Class II MHC Antigen Expression in Bronchial Lavage Cells in a Canine Lung Allograft Model using FK506 Immunosuppression

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Abbreviations: MHC: Major histocompatibility antigens, CsA: Cyclosporine A, BAL: Bronchoalveolar lavage, APC: Antigen presenting cell

To assess the effect of FK506 on class II MHC antigen expression on lymphocytes recovered from bronchoalveolar lavage (BAL) in dogs following left lung allotransplantation, flow cytometric analysis with OKIa-1 monoclonal antibody was performed. Dogs were divided into two groups: Group 1 (n = 6), control group (dogs without surgery); Group 2 (n = 23), dogs receiving left lung allotransplantation and immunosuppression using FK506 (0.1mg/kg/day intramuscularly). No significant difference existed in the percentage of OKIa-1 positive lymphocytes recovered from control animals (31.2 ± 11.2%) vs. normal allografts in Group 2 (34.8 ± 8.5%, n = 16). With rejection, however, the percentage of OKIa-1 positive lymphocytes increases significantly in Group 2: 56.2 ± 10.3% in mild rejection (n = 6) and 91.4 ± 4.3% in moderate or severe rejection (n = 4) (p < 0.01). Chest radiographs appeared normal in allografted lungs with histologically mild rejection. The percentage of the OKIa-1 positive lymphocytes in BAL did not significantly change during the first, second, or third week following transplantation, ((32.2 ± 6.4 (n = 5), 36.4 ± 4.2 (n = 4), and 35.8 ± 12.3 (n = 7), respectively)) in the allografted lungs without rejection.

FK506 does not affect the class II MHC antigen expression of lymphocytes recovered from BAL in canine allografted lungs without rejection. Furthermore, this compound does not change in class II antigen expression seen with allograft rejection.

Key Words: lung transplantation, rejection, bronchoalveolar lavage, class II MHC antigen

Introduction

Early recognition of rejection is one of the most important factors in maintaining functional lung allografts. Transbronchial lung biopsy (1), immunohistochemical staining of transbronchial biopsy specimens (2), and analysis of cells derived from bronchoalveolar lavage (BAL) (3-6) have been reported as methods of detecting early rejection. However, only open lung biopsy currently allows reliable determination of lung allograft rejection.

Class II major histocompatibility antigens (class II MHC antigens), play an important role in the initiation of immunologic rejection (7). Previously, we have reported that the expression of class II MHC antigen on BAL lymphocytes, indicated by staining with a monoclonal antibody (OKIa-1), increased during rejection of canine allograft lungs without immunosuppression, and was not affected by cyclosporine A (CsA) (8).

In this study, we examined class II MHC antigen expression by flow cytometry in a canine lung allograft model with FK506 immunosuppression. FK506 is a macrolide antibiotic extracted from the fungus Streptomyces tsukubaensis (9) that has been found to be an effective immunosuppressive agent in animal as well as human kidney and liver transplantation (10). We also assessed the effectiveness of evaluating class II MHC antigen expression for the diagnosis of lung allograft rejection in the recipient dogs.

Materials and Methods

Adult mongrel dogs weighing 10 to 15kg were used in these examinations. Dogs were classified into two groups: Group 1 (n = 6): control dogs without surgery or immunosuppression, and Group 2 (n = 23): dogs which underwent left lung allotransplantation and received 0.1mg/kg FK506 (Fujisawa Pharm. Ltd., Japan) each day intramuscularly. Chest radiography was performed on days 1, 3, 5 and 7, and then twice a week until the animal was sacrificed.

Bronchoalveolar Lavage

Bronchoalveolar Lavage was performed weekly, or when consolidation was found on the chest radiograph, from postoperative day 7 to 28. After general anesthesia was
obtained via intravenous pentobarbital, a flexible fiberoptic bronchoscope was introduced into a subsegmental orifice of the lower lobe. Fifty milliliters of sterile normal saline was infused and then aspirated gently with a syringe. Lavage was repeated three or four times, and a total of 100 ml of fluid was collected.

**Cell Preparation**

The lavage fluid was filtered through one layer of sterile cotton gauze and a nylon mesh filter with a pore size of 50 μm, then centrifuged at 300 x g for 6 minutes. The supernatant was shaken and overlaid onto 4 ml Lymphoprep-R solution in 15 ml conical centrifuge tubes and centrifuged at 300 x g for 30 minutes. The pellet was resuspended with 2 ml of distilled water, 2 ml of 1.8% NaCl and 2 ml of phosphate buffered saline (PBS), and centrifuged at 300 x g for 10 minutes. The pellet was washed with 2 ml of PBS, and the mononuclear cells were separated and resuspended in 2 ml of PBS. The final cell suspension contained 5-10 x 10⁶ cells/ml.

**Immunofluorescent Staining**

Two hundred microliters of cell suspension were placed into each of two 12 x 75 mm test tubes. Ten microliters of mouse OKIa-1 monoclonal antibody to B lymphocytes and monocytes (Ortho Diagnostic Systems Inc., U.S.A.), were added to one tube, and 10 μl of wash media to the other as a control. The cell mixture was incubated in an ice water bath for 30 minutes with agitation every 10 minutes. Two milliliters of PBS were added to each tube, the cells washed by centrifugation at 300 x g for 10 minutes, and the supernatant removed with a Pasteur pipette, leaving approximately 100 μl of media in the tube. Tubes were shaken to resuspend the cell pellets. One hundred microliters of diluted (1:100) FITC-conjugated goat-anti-mouse IgG was added to each tube, incubated in ice water, and washed by centrifugation as described above. For flow cytometric analysis, the cells were resuspended in 2 ml of PBS, and maintained at 0-4 °C until analyzed.

**Flow Cytometric Analysis**

The samples were analyzed on an Ortho Spectrum III Laser Flow Cytometry System (Ortho Diagnostic Systems, U.S.A.) at a wavelength of 720 nm. Lymphocytes were differentiated by the forward and right angle scattering scale. Samples were evaluated on single-parameter histograms displayed on a linear scale of 256 channels through a green filter. The presence of marker positive cells in the control samples was less than 2%. Results were presented as a percentage of marker positive cells ± the standard deviation.

**Open Lung Biopsy**

Open lung biopsy of allograft lungs was performed after BAL, and graft rejection confirmed microscopically with hematoxylin-eosin staining of biopsy specimens. Rejection was histologically graded mild, moderate, or severe, according to the Yousem classification (11). Mild rejection was identified by edema of the interstitium and mild perivenular and peripaternal cuffing by small round transformed lymphocytes. Intense perivenular, peribronchial, and peripaternal cuffing by lymphocytes and immunoblasts with endothelial inflammation constituted moderate rejection. Severe rejection was characterized by fibrinoid necrosis and thrombosis of veins and arteries with hemorrhage and parenchymal necrosis.

The animals received care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society of Medical Research. Data between groups were compared using the Mann-Whitney U nonparametric test. A p-value of less than 0.05 was considered significant.

**Results**

Allografted dogs survived from 7 to 86 days following surgery. Thirteen dogs were sacrificed after 14 days. The causes of death in the remaining dogs are listed in Table 1. Serious toxic effects of FK 506 such as severe diarrhea, vomiting, or emaciation accounted for the death of 3 dogs.

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>Number of dogs (survival days)</th>
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<tbody>
<tr>
<td>Sacrifice</td>
<td>11 (day 16, 21, 26, 29, 29, 31, 41, 41, 47, 86)</td>
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<tr>
<td>Rejection</td>
<td>3 (day 20, 32, 48)</td>
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<tr>
<td>Toxicosis of FK-506</td>
<td>3 (day 21, 54, 76)</td>
</tr>
<tr>
<td>Acute lung edema after BAL</td>
<td>3 (day 7, 15, 18)</td>
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<tr>
<td>Pneumonia</td>
<td>2 (day 15, 17)</td>
</tr>
<tr>
<td>Pulmonary vein thrombosis</td>
<td>1 (day 9)</td>
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<tr>
<td>Total</td>
<td>23</td>
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</tbody>
</table>

BAL followed by open lung biopsy was performed 44 times in 21 dogs from 7 to 28 days after transplantation. Twenty-two BAL and biopsies were obtained in dogs with normal appearing chest roentgenograms, and 15 specimens were collected in dogs with infiltrates on radiograph. Rejection was histologically confirmed 21 times in lung biopsy specimens of 21 dogs. The number of BAL mononuclear cells was higher in grafted vs. control animals. The percentage of OKIa-1 positive lymphocytes in allografted immunosuppressed dogs with rejection was significantly higher than in dogs without rejection (Table 2). However,
Table 2. Number of BAL mononuclear cells and percentage of lymphocytes or macrophages in BAL.

<table>
<thead>
<tr>
<th></th>
<th>Total number of cells (x10^6/BAL100ml)</th>
<th>Lymphocytes (%)</th>
<th>Macrophages (%)</th>
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</thead>
<tbody>
<tr>
<td>Control lungs</td>
<td>15.7 ± 21.3 (n = 14)</td>
<td>17.4 ± 7.2 (n = 9)</td>
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<tr>
<td>Grafted lungs</td>
<td></td>
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<tr>
<td>Rejection (-)</td>
<td>60.8 ± 73.1 (n = 19)</td>
<td>9.3 ± 4.5 (n = 16)</td>
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<tr>
<td>Rejection (+)</td>
<td>40.4 ± 45.6 (n = 15)</td>
<td>18.9 ± 13.9 (n = 14)</td>
<td></td>
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</tbody>
</table>

*p < 0.01

This percentage was not different from control animals.

There was no significant difference in the percentage of OKIa-1 positive lymphocytes between control lungs and grafted lungs without rejection ((31.2 ± 11.2% (n = 6) vs. 34.8 ± 8.9% (n = 16)). In the lungs with rejection, the percentage of OKIa-1 positive cells was significantly higher than in control lungs or lungs without rejection, and reflected the severity of rejection (Fig. 1, 2, 3, 4).

Fig. 1. Percentage of BAL lymphocytes expressing antigen recognized by OKIa-1 monoclonal antibody. Values expressed as mean ± standard deviation.

Chest radiographs appeared normal in the allografted lungs with histologically mild rejection, but infiltrates were observed in lungs with moderate and severe rejection. Despite the normal appearance on chest roentgenograms in some episodes of mild rejection, the percentage of OKIa-1 positive lymphocytes was higher than lungs without rejection ((59.4 ± 11.0% (n = 10) vs. 38.6 ± 8.0% (n = 6)). This difference persisted even in lungs without rejection which had infiltrates on chest radiographs (32.6 ± 9.3% (n = 9) p < 0.01) (Fig. 5). There was no variation in the percentage of OKIa-1 positive lymphocytes in the allografted lungs with or without rejection over the first three weeks following transplantation (Fig. 6).

Fig. 2. The chest roentgenogram, photomicrograph (x 40), and cytogram and histogram of the BAL cells in a normal lung. BAL and open lung biopsy performed on postoperative day 7. There was no infiltration on chest radiograph, and no rejection microscopically. The percentage of lymphocytes in the BAL mononuclear cells was 14.3%. OKIa-1 positive lymphocytes accounted for 26.2%.
Fig. 3. The chest roentgenogram, photomicrograph (x 40), and cytogram and histogram of the BAL cells in a lung with mild rejection in a dog with BAL and open lung biopsy performed day 14 postoperatively. The chest roentgenogram showed a virtually normal appearance. Perivascular cuffing was detected microscopically. The percentage of lymphocytes in the BAL mononuclear cells was 14.4%. OKIa-1 positive lymphocytes was 60%.

Fig. 4. Chest roentgenogram, photomicrograph, and cytogram and histogram of the BAL cells in a lung with severe rejection in a dog with BAL and open lung biopsy performed on day 21 postoperatively. The chest roentgenograph showed diffuse consolidation, and biopsy showed diffuse, small, round cell infiltration with destruction of alveolar structure. The percentage of lymphocytes in the BAL mononuclear cells was 66.4%. OKIa-1 positive lymphocytes accounted for 95.6%.

Fig. 5. Percentage of the BAL OKIa-1 positive lymphocytes in canine allografted lungs categorized by infiltration on chest roentgenography and histologic rejection.

Fig. 6. Percentage of BAL OKIa-1 positive lymphocytes in canine allografted lungs over time. Closed circles indicate lungs with rejection; open circles indicate normal allografted lungs.
Discussion

Bronchoalveolar lavage offers a unique opportunity for safe and repetitive sampling of large quantities of graft-infiltrating immunocompetent cells. The normal canine cellular profile of BAL fluid is 83% macrophages, 9.9% lymphocytes, and 4.1% neutrophils (12). After lung allograft transplantation without immunosuppressant therapy, the percentage and absolute number of lymphocytes increases significantly, and the predominant cell type changes from macrophages to lymphocytes (8, 12). In this study, the absolute number of mononuclear cells in the BAL fluid of grafted lungs increased after transplantation. Although, the ratio of BAL lymphocytes to macrophages decreased in the BAL fluid of grafted lungs without rejection, the ratio in lungs with rejection was not different from control animals. This suggests that FK506 decreases the ratio of BAL lymphocytes to macrophages, but does not affect the profile of BAL cells in active rejection.

This study also documents that the typical increase of canine BAL lymphocytes expressing class II MHC antigen (as measured via OKIa-1 reactivity) is not impaired in normal allografted lungs, and increased during acute rejection despite treatment with FK506. OKIa-1 is a cross reactive murine monoclonal antibody to class II MHC antigen (13) and IgG2. OKIa-1 identifies B lymphocytes, activated T lymphocytes, and some monocytes. Paradis (14) reported that there was rapid replacement of the donor B-cells with those of the recipient in BAL fluid of allografted lungs within 4 weeks unless allograft rejection occurred (3). Recently, experimental and clinical studies utilizing phenotypic analysis of BAL cells via monoclonal antibodies in lung allograft infection and rejection were reported. Shennib et al. (6) showed that the percentage of DT2-labeled cells (which is a cross-reactive murine monoclonal antibody reacting specifically with canine T-cells (15)) was significantly higher in BAL samples from canine lungs with rejection than in BAL from infected lungs. DT2-positive cells proliferate in response to allogeneic cells and mitogens, but are not able to stimulate allogeneic cells in MLC (15). Zeevi et al. (3) found that during acute lung rejection, there was an increase in numbers of macrophages, CD8+ cells (cytotoxic/suppressor T lymphocytes), and neutrophils. During infection, especially with Pneumocystis carinii and cytomegalovirus, both CD4+ (inducer/helper T-cells) and CD8+ subsets were increased markedly (5). In the present study, the origin or subtype of lymphocytes with expression of class II MHC antigen is not known. However, we speculate that the increased OKIa-1 positive lymphocytes in rejected lungs without infection are likely to be activated T lymphocytes originating from the recipient dogs.

FK506 is immunosuppressive in vitro at about 100 times lower concentrations than CsA (16). FK506 is exceptionally potent in inhibiting T lymphocyte-mediated immu-

References