

## Specific Tolerance to Neural Allografts Induced with an Antibody to the Interleukin 2 Receptor

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### Summary

Despite considerable evidence documenting the central nervous system as a site of immunological privilege, immune responses do occur within the brain and neural allografts between major histocompatibility complexes (MHC) and minor antigen incompatible rat strains may be rejected. The survival of completely MHC incompatible neural allografts has been found to be prolonged indefinitely after administration of a monoclonal antibody (mAb) to the interleukin 2 receptor (IL-2R) for 10 d after transplantation. Here we present evidence that rats with long-term surviving lateral ventricular neural allografts, after anti-IL-2R treatment, accept subsequent neural allografts from the same donor strain, placed in a peripheral nonprivileged site, but rapidly reject third-party grafts. Thus, treatment with a mAb to the p55 chain of the IL-2R has resulted in the specific acceptance of second grafts of fully allogeneic neural tissue. These results suggest that ongoing interaction between elements of the host immune system and alloantigen within the brain maintains the tolerant state and furthermore, that interruption of signaling through the IL-2R may be important in allospecific tolerance induction.

The central nervous system (CNS)<sup>1</sup> has been well characterized as a site of immune privilege (1, 2), nonetheless, immune responses, such as those observed in multiple sclerosis, do occur within the brain. This apparent paradox remains unresolved. Further, the rejection of completely MHC incompatible neural allografts placed within the brain does occur (3, 4), albeit at a slower rate compared with similarly mismatched skin allografts (5). In the case of neural allograft rejection, the precise mechanism of host sensitization is not known, however, local neural elements, in the form of microglia and astrocytes with antigen-presenting capacity, do exist (2). The chronic nature of the immune response to neural allografts is thought to be the result of an afferent arc defect, indirect evidence for which is the observation that a second allograft of neural tissue placed in a peripheral nonprivileged site can elicit the rapid rejection of an earlier allograft transplanted into the CNS (6). In addition, the strength of the immune response to transplants within the CNS is site dependent, transplants in the ventricles being more vulnerable than those within the brain parenchyma (6), suggesting different degrees and possibly modes of host sensitization in these cases.

Lateral ventricular neural allografts are normally rejected within 150 d but have been observed to survive indefinitely in anti-IL-2R mAb treated recipients (7). IL-2 is a pivotal cytokine in the generation of immune responses (8) and furthermore, its mRNA is detectable at sites of allograft rejection *in vivo* (9). Evidence suggests that a lack of IL-2 and other cytokines after antigenic stimulation may be critical in the induction of T cell clonal anergy (10), and work *in vitro* has demonstrated suboptimal proliferative responses and marked reductions in the levels of IL-2 message in anergized T cells (11). In addition, studies *in vivo* have suggested that altered regulation of the IL-2 pathway may be implicated in the induction of allospecific tolerance (12).

In the present study, the status of long-surviving neural allografts in anti-IL-2R mAb treated animals has been investigated. We present evidence to show that treatment with the mAb NDS 63 to the p55 chain of the IL-2R can induce specific tolerance to fully MHC mismatched allografts of neural tissue. In the PVG RT1<sup>c</sup> (PVG) to AO RT1<sup>u</sup> (AO) rat strain combination treatment with NDS 63 results in indefinite lateral ventricular neural allograft survival. Animals with long-surviving (60–300 d) brain allografts accepted second grafts of donor neural tissue placed in a peripheral site beneath the kidney capsule (KC), whereas third-party Lewis - RT1<sup>l</sup> (Lew) allografts were rejected rapidly within 10 d. These data suggest that interruption of the IL-2 pathway at the level of

<sup>1</sup> Abbreviations used in this paper: CNS, central nervous system; KC, kidney capsule.

the IL-2R can result in the generation of specific peripheral transplantation tolerance after allografts of neural tissue within the CNS.

## Materials and Methods

**Animals.** 8–12-wk-old AO RT1<sup>u</sup> rats were maintained in our own animal research facility and used as recipients in all allograft experiments. PVG RT1<sup>c</sup> and Lewis RT1<sup>l</sup> pregnant rats were obtained from Harlan Olac (Bicester, Oxon., UK), and rat pups 24-h-old were used as donors in all experiments. PVG adults (8–12-wk-old) obtained from our own animal unit were used as recipients for control syngeneic grafts.

**mAb Preparation.** Anti-IL-2R mAb cell lines NDS 63 (isotype IgG1), which functionally inhibit the IL-2R, and NDS 66 (isotype IgG2a), a control anti-IL-2R mAb which does not block the receptor, were used *in vivo* (13). mAb activity and specificity were tested in immunofluorescence assays. These hybridoma cell lines were grown as ascites in 3-mo-old BALB/c × DBA (CD2) F<sub>1</sub> hybrid mice. The IgG fractions were obtained by DEAE ion exchange chromatography and purity determined by SDS-PAGE analysis. mAbs were dialyzed against PBS and administered at 750 µg/kg/d *i.p.* from days 0 to 9 after the initial transplant.

**Neural Transplantation Surgery.** Donor tissue for transplantation consisted of 1–2-mm cubes of neocortical neural tissue dissected from neonatal rat pups <24-h-old. The tissue was placed into sterile physiological saline cooled to 0–4°C on ice and then taken up in a stainless steel cannula of ~12 µl volume. Recipient rats were anaesthetized with fentanyl citrate and fluanisone (Hypnorm; Janssen Pharmaceutical LTD, Oxford, UK) and midazolam (Hypnovel; Roche Products, Welwyn Garden City, UK), placed in a stereotaxic frame, and the transplant placed in the lateral ventricle at the following coordinates: A, -1 mm; L, 1.5 mm; and V, 3 mm (A, anterior from bregma; L, lateral from bregma; and V, ventral from dura).

**Kidney Capsule Grafts.** Neocortical neural tissue was prepared and anaesthetic was administered as described above. A cannula of volume 25 µl was used. A dorso-ventral incision was made beneath the lowest rib on the recipient animal's left flank. The left kidney was exposed and externalized and the KC pierced. The cannula was inserted beneath the KC, and the transplant tissue expelled and gently massaged towards the upper pole of the kidney. The kidney was replaced in the abdominal cavity and the peritoneum and skin closed.

**Experimental Design.** Experimental allograft groups were treated with either NDS 63 mAb (group 1), control mAb NDS 66 (group 2), or were untreated (group 3), as detailed in Table 1. Neural allografts were transplanted into the CNS on day 0, and at times of 60, 120, or 300 d thereafter animals were challenged with peripheral KC allografts of neural tissue. Recipients receiving allografts into the CNS alone were included in each group at all time points for comparison with the combined CNS and KC allografted animals. Third-party control KC allografts were of the fully MHC histoincompatible Lew strain.

**Immunocytochemistry.** Animals were killed at 70, 155, or 350 d after initial surgery. Brains were removed and quick frozen in OCT embedding compound (Miles Laboratories Inc., Elkhart, IN), and 10 µm -cryostat sections were prepared and stained by the immunoperoxidase technique of Barclay (14). Secondary antibody used was a horseradish peroxidase-conjugated rabbit anti-mouse polyclonal Ig (Dako, High Wycombe, UK) with diaminobenzidine (Sigma Chemical Co., Dorset, UK) used as the chromogen. Coun-

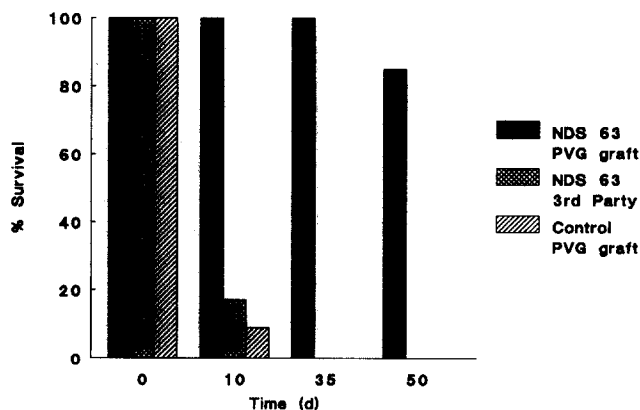
terstaining was performed with 0.5% Toluidine blue (BDH Chemical Co., Dorset, UK).

**mAbs for Immunocytochemistry.** The following mAb were used: MRC OX-27, polymorphic anti-rat MHC class I, RT1<sup>c</sup> haplotype only (15); MRC OX-7 anti-Thy-1.1 antigen found on mature rat CNS neurons (16); MRC OX-6, anti-rat MHC class II (17); MRC OX-1+30, leukocyte common antigen (18); MRC OX-19 (19) +52 (20), anti-rat T cell; MRC OX-10, anti-rat CD8 (21); MRC OX-39, anti-rat IL-2R (22); MRC OX-42, anticomplement receptor type 3 (23), and a negative control MRC OX-21, against human factor I. The MRC OX mAbs were kind gifts of Prof. A. F. Williams and Dr. D. W. Mason (Medical Research Council Cellular Immunology Unit, Oxford, UK).

**Allograft Analysis.** The survival of neural grafts within the brain and beneath the KC was determined by immunostaining with MRC OX-7 for the Thy-1.1 antigen, a cell surface marker expressed at high density on mature neurons of the CNS (24).

## Results

Syngeneic grafts were performed to confirm tissue viability *in vivo*, and all such grafts to the lateral ventricle and KC survived indefinitely. Results of neural allograft survival into the CNS and beneath the KC are shown in Table 1. In recipients treated with the anti-IL-2R mAb NDS 63 (group 1a) 100% survival of neural allografts in the brain was observed at day 350, whereas 37 and 67% of control grafts (NDS 66 treated and untreated; groups 2a and 3a) were rejected at days 70 and 155, respectively. To further investigate the tolerant status of recipients with long-surviving allografts, second allografts of neural tissue, syngeneic with the original donor tissue, were placed in a peripheral site beneath the KC at 60 and 120 d after the initial brain graft, without the administration of any further immunosuppression. 100% of such peripheral neural allografts were accepted (group 1b), for



**Figure 1.** The survival of second neural allografts to the kidney capsule. AO RT1<sup>u</sup> rats received PVG RT1<sup>c</sup> neural allografts transplanted into the lateral cerebral ventricle on day 0 and were treated either with NDS 63 mAb or were control treated (NDS 66 mAb or untreated). At times thereafter (days 60, 120, 300), animals received a second allograft of either donor-specific (PVG) or third-party (Lew RT1<sup>l</sup>) neural tissue transplanted to the kidney capsule. The survival of these second kidney capsule neural allografts was examined at days 70, 155, and 350 (10, 35, and 50 d after transplantation, respectively).

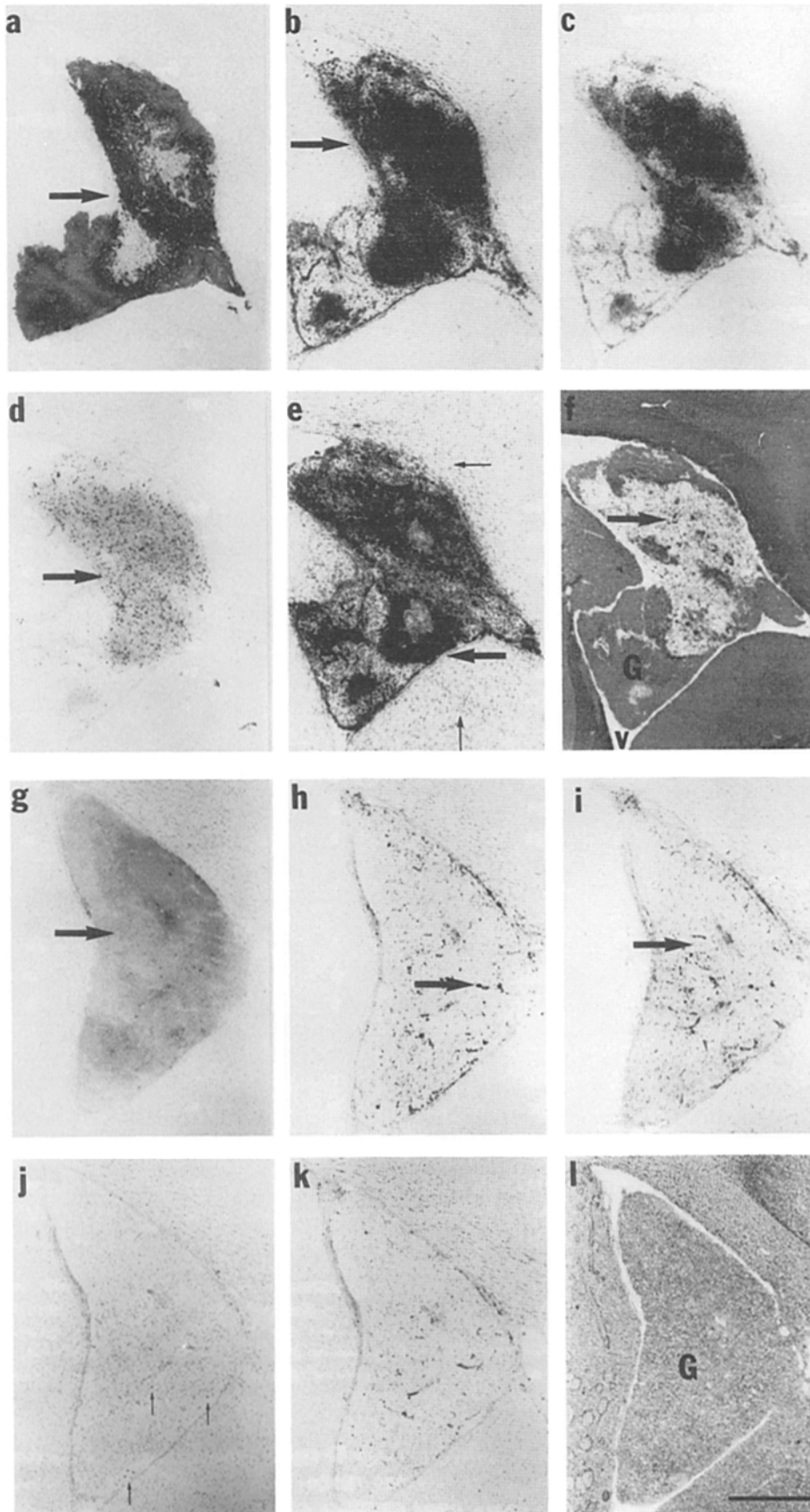
**Table 1.** Brain and Kidney Capsule Neural Allograft Survival

Experiment	Day of assessment	No.	Percent survival	
			Brain	Kidney
Group 1: NDS 63 mAb treatment				
a: PVG RT1 <sup>c</sup> >AO RT1 <sup>a</sup> V <sub>L</sub> graft alone	70	9	100	-
	155	10	100	-
	350	8	100	-
b: PVG V <sub>L</sub> graft + PVG RT1 <sup>c</sup> KC graft at	d 60	6	100	100
	d 120	7	100	100
	d 300	5	100	80
c: PVG V <sub>L</sub> allograft + third-party Lew RT1 <sup>1</sup> KC allograft at	d 60	6	100	16
	d 120	5	100	0
	d 300	6	100	0
Group 2: NDS 66 mAb control treatment				
a: PVG >AO V <sub>L</sub> neural allograft alone	70	9	66	-
	155	11	35	-
	350	8	0	-
b: PVG V <sub>L</sub> allograft + PVG KC graft at	d 60	5	40	20
	d 120	8	0	0
	d 300	5	0	0
c: PVG V <sub>L</sub> allograft + third-party Lew KC allograft at	d 60	7	56	28
	d 120	6	33	0
	d 300	5	0	0
Group 3: No treatment control				
a: PVG >AO V <sub>L</sub> neural allograft alone	70	7	57	-
	155	7	28	-
b: PVG V <sub>L</sub> allograft + PVG KC graft at	d 60	6	33	0
	d 120	6	0	0

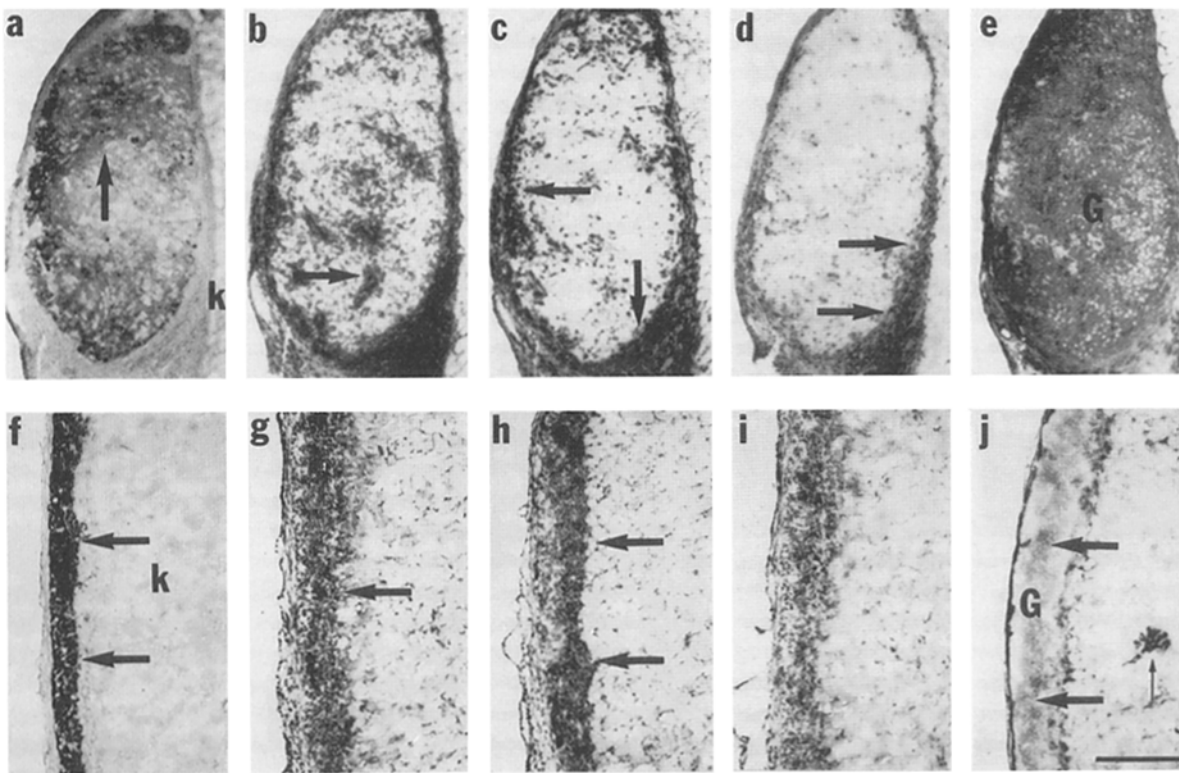
AO RT1<sup>a</sup> rats received PVG RT1<sup>c</sup> neural allografts to the lateral cerebral ventricle (V<sub>L</sub>) on day 0 and recipients were treated with either NDS 63 mAb, NDS 66 mAb, or were untreated. At time point days 60, 120, or 300, these animals (except those in groups 1a, 2a, and 3a) received second allografts to the KC of either donor-specific (PVG) or third-party (Lew) neural tissue, as indicated in column one. Brain and KC neural allografts were examined at the times (days 70, 155, and 350) indicated in column 2, the times after KC transplantation being 10, 35, and 50 d, respectively. Brain and KC neural allograft survival is shown in columns 4 and 5 and was determined by the expression of the Thy-1.1 neuronal cell surface antigen as described in Materials and Methods.

greater than 35 d in the case of those transplanted at day 120, whereas third-party KC allografts were rejected rapidly (group 1c and Fig. 1). In these groups (1b and 1c), the initial neural allografts within the CNS survived indefinitely. In both

control groups (groups 2b and 3b), peripheral KC allografts were acutely rejected, within 10 d in most cases, themselves provoking rapid rejection of the original brain allografts within the same 10-d period. As predicted, third-party pe-



**Figure 2.** Immunohistological features of lateral ventricular neural allografts. PVG to AO lateral ventricular neural allograft in an NDS 66 treated recipient at day 70, 10 d after the transplantation of an AO KC neural allograft (a-f). (a) MRC OX-27 expression indicating strong donor-specific MHC class I induction across much of the graft. Patches of absent expression are noted as well as an area of elevated expression (arrow). (b) MRC OX-6, indicating strong expression of MHC class II antigens within the graft (arrow). (c) MRC OX-1/30, showing strong leukocytic infiltration within the graft. (d) MRC OX-39, indicating numerous IL-2R<sup>+</sup> cells within the infiltrate. (e) MRC OX-42. Upregulation of this antigen is noted upon activated microglia within the host brain (small arrows) and upon macrophages within the graft (large arrow). (f) MRC OX-7. The allograft (G) is noted within the lateral cerebral ventricle (V). Uniform Thy-1 expression is seen upon the host brain and on the inferior part of the graft, but large areas of reduced or absent expression are noted at the allograft apex (arrow), indicative of allograft rejection. The latter areas correlate closely with those regions of intense MHC class II expression and strong leukocytic infiltration. PVG to AO lateral ventricular neural allograft in an NDS 63 treated recipient at day 350, 50 d after the transplantation of a donor-specific AO KC neural allograft (g-l). (g) MRC OX-27. Weak induction of donor-specific MHC class I expression is noted (arrow). (h) MRC OX-6. Small clusters of MHC class II antigen expressing cells are seen within the graft (arrow). (i) MRC OX-1/30. Small numbers of infiltrating leukocytes are seen (arrow). (j) MRC OX-39. Very few IL-2R<sup>+</sup> cells are noted (arrows). (k) MRC OX-42. Very few activated microglia or macrophages are seen within the graft or host brain. (l) MRC OX-7. Uniform Thy-1.1 antigen expression is noted within the allograft (G) and host brain, indicative of excellent allograft survival. Bar (a-l), 550  $\mu$ m.



**Figure 3.** Immunohistological features of second neural allografts to the kidney capsule. PVG to AO KC neural allograft in an NDS 63 treated recipient 50 d after transplantation (a–e). (a) MRC OX-27. Weak to moderate donor-specific MHC class I expression is noted (arrow). The host kidney parenchyma (k) is noted at the medial edge. (b) MRC OX-6. Patches of MHC class II expression are noted within the allograft (arrow) and at the graft borders. (c) MRC OX-1/30. Some infiltrating leukocytes are noted within the graft but most are seen at the graft borders (arrows). (d) MRC OX-10. Very few CD8<sup>+</sup> cells are noted within the graft, but most of these cells are seen at the inferior graft/host interface (arrows). (e) MRC OX-7. Strong, uniform Thy-1.1 expression is noted upon the entire allograft (G), indicative of excellent allograft survival. PVG to AO KC neural allograft in an NDS 66 treated recipient 10 d after transplantation (f–j). (f) MRC OX-27. Very strong donor-specific MHC class I induction is noted upon a thin rim of graft tissue. The adjacent host kidney parenchyma (k) is noted. (g) MRC OX-6. Strong expression of MHC class II antigens are seen within the allograft (arrow) and some positive cells are seen within the host kidney parenchyma. (h) MRC OX-1/30. Numerous infiltrating leukocytes are noted within the graft (arrow). (i) MRC OX-10. Many CD8<sup>+</sup> cells are seen within the allograft. (j) MRC OX-7. Very weak and patchy Thy-1.1 expression is noted upon the allograft (G) indicative of graft rejection (large arrows). An area of stronger expression is noted within the host kidney parenchyma upon a glomerulus (small arrow). Bar (a–j), 500  $\mu$ m.

ripheral KC allografts administered to control animals (group 2c) were rejected rapidly, whereas allografts within the brains of these animals remained relatively intact resembling those in similarly treated recipients without a peripheral stimulus (group 2a).

The essential immunohistological features shown in Figs. 2 and 3 are typical of our findings. In NDS 63 treated recipients without a second KC transplant, grafts in the CNS demonstrated homogeneous Thy-1.1 expression (indicative of neuronal survival), in the absence of observable MHC antigen induction, although occasional weak cell infiltration was noted. MHC antigens are not normally detectable on healthy neuronal tissue (25). However, in recipients of second KC allografts at all timepoints (group 1b), original brain graft survival was not compromised (as indicated by uniform Thy-1.1 staining), but it is interesting that weak donor MHC class I and II expression and patchy cell infiltration, consisting of some IL-2R<sup>+</sup> and CD8<sup>+</sup> cells, were consistently observed (Fig. 2, a–f). It was thus readily apparent that administration of subsequent KC allografts had increased immune cell infiltra-

tion and MHC antigen expression within the original lateral ventricular neural allografts. Accepted KC allografts in NDS 63 treated animals (group 1b) demonstrated high levels of uniform Thy-1.1 expression (indicative of neuronal survival) and mildly elevated donor MHC class I induction. Cell infiltration was observed at the periphery of these allografts without, however, any evidence of allograft destruction (Fig. 3, a–e). Numerous CD8<sup>+</sup> and IL-2R<sup>+</sup> cells were noted within the infiltrate.

In contrast, allografts within the CNS of control animals after the administration of a peripheral KC neural allograft stimulus (groups 2b and 3b), showed strong and rapid donor MHC class I and II induction. This was accompanied by massive cell influx (predominantly CD8<sup>+</sup> and IL-2R<sup>+</sup> lymphocytes and OX-42<sup>+</sup> macrophages) with evidence of marked graft destruction (eroded and nonuniform Thy-1.1 staining) detectable within 7 d (Fig. 2, g–l) and complete allograft rejection in all cases by day 35. Furthermore, these control recipients (groups 2b and 3b) showed rapid rejection of peripheral KC allografts. In these grafts, strongly upregulated

donor MHC class I expression was noted in the absence of uniform Thy-1.1 expression, and in most cases, only a heavy band of infiltrating leukocytes was detectable 10 d after transplantation (Fig. 3, *f-j*).

In the final experiments, the duration of the induced state of specific unresponsiveness was examined. 100% survival of neural allografts within the CNS of NDS 63 treated recipients was noted at 350 d after transplantation. Peripheral KC allografts administered to such long-term brain-grafted animals (group 1b) were accepted for >50 d in all but one case (80%), in which no evidence of KC allograft survival nor of residual immune response could be detected. The original allograft in the brain of this animal survived intact, resembling others in this group, which suggested a technical failure of the KC transplant.

### Discussion

The data presented demonstrate that a state of unresponsiveness has been induced to allografts of neural tissue in rats treated with a mAb to the p55 chain of the IL-2R, NDS 63. This tolerance was allospecific as third-party peripheral KC allografts were rapidly injected with normal kinetics. In contrast, control (NDS 66 and untreated) recipients rejected original brain allografts chronically, and rapidly rejected second peripheral KC allografts, demonstrating that the tolerogenic effect was specific to inhibition of IL-2R function. Furthermore, this alloantigen-specific nonresponsive state persisted for >350 d. Of particular interest is that administration of short course cyclosporin A treatment in this rat strain combination does not result in indefinite neural allograft survival (our unpublished results), and thus the use of mAb immunotherapy for neural allotransplantation may have certain advantages.

It is known that NDS 63 treatment leads to modulation of the IL-2R and functional inactivation of the receptor without cell depletion (13), and thus tolerance in this model is not maintained by the deletion of alloreactive T cells. From our observations, it is clear that small numbers of circulating lymphocytes have the capacity to enter long-surviving neural allografts within the brain, an immunologically privileged site, such recirculation through the healthy brain not normally being observed. After administration of second KC al-

lografts to NDS 63 treated recipients, increased lymphocyte numbers were noted within the original brain grafts in the absence of allograft rejection. These lymphocytes were predominantly of the CD8<sup>+</sup> phenotype and the induced levels of class I and II MHC antigens observed within the allografts suggested that these infiltrating lymphocytes were not entirely quiescent but capable of some cytokine production. This supports the finding by others of a degree of cytokine production by anergic T cells in vitro (11). It has been suggested that the persistence of antigen is required to maintain an unresponsive state (26, 27). Our observations support this hypothesis and indicate that alloantigen within the CNS may be continually monitored by small numbers of lymphocytes sufficient to maintain the tolerant state. These findings may be relevant to the maintenance of self tolerance to antigens within the brain. Further, the present model may be useful to investigate the circumstances in which the breakdown of self tolerance to such brain-derived antigens occurs.

Our findings resemble other models of T cell anergy in that inhibition of normal flux through the IL-2 pathway appears to be an important event leading to the tolerant state. Furthermore, this inhibition has been found to occur at different levels within the pathway. Cell culture evidence has suggested that a >93% reduction in the level of IL-2 message accompanies T cell inactivation (11), whereas in vivo evidence from a pretransplantation blood transfusion model of renal transplant tolerance has indicated that normal levels of IL-2 mRNA are present in graft-infiltrating cells (12). However, in the latter case, biologically active IL-2 was not produced and tolerance could be overcome by IL-2 administration, suggesting the possibility that IL-2 antagonists maintained the unresponsive state in vivo. A decrease in effective IL-2 production has also been found in states of anti-CD4 mAb induced tolerance (28). In the present report, interruption of the IL-2 pathway at the level of IL-2R signaling has been shown to give rise to a state of allospecific tolerance in which some cytokines are produced. Hence, prevention of the normal interaction between IL-2 and its receptor would seem to be critical to the generation of peripheral transplantation tolerance. Direct analysis of intragraft events with the examination of cytokine production in situ should shed light on the nature of the tolerance induced in this model.

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