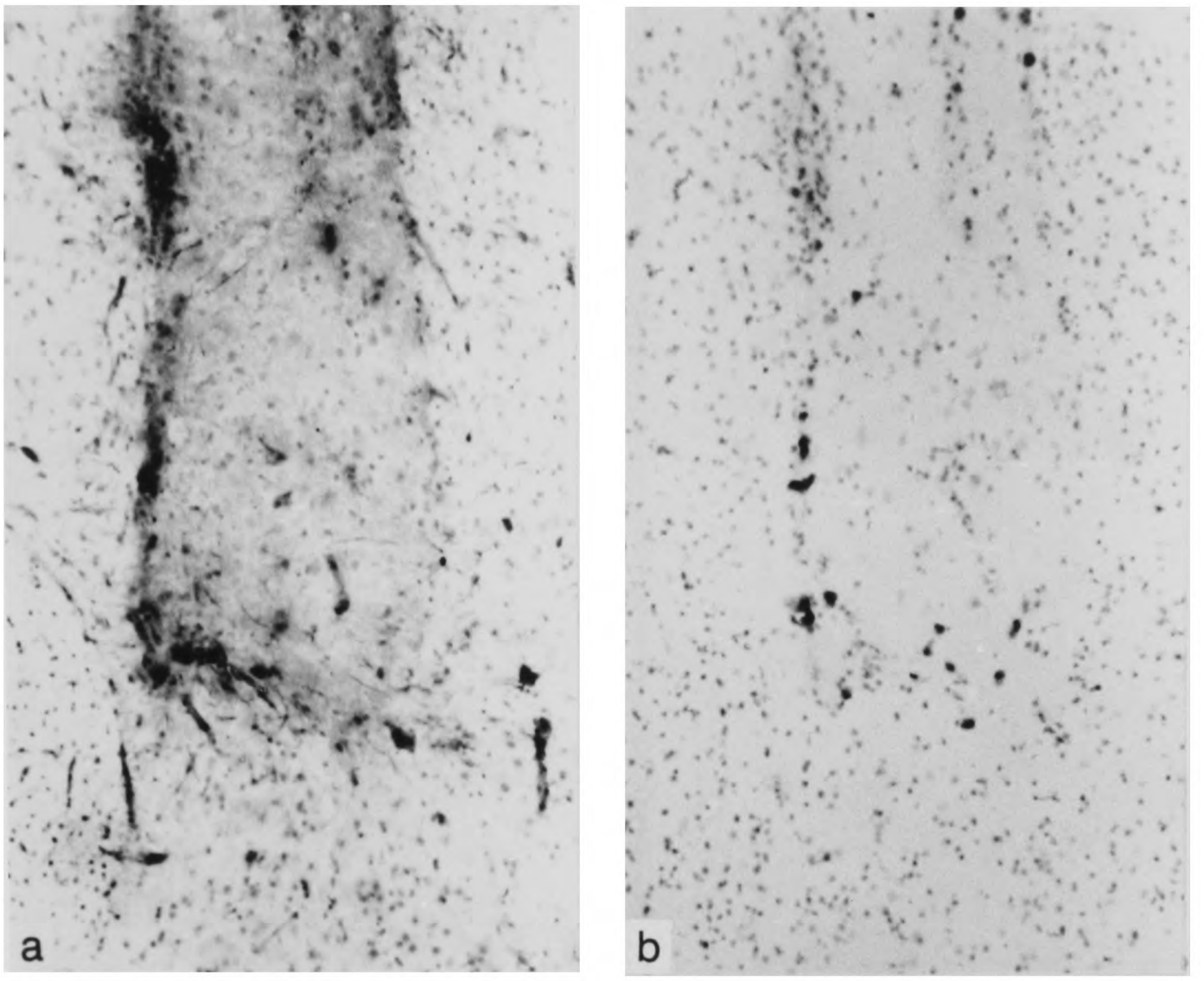
c Rat pan T-cell Staining

A cocktail of MRC OX-19 and MRC OX-52 antibodies were used. There were very few T-cells within the xenograft. *x116*

d Rat IL2R Staining

Activated T-cells were stained with NDS 63 antibody. There were very few positive staining cells. *x116*

FIGURE 8.4



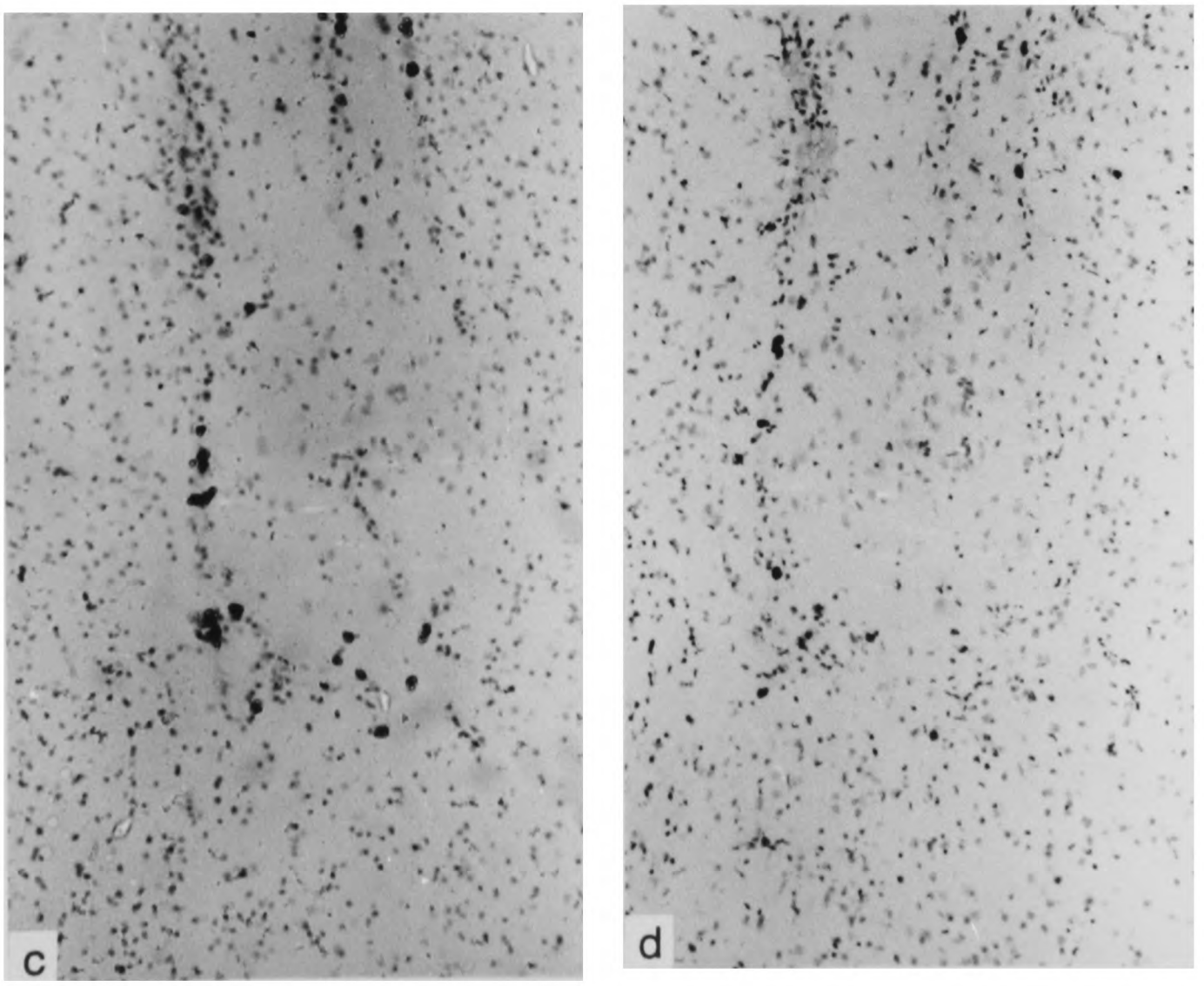


Figure 8.5 **Histology of Human Neural Xenografts in Rat Striatum**

a Rat MHC Class I Staining

The MRC OX-18 antibody showed positive staining rat blood vessels throughout the xenograft but there was very little induction of these antigens on the surrounding host brain. *x116*

b Rat MHC Class II Staining

The MRC OX-6 antibody showed some positive staining cells within the xenograft. *x116*

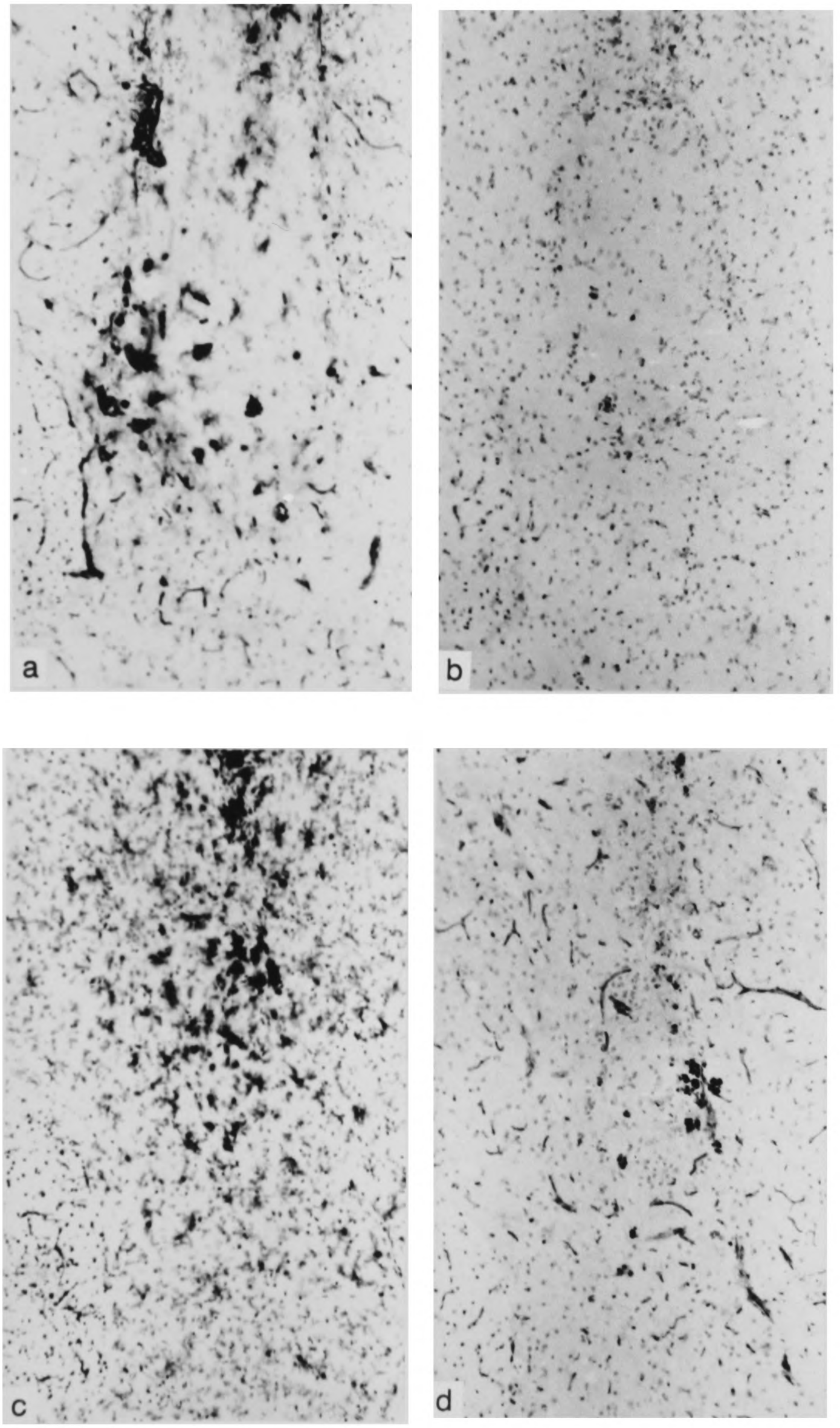
c MRC OX-42 Staining (Microglia)

There was no dramatic glial reaction around the xenograft. *x116*

d MRC OX-26 Staining

This antibody bound the rat transferrin receptor found on rat cerebral endothelial cells. There were numerous rat blood vessels within the xenograft. *x116*

FIGURE 8.5



Chapter 9

General Discussion

The four original aims of this thesis were (i) to determine if anti-L3T4 monoclonal antibody treatment could prolong neural allograft and xenograft survival in the mouse, (ii) to determine if anti-IL2R monoclonal antibody treatment could prolong neural allograft and xenograft survival in the rat, (iii) to determine if neural graft function could also be prolonged with these two treatments, and (iv) to determine if there are any detrimental effects of these two treatments. The results presented have shown that both these antibodies can prolong neural allograft/xenograft survival and function in mice and rats without any serious side-effects.

Immunosuppression with anti-L3T4 MAb appeared to provide only temporary prolongation of neural grafts (although a subset of allografts in the parenchyma appeared to be surviving well at 24 weeks). There are several possible reasons why this technique failed to provide complete tolerance to neural grafts. First, there may not have been complete helper T-cell depletion. Examination of spleen and lymph node sections revealed the presence of occasional L3T4-positive cells even at the peak of cellular depletion. Second, unusual cells of Lyt-2 phenotype (and thus exempt from anti-L3T4 depletion) with 'helper' function have been demonstrated by Swain and Panfili (1979).

The results using the anti-IL2R MAb in rats were very promising. The human fetal xenografts showed no signs of rejection (ie. infiltrate or MHC expression) even six months post-transplant. Studies in our laboratory are continuing to determine if the rats are made tolerant to the neural xenografts with this treatment.

The success of these two monoclonal antibodies suggests that other antibodies or combinations of antibodies to various components of the immune

system could provide further prolongation of neural graft survival. Cobbold *et al.* (1986) showed that a cocktail of monoclonal antibodies against the L3T4 and the Lyt-2 surface antigens provided greater delay in skin allograft rejection than the anti-L3T4 MAb alone. In our initial experiments (Chapter 3), we found a significant increase in mortality in mice treated with both MAb. The experiments could, however, be conducted in a specific pathogen free centre to avoid any opportunistic infections. Other possible protocols of immunosuppression could include using cocktails of antibodies against different epitopes on the same target. Qin *et al.* (1987) demonstrated that pairs of IgG2b monoclonal antibodies to non-overlapping epitopes of the CD4 molecule were more lytic *in vitro* and *in vivo* than either one alone.

Other receptors or molecules of the immune system could be targeted with monoclonal antibodies for immunosuppression. For example, the interleukin-1 receptor found on activated T-cells or the adhesion molecules used by lymphocytes to attach to their targets.

Monoclonal antibodies could be used to aid neural transplantation in other areas beside immunosuppression. Their high specificity and ability to trigger destruction of their targets could be used to purify neuronal populations for transplantation. Bartlett, Rosenfeld and Kerr (1989) have immunoselected populations of non-MHC Class I expressing cells (neurons without glia) for transplantation. These cells are not rejected by allogeneic mice even after 30 weeks. Presumably, the host immune system was not fully activated because there are no foreign MHC antigens to trigger it. This is a promising field of research and one which will be investigated in our laboratory in the coming years. It will be interesting to see if these neurons can function effectively as well as survive.

Like most early investigations, this thesis has raised as many questions as it has answered! Were there any subtle deleterious effects of the treatment not seen originally? Are the rats truly tolerant to their neural grafts? If the hosts were tolerant to their grafts, how could they be destroyed in case of over-function? Could this method of immunosuppression work in higher animals such as the monkey or even man? Could xenografts become a source of neural tissue for clinical purposes?

These questions can only be answered in future studies.

Future Studies

1. The prime target for any deleterious effects of this treatment would be the kidney. This is where any trapped immune complexes could be damaging. Renal histology in the current experiments showed no structural damage but could give no indication of possible subtle changes in functional capacity. The function of the kidneys could be followed more closely in future experiments by determining serum urea and creatinine concentrations throughout the experiment.

2. In order to determine if the rats were made tolerant to their neural grafts, inbred strains of mice could be used as a source of graft tissue. Once a rat had received an initial transplant with anti-IL2R MAb immunosuppression, a second transplant (antigenically identicle to the first) could be given without immunosuppression. If the second graft enjoyed prolonged survival compared to third party control tissue, then the rats could be said to be truly tolerant to the initial grafts.

3. If animals were tolerant to their neural grafts, it would be difficult to block graft function in case of over-function, short of surgical extirpation. Previously, long surviving (but not tolerant) neural grafts could be destroyed by

triggering the immune system with skin grafts syngeneic with the neural transplants. No studies, however, have examined the extent of non-specific damage to the surround brain with this method.

4. Immunosuppression with anti-Il2R MAb could be extended to higher animals. There are monoclonal antibodies specific for the monkey and human IL2R. Transplantation of neural allografts under anti-IL2R MAb immunosuppression could determine if the volume as well as the survival time of graft tissue could be increased.

5. If xenografts could be used in the clinical setting then the neurosurgeon could avoid the ethical problems inherent in using human fetal tissue. Breeding colonies could be set up to provide a constant, predictably uniform supply of fetal xenogeneic neural tissue. If the patients were tolerant to their first graft, then any subsequent graft could be given without immunosuppression.

Nicholas and Arnason (1989) point out the possibility that xenogeneic neural transplants could have a deleterious effect on patients. In an extraordinary experiment, Bauer (1983) inadvertently showed that 5 out of 24 patients with multiple sclerosis suffered neurologic complications (including one death) when porcine brain fragments were transplanted to their subcutaneous abdominal fat. In the one patient that was examined for an immunologic etiology, Knorr-Held *et al.* (1986) showed an immune response directed against gangliosides shared by the patient and the neural implant. The neuroparalytic accidents following the administration of brain-derived rabies vaccines have also been shown to be of immunologic etiology (Arnason, 1987). Both these complications occurred after peripheral transplantation of CNS tissue, however. Widner and Brundin (1988)

point out that no such autoimmune disease has been seen with central transplantation of CNS tissue.

In summary, immunosuppression with monoclonal antibodies can be very effective in prolonging neural allograft and xenograft survival and function within the CNS of mice and rats. These results may be of immediate benefit to the basic scientist studying neural transplantation in rodent models and may become beneficial to the neurosurgeon in the future.

Appendix

Determining the antibody concentration in Tissue Culture Supernatant

The following reagents were used:

control rat IgG2b (Serotec)

BSA: bovine serum albumin (BDH)

D(A+B): Dulbeco's A + B (see section 2.4)

RAR-I^{125}: iodinated rabbit-anti-rat immunoglobulin diluted in D(A+B) to give 400,000 counts/minute/100 µL

(RAR (Serotec) was iodinated by Dr. K. Wood)

Purified rat IgG2b was diluted in tissue culture medium to give concentrations of 0.05, 0.1, 0.5 and 1 µg/100 µL. Serial dilutions of the supernatants from the anti-L3T4 and anti-Lyt-2 cell cultures were made (neat, 1:2, 1:4, 1:8, 1:16).

100 µL of each antibody solution was incubated in a standard well for one hour at 4 °C and then washed three times with 0.1% BSA in D(A+B). This allowed the antibody to bind to the walls of the well. 200 µL of 1% BSA in D(A+B) was added for one hour at 4 °C to block all the remaining binding sites in the well and washed as before. Finally, 100 µL of RAR-I^{125} was added, incubated for one hour at 4 °C and washed as before. This tagged the original antibody bound to the well walls with a gamma emitter. The wells were cut out and placed in LP3 tubes and counted in a gamma counter.

Figure 10.1 shows the results from the control rat IgG2b.

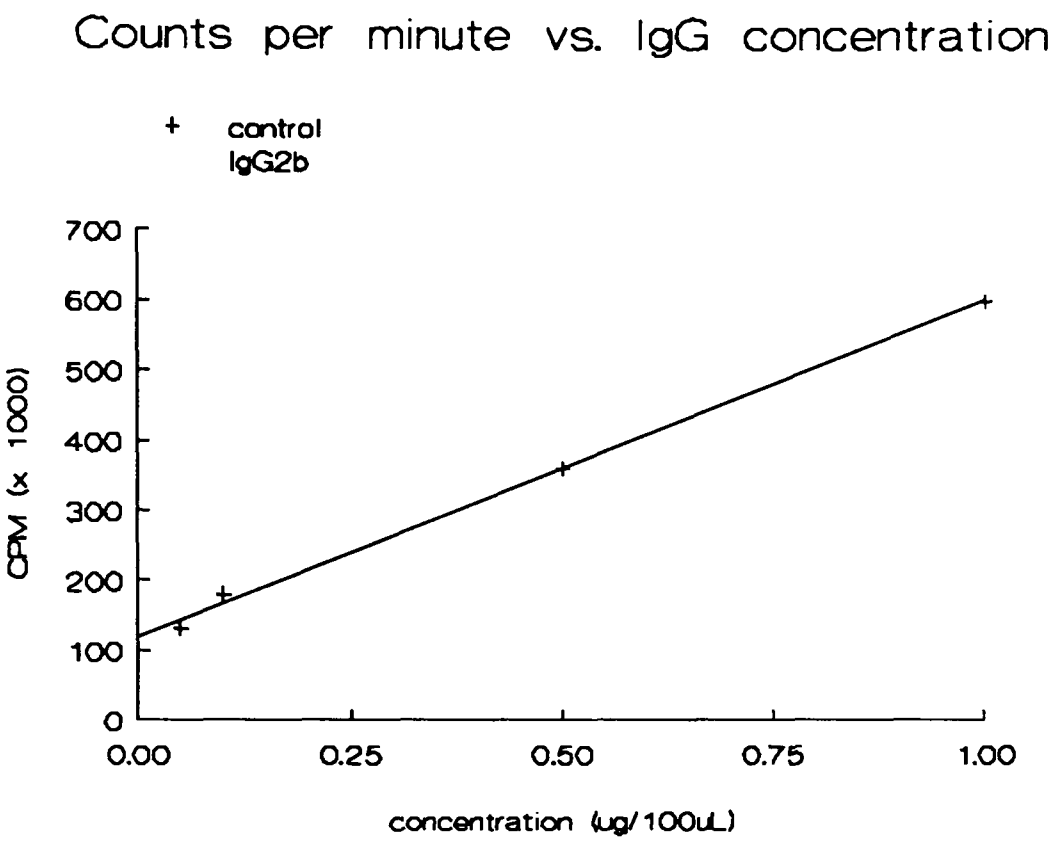


Figure 10.1

The control rat IgG2b showed 350,000 counts per minute at a concentration of 0.5 μg/100 μL or 5 μg/mL.

Figure 10.2 shows the results for the anti-L3T4 and anti-Lyt-2 supernantant.

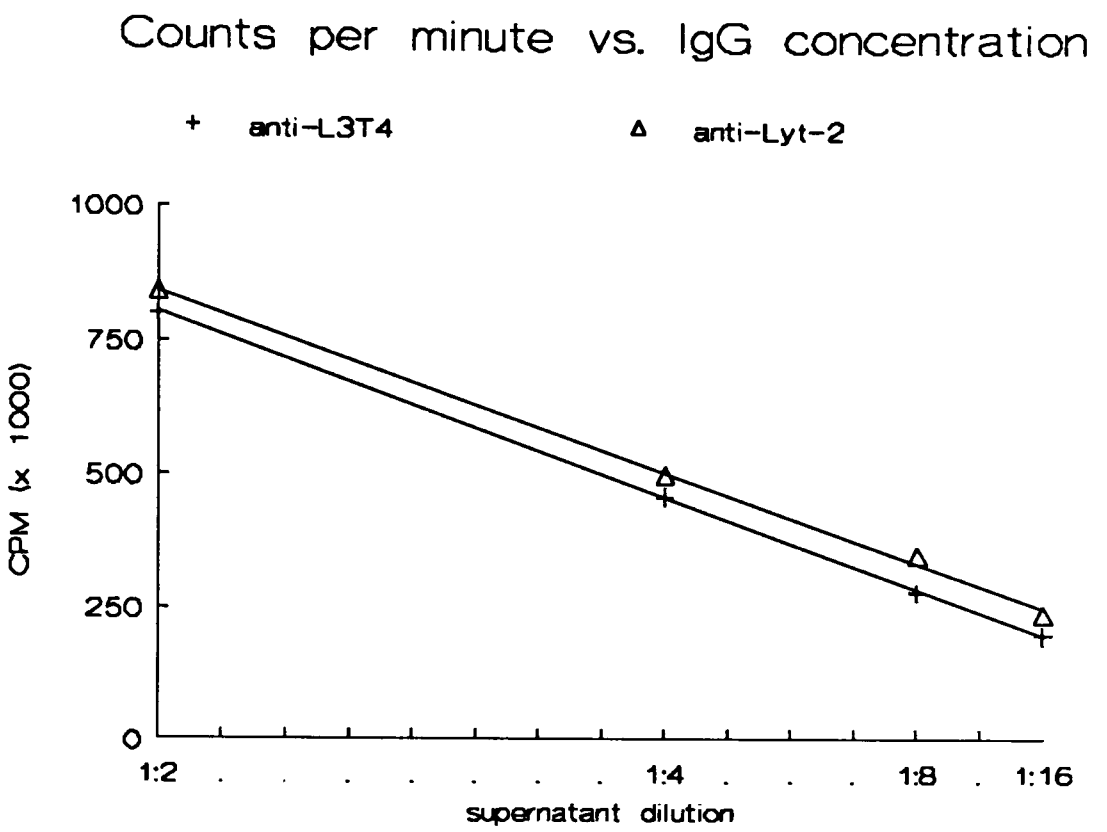


Figure 10.2

The anti-L3T4 supernatant had a count of 350,000 (and thus 5 μg/mL) at a dilution of 5:32. Therefore its original concentration was approximately 32 μg/mL. Similarly, the concentration of anti-Lyt-2 was calculated to be 40 μg/mL.

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