Immune Tolerance Induced by Donor Antigen and Cyclophosphamide in Rat Fetal Small Bowel Transplantation

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https://doi.org/10.15017/19259

出版情報：福岡医学雑誌. 96 (2), pp.49–57, 2005-02-25. 福岡医学会
バージョン：published
権利関係：
Immune Tolerance Induced by Donor Antigen and Cyclophosphamide in Rat Fetal Small Bowel Transplantation

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Abstract Background / Purpose: Donor specific immune tolerance is thought to be the ideal state for the recipient after organ transplantation. The administration of donor antigens and cyclophosphamide has been reported to induce donor specific immune tolerance in heart or liver transplantation. However, the effectiveness of this method for small bowel transplantation has not yet been studied. We assessed the cyclophosphamide induced immune tolerance on rat fetal small bowel transplantation.

Methods: Lewis rats (RT1', n=99) were used as recipients while either F344 (RT1', n=44) or WKAM (RT1u, n=47) rats were used as donors. The combination of F344 and Lewis rats produces an immunologically low responder, while that of WKAM and Lewis rat produces a high responder. Bone marrow and spleen cells were harvested from the donor rats and 3x10^8 / kg of each were administrated to the recipient rats intravenously on day 0. Next, cyclophosphamide was given either divisionally or bolously. The fetal small bowel of the same strain as the donor was transplanted into the rectus muscle of the recipient abdominal wall on day 10. On day 17, all grafts were taken out and graft survival was thereafter evaluated. The body weight of recipient was also assessed.

Results: Most of the grafts (87.5 %) survived in the F344-Lewis rat (low responder) combination using the divisional administration of 120 mg / kg of cyclophosphamide. Histologically, most of them showed the whole layers of the intestinal architecture to be well preserved. The weight loss of the recipient was minimal after divisional administration. In contrast, no graft survived in the WKAM-Lewis rat (high responder) combination. Conclusions: Immune tolerance is considered to be induced by the administration of donor specific antigen and cyclophosphamide in an immunologically low responder combination. Therefore, this method is expected to be useful as an adjuvant therapy and may also be able to reduce the dose of immunosuppressive agents in living-related clinical small intestinal transplantation.

INTRODUCTION

The small bowel has a strong degree of immunogenicity, such as a large amount of lymphocytes in lamina propria mucosae, Pyel patches, and mesenteric lymph nodes.

As a result, immune responses such as rejection and GVHD, still remain huge obstacles in small bowel transplantation. It is essential to maintain a high blood level of immunosuppressant to avoid rejection and, as a result, the risk of infection and nephrotoxicity is very high. Nonspecific immunosuppression using immunosuppressant agents always contains potential risks such as severe infection, post transplantation lymphoproliferative disorder, and second
malignancy. Such adverse effects tend to be more serious in small bowel transplantation, than after other types of solid organ transplantation, because small bowel transplantation requires strong immunosuppression.

Donor specific immune tolerance is thought to be the ideal state for recipients after transplantation. Various protocols for the induction of immune tolerance have been investigated in experimental organ transplantation. Although simultaneous bone marrow transplantation has been attempted in clinical small bowel transplantation, so far no improvement in the outcome has been observed.

The administration of bone marrow and spleen cells of the donor and cyclophosphamide has been reported to induce donor specific immune tolerance in mouse heart and skin transplantation models. Although this method is relatively simple and easy to perform, there have so far been no reports concerning small bowel transplantation using this method. In our previous study the regional differences of immunogenicity in rat small bowel transplantation and ileum demonstrated a slightly stronger immunogenicity than the jejunum regarding the histological findings and cytokine response.

Cyclophosphamide is known to be an alkylating agent that has an anticancer effect. It has been reported to be useful for the treatment of pediatric malignancy, especially neuroblastoma and thus this drug remains popular among pediatric surgeons. Cyclophosphamide also has an immunosuppressive effect and it is sometimes used for autoimmune diseases and organ transplantation such as clinical xeno-liver-transplantation and small bowel transplantation. We thus investigated the induction of donor specific immune tolerance using this method in rat fetal small bowel transplantation.

MATERIALS AND METHODS

Inbred Lewis (RT1\(^{a}\): weighing 190g to 210g) and WKAM (RT1\(^{b}\): weighing 300g to 450g) rats were obtained from Seac Yo-shitomi Inc (Fukuoka, Japan). F344 (RT1\(^{c}\): weighing 300g to 450g) rats were obtained from Charles River Japan, Inc. (Yokohama, Japan). Lewis rats were used as the recipient. F344 and WKAM rats were used as the donor of bone marrow and spleen cells. Pregnant The F344 and WKAM rats at 20 days of gestational age were also used in order to harvest the fetal intestine. (The gestation period of the rats was 21 days.) They were kept in cages and fed standard rodent chow and tap water ad libitum. Lewis and F344 rats hold the same major histocompatibility antigen (MHC) and the different minor histocompatibility antigen. Therefore, only a weak immune response occurs in organ transplantation using this combination. (Low responder combination) On the other hand, Lewis and WKAM rats possess a different MHC. A strong immune response occurs in the organ transplantation of this combination (High responder combination).

The donor rats were killed by an overdose of anesthesia with ether. The spleens, humerus, and femur bones were harvested from either the F344 rats (Low responder combination with Lewis rats) or WKAM rats (High responder combination with Lewis rats). The spleens were disrupted in the cold RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) by pressing fragments between two glass slides. The bone marrow in the humerus and femur bones was flushed out using a 5-ml syringe with a 21-gauge needle. Cell suspensions
were filtered through cotton gauze and washed twice with RPMI 1640 medium. Viable cells were then counted using a standard trypan blue dye exclusion system. Both spleen and bone marrow cells at 3x10^8 / kg were thereafter injected intravenously to Lewis rats and this day was called day 0.

The rats were divided into 6 groups, according to the dose and the method of cyclophosphamide administration. In group 1, cyclophosphamide was administrated intraperitoneally at 30 mg / kg a day on days 1, 2, and 3. In group 2, cyclophosphamide was administrated intraperitoneally at 40 mg / kg a day on days 1, 2, and 3. In group 3, cyclophosphamide was administrated intraperitoneally at 50 mg / kg a day on days 1, 2, and 3. In group 4, cyclophosphamide was administrated intraperitoneally at 150 mg / kg on day 2. In group 5, cyclophosphamide was administrated intraperitoneally at 200 mg / kg on day 2. In group 6, no treatment was performed and this group served as a control. These protocols are summarized in Table 1.

On day 10, the fetuses were removed by cesarean section from the pregnant rat within 48 hours before delivery. The small bowels were harvested from the fetuses of the same strain rats as bone marrow and spleen cells donor and 2 cm of the small bowel was transplanted into the rectus muscle of the recipient abdomen without vascular anastomosis. On day 17, the rats were sacrificed and the grafts were observed. An enlarged intestine with mucus inside was a macroscopic finding of graft survival. Graft survival was also histologically confirmed, based on the rejection grade score by Ogita et al. (Table 2). Grades 0, 1, and 2 were considered to indicate "survival", while grades 3 and 4 were considered demonstrate "non-survival". We also weighed all the rats in order to evaluate the toxicity of cyclophosphamide. Weight changes between the day 0 and the day at small intestinal transplantation (day 10) were compared in each group. The flowchart of these procedures is summarized in Figure 1.

This study was approved by the Ethical Committee of the Animal Laboratory Center, Kyushu University School of Medicine. A statistical analysis was performed using Student's t-test and the chi-square test. A p-value less than 0.05 was considered to be statistically significant.

## RESULTS

In the low responder combination (F344 to Lewis), all the rats survived until day 17 in Groups 1 and 2. One rat of Group 3 died due to cyclophosphamide toxicity. In Groups 4, 5, and 6, the recipient survival rates were 62.5 %, 66.7 %, and 100 %, respectively. Graft survival was also esti-

### Table 1  The administration protocol of cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg/day)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>X 3 days (on days 1, 2, 3)</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>X 3 days (on days 1, 2, 3)</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>X 3 days (on days 1, 2, 3)</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>X 1 day (on day 2)</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>X 1 day (on day 2)</td>
</tr>
<tr>
<td>6</td>
<td>No treatment</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2  Rejection grade score based on the histologic findings by Ogita et al.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The complete intestinal structure remains</td>
</tr>
<tr>
<td>1</td>
<td>Over 50% of the villi remain</td>
</tr>
<tr>
<td>2</td>
<td>Less than 50% of the villi remain</td>
</tr>
<tr>
<td>3</td>
<td>The villi are lost but the other intestinal layers remain</td>
</tr>
<tr>
<td>4</td>
<td>Loss of intestinal tissue</td>
</tr>
</tbody>
</table>
mated on day 17. In Group 1, only 37.5% of the grafts survived and the dose of cyclophosphamide was not thought to be sufficient for tolerance induction. In Groups 2 and 3, 87.5% and 85.7% of the grafts survived and the mortality rate after cyclophosphamide administration was negligible. The histological findings showed a normal structure of the small intestine of Group 2 with a mild infiltration of lymphoid cells in the mucosa and the smooth muscle layer (Fig. 2a). Ganglion cells were also found in the myenteric plexus. This finding is Grade 1 based on the rejection grade score. In Groups 4 and 5, the graft survival rates were 20.0% and 75.0%, respectively. The divisional administration was significantly more advantageous than the bolus one regarding the graft survival rate. (Group 3 vs. Group 4: p<0.05) However, no significant advantage in the recipient survival was observed.

In the high responder combination (WKAM to Lewis), in Groups 1, 2, 3, 4, 5, and 6, the recipient survival rates were 85.7%, 75.0%, 75.0%, 50.0%, 25.0%, and 100%, respectively. No statistically significant differences were observed in the recipient survival rate between the low responder and the high responder combination. However, all of the grafts in Groups 1, 2, 3, 4, 5, and 6 disappeared on day 17. In the histological findings, significant cellular infiltration was observed in the recipient abdominal muscle and the structure of the small intestine completely disappeared and the rejection grade score was Grade 4 (Fig. 2b).

The results of this experiment are summarized in Tables 3 and 4. The higher

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Fig. 1 A schematic drawing of the experimental flowchart

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Fig. 2a Transplanted graft of Group 2 in the low responder combination (F344 to Lewis) showed an almost normal architecture of the small intestine with intact enteric plexuses. Mild lymphocyte infiltrations were observed in the lamina propria mucosae and the proper muscle in some grafts. The rejection grade score was Grade 1.

Fig. 2b Transplanted graft of Group 2 in the high responder combination (WKAM to Lewis) disappeared and a prominent degree of cellular infiltration was observed in the rectus muscle of the recipient. The rejection grade score was Grade 4 (H.E. staining, high magnification, LPM: lamina propria mucosae, PM: proper muscle).
Table 3  The results of graft survival in the low responder combination (F344 → Lewis)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cyclophosphamide</th>
<th>No.</th>
<th>Recipient survival on day 17</th>
<th>Recipient survival Rate</th>
<th>Graft survival</th>
<th>Graft survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30mg/kg × 3</td>
<td>n= 8</td>
<td>8</td>
<td>100%</td>
<td>3</td>
<td>37.5%</td>
</tr>
<tr>
<td>2</td>
<td>40mg/kg × 3</td>
<td>n= 8</td>
<td>8</td>
<td>100%</td>
<td>7</td>
<td>87.5%</td>
</tr>
<tr>
<td>3</td>
<td>50mg/kg × 3</td>
<td>n= 8</td>
<td>7</td>
<td>87.5%</td>
<td>6</td>
<td>85.7%</td>
</tr>
<tr>
<td>4</td>
<td>150mg/kg × 3</td>
<td>n= 8</td>
<td>5</td>
<td>62.5%</td>
<td>1</td>
<td>20.0%</td>
</tr>
<tr>
<td>5</td>
<td>200mg/kg × 1</td>
<td>n= 6</td>
<td>4</td>
<td>66.7%</td>
<td>3</td>
<td>75.0%</td>
</tr>
<tr>
<td>6</td>
<td>No treatment</td>
<td>n= 6</td>
<td>6</td>
<td>100%</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

dosage of cyclophosphamide leads to a higher mortality rate due to the toxicity of cyclophosphamide in both combinations. Weight changes, including both combinations, during the experiment are summarized in Table 5. In Groups 4 and 5, weight loss was significantly larger than that in Groups 1, 2, and 3. The divisional administration was thought to be more advantageous than a bolus one regarding the recipient mortality rate as well as weight loss.

**DISCUSSION**

Medawar PD reported the first experimental immune tolerances. He administered allogeneic bone marrow to the newborn mice and thus induced long-term immune tolerance using skin grafts. Thereafter, various strategies for the induction of immune tolerance have been reported. Bone marrow or splenocyte transfusion is one of the most common methods and but controversy remains regarding the amount, timing, the route of administration, such as the intravenous, intrathymic, or portal injection of tolerogen, and additional procedures, such as radiation or chemotherapy. Blocking costimulatory signals concerning CD28 and CD40 was also reported to be effective for tolerance induction. Immunomodulation by graft irradiation was also investigated for tolerance induction. Recently, the control of the dendritic cells has attracted a great deal of attention regarding tolerance induction. Both the stem cell and the gene transfer techniques have been investigated regarding tolerance induction. However, most of these methods have still not been applied for human organ transplantation.

The first report on cyclophosphamide-induced immune tolerance was made by Mayumi in 1985. He confirmed the induction of immune tolerance in mouse skin.

Table 4  The results of graft survival in the high responder combination (WKAM → Lewis)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cyclophosphamide</th>
<th>No.</th>
<th>Recipient survival on day 17</th>
<th>Recipient survival Rate</th>
<th>Graft survival</th>
<th>Graft survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30mg/kg × 3</td>
<td>n= 7</td>
<td>6</td>
<td>85.7%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>40mg/kg × 3</td>
<td>n= 8</td>
<td>6</td>
<td>75.0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>50mg/kg × 3</td>
<td>n= 8</td>
<td>6</td>
<td>75.0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>4</td>
<td>150mg/kg × 1</td>
<td>n= 8</td>
<td>5</td>
<td>50.0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>5</td>
<td>200mg/kg × 1</td>
<td>n= 6</td>
<td>2</td>
<td>25.0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>6</td>
<td>No treatment</td>
<td>n= 6</td>
<td>6</td>
<td>100%</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>
transplantation. Since then this protocol has been improved and applied to other organ transplantation such as the mouse heart and rat liver\textsuperscript{16}\textsuperscript{17}\textsuperscript{18}). The mechanisms of immune regulation in this system are thought to be as follows: (1) the destruction of proliferating allo-reactive T cells by cyclophosphamide; (2) the intrathymic clonal deletion of immature allo-reactive T cells associated with intrathymic mixed chimerism; (3) the anergy induction of T cells which avoided clonal destruction; (4) the appearance of donor specific regulatory T cells in the late phase\textsuperscript{19}\textsuperscript{20}\textsuperscript{21}\textsuperscript{22}\textsuperscript{23}). Peripheral chimerism is thought to play an important role in the clonal deletion of donor specific regulatory T cells in the late phase. Accurately determining of the chimerism in the recipient is essential in the late phase. However, we sacrificed all recipients one week after small bowel transplantation, a time period which we thought to be before late phase. Therefore, we did not confirm the level of recipient chimerism in this experiment.

We investigated the effect of cyclophosphamide induced immune tolerance on rat fetal small bowel transplantation. Fetal bowel transplantation was chosen as a mode of transplantation because this method is less invasive. In our preliminary study, the rats administered high doses of cyclophosphamide could not endure the standard vascularized bowel transplantation with vascular anastomosis, probably due to surgical stress. Therefore, in the future, an adequate dose of cyclophosphamide should be found out using a vascular anastomosis model.

Our results revealed that immune tolerance was only induced in the low responder rat combination. In the high responder rat combination, no grafts survived in any of the protocol groups. In mouse skin transplantation, this protocol was only effective in the minor histocompatibility mismatched combination\textsuperscript{1}). This result was very similar to ours. Both the small bowel and skin have been reported to have a high degree of immunogenicity. Therefore, split tolerance may be induced in the high responder combination. Additional immunomodulation, such as low-dose immunosuppressants may help to effectively maintain the graft in the high responder rat combination.

In the low responder rat combination, the optimal medication method of cyclophosphamide was a trisected administration with a total dosage of 120 mg/kg. Smaller dosages of cyclophosphamide than this

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight change at small bowel transplantation</th>
<th>Weight change</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30mg/kg x3</td>
<td>+1.08%</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>40mg/kg x3</td>
<td>-0.45%</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>50mg/kg x3</td>
<td>-1.33%</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>150mg/kgx1</td>
<td>-9.16%</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>200mg/kgx1</td>
<td>-14.78%</td>
<td>10</td>
</tr>
</tbody>
</table>

\* p<0.01
\** p<0.03
Immune tolerance induced by cyclophosphamide

decreased the graft survival rate while larger dosages increased the recipient mortality rate and weight loss due to the toxicity of cyclophosphamide. The divisional administration of cyclophosphamide was more effective for tolerance induction than a bolus administration. The toxicity of cyclophosphamide was also enhanced by the bolus administration on both mortality rate and weight loss (Group 3 vs. Group 4). In Group 5, the graft survival rate was better than that in Group 4. Therefore, the bolus administration of 150 mg/kg of cyclophosphamide was not considered to provide sufficient tolerance induction. The divisional administration of 120 mg/kg of cyclophosphamide was more effective than the bolus administration of 150 mg/kg of cyclophosphamide (87.5% vs. 75.0%). The recipient survival rate was also worse in the bolus administration group than in the divisional administration group.

Our results were different from those for mice. In particular, the rats were found to be more sensitive to cyclophosphamide than the mice and tolerance could be induced with smaller dosages of cyclophosphamide than in mice. Mayumi reported that mouse LD50 of cyclophosphamide was 500 mg/kg. In our experiment, rat LD50 of cyclophosphamide was thought to be 200 mg/kg.

Reyes J reported the clinical application of cyclophosphamide for tolerance induction in human small bowel transplantation. He decided to discontinue this trial due to the insufficient clinical advantage and severe side effects such as bone marrow toxicity and infection. Our experiment is different from his trial regarding the volume and mode of administration (simultaneous vs. asynchronous with transplantation).

Further investigations using larger sized animals are required to clarify the effectiveness of cyclophosphamide induced immune tolerance to human organ transplantation. In conclusion, we were able to induce donor specific immune tolerance for fetal intestinal transplantation in a low responder rat combination using donor bone marrow and spleen cells and cyclophosphamide. While in a high responder rat combination, our protocol showed no effectiveness in tolerance induction. This method is therefore expected to be useful as an adjuvant therapy. In addition, this method may also help to reduce the dosage of immunosuppressive agents in living-related small intestinal transplantation.

ACKNOWLEDGEMENTS

This work was supported in part a Grant-in-Aid for Scientific Research B2 from the Japanese Society for the Promotion of Science.

The authors also thank Mr. Brian Quinn for reading the manuscript.

References


(Received for publication January 13, 2005)
背景/目的：ドナー特異的免疫寛容は臓器移植において理想的な状態と考えられる。ドナー抗原とサイクロホスファミド投与による免疫寛容誘導はいまだに心移植や肝移植では報告がみられるが、小腸移植における有用性は全く報告がない。我々はラット胎児を用いた小腸移植モデルにおいてサイクロホスファミド投与による免疫寛容誘導を検討した。

方法：ルイスラット（RT 1a, n=99）をレシピエント、F344（RT 1a, n=44）またはWKAM（RT 1a, n=47）をドナーとした。F344からルイスラットへの移植は弱免疫反応群として、WKAMからルイスラットへの移植は強免疫反応群として用いた。ドナーから骨髄および脾細胞を採取しそれぞれ3x108/ kgずつレシピエントへ第0日に静脈投与した。次ぎにサイクロホスファミドを分割または一括投与した。第10日にドナーと同じストレインのラットの胎児の小腸を採取し、レシピエントの腹直筋内へ移植した。第17日にグラフトを摘出し生着を判定した。レシピエントの体重変化も評価した。

結果：弱免疫反応群のサイクロホスファミド120 mg/ kgの分割投与群では大部分のグラフトが生着した。この群では組織学的に腸管の全層構造が維持されていた。体重減少も分割投与群が一括投与群よりも少なかった。一方、強免疫反応群ではグラフト生着は全くみられなかった。

結論：ドナー抗原とサイクロホスファミド投与による免疫寛容誘導はラット小腸移植において、強免疫反応群では得られないが、弱免疫反応群では得られると考えられる。したがってこの方法は臨床の生体間小腸移植では免疫抑制剤の量を減らせる補助療法としての有用性が期待できる。