Long-term restoration of gonadal activity with xenografts of preoptic area tissue in hypogonadal (*hpg*) mice

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1. Introduction

The identification of novel mutants and their genetic and phenotypic characterization leads initially to fairly well-defined areas of experimentation. However, some mutant models lend themselves to investigations in fields that at first glance may appear remote from the original observation.

This has been true of the hypogonadal (*hpg*) mouse first discovered by Bruce Cattanach (Cattanach *et al*., 1977). In these mutant mice there is a failure of postnatal gonadal development such that paired testicular weight in 60-d-old *hpg* males is less than 10 mg whilst in normal littermates the testes weigh nearly 200 mg. The seminal vesicles of the mutants are extremely atrophic, indicating a failure of androgen production by the testes. In female mutants ovarian follicles rarely advance beyond the pre-antral stage and the uterus is thin and thread-like.

The initial characterization of the *hpg* mutant indicated that the proximate cause of the hypogonadism lay in a failure to produce the decapeptide gonadotrophic hormone-releasing hormone (GnRH) within the hypothalamus of the brain. This decapeptide is essential for normal synthesis and secretion of the pituitary gonadotrophic hormones – luteinizing hormone (LH) and follicle stimulating hormone (FSH) – which themselves stimulate the gonads. We now know that there is a 33·5 kilobase deletion in the 3′ region of the gene encoding GnRH (Mason *et al*., 1986*a*) and that normal gonadal function can be restored in transgenic *hpg* mice after pronuclear injection of copies of the normal GnRH gene (Mason *et al*., 1986*b*).

We have utilized the *hpg* mouse to investigate the effects of injecting synthetic decapeptide GnRH upon pituitary and gonadal structure and function and to dissect out the actions of LH and FSH themselves upon the gonads. Multiple daily injections of GnRH increased the numbers of GnRH receptors on pituitary gonadotrophins and stimulated both the synthesis and secretion of LH and FSH (Charlton *et al*., 1983, Naik *et al*., 1985; Young *et al*., 1985). The total number of gonadotroph cells within the pituitary was significantly increased, as was the size of these cells and the number of hormone granules they contained (McDowell *et al*., 1982). Treatment of adult male *hpg* mice with LH increased both Leydig cell numbers and steroidogenic activity and stimulated full spermatogenesis (Scott *et al*., 1990). Treatment with FSH had little or no effect on Leydig cell function but allowed spermatogenesis to advance with formation of round spermatids and an increased seminiferous tubule diameter after 10 d of hormone injections (O'Shaughnessy *et al*., 1992). The use of the *hpg* mutant in such endocrine studies is an obvious one, but other lines of investigation have also proved fruitful.

The strategy of grafting normal neural tissue into the central nervous systems (CNS) of experimentally lesioned or genetically deficient animals has been adopted in attempts to restore aberrant neural function (Perlow *et al*., 1979; Bjorklund & Stenevi, 1979; Gash *et al*., 1980). The hypogonadal (*hpg*) mouse mutant has allowed us to investigate survival and function of neural grafts. Functional recovery in female mice is readily assessed externally by vaginal opening and the presence of oestrus smears and internally by an increase in both ovarian and uterine weight. In males testicular growth is evident externally by swelling of the scrotum and on autopsy by an increased testicular weight, histological evidence of spermatogenesis, and an increase in seminal vesicle weight. We have successfully reversed the hypogonadal phenotype with neural grafts of late fetal/early neonatal hypothalamic preoptic area (POA) tissue derived from normal colony mates and transplanted into the third ventricle (Krieger *et al*., 1982).

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In such implants GnRH-positive axons extend specifically to the median eminence (Silverman *et al.*, 1985), resulting in detectable pituitary and gonadal stimulation by 14 d after grafting (Charlton *et al.*, 1985). However, the capacity of these grafts correctly to transduce hormonal feedback signals has been shown to be impaired (Charlton *et al.*, 1987).

The high success rate of these neural allografts in restoring function led us to look at strategies that might prolong the survival of xenogeneic material and perhaps allow restoration of gonadal function in *hpg* mice using rat POA xenografts.

The CNS has traditionally been though of as an immunologically privileged site (Medawar, 1948; Barker & Billingham, 1972). However, it is now accepted that this privilege is not absolute and that the rejection of allogeneic and xenogeneic neural grafts does occur (Mason *et al.*, 1986; Sloan *et al.*, 1991). Thus unless syngeneic donor neural tissue is used for transplantation into the CNS, graft survival cannot be absolutely guaranteed. Furthermore, genetically engineered cells (Rosenberg *et al.*, 1988) or immortalized neural cell lines (Cepko, 1988), neither of which are entirely normal cells, or human fetal neural donor tissue used in clinical transplantation trials (Lindvall *et al.*, 1997), being of allogeneic origin, may all be subject to immune responses of varying degrees that may necessitate the use of some form of immunosuppression.

The fungal metabolite cyclosporin A has been demonstrated to provide successful immunosuppression for xenogeneic neural transplants (Inoue *et al.*, 1985; Brundin *et al.*, 1985) but is not without deleterious side-effects in both human and rodent subjects. An alternative approach to suppression of the immune system is the use of monoclonal antibodies (MAbs) directed at T lymphocyte cell surface molecules, which can provide specific and effective immunomodulation (Cobbold *et al.*, 1984; Wood *et al.*, 1992). In addition, the role that different components of the immune system play in immune responses in the nervous system can be investigated by depletion of particular T cell sub-populations and evaluating the outcome following challenge with an immunogenic stimulus placed in the brain.

It is now clear that the CD4 molecule is intimately involved in the process of T cell activation, not only in augmenting low-affinity T cell responses as an adhesion molecule (Sleckman *et al.*, 1987) but also in contributing to intracellular signal transduction after antigen recognition (Veillette *et al.*, 1989).

The hypogonadal mutant represents an ideal model to test out strategies of immunosuppression. Preliminary work has indicated that a single course of injections of an anti-CD4 MAb may allow POA xenograft function in the *hpg* mouse for up to 30 d (Honey *et al.*, 1991). This has been extended in the present study in which a second course of antibody was given at 30 d and the mice left for a further 30–80 d.

2. Materials and methods

(i) *Animals*

Male and female *hpg* mice (H-2^k) 10–20 weeks old that had been bred in our own animal unit were used as recipients in all experiments. This mutant strain arose as a result of an F1 backcross between the C3H/HeH and 101/H strains and consequently is itself not an inbred strain; thus grafts between colony members may involve some minor antigen incompatibilities. The use of a polymerase chain reaction assay to detect heterozygotes for the *hpg* gene (Lang, 1991) has enabled breeding of the mutant animals within our animal unit to be highly efficient. Heavily pregnant PVG rats (RT1^c) were obtained from Harlan Olac (Bicester, UK) and neonates used as a source of xenogeneic tissue. Animals were housed in a 14 h light/dark cycle with free access to food and water.

(ii) *Depletion of T lymphocyte subsets*

MAbs used in the subset depletions were anti-CD8 (YTS 169.4) (Cobbold *et al.*, 1984) and anti-CD4 (YTA 3.1) (Qin *et al.*, 1987), both of the IgG2b isotype. MAb activity and specificity were tested in immunofluorescence assays. Hybridoma cell lines were grown as ascites in 3-month-old DA × LOU F1 hybrid rats. The IgG fractions were purified by diethylaminoethyl ion exchange chromatography and purity determined by SDS-PAGE analysis. Thereafter MAbs were dialysed against phosphate-buffered saline (PBS) and administered by intraperitoneal injection at either a low dose of 4 μg/g per d or a higher dose of 20 μg/g per d from day -1 to day 9. In the case of a second course being administered, MAb was given from day 30 to day 34.

(iii) *Transplantation surgery*

POA tissue was dissected from the brains of neonatal (less than 24 h old) rats and mice and taken up into a stainless steel cannula approximately 4 μl in volume. Recipient *hpg* mice were anaesthetized with Rompun (xylazine, Bayer UK, Bury St Edmunds, Suffolk, UK) and Vetalar (ketamine hydrochloride, Parke Davis, Pontypool, Gwent, UK) placed in a stereotaxic frame and the implant placed in the third ventricle at a depth of 5·5 mm in the midline, 1 mm posterior to bregma.

(iv) *Experimental groups*

Various treatment groups are shown in detail in the results tables. Briefly, *hpg* recipients received grafts of colony member or xenogeneic POA into the third

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ventricle. Recipients in each group were either untreated, anti-CD8 MAb treated, anti-CD4 MAb treated or treated with a combination of anti-CD4 and anti-CD8 MAbs.

At autopsy the ovaries and uterus were removed from females and the testes and seminal vesicles from males, the organs weighed and then fixed in 4% formaldehyde for histological studies. The brains were also fixed and prepared for subsequent histological analysis.

Graft survival was determined by positive staining for the presence of either GnRH or donor Thy-1 antigen, a neuronal cell surface glycoprotein (Charlton *et al*., 1984; Morris *et al*., 1985, within the graft.

3. Experiments

(i) *Colony member grafts*

In order to have a baseline against which to measure the success of xenotransplants, day 1 postnatal POA grafts from normal C3 He.101 mice were given to *hpg* recipients. Groups of mice were killed at 30, 60 and 110 d to measure alterations in gonadal weight, and to assess graft survival.

(ii) *Rat xenografts*

(*a*) *Single-course injections*

In these experiments both male and female *hpg* mice were given a third ventricular transplant of day 1 POA from rats of the PVG strain. They were divided into groups, with the first group receiving a single course of intraperitoneal injections of either YTA 3.1 or YTS 169.4 at a dose of 4 mg/kg per day from the day before surgery and for the following 9 d. The animals were killed at 30 and 60 d, the gonads and accessory sexual tissue removed and weighed, and the brain examined for the presence of xenogeneic transplant tissue. A further group of *hpg* males was given a single course of high-dose YTA 3.1 (20 mg/kg per d for 10 d); these were killed at 60 and 110 d.

(*b*) *Double-course injections*

In a further set of experiments in male mice the anti-CD4 antibody (YTA 3.1) was given during the first 10 d and then a second course of injections was given between days 30 and 34. In one experiment the mice received two courses of the low dose and, in a second experiment, two courses of the high dose. Finally one group of male *hpg* mice received a single high dose of YTA 3.1 coupled with a concomitant injection of the anti-CD8 MAb YTS 169.4 from day −1 to day 9. These groups were killed at 60 and 110 days.

4. Results

(i) *Colony member grafts*

There was histological evidence of graft survival in all the transplanted mice, with 65% of the grafts resulting in stimulation of gonadal and accessory sexual tissue growth in females (Table 1) and 63% in males (Table 2).

(ii) *Xenogeneic grafts*

(*a*) *Single-course injection* (*Tables 1*, *2*)

In those mice that were untreated, all grafts had been rejected by 30 d and there was no evidence of gonadal stimulation. This was also true of the group that received a single injection of the anti-CD8 MAb YTS

Table 1. *Graft survival and organ weights in* hpg *female mice after single course injection*

Experiment	Day	No.	Physiological success (%)	Graft survival (%)	Uterine weight (mg ± SEM)	Ovarian weight (mg ± SEM)
Colony member grafts						
C3 He.101	30	20	62	100	30·7 ± 2·5	2·5 ± 0·4
Untreated	60	21	65	100	68 ± 10·3	3·1 ± 0·3
Xenografts						
PVG → C3 H	30	15	0	0	5·14 ± 1·4	0·4 ± 0·1
Untreated	60	18	0	0	6·08 ± 0·9	0·5 ± 0·1
PVG → C3 H	30	15	0	0	6·33 ± 1·3	0·5 ± 0·1
Anti-CD8	60	12	0	0	8·1 ± 1·6	0·6 ± 0·1
YTS 169.4 low dose						
PVG → C3 H	30	27	43	100	36·8 ± 3·1	1·5 ± 0·2
Anti-CD4	60	25	40	52	19·8 ± 2·9	1·4 ± 0·4
YTA 3.1 low dose						

In this and subsequent tables the percentage of physiological success is based on the numbers of animals with positive stimulation of the gonads, and the percentage of graft survival is based on an analysis of Thy 1.1 staining of xenogeneic tissue within the ventricle.

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Table 2. *Graft survival and organ weights in male* hpg *mice after single course MAb treatment*

Experiment	Day	No.	Physiological success (%)	Graft survival (%)	Seminal vesicle weight (mg ± SEM)	Mean paired testis weight (mg ± SEM)
Colony member grafts						
C3 He.101	30	10	60	100	31·2 ± 5·9	53 ± 11·7
Untreated	60	11	63	100	77·8 ± 6·6	104 ± 9·9
Xenografts						
PVG → C3 H	30	6	0	0	2·2 ± 0·8	6·1 ± 0·3
Untreated	60	8	0	0	1·9 ± 0·6	5·9 ± 0·8
PVG → C3 H	30	7	0	0	1·8 ± 1·3	4·5 ± 0·7
Anti-CD8 YTS 169.4 × 1	60	9	0	0	2·1 ± 1·6	5·7 ± 0·4
PVG → C3 H	30	13	45	100	31·1 ± 3·5	49·6 ± 4·9
Anti-CD4 YTA 3.1 Low dose × 1	60	11	45	52	4·5 ± 0·7	12·1 ± 3·2
PVG → C3 H	60	12	48	90	28·2 ± 4·9	45·9 ± 4·7
Anti-CD4 YTA 3.1 high dose × 1	110	10	42	39	23·5 ± 6·1	31·5 ± 6·5

Table 3. *Graft survival and organ weights in male* hpg *mice after double course MAb treatment*

Experiment	Day	No.	Physiological success (%)	Graft survival (%)	Seminal vesicle weight (mg ± SEM)	Mean paired testis weight (mg ± SEM)
Allografts						
C3 HE.101	60	12	67	100	88·5 ± 9·7	92·6 ± 12
Untreated	110	8	63	100	92·5 ± 8·8	113 ± 9·8
Xenografts						
PVG → C3 H	60	11	47	79	20·4 ± 3·7	41·6 ± 5·1
Anti-CD4 YTA 3.1 Low dose × 2	110	9	44	24	9·5 ± 2·9	14·2 ± 7·5
PVG → C3 H	60	11	48	100	36·8 ± 3·8	53·4 ± 8·4
Anti-CD4 YTA 3.1 High dose × 2	110	9	48	91	39·8 ± 4·1	74·4 ± 11·6
PVG → C3 H	60	14	47	100	34·7 ± 4·5	45·4 ± 2·7
Anti-CD4 YTA 3.1 High dose + Anti-CD8 YTS 169·4	110	8	50	63	29·9 ± 6·2	61·2 ± 7·8

169.4. In the mice that received a single low-dose injection of YTA 3.1, 100% of grafts had survived at 30 d with between 40% and 50% resulting in significant gonadal stimulation. However, by day 60 only 50% of grafts had survived, with a dramatic fall in both testicular and seminal vesicle weights in the males and in uterine weight in the females.

We can be certain that there was a decrease in gonadal size from day 30 to 60, and that we were not just sampling a less stimulated population, because we undertook laparotomies at day 30 to measure testis diameter and in all cases this was reduced by day 60.

In those males that received the single high-dose YTA 3.1 treatment, 90% of grafts had survived to 60 d and testis weight was maintained in the positive responders. However, by 110 d total graft survival had dropped to 39% and, in responding animals, mean testis weight had fallen (Table 2).

(*b*) *Two-course injections* (*Table 3*)

In the low-dose YTA 3.1 anti-CD4 two-course treatment group, testis weight was high at 60 d compared with the single low-dose group (41·6 ±

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5·1 mg vs 12·1 ± 3·2 mg), indicating prolonged graft survival. However, by 110 d testis weight was reduced to 14·2 ± 7·5 mg and total graft survival was down to 24%.

The high-dose two-course treatment was very successful, with 100% graft survival at 60 d and 91% at 110 d. In this group, testis weight increased further from 53·4 ± 8·4 mg to 74·4 ± 11·6 mg.

Graft survival and gonadal stimulation were also enhanced in the group of mice receiving a single high dose of anti-CD4 MAb plus concomitant injections of the anti-CD8 antibody.

5. Discussion

Neural transplantation has the potential to provide treatment for a variety of neurodegenerative and other disorders of the CNS. The most widely reported protocol has been the use of fetal substantia nigra cells transplanted to the striatum in Parkinson's disease patients (Lindvall, 1997). At the moment, fetal tissue has to be used and be collected from several fetuses at the time of transplantation, giving rise to difficult ethical and logistical problems. Some of these problems could be addressed if xenogeneic material were available and strategies devised to ensure long-term survival. In this regard the use of porcine material is being considered (Dunnett *et al*., 1997).

For several years we have been involved in investigating the immune response to both allogeneic and xenogeneic neural grafts. Allografts placed within the parenchyma of the brain are less susceptible to rejection than those placed within the ventricles; however, xenografts are rapidly rejected in either location.

The $CD4^+$ T lymphocyte subset has been shown to play a critical, central role in the rejection of fully histoincompatible organ and skin allografts (Cobbold *et al*., 1986; Madsen *et al*., 1987; Shizuru *et al*., 1987). These T cells provide 'help', which in effect means that they produce the relevant cytokines, particularly interleukin 2 (IL-2), to initiate an alloreactive immune response. In some exceptional cases the $CD8^+$ subset can provide this function, most commonly in cases of second-set rejection in which the recipients have been previously immunized (Madsen *et al*., 1989). The importance of T cells of $CD4^+$ phenotype in the early phase of neural allograft rejection has been suggested by observations of their numerical prominence at early times in comparison with the $CD8^+$ cells that appear to dominate the later phases of rejection (Nicholas *et al*., 1990). It was thus reasoned by Nicholas *et al*. that elimination of cells of the $CD4^+$ subset might diminish the immune response to allogeneic neural grafts. Such a reduced immune response was demonstrated in recipients depleted of $CD4^+$ cells (Nicholas *et al*., 1990).

An earlier study (Honey *et al*., 1991) had demonstrated functional recovery and xenograft survival in *hpg* male mice for up to 30 d; however, these grafts within the third ventricle were being rejected at this time point. Only one course of anti-CD4 treatment was given in these experiments.

Utilizing a two-course injection strategy, indefinite survival of neural xenografts placed within the CNS parenchyma has been reported (Wood *et al*., 1996). Grafts within the brain parenchyma are less susceptible to rejection than those placed within the ventricles (Sloan *et al*., 1991). Utilizing similar protocols we have now succeeded in maintaining the survival and physiological function of xenografts within the third ventricle, again confirming the observation that $CD8^+$ lymphocytes alone appear unable to mediate acute rejection of xenografts. The immunological background to this observation has been addressed extensively by Wood *et al*. (1996).

Neither high- nor low-dose anti-CD4 treatment elicited total CD4 cell depletion. However, with the high dose there was a significant reduction in intrathymic CD4 cells compared with the low dose (Wood *et al*., 1996). This could provide some explanation for the greater success of the high-dose treatment. There is evidence that anti-CD4 treatment may also induce functional inactivation of many of the $CD4^+$ T cells that remain in the circulation. We have not addressed the question of how long residual antibody remains in the circulation after the final injection, but it is likely that in the high-dose treatment this could be for several days.

Reconstitution of lymphoid tissue by $CD4^+$ T cells reaches about 30% by day 30 following depletion (Bushell *et al*., 1995) and Wood *et al*. (1996) reported that neural xenograft rejection was occurring around this time after single-injection low-dose treatment. Vine (1997) has shown that, whilst anti-CD4 antibody treatment prevents the gene expression of many pro-inflammatory cytokines in the region of neural xenografts, by 30 days mRNA for interferon γ, interleukin 4 and interleukin 10 are detectable, and T cells are present within and around grafted tissue.

The failure of the single low-dose YTA 3.1 treatment ultimately to prevent rejection may relate to the time required for re-formation of the blood–brain barrier (BBB) in the vicinity of the transplant. The importance of BBB disruption in the rejection of neural xenografts has been demonstrated (Pollack *et al*., 1990) and it can take up to 30 d to be re-formed after transplantation (Broadwell *et al*., 1989). It is therefore possible that repletion of $CD4^+$ T cells could be taking place before the barrier is in place.

The obvious strategy of giving a second course of low-dose treatment at 30 d was successful in maintaining testicular stimulation at least up to day 60; however, by day 110 both mean testicular and seminal

vesicle weights were significantly reduced and graft survival assessed histologically was down to 24%. In these latter experiments it may be that sufficient xenoreactive $CD8^+$ T cells had been activated before the second injection to bring about protracted graft rejection.

The results of the single high-dose regimen are interesting. As assessed by testicular weight, grafts were still functioning at 60 d compared with the single low-dose group ($45{\cdot}9 \pm 4{\cdot}7$ mg vs $12{\cdot}1 \pm 3{\cdot}2$ mg). Histological evidence of graft survival at this time demonstrated that in 90% of animals grafts were present within the third ventricle, compared with 52% in the low-dose treatment. A possible explanation for these differences could be that the higher dose resulted in a greater delay of $CD4^+$ cell repletion as a result of a more prolonged presence of antibody in the circulation.

Although the experiments reported in this paper and elsewhere (Wood *et al*., 1996) indicate that $CD8^+$ T cells alone are unable to mediate acute graft rejection, the fact that single high-dose anti-CD4 and anti-CD8 treatment was more successful than the high-dose anti-CD4 treatment alone argues that $CD8^+$ cells are involved in the rejection response. Indeed the fact that $CD8^+$ cells are present at sites of neural xenograft rejection suggests that they play a role in the rejection process.

When only a single treatment with anti-CD4 is given it is possible that some of the remaining $CD4^+$ lymphocytes could provide early help to the full complement of $CD8^+$ cells to provide a nucleus of xenoreactive clones capable of further expansion when the $CD4^+$ population is reconstituted, more particularly if this occurs before full restoration of the BBB. The additional depletion of $CD8^+$ cells at the time of grafting would result in far fewer cells available for stimulation against a background of far fewer $CD4^+$ helper T cells. It certainly seems worth exploring the possibility that double antibody treatment at the time of transplantation and at 30 d might provide the greatest protection against xenograft rejection.

From the physiological standpoint several other interesting observations have emerged. Female and male *hpg* recipients of POA neural grafts from normal colony mates demonstrated high success rates of functional recovery and indicated the maximum rates of graft-driven gonadal stimulation that could be expected. The finding that recovery did not occur in every case corroborated similar observations in previous studies (Krieger *et al*., 1982; Silverman *et al*., 1985). The reasons for the failure of functional recovery in a proportion of recipients were probably related to factors such as inadequately dissected graft tissue, suboptimal placement of the transplant or an unfavourable location of GnRH cell bodies within the graft.

Despite the fact that overall graft survival was excellent at 30 d, nevertheless functional recovery was found in less than 50% of recipients compared with over 60% with colony member grafts. There are several possible reasons that might account for this observation. The donor P1 rat neonatal brains were larger than their murine counterparts and hence a similar volume of rat POA tissue would be likely to contain fewer GnRH-positive neurons and this may have reduced the probability of functional recovery. The immediate environment of the mouse third ventricle may be unfavourable for the growth of rat neurons, possibly lacking requisite species-specific growth factors, and thus only a small proportion of rat GnRH neurons might receive sufficient appropriate stimulation both for survival and pathfinding. This again might reduce the probability of successful restoration of function. A subpopulations of cells, for example GnRH neurons, may be more vulnerable to early immune attack.

These studies have demonstrated that a genetic mutant, the hypogonadal mouse, discovered by Bruce Cattanach, has provided a means whereby strategies to enhance neural xenograft survival of potential therapeutic relevance to CNS pathology in the human can be investigated and fundamental information obtained on the immune privilege in the brain.

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