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# Treatment with total lymphoid irradiation, cyclosporin A and a monoclonal anti-T-cell antibody in a hamster-to-rat heart transplantation model: Graft survival and morphological analysis

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**Abstract.** Treatment with preoperative total lymphoid irradiation and post-transplant cyclosporin A has been shown to have a synergistic effect on graft survival in allo- and xenotransplantation. Specific monoclonal antibodies against T cells and T cell subpopulations could offer new ways of preventing graft rejection in xenotransplantation. Graft survival and histology were examined after total lymphoid irradiation plus cyclosporin A treatment versus cyclosporin A plus a monoclonal antibody in a concordant, heterotopic, hamster-to-rat heart transplantation model. Preoperative total lymphoid irradiation was given at a dose of 1.25 Gy, 12 times over a period of 3 weeks. Cyclosporin A at a dose of 12.5 mg/kg per day was administered perorally and OX-19, a pan T cell monoclonal antibody, was given as intraperitoneal injections at doses of 100 μg or 500 μg/kg per day from day 0 until graft rejection. While total lymphoid irradiation alone prolonged graft survival to 9.4 days, total lymphoid irradiation plus cyclosporin A extended graft survival to a mean of 22 days. Cyclosporin alone or combined with the monoclonal antibody could not increase graft survival significantly when compared to untreated animals, which rejected their grafts within 3.7 days. Vascular rejection was the characteristic morphological finding, even after some weeks of excellent graft function. In conclusion, total lymphoid irradiation and cyclosporin A had a synergistic effect on graft survival in this concordant xenotransplantation model, although recent impressive results from other groups could not be reproduced. Total lymphoid irradiation combined with cyclosporin A appears to delay a primary humoral graft rejection, while the mechanism of rejection, judged by histology, stays the same.

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In allogeneic and concordant xenogeneic heart transplantations, a synergistic immunosuppressive effect of total lymphoid irradiation (TLI) and cyclosporin A (CyA) has been reported [3, 10, 18, 19]. The mechanisms of rejection in concordant xenotransplantation are complex, involving preformed natural antibodies, incompatibility of enzyme systems, and most likely the sensitization and induction of specific antibodies [7].

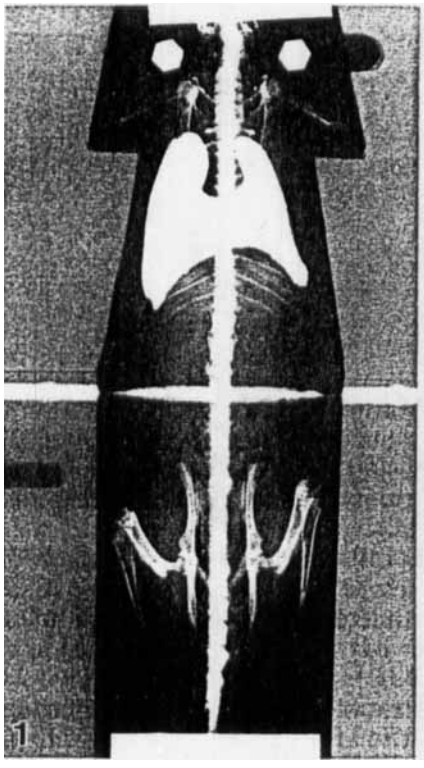
During recent years, monoclonal antibodies (MAB), acting as specific T cell or T-subpopulation markers and inhibitors, have been used clinically in the treatment of graft rejection [15] and in different experimental allotransplantation models with the aim of improving conventional immunosuppressive treatment strategies [9, 11, 23]. These specific MABs could possibly influence the sensitization and induction phases in concordant xenotransplantation.

We report here on the results of studies of graft survival and histology in a hamster-to-rat model for evaluation of CyA and TLI versus CyA and MAB treatment.

## Materials and methods

### *Animals*

Outbred Sprague-Dawley rats (SPF, Møllegard Breeding Center, Copenhagen, Denmark) served as recipients and inbred Syrian hamsters (albino variant, Department of Odontological Research, Göteborg University, Göteborg, Sweden) as donors. All animals were male, 3–5 months of age, and were maintained under standard laboratory conditions, receiving humane care.



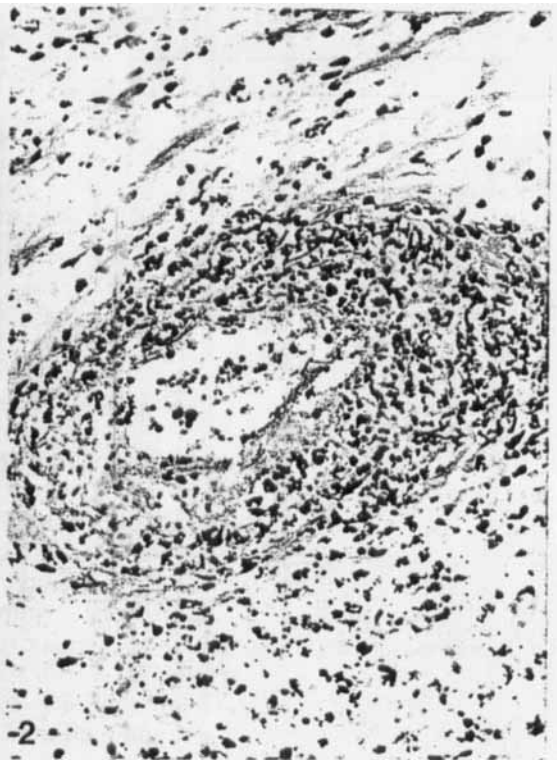


**Fig. 1.** Exposure of rats under total lymphoid irradiation, where head, most of the lungs and heart, half of the liver, and the tail are shielded by lead alloys

**Fig. 2.** Central artery with pronounced granulocyte infiltration and bleeding in the vessel wall and marked perivascular edema

### *Surgical procedure*

Fifty-three concordant xeno heart transplantations were performed using a modified microvascular technique described by Ono and Lindsey [14]. Briefly, the donor hamsters were anesthetized with fentanyl/fluanisone (1 mg/0.02 mg per 100 g body weight) and diazepam (0.5 mg per 100 g body weight).

Heparin (500 IU) was injected into the inferior vena cava, after which the animals were bled and the anterior chest wall was opened. The inferior and superior venae cavae were clamped and the donor heart was flushed with 5 ml aqueous heparin solution (50 IU/ml) via the inferior vena cava. After ligation of the venae cavae and the pulmonary veins, the aorta and the pulmonary artery were divided.

Recipient rats were anesthetized with pentobarbital (3.5 mg/100 g body weight). A segment of the infrarenal aorta and inferior vena cava was freed and clamped. End-to-side anastomosis was performed with 9-0 running sutures. In contrast to the original description by Ono and Lindsey, the heart was turned 180 degrees with the heart apex pointing to the right, resulting in our experience in an improved venous outward flow.

### *Irradiation technique*

Preoperatively, TLI was performed using a 4 MV linear accelerator (Philips SL 75-5) with pentobarbital-sedated rats in a custom-built perspex box. The head, most of the lungs and heart, half of the liver and the tail were shielded by lead alloys (Fig. 1). Treatment was given four times a week for 3 weeks; 1.25 Gy per session to a total central dose of 15 Gy. The dose rate was 0.41 Gy/min at a source-animal distance of 211 cm. The dose was calculated using a perspex phantom and continually checked by Baldvin-Farmer air ionization chambers during the irradiation sessions.

### *Monoclonal antibodies*

Hybridoma cells secreting MRC OX-19, a MAB directed against all rat T cells [2], was a gift from Dr. A. F. Williams, MRC Cellular Immunology Unit, Oxford, UK.

Recloning was performed when necessary. BALB/c x CF 1 mice served for the production of ascites. After protein A purification, the antibody was dialyzed against PBS and sterilized by filtration.

The immunoglobulin concentration was determined by ELISA, and the antibodies were stored at 4°C until use.

### *Immunosuppressive treatment*

CyA (Sandoz), dissolved in oleum tenue vegetabile to a concentration of 7.5 mg/ml, was given perorally from day-8 to graft rejection.

CyA levels were analyzed by radioimmunoassay (RIA; Sandoz method) on day 0 and at 14-day intervals after transplantation or at the time of graft rejection.

MRC OX-19 was administered intraperitoneally from day 0 to graft rejection.

Total white blood cells and differential counts were determined before TLI treatment, on day 0, and with 14-day intervals after transplantation or at the time of rejection.

### *Experimental groups*

The following eight groups were studied:

Group 1: Control group, no immunosuppressive treatment ($n = 11$)
Group 2: 12.5 mg CyA/kg per day ($n = 5$)
Group 3: 25 mg CyA/kg per day ($n = 4$)
Group 4: 50 mg CyA/kg per day ($n = 7$)
Group 5: 5 Gy TLI ($n = 7$)
Group 6: 5 Gy TLI plus 12.5 mg CyA/kg per day ($n = 9$)

**Table 1.** Hamster-to-rat heart xenograft survival

| | Treatment | Graft (survival in days) | *P* |
|---|---|---|---|
| Group 1 | None | 3, 4, 3, 4, 4, 4, 3, 5, 3, 4, 4 | |
| Group 2 | CyA: 12.5 mg/kg per day | 4, 5, 4, 5, 5 | NS |
| Group 3 | CyA: 25 mg/kg per day | 5, 4, 7, 5 | NS |
| Group 4 | CyA: 50 mg/kg per day | 4, 4, 4, 5, 3, 7, 6 | NS |
| Group 5 | TLI | 5, 10, 15, 5, 14, 5, 12 | 0.002 |
| Group 6 | TLI + CyA 12.5 mg/kg per day | 14, 15, 15, 11, 14, 13, 57, 42, 22 | 0.002 |
| Group 7 | CyA: 12.5 mg/kg per day + OX-19 100 μg/kg per day | 7, 7, 7, 4, 3 | NS |
| Group 8 | CyA: 12.5 mg/kg per day + OX-19 500 μg/kg per day | 5, 4, 3, 3, 4 | NS |

Group 7: 12.5 mg CyA/kg per day plus 100 μg OX-19/kg per day ($n = 5$)
Group 8: 12.5 mg CyA/kg per day plus 500 μg OX-19/kg per day ($n = 5$)

### *Graft function*

Graft function was controlled daily by direct transabdominal palpation, supplemented with Doppler sound technique and laparotomy, in case of doubt.

### *Histology*

Rejected grafts were examined histologically by routine staining with eosin/hematoxylin of formalin-fixed tissue.

Immunohistology was performed using an alkaline-phosphatase-antialkaline-phosphatase technique (APAAP) on snap-frozen and tissuetek-embedded tissue. MRC OX-19 served as the primary and rabbit-anti-mouse immunoglobulins as the secondary antibodies. Rat serum (5% v/v) was added to the secondary antibodies to prevent nonspecific binding.

Immunohistology also included immunofluorescence microscopy, where fluorescine-labelled goat-anti-rat antibodies were used in a one-step procedure to demonstrate IgM, complement factor C3, or fibrinogen deposits in the rejected grafts.

### *Statistics*

The Mann-Whitney U-test (two-tailed) and Krushal-Wallis (one-way) analysis of variance were used for statistical evaluation of graft survival data. *P* values at or below 0.02 were considered significant.

## Results

Preoperative TLI treatment was tolerated without any problems. Apart from an average weight loss of 12.5%, no side effects or infections were observed during TLI treatment.

In the postoperative course, none of the animals died due to the different immunosuppressive regimens. No increased fragility of vessel walls was noticed when performing the vascular anastomoses, wound healing was completely normal, and the preoperative weight was regained within a few days.

Graft survival in shown in Table 1. Treatment with CyA at 12.5, 25, or 50 mg/kg per day was not able to extend graft survival compared to untreated animals, where the spontaneous graft survival was 3.7 days.

TLI alone resulted in a statistically significant prolongation of graft survival to 9.4 days, but the results were heterogeneous, with some animals not responding to TLI in terms of graft survival. The effect of TLI on total white blood count and differential counts was, in contrast, constant and reproducible. Under TLI treatment, average leukocyte counts of $10.4 \times 10^6$/ml (range 8.8–11.5 × $10^6$/ml) with a lymphocyte fraction of 85% (range 72%–91%) were reduced to leukocyte counts of $0.42 \times 10^6$/ml (range 0.12–0.78 × $10^6$/ml) with a lymphocyte fraction of 47% (range 37%–62%).

Combined treatment with CyA plus TLI resulted in a prolongation of graft survival to an average of 22 days. This was significant, compared both to the control group and to the group receiving only TLI treatment. All animals in this group responded to the given treatment, two recipients with excellent graft function for 42 and 52 days. The concentration of CyA in whole blood, determined at 14-day intervals after transplantation and at the time of rejection, showed an average CyA trough level of 854 ng/ml (range 462–> 1600 ng/ml) when using specific antibody.

Treatment with CyA and OX-19 for 5 days reduced the number of peripheral leukocytes from an average of $9.0 \times 10^6$/ml (range 7.6–11.5 × $10^6$/ml) to $2.5 \times 10^6$/ml (range 1.4–3.8 × $10^6$/ml), while differential analysis showed a slight decrease in the lymphocyte fraction from 79% (range 76%–83%) to 64% (range 46%–73%). Immunohistological analysis of the thymus of OX-19-treated animals showed a threefold reduction in OX-19-positive cells compared to untreated controls. But this treatment modality had no beneficial effect on graft function. Increasing the OX-19 dose from 100 to 500 μg/kg per day did not make any difference.

In both groups – TLI alone and TLI plus CyA – the rejection process was clinically quite characteristic and defined. Within 1–2 days, the previously well-functioning grafts became enlarged, pronounced intestinal adherence developed, and heart function stopped.

The histological examination of rejected heart grafts showed no essential differences between the different experimental groups. Sections were characterized by total acute infarction, muscular necrosis, pronounced subendocardial and epicardial inflammation, and vascular rejection with endothelial cell proliferation and granulocyte infiltration in the vessel walls (Fig. 2). Myocardial infiltration by mononuclear cells was modest and almost absent in the groups treated with pan T-cell MAB.

The overall dominant impression of vasculopathy and vascular rejection was also found in the best functioning

grafts, where the morphological changes after several weeks of excellent graft function were quite similar to the changes found in grafts rejected after a few days.

No immunoglobulin, C3, or fibrinogen deposits could be demonstrated by immunofluorescence microscopy in histological sections of rejected transplants in treated or untreated animals.

## Discussion

During the last decade TLI, as an immunosuppressive treatment in relation to organ transplantation, has been shown to be a substantial supplement to conventional immunosuppression. The clinical experience obtained through the treatment of Hodgkin's disease [4] has led to the use of TLI in quite different experimental allo- and xenogeneic transplantation models [16, 21]. The improved application of dose and fractionation of TLI [6, 18, 19] has resulted in clinical use at several centers [5, 8, 13, 20, 22].

In 1986, Knechtle et al. [10] reported a pronounced synergistic effect of pretransplant TLI and CyA in a hamster-to-rat heart transplantation model. Our results confirm the synergistic effect of TLI plus CyA, but we were unable to reproduce the remarkable results in terms of graft survival time, presumably because of a different hamster-to-rat combination.

Another possible explanation could be that the pentobarbital sedation during TLI induced the P 450 microsomal system in the liver [1, 24], leading to an increased CyA catabolism and insufficient CyA levels after transplantation. Yet, recent experiments (not reported here) have resulted in the same heart graft survival times in this model when using fentanyl/fluanisone sedation during TLI, and the actual CyA levels do not indicate an impaired gastrointestinal absorption or increased CyA catabolism. Contrary to Knechtle et al. [10], we found that TLI had some, albeit a variable, effect on graft survival.

We have, in an allograft heart transplantation model, found prolongation of graft survival by treatment with MAB OX-19, which is an anti-CD5 antibody (manuscript in preparation), and we therefore wanted to examine the effect of this antibody in the xeno model. However, adding treatment with OX-19 (at a dose of 100–500 μg/kg per day) to the CyA treatment had no significant effect on graft survival. This could lead to the assumption that predominantly humoral and not T-cell-dependent effector mechanisms are responsible for graft rejection in this model. Preliminary results on serological crossmatching with affinity-purified IgG from xenograft recipients and donor lymphocytes show a strongly positive crossmatch at the time of rejection, while the crossmatch on day 0 was negative. A recent investigation by Monden et al. [12], in which a similar hamster-to-rat heart transplantation model was used, demonstrated a clear correlation between raising lymphocytotoxic antibody levels and the time of rejection, which is in accordance with our findings.

Histological examination showed that the rejected grafts, independent of the kind of immunosuppression, were characterized by vasculopathy and vascular rejection. This would support the impression of humoral rejection mechanisms even after some weeks of excellent graft function. Similar observations were made by Rosengard et al. [17] in a sequential morphological analysis of xeno heart grafts. Immunohistological analysis, in contrast, did not reveal signs of antibody-mediated rejection in our model. We conclude that TLI plus CyA appears to delay the onset of acute, primarily humoral xenograft rejection in this concordant heart transplantation model; however, the morphological pattern of rejection, as assessed by histological analysis, remains unchanged, compared to the spontaneous rejection in untreated animals. Our results confirm that TLI in combination with CyA has a synergistic effect on graft survival in a hamster-to-rat heart transplantation model. We cannot, however, share the optimism on TLI plus CyA as the treatment of choice in concordant xenotransplantation since earlier impressive results could not be reproduced. Further investigations on this subject are in progress.

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