Rejection of the transplanted uterus is suppressed by cyclosporine A in a semi-allogeneic mouse model

C.A.Wranning¹, R.R.El-Akouri¹, K.Groth¹, J.Mölne², A.K.Parra³ and M.Brännström¹

¹Department of Obstetrics and Gynecology, ²Department of Clinical Pathology and Cytology and ³Department of Clinical Immunology, Institute for Clinical Sciences, The Sahlgrenska Academy at Göteborg University, Sahlgrenska University Hospital, Göteborg, Sweden

BACKGROUND: A mouse uterus transplantation model has previously been developed for studies of various aspects of uterine transplantation, which in the future may be used as treatment for uterine infertility. The aim of the study was to evaluate the effect of the immunosuppressant cyclosporine A (CyA) on the rejection of the allotransplanted uterus in the mouse. METHODS: C57BL/6 mice were recipients of uteri from F1 hybrids (C57BL/6 × CBA/ca). Transplanted mice received vehicle (control, n = 5), 10 or 20 mg/kg/day of CyA (CyA10, n = 5 and CyA20, n = 5). Untreated F1 hybrids with syngeneic transplants (n = 3) were negative controls. On day 10 post-transplantation, the grafted uteri were examined, and biopsies were taken for histology and quantification of T cells. RESULTS: Histology analysis revealed necrosis of the uterine transplants in controls and to a lesser extent in the CyA groups. Apoptosis and inflammation was prominent in grafts from the CyA10 group but suppressed in the CyA20 group. A similar increase of CD4+ cells was seen in all groups, whereas the number of CD8+ cells was higher (P < 0.05) in the two allogeneic groups receiving CyA compared with the allogeneic vehicle group. CONCLUSIONS: CyA treatment clearly delays the progress of rejection of grafted uteri but is insufficient to suppress T cell infiltration. Interestingly, the number of CD8+ cells was higher in groups receiving CyA, possibly reflecting a CyA-dependent depression of activation-induced cell death (AICD) of cytotoxic T cells.

Key words: cyclosporine A/mice/rejection/transplantation/uterus

Introduction

There have been enormous developments in assisted reproductive techniques during the past decades, and many causes of both male and female infertility can now be circumvented. However, absolute uterine factor infertility, usually due to congenital absence of the uterus or previous hysterectomy, is still untreatable. For younger women with this type of infertility the options to form a family are by adoption and—in some countries—by the use of a gestational surrogate mother. If transplantation of the uterus was a safe procedure with reasonable chances of a positive outcome, these women would have the opportunity to carry their own child during the entire pregnancy.

The idea of transplantation of the uterus as infertility treatment is not new. Several experimental studies were reported in the 1960s and 1970s (for review see Brännström et al., 2003), and in the year 2000, an attempt to transplant a human uterus was performed (Fageeh et al., 2002). In this case, the patient received a uterus from a live donor, and transplantation was performed by vascular anastomosis of the uterine vessels to the external iliac vessels by the use of venous grafts. Rejection was controlled by standard immunosuppression with cyclosporine A (CyA), azathioprine and prednisolone. However, 3 months after the transplantation, the uterus was found to be necrotic and had to be removed. This was reported to be caused by vascular thrombosis because of inadequate support of the uterus and torsion of the vessels, rather than rejection (Fageeh et al., 2002).

In the light of the negative results of this first uterus transplantation in the human and the complicated medical and ethical nature of using transplantation as infertility treatment, it is of uttermost importance to create a solid scientific foundation before any further attempts are made in the human. For this purpose, uterus transplantation is developed in different animal models such as the mouse (Racho El-Akouri et al., 2002), the rat (Jiga et al., 2003) and the pig (Wranning et al., 2006) for investigation of different aspects of both the surgical technique and the function of the transplanted uterus. Our experience of a heterotopic uterus transplantation mouse model (Racho El-Akouri et al., 2002) demonstrated healthy offspring from a syngeneic donor–recipient combination (Racho El-Akouri et al., 2003a). This syngeneic model was also used to investigate the effect of cold ischaemic preservation on the function of the transplanted...
Materials and methods

Animals

Females (6–8 weeks of age) of inbred C57BL/6 mice (haplotype H-2^b\) and F1 hybrids of inbred C57BL/6 and CBA/ca mice (B6CBAF1, H-2^b\) were used. The animals were housed in controlled conditions (21–23°C, relative humidity of 50–60%, illumination between 0700 and 1900 h) and had free access to water and pelleted food. The study was approved by the animal ethics committee in Göteborg and was carried out according to the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institute of Health, USA).

C57BL/6 mice were recipients of uteri from the semi-allogeneic F1 hybrid. The transplanted mice were divided into three groups: controls received no CyA; 0 mg/kg (vehicle, n = 5), 10 mg/kg (CyA10, n = 5) and 20 mg/kg (CyA20, n = 5). Untreated F1 hybrids with syngeneic transplants (n = 3) were used as negative control.

Drugs

As post-operative analgesia, buprenorphin at 0.05 mg/kg (Temgesic®, Schering-Plough, New Jersey, USA) and carprofen at 5.0 mg/kg (Rimadyl®, Orion Pharma AB, Esbo, Finland) were given as single, s.c. injections. The antibiotic cefuroxime (Zinacef, Glaxo Smith Kline, Uxbridge, UK) was given once immediately post-operatively, s.c. at 40 mg/kg. CyA (Sandimmun, Novartis Pharma AG, Basel, Switzerland) was diluted in 90% propylene glycol (Fluka, Buchs, Switzerland) to desired concentrations (10 or 20 mg/kg daily dose was calculated for 5-g intervals of mouse weight). Mini-osmotic pumps (model 1007D, Alzet Osmotic Pumps, Cupertino, CA, USA) were loaded with CyA or 90% propylene glycol (vehicle group), according to the manufacturer’s instruction. To activate the pumps, they were placed in sterile saline at 37°C overnight before use.

Surgery

The surgical procedures were performed essentially as previously described in detail (Racho El-Akouri et al., 2002, 2003a). Briefly, the donor was anesthetized with isoflurane, and the right uterine horn and the cervix with its feeding and draining vessels up to the level of the aorta and vena cava were dissected, and all minor vessels were ligated or cauterized. Ligatures were tied around the aorta and vena cava just cranial of the intended graft, and the uterus was flushed with saline solution (0.154 M), supplemented with heparin sulphate (100 IU/ml; Leo Pharma AB, Malmö, Sweden) and Xylocaine (0.2 mg/ml; Astra Zeneca, Göteborg, Sweden) through a small incision in the aorta. The vena cava was cut to allow the fluid to escape during flushing and when the fluid from the vena cava side (∼1 ml) was clear, the aorta was cut, the graft put on ice, and the donor was euthanized. In the recipient, the aorta and vena cava were mobilized, and haemostatic clamps were placed en bloc around the vessels. The grafted uterus was placed in a heterotopic position in the recipient, further cranial and parallel to the native uterus. The aorta and vena cava of the graft were anastomosed end-to-side to the aorta and vena cava of the recipient by 1-0 nylon sutures. The native uterus of the recipient was left in place, and the cervix of the grafted uterus was exteriorized and sutured to a stoma to the skin of the abdomen. Small pieces of resorbable haemostatic matrix (Surgicel®, Ethicon, Brussels, Belgium) were wrapped around the two anastomosis sites, and the clamps were released. The graft was observed through the uterine vessels until pulsation was established and the uterus had gained normal coloration. The laparotomy scar was closed with 6-0 sutures, and the animal was given s.c. injections of 1 ml of 0.154 M NaCl and 1 ml of glucose (50 mg/ml) to correct for fluid loss and potential hypoglycaemia. Antibiotics and analgesia, as described above, were also given by s.c. injections, and a mini-osmotic pump loaded with CyA of desired concentration or 90% propylene glycol was placed s.c. dorsolateral on the thorax of the animal. The animal was placed under a heating lamp until fully awake. Heparin sulphate (50 IU) was given as s.c. injections to each animal, 6 and 24 h after surgery.

On day 10 after transplantation, blood samples were taken, the animals were anesthetized and a midline laparotomy was performed. The gross appearance of the stoma and the grafted and native uteri were examined with the aid of an operating microscope, tissue samples were taken for analysis and the animal was euthanized.

CyA analysis

Venous blood from the tail (∼250 μl) was sampled into an EDTA-coated microtube (Microtainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) before the mouse was sacrificed. Analysis of free CyA was performed on whole blood by enzyme immunochemistry using a CyA-specific assay (Emit®2000, Dade Behring, Milton Keynes, UK), according to manufacturer’s instruction. Where appropriate, the samples were diluted before analysis and results were expressed as ng/ml. The lower detection limit of the assay was 30 ng/ml.
and the inter-assay coefficient of variation was below 9% for the targeted concentration at analysis (200–400 ng/ml).

The doses of CyA administered were calculated in 5-g intervals of mouse weight before placement of the mini-osmotic pump. When blood samples were taken the mice were weighed again, and the average actual dose received during treatment was calculated for each animal.

**Gross appearance and histology by light microscopy**

At examination of gross appearance, parameters such as coloration, size, texture and bleeding from the myometrium were evaluated, and the grafted uterus was compared with the native uterus. After gross appearance had been examined, the grafted and native uteri were dissected out, and each uterine horn was divided into two. One bissection of the grafted uterine horn and one bissection of a native uterine horn were fixed in cacodylate-buffered, 4% formaldehyde (Kidney biopsy solution, Bie & Berntsen A-S, Rodovre, Denmark), dehydrated and embedded in paraffin. Tissue sections (4–5 μm) were stained with eosin and haematoxylin and periodic acid-Şiff and examined by light microscopy. Signs of stasis, haemorrhage, oedema and vacuolization of glandular epithelial cells as well as the presence of necrotic cells, apoptotic bodies and immune cells were used as parameters in the histological assessment of the rejection process.

**Immunohistochemistry for CD4+ and CD8+ cells**

One bissection of the grafted uterus and one bissection of a native uterine horn were frozen in glycerol and resin compound (Tissue-Tech, Sakura Fintech Europe, Zoterwoude, Netherlands). Tissue sections (4–5 μm) were fixed on glass slides in acetone, blocked with normal rabbit serum in phosphate-buffered saline, incubated with a rat anti-mouse primary anti-CD4 (dilution 1:500) or anti-CD8 (dilution 1:500) antibody (BD Pharmingen, Franklin Lakes, NJ, USA) washed and incubated with biotinylated, mouse adsorbed, rabbit anti-rat immunoglobulin G and Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA). After incubation with diamino benzidine substrate, the sections were counterstained with haematoxylin and mounted.

The numbers of CD4+ and CD8+ cells were estimated by counting positively stained cells within a grid (10 × 10 squares, 0.125 mm² in ×20 objective and ×10 eyepieces). One tissue section of the native and one tissue section of the grafted uterus from each animal were examined independently by two observers in a blinded fashion, and in each section, five randomly chosen areas were counted. The average of these values for each grafted uterus was related to the average value of the native uterus in the same treatment group and set as a data point.

**Statistics**

For analysis of the quantitative data of CD4+ and CD8+ T lymphocytes, Kruskal–Wallis analysis of variance followed by Mann–Whitney U-test was performed. A P-value of less than 0.05 was considered significant.

**Results**

**Cyclosporine A**

The concentration of unmetabolized CyA in blood was analysed in vehicle, CyA10 and CyA20 mice. A small number of animals were excluded for this analysis because of inadequate volume of blood (n = 1) or clotting (n = 2). The vehicle animals had CyA concentrations below or close to the detection limit (30 ng/ml) for the analysis. Mice that received 10 mg/kg/day had between 180 and 340 ng/ml of unmetabolized CyA and mice that received 20 mg/kg/day had between 360 and 1066 ng/ml of unmetabolized CyA in whole blood at the end of the experiment. The actual dose given to each animal was related to free CyA concentration in blood, and the results are shown in Figure 1.

![Figure 1](https://academic.oup.com/humrep/article-abstract/22/2/372/2939490)

**Gross appearance**

Visual and tactile examinations of grafted and native uteri were performed on day 10 post-transplantation for all groups. In the untreated F1 hybrid syngeneic group, no differences were seen between the grafted and native uterus with respect to colour, texture and size of the stoma and graft, pulsation in the graft segment of the aorta and vena cava and bleeding from the myometrium of the grafted uterus.

In the vehicle group, the stomas were slightly shrunken and hardened compared with the syngeneic group, and the grafted uteri were of markedly darker colour, harder texture and larger size compared with the native uterus. One transplant in the vehicle group showed no pulsation in the aortic segment, and in the remaining four a reduced pulsation was seen. The blood flow through the vena cava segment of the grafted uterus was reduced in three of the vehicle animals, and when the myometrium was cut, only one animal showed normal bleeding, whereas the others showed reduced bleeding.

In the CyA10 and CyA20 groups, the uterine stomas of three out of five animals in each group were soft and of normal size and colour compared with the stomas of the syngeneic grafts. The stomas of the remaining two animals in each group were less hard, darkened and shrunken than the stomas of the grafts in the vehicle group. Also, all grafts in the CyA10 and all but one graft from the CyA20 group showed pulsation in the aortic segment and normal bleeding from the cut myometrium. This single graft that lacked pulsation in the grafted vessels had a thrombus in the aortic segment. All grafted uteri in the CyA10 group were moderately darkened in comparison with the native uteri, whereas three of these five grafts were of normal size and texture. In the CyA20 group, only two of five grafts were considered as being darker than the native uteri, and one of those was slightly hardened and swollen. This specimen was the previously mentioned thrombotic graft. The remaining four grafts...
were of normal size and texture, and the serosal vessels of
grafted uteri in the CyA20 group were better preserved in com-
parison with the CyA10 group. The results are summarized in
Table I, and representative examples of the gross appearance of
stomata and transplants from the different groups are shown in
Figure 2.

**Histology**

Histological analysis of grafted uteri from the vehicle group
showed that four of the five transplants had oedema, stasis and
extravasation of erythrocytes. One transplant was necrotic with
extensive bleeding. In the glandular epithelial cells of all
grafts, degenerative changes such as cytoplasmic vacuolization
were seen. There was a less number of lymphocytes and apop-
otic bodies. In the endometrial stroma and muscle layer, occa-
sional infiltrating immune cells were seen.

In the CyA10 group, there were less signs of oedema, stasis
and focal bleeding than in the vehicle group. In all grafts, a
moderate infiltration of inflammatory cells was seen in the
stroma and muscle layer, and only small areas of focal necrosis
were seen. In the glandular and endometrial epithelia, numer-
ous apoptotic bodies were present. The status of the glandu-
lar epithelia varied between specimens in this group, from rejected
epithelium showing degraded cells, apoptotic bodies and infil-
trating lymphocytes to well-preserved epithelial glands.

In the CyA20 group, no stasis, bleeding or oedema was seen
in four of the five specimens. The one specimen that was
thrombotic displayed features of erythrocyte extravasation, sta-
sis and necrosis. In the other four grafted uteri, only occasional
apoptotic bodies were seen but no necrotic areas. The number
of infiltrating immune cells was lower compared with the vehi-
cle and CyA10 groups, and the glandular epithelia were better
preserved. Representative slides from the different groups are
shown in Figure 3.

**CD4+ and CD8+ cells**

Phenotypic analysis of infiltrating lymphocytes in grafted uteri
showed a similar increase of CD4+ cells in the different allogene-
ic groups, with or without CyA treatment. However, the
increase compared with the syngeneic group was not statisti-
cally significant. The number of CD8+ cells was significantly
higher in the two allogeneic groups receiving CyA compared
with the allogeneic vehicle group. Both CD4+ and CD8+ cells
were seen in the stroma between glands, but cells infiltrating
glandular epithelia were generally CD4+. Average ratios of
CD4+ to CD8+ cells in non-rejected, syngeneic grafts were
0.72 (SD ±0.40), vehicle grafts 2.59 (SD ±0.77), CyA10 grafts
1.78 (SD ±1.26) and CyA20 grafts 1.10 (SD ±0.47). The results
from the quantification of CD4+ and CD8+ cells are
shown in Figure 4, and representative micrographs from the
different groups are shown in Figure 5.
process in a fully allogeneic model (El-Akouri et al., 2006), it was found that the rejection in the vehicle group in the present study had not progressed to the same stage as in transplants taken at the same day in the previous study. This might be a consequence of several factors, alone or in combination. The donor–recipient strain combination is known to influence the
immunological alloresponse in mice (Zhang et al., 1996; Xia and Kao, 2005), and the use of a semi-allogeneic donor in the present study is likely to have modified the response of the recipient’s immune system. It is also possible that carprofen (a non-steroid anti-inflammatory drug) that was used as analgesia in the present study might dampen the rejection process, because it has been shown that cyclooxygenase-2 inhibition can be favourable for graft survival (Ma et al., 2002).

Because the infiltration of CD4+ T cells was not altered by 10 and 20 mg/kg/day of CyA, it can be assumed that these doses are suboptimal for inhibition of CD4+ T-cell activation after uterus transplantation in this donor–recipient strain combination. The finding of a higher number of CD8+ T lymphocytes under CyA treatment compared with the allogeneic vehicle group is interesting. A potential explanation for this increase of CD8+ T cells could be a CyA-dependent depression of activation-induced cell death (AICD) among the activated, alloreactive CD8+ T cells. Such hypothesis is consistent with the findings by Cebecauer et al. (2005), who showed that soluble major

Figure 3. Representative histological sections (PAS staining) from native uterus (a) and semi-allogeneic grafted uteri from animals given 20 mg/kg per day cyclosporine A (CyA20) (b), 0 mg/kg per day (vehicle) (c) and 10 mg/kg/day (CyA10) (d) of CyA on day 10 post-transplantation. There are very few leukocytes in the native control tissue (a). In the CyA20 group, there is a moderate increase in the number of infiltrating leukocytes (b). In both the vehicle group and the CyA10 group, there are dense infiltrates of leukocytes (c and d). Apoptotic figures (arrows) are seen in both CyA10 and vehicle specimen.

Figure 4. Average ratios (graft-native/native) of CD4+ and CD8+ T-lymphocyte density in grafted uteri in syngeneic, vehicle and 10 mg/kg of cyclosporine A (CyA10) per day and 20 mg/kg per day (CyA20) groups. Error bars represent SEM and significant differences (P < 0.05) by Kruskal-Wallis analysis of variance followed by Mann-Whitney U-test which are indicated by asterisks.
histocompatibility complex–peptide complexes, normally inducing AICD in T cells in vitro, failed to do so in the presence of CyA. Whether the surviving CD8+ T lymphocytes in the CyA-treated grafts have retained their cytotoxic activity and thus capability to destruct allogeneic cells or if they produce cytokines that promote rejection by modes other than CD8+ cytotoxicity remains to be evaluated.

In the present study, CyA was able to partly suppress rejection of the transplanted uterus in the mouse. However, at the doses used, CyA did not reduce the morphological and histological signs of graft rejection to the extent that long-term graft survival could be expected. It is likely that CyA should be administered at higher doses as monotherapy for prevention of rejection, and we are currently studying this possibility. It might also be possible that CyA should be used in combination with other immunosuppressive agents or that induction of allostolerance (Wallgren et al., 2006) is needed to establish a protocol for long-term survival of the allotransplanted uterus in the mouse.

Acknowledgement
This research was supported by grants from the Swedish Research council (11607 to M.B.) and Hjalmar Svensson’s Research Foundation.

References
Cyclosporine and uterine transplantation


Submitted on June 15, 2006; resubmitted on August 31, 2006; accepted on September 11, 2006.