Introduction

The first human total laryngeal transplantation was successfully performed in 1998 by the senior author. Success of this procedure was largely dependent upon the development of a reliable rat transplantation model. Our lab recently developed a novel mouse laryngeal transplant model to fully evaluate the immune response to various immunosuppressive agents and explore cellular immunosuppression tactics. Knowledge of the mouse immune system and availability of reagents are far superior to the rat, making the mouse the ideal model with which to study transplant immunology. The model has proven effective and reliable, with syngeneic transplants to surmount inbred deficiencies.

Our purpose was to develop a rejection grading system based on a study of the natural laryngeal allograft rejection process in immunocompetent mice. The grading system will provide objective criteria with which to assess rejection severity for a given allograft and to compare the efficacy of various immunosuppressive agents.

Study Design

- Thirty-one mouse laryngeal transplants (C57 BL/6 into C3H) were performed and postoperative immunosuppression was given.
- Five animals were sacrificed at each of 6 time points (1, 2, 3, 5, 7, and 10 days posttransplant).
- All animals were sacrificed on day 15.
- The time points were considered to be 6 separate time groups, numbered 1-6 respectively.
- Langrenger and sent for H&E staining.
- Each larynx was evaluated in a blinded fashion for eight histological criteria (Figure 1).
- Three criteria were assigned a range of point values based on the severity of cell damage.

Table 1. Histological Criteria

<table>
<thead>
<tr>
<th>Histological Criteria</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiak</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Epithelium</td>
<td>Absent</td>
<td>Moderate</td>
<td>Severe</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Fat</td>
<td>Absent</td>
<td>50% Nucleated</td>
<td>50% Nucleated</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Minor Salivary Glands</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Lymphocytic Infiltration</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Muscle</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>50% Nucleated</td>
<td>25% Nucleated</td>
</tr>
<tr>
<td>Pneumothorax</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Thyroid Gland</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Table 2. Surgical Procedure Outlines (Figures 2 & 3)
- Donor laryngotracheal complex harvested and transplanted heterotypically into the recipient mouse.
- Vascular supply based on superior thyroid artery pedicled via the external carotid artery.
- Anterior Inflow: Donor common carotid artery anastomosed to the recipient common carotid artery.
- Venous Outflow: Donor contralateral common carotid artery anastomosed to the recipient internal jugular vein.

Figure 2

Statistical Methods

Model Building
- The 6 separate plates of sacrifice were considered as 6 separate time points.
- After each larynx had been graded in the 8 histological categories, a multivariate linear regression model was constructed to determine which characteristics were the most meaningful in predicting from which time group the specimen had originated.

Accuracy Verification
- The mean of each particular was assessed for accuracy by plotting the equation with the results of the remaining 30 specimens and testing the one specimen out of the analysis.
- Every specimen was tested to determine how similar the predicted group would be with its own basis in the equation.

Group Classification
- Adjacent groups were combined in a manner that would preserve the maximum predictability of the category.

Results

Allotraft Status
- All 30 specimens between day 1 and day 12 were noted to have blood flow throughout the anastomosed system.
- None of the specimens at day 15 exhibited any blood flow.

Histological findings demonstrated predictable progressive degradation of all structures (Figure 4), with the exception of epithelium.
- Epithelium displayed great variability in days 1-5 but was uniformly absent beginning on day 7.
- The thyroid gland demonstrated >50% follicular density and increased fibrous tissue beginning at day 1.
- This remained unchallenged at all time points until day 15, when all thyroid follicles were uniformly absent.

Figure 4

Comparison of Histological Criteria at Different Time Points

Table 3. Rejection Severity Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Corresponding Factors</th>
<th>Rejection Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Day of Sacrifice 1</td>
<td>Sum of Factors 3</td>
</tr>
<tr>
<td>Minimal Rejection</td>
<td>Day of Sacrifice 3</td>
<td>Sum of Factors 5</td>
</tr>
<tr>
<td>Moderate Rejection</td>
<td>Day of Sacrifice 5, 7</td>
<td>Sum of Factors 4</td>
</tr>
<tr>
<td>Severe Rejection</td>
<td>Day of Sacrifice 15</td>
<td>Sum of Factors 3</td>
</tr>
</tbody>
</table>

Discussion

- Degradation of the laryngeal allotraft occurred in a predictable fashion over time in both models.
- Lymphocytic infiltration, fat, muscle, and cartilage were found to significantly reflect rejection severity.

Lymphocytic Infiltration
- The degree of lymphocytic infiltration appears to govern the severity of allotraft rejection.
- Infiltration reached a maximum on day 9 and remained profound throughout the remainder of the rejection process.
- In our previous rat model, maximum infiltration occurred between days 5 and 7, then settled to a persistent minor infiltration as rejection completed.
- These differences may be due to smaller vesicle size in the mouse, causing a relative delay in cell accumulation at response initiation and cell backsliding at the cessation of rejection completion.

- Inter-species immunologic response variation also likely plays some role.

Fat
- Fat presence was the only characteristic factor that differentiated no rejection from mild rejection.
- Suggests fat tissue is relatively resistant to ischemia but acutely sensitive to lymphotoxic destruction.

Muscle
- Muscle necrosis corresponded to the difference between mild and moderate rejection.
- Muscle exhibited progressive loss of nuclei until absent at day 9.
- Necrosis decreased to 50% between day 3 and days 5-9, corresponding to the transition from mild to moderate rejection.
- By day 9, lymphocytic infiltration became profound, muscle necrosis was completely absent.

Cartilage
- Cartilage underwent progressive cell death between moderate and severe rejection.
- No change in cellularity seen until day 15, when lymphocytic infiltration had been profound for almost one week and all other tissues had been destroyed.

Rejection Severity Equation
- This four factors were incorporated into an equation that allowed for the classification of allotraft rejection severity based on the natural rejection course in immunocompetent mice.
- The equation achieved 100% accuracy in predicting the rejection severity group for the 31 specimens in the study.

Conclusion

To summarize our findings, our mouse laryngeal transplant rejection grading system is based on chronological allotraft rejection observed at the histological level with no immunosuppression given. Using this histological criteria, one is able to predict the rejection severity with 100% accuracy among the four different rejection groups. This new system enables reliable comparison of allotraft rejection allowed by different immunosuppressive agents.

References