Prolongation of Canine Renal Allograft Survival in Combined Therapy with Rabbit-anti-dog Antilymphocytic Serum, Azathioprine and the Allotransplantation of the Donor's Bone Marrow*

MINORU YAMAMOTO
The First Department of Surgery,
Hirosaki University School of Medicine, Hirosaki

YAMAMOTO, M. Prolongation of Canine Renal Allograft Survival in Combined Therapy with Rabbit-anti-dog Antilymphocytic Serum, Azathioprine and the Allotransplantation of the Donor's Bone Marrow. Tohoku J. exp. Med., 1970, 101 (4), 333-338 — Seventy-four dogs received renal allografts after simultaneous bilateral nephrectomy. Five recipients were untreated, another five were given daily subcutaneous injections of normal rabbit serum, twenty-five received daily injections of rabbit-anti-dog antilymphocytic serum (ALS) at varying intervals before and after transplantation, ten were treated daily by mouth with azathioprine, fourteen received daily injections of ALS combined with azathioprine, and fifteen were treated with daily injections of ALS combined with both azathioprine and the allotransplantation of the donor's bone marrow. The mean survival time of the untreated control animals and that receiving normal rabbit serum were 9.0±1.0 and 10.0±0.3 days, respectively. No animals in these groups survived more than eleven days. In the ALS-treated dogs, some prolongation of survival was achieved when the serum was administered subcutaneously twice a day in a total dose of 1.0 ml per kg per day from 4 days before renal transplantation to 14 days after the transplantation and then once a week, and the survival time averaged 20.9±2.7 days. On the other hand, azathioprine combined with ALS and allotransplantation of the donor's bone marrow produced a mean survival of 38.4±4.7 days, a significant prolongation of functional survival over those of the other series. Accordingly, a considerable synergism was found in the combined therapy with ALS, a small dose of azathioprine and allotransplantation of the donor's bone marrow.

— renal allograft; antilymphocytic serum; bone marrow transplantation

The role of lymphocytes in the immune response and in particular its role in tissue allograft rejection is well established. For the destruction or suppression of the lymphoid tissue, different methods have been used, such as chemotherapeutic agents, total and subtotal irradiation, surgical ablation, extracorporeal irradiation of the circulating blood, thoracic duct fistula, and heterologous antisera. The first two modalities, which have been used most extensively in clinical situations, have widespread effects on the host in causing complications which limit the success of these agents. Accordingly, there has been an increasing interest in more selective approaches.

Received for publication, October 15, 1969.
* Studies on Prolongation of Canine Renal Allotransplant Survival, Report II.
Suppression of the lymphoid tissue by heterologous antisera has been attempted since 1899 when Metchnikoff prepared antiserum in the rabbit against leucocytes of rat and guinea pig. Since then, many attempts have been made by different investigators to produce antilymphocytic serum. Recently, the successful prolongation of skin allograft survival in rats and mice utilizing the serum as an immunosuppressive agent has been reported.

The present paper describes the preparation of rabbit-anti-dog lymphocytic serum and its effect on the prolongation of canine renal allografts. Previous studies in our laboratory demonstrated a considerable value of the allotransplantation of the donor's bone marrow combined with the administration of azathioprine in the prolongation of canine renal allograft survival. Further experiments have been carried out to determine the effect of the selective immunological tolerance against the donor's kidney by administering ALS combined with both the administration of azathioprine and the allotransplantation of the donor's bone marrow.

**MATERIALS AND METHODS**

Suspensions of canine lymph-node lymphocytes were prepared from the mesenteric lymph-nodes of dogs previously exsanguinated. Exsanguination markedly reduced the amount of blood in the nodes, thus producing lymphocyte suspensions with a minimum number of contaminating erythrocytes. Lymph-nodes were cleaned of perinodal fat and adventitia and minced with coarse scissors. Node fragments were then passed through stainless steel wire meshes into saline. The resulting suspension was washed three times in saline. The ratio of lymphocytes to contaminating erythrocytes usually varied from 10:1 to 4:1.

A rabbit was immunized by intravenous injection of this saline suspension (10 ml) of canine lymphocytes (300 × 10⁶ lymph-node cells per milliliter) 4 times at weekly intervals. After 4 weeks, one subcutaneous injection was given, and the rabbit was bled in a week after the last injection. The blood was allowed to remain at 4°C overnight. The serum was then separated and centrifuged to eliminate contaminating red cells. The serum was inactivated by heating at 56°C for 30 minutes and, to remove hemolysins, absorbed twice with dog erythrocytes which had been washed three times until the agglutinin titer was reduced to 1:4. The serum was stored in 10 or 20 ml ampoules at -20°C. The agglutination of dog lymph-node cells with rabbit-anti-dog lymphocytic serum was determined by the leucoagglutination technique described by Amos and Peacocke. The titers ranged from 1:32 to 1:256. Normal rabbit serum with a lymphagglutinin titer of 1:4 was obtained from this animal before immunization.

ALS was administered subcutaneously twice a day in a total dose of 1.0 ml per kg per day, before and after the renal allotransplantation for 18 days, then once a week. Azathioprine was given by mouth and the dosage schedule remained the same in all experiments as follows: 5 mg per kg per day for a week before and after the transplantation and then 3 mg per kg per day. Bone marrow cells were obtained by flushing out the long bones and ribs of the dog which had donated the kidney transplants. A saline suspension of the bone marrow cells (about 8.4 × 10⁶ nucleated cells) was injected intravenously 4 days before the transplantation and once a week for 4 weeks after the transplantation.

Seventy-four dogs were divided into 8 groups, and all were given renal allotransplants. The details of treatment are summarized in Tables 1 and 2. The kidney donors and recipients were adult mongrel dogs weighing between 8 and 12 kg. The details of operative technique were described in the previous paper. Ischemic periods were between 18 and 32 minutes. Bilateral nephrectomy of the recipient was carried out after completion of the anastomoses.
Blood samples were obtained before the injection of serum, before operation, and at intervals of 3 days thereafter. Total and differential white cell count, hemoglobin, platelet count and blood urea were estimated on these samples. Autopsies were carried out when the animals died, and histological sections were prepared from the kidneys, lymph glands, spleen, liver and lung.

RESULTS

Survival times in the present experiments are summarized in Tables 1 and 2. Neither the untreated controls nor the dogs receiving normal rabbit serum survived over 11 days, and these two groups showed a mean survival of 9.5±4.3 (s.d.) days. The transplanted kidney in these groups showed typical features of allograft

<table>
<thead>
<tr>
<th>Table 1. Survival of renal allograft recipients treated with ALS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Survival of renal allograft recipients in combined therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>
rejection: marked swelling, interstitial hemorrhage, round cell infiltration and tubular necrosis. No recognizable difference was found between the two groups.

In the ALS-treated dogs, only slightly more prolongation was achieved when the serum was administered both pre- and postoperatively than when the serum was administered postoperatively alone, but the best results showing a mean survival of 20.9±2.7 days were obtained with 4 days' pretreatment and continued postoperative administration of the serum. The appearance of the kidneys was similar to that of the controls and the histological pattern of rejection was less acute than in the control groups showing only focal tubular necrosis, with little interstitial hemorrhage but marked mononuclear infiltration.

The degree of lymphopenia produced was variable, but all dogs which received ALS showed a fall in peripheral lymphocyte count in spite of a marked initial granulocytosis.

In cases pretreated with ALS, the initial degree of lymphopenia was not related to eventual survival of the transplant, but all dogs showed a definite rise in count at the time of rejection. Most of the dogs receiving ALS showed a slight depletion of small lymphocytes in the spleen and lymph-nodes, but there was no evidence of the large central hyperplastic cells noted by Iwasaki et al.10

The dogs showed no signs of general upset following subcutaneous administration of ALS. The injections were of large volume and caused slight local swelling which persisted for 24 hours. No sepsis was seen in these dogs. The general condition of the dogs seemed satisfactory while the renal function was good, and the longer surviving dogs ate well and gained weight well after slight initial weight loss. All dogs showed an initial postoperative anemia apparently due to blood loss, but the hematocrit returned to normal levels within 3 weeks when the renal function remained satisfactory. Wound infection did not occur in any dogs in this series, but 7 dogs developed urinary tract infections. The pneumonia seen in several dogs was terminal in type and probably secondary to uremia. No evidence of hepatitis was seen.

Azathioprine combined with ALS administered on this schedule produced a mean survival of 38.4±4.7 days. On the other hand, the dogs receiving azathioprine combined with both the administration of ALS and the allotransplantation of the donor's bone marrow showed a mean survival of 50.2±8.1 days. There was a highly significant difference in the survival time between group 8 and the other groups.

In combined therapy, the histological pattern of rejection was similar to those of the longer surviving dogs of Group 6.

Although the results in cases of administration of the two or three combined agents were better than those obtained when ALS was used alone, wound infection and urinary tract infection occurred in several dogs.

**DISCUSSION**

The present experiments provide further evidence of the immunosuppressive property of antilymphocytic serum and demonstrate its capacity to prolong
the survival of canine renal allotransplants. The degree of immunosuppression obtained in the group of dogs receiving ALS both before and after operation, with a mean survival of 20.9±2.7 days, is comparable with that achieved in our laboratory by the administration of azathioprine (mean survival: 17.5±5.2 days), and there was a striking freedom from the usual infection, marrow depression, gastrointestinal hemorrhage and weight loss seen in the drug-treated dogs.

Using a mouse skin graft system, Levey and Medawar\textsuperscript{11} found that one or two intraperitoneal injections given 2 or 3 days after operation produced a greater effect than the same dose given preoperatively, but according to Monaco and his colleagues,\textsuperscript{12} when repeated doses of ALS were administered both pre- and postoperatively in a similar mouse system, pretreatment with continued postoperative administration produced a significantly longer graft survival than postoperative administration alone. This finding has been confirmed in the canine renal allografting in the present experiments. A slight prolongation of survival in the dogs not receiving pretreatment suggests that ALS might also be of value in the treatment of established rejection crisis, as suggested by Levey and Medawar.

The effect of ALS in producing marked lymphocytopenia is well demonstrated by the present experiments. But the degree of immunosuppression may not be due to the lymphopenia alone. The mechanism of action of ALS is uncertain. One view is that the effect is produced by depletion of lymphoid tissue. The profound reduction in circulating lymphocytes is not accompanied by such changes in lymphoid tissue, at least in the first 3 weeks of treatment with antiserum. Levey and Medawar\textsuperscript{13} suggested that ALS blocks the recognition of antigen by lymphocytes.

However, the value of ALS is offset to some degree by several disadvantages. ALS is locally irritative and occasionally associated with abscess formation, perhaps based initially on an Arthus phenomenon. Certainly profound anemia is seen when multiple ALS injections are given, presumably attributable in part to high titers of hemagglutinins in the impure serum. This can be reduced by absorption with erythrocytes. Anaphylactic reactions are not seen commonly in dogs.

Sufficient investigation of the nature and mode of action of ALS has been completed and ALS is accepted in general as a most powerful immunosuppressive agent. Previous studies in our laboratory demonstrated the value of the administration of azathioprine combined with the allotransplantation of the donor’s bone marrow. The present author attempted to induce a selective immunological tolerance against the donor’s kidney by administering azathioprine and ALS combined with the allotransplantation of the donor’s bone marrow cells, and found synergism in the combined therapy with a very low dose of azathioprine, ALS and allotransplantation of the donor’s bone marrow cells.

\textbf{Acknowledgment}

I wish to express my thanks to Prof. Y. Ishikawa for his guidance.
References


