Cellular Chimerism of the Lung After Transplantation

An Interphase Cytogenetic Study

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Abstract

The present study evaluated the origin of endothelial and epithelial cells, as well as of lymphocytes and macrophages, after lung transplantation. Biopsy specimens from patients who underwent lung and heart-lung transplantation and received organs of sex-mismatched donors were studied by means of nonisotopic in situ hybridization with DNA probes of the X and Y chromosome. By means of monoclonal antibodies against leukocytes, T and B lymphocytes, and macrophages, the various infiltrating cell types were analyzed.

In all allografted lungs, the endothelial cells and bronchial and alveolar epithelium retained the donor sex type. The lymphocytes of the donor were almost completely replaced by recipient cells 1 month after transplantation. Low numbers of alveolar macrophages of the donor were present during the entire period under study. Low numbers of donor lymphocytes and high numbers of donor alveolar macrophages in the allografted lung seem to be correlated with a worse clinical course.

An integrated network of defense mechanisms safeguards the gas-exchanging surface of the lung. The lung has a critical role in the homeostatic regulation of systemic immunity, as judged by the demonstration that the pulmonary vascular bed and interstitium contain about half of the body complement of T lymphocytes. The pulmonary lymphoid tissue shows decreasing levels of histologic organization as one proceeds distally from the trachea. Encapsulated lymph nodes are seen adjacent to the trachea and major bronchi. The bronchial-associated lymphoid tissue may have an important role in pulmonary antigen presentation. It is developed only poorly in human beings, but it may become prominent in patients with chronic airway disease or persistent antigenemia. Pulmonary macrophages are essential for maintaining the integrity of the gas-exchanging surface of the lung. Resident alveolar macrophages (AMs) scavenge inhaled particles and potentially toxic microorganisms.

Besides lymphocytes and macrophages, the vascular endothelium and epithelial cells are involved in the immunologic control mechanisms of the lung. These mechanisms are especially relevant after allogeneic lung transplantation.

When Medawar1 hypothesized in 1965 that vascular endothelium could be replaced after transplantation by endothelial cells of host origin, it was suggested that this might result in increased tolerance of the transplanted organ. As in other organs like kidney2 and heart,3 this hypothesis could not be confirmed by Yousem and Sonmez Alpan4 in the lung in a study after application of the nonisotopic in situ hybridization (NISH) technique using only 1 DNA probe for the Y chromosome.

Starting from these questions, which were not sufficiently or even controversially answered, we comprehensively evaluated lung specimens of patients who had received lung allografts of sex-mismatched donors. The aim of the
study was to determine whether lymphocytes, macrophages, vascular endothelial cells, and epithelial cells in the donor lung are replaced by the recipient’s own cells or whether a chimeric status developed, and if a chimeric status developed, how long it would take.

The study was performed on lung tissue sections after formalin fixation and paraffin embedding by means of NISH using biotinylated centromere-specific DNA probes for the human Y and X chromosomes. This technique allows the clear-cut discrimination of donor and recipient cells after sex-mismatched organ transplantation. By means of monoclonal antibodies, the various infiltrating cell types were identified.

Materials and Methods

Patients

The Munich Lung Transplant Group has performed a total of 100 procedures involving lung transplantation in 84 patients since 1991. Of these, 12 lung transplant recipients who received organs of sex-mismatched donors were studied. There were 3 men and 9 women, ranging in age from 19 to 59 years (mean, 30 years). The primary diagnoses were emphysema (n = 3), cystic fibrosis (n = 1), idiopathic fibrosis (n = 4), lymphangioleiomyomatosis (n = 1), and pulmonary hypertension (n = 3). Five patients underwent right-sided single lung transplantation; 2, left-sided single lung transplantation; 2, bilateral sequential lung transplantation; and 3, combined heart and lung transplantation.

Table I. Forty-nine biopsy specimens fulfilled the requirements for histologic diagnosis. There were 31 transbronchial biopsy specimens and 18 open lung biopsy specimens. In 2 cases, an autopsy was performed. Each transbronchial biopsy specimen contained 3 to 8 pieces of alveolar lung parenchyma. The biopsy specimens were obtained from posttransplant day (PTD) 1 to PTD 781 (mean ± SD, 201 ± 225) (Table I). The rejection was graded according to the revised working classification of the International Society for Heart and Lung Transplantation in 1996. The diagnoses obtained by light microscopy included 15 biopsy specimens with no rejection or infection (A0, B0), 27 biopsy specimens that revealed acute rejection (A0-A1, B1-B2, 15; A2, B1-B2, 7; A3, B2-B3, 2; A4, B2-B4, 3), and 2 biopsy specimens that revealed chronic rejection with bronchiolitis obliterans. Other diagnoses included 5 biopsy specimens with diffuse alveolar damage and 2 with organizing pneumonia.

Tissue Preparation

All lung specimens were fixed routinely in 4% buffered formalin and paraffin embedded according to standard procedure.

Nonisotopic In Situ Hybridization

For NISH, commercial biotinylated DNA probes (ONCOR, Amersham Buchler, Gaithersburg, MD) binding to highly repeated alphoid DNA located at the centromeres of human chromosomes X (DXZ1) and Y (DYZ1/DYZ3) were used. NISH was performed following the protocol of Hopman et al with our own modifications, described elsewhere, using the probes for the X and the Y chromosomes on consecutive individual serial sections. Briefly, 6-µm-thick slides were cut from the paraffin blocks and mounted on poly-L-lysine–coated slides. Sections were dried overnight at...
56°C. After dewaxing in xylene, the sections were rinsed in absolute methanol (10 minutes), and endogenous peroxidase activity was blocked in 1% hydrogen peroxide in methanol for 30 minutes. To make DNA accessible for hybridization, the sections were pretreated with a 1-mol/L concentration of sodium thiocyanate (NaSCN, Sigma, Deisenhofen, Germany) for 0 to 4 minutes (average, 0-2 minutes) at 80°C and digested with proteinase K (Sigma) in phosphate-buffered saline (5 mg/mL; Sigma) for 30 to 60 minutes (average, 60 minutes) at room temperature. After washing, the slides were again in phosphate-buffered saline, passed through a series of ethanols in ascending concentrations, air dried, and heated for 30 minutes at 80°C. The slides were incubated overnight at 37°C with the hybridization mix containing 0.4 ng/µL of the DNA probe, 60% formamide 10%, 20× standard saline citrate (sodium chloride and a 30-mmol concentration of sodium citrate, pH 7), 5% dextran sulfate, and 1% herring sperm (Sigma). The hybridization reaction was visualized by means of a 3-step immunoperoxidase technique.

**Evaluation of In Situ Hybridization Signals**

Criteria for the evaluation of NISH signals were as follows: (1) Overlapping interphase nuclei were not counted. (2) Signals within one nucleus were to have more or less the same size and intensity. (3) Paired NISH spots (split spots) were counted as 1 signal.

Hybridization signals were counted, if possible, within 100 interphase nuclei each of bronchial and alveolar epithelial cells, endothelial cells, macrophages, and lymphocytes. Interstitial and alveolar macrophages were evaluated separately. In all cases, hybridization of the X and Y chromosomes was performed.

**Immunohistochemical Study**

Immunohistochemical study was performed by means of the alkaline phosphatase–antialkaline phosphatase technique, applying a panel of monoclonal antibodies (Table 2). Monoclonal antibodies used were against leukocyte-common antigen (CD45), T lymphocytes (CD3), B lymphocytes (CD20), and macrophages (Mac387).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary Concentration</th>
<th>Dilution</th>
<th>Identified Tissue Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>375 mg/L</td>
<td>1:90</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>CD3</td>
<td>0.5 g/L</td>
<td>1:300</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>CD20</td>
<td>2 mg/mL</td>
<td>1:20</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>Mac387</td>
<td>86 µg/mL</td>
<td>1:500</td>
<td>Macrophages</td>
</tr>
</tbody>
</table>

* All from Dakopatts, Hamburg, Germany.

**Combined Immunohistochemical Study and NISH**

Before NISH, immunohistochemical study was performed according to standard procedure. In 23 specimens, immunohistochemical study with CD45 and Mac387 was combined with hybridization of the Y chromosome to facilitate typing of cells in cases of doubt.

**Data Analysis**

Results were expressed as mean ± SEM unless otherwise mentioned.

Following a study of O’Connell et al., we analyzed the outcome of patients with a low (<10%) and high (10% or more) median content of donor lymphocytes within the first 200 days after transplantation and the outcome of patients with a low (<20%) and high (20% or more) median content of donor AMs within the first 200 days after transplantation. The percentages of donor cells were calculated from the total amount of lymphocytes and macrophages. The patients were divided in 2 groups: (1) patients in whom bronchiolitis obliterans syndrome (BOS) developed within the first year after transplantation and patients who died; (2) patients in whom BOS developed more than 1 year after transplantation.

Because our study group included only 12 patients, we used the Pearson chi-square test to compare the groups. Differences between groups were considered significant if P values were less than .05. All data were analyzed using SPSS (Superior Performance Software System, Chicago, IL).

**Results**

In all allografted lungs, the endothelial cells and bronchial and alveolar epithelium Image 1A retained the donor sex type. The majority of inflammatory cells were T lymphocytes and macrophages.

**Lymphocytes**

Besides dominant T lymphocytes, a few biopsy specimens contained small lymphoid follicles with B cells. Seven transbronchial biopsy specimens did not contain sufficient lymphocytes for evaluation by NISH.

Almost all biopsy specimens (15 of 18) obtained until PTD 39 contained lymphocytes of the donor (2%-30%; mean, 11.4% ± 6.4%) (Table 3). The remaining detectable lymphocytes showed the sex of the recipient, and 3 biopsy specimens showed no donor lymphocytes.

For example, on PTD 10, 1 biopsy specimen from a male donor lung showed several perivascular lymphoid cuffs indicative of acute rejection grade 2. There were no signs of endothelitis. NISH demonstrated 1 signal of the Y chromosome in the nuclei of the lymphocytes, confirmed by a combination of NISH and immunohistochemical study, indicating...
that the lymphocytes were of donor origin and that no rejection episode occurred.

Most of the biopsy specimens (21 of 26) obtained later than PTD 39 no longer contained donor lymphocytes. The exceptions were biopsy specimens (n = 5) from 2 female recipients, which contained small aggregates of donor lymphocytes with nuclei demonstrating 1 signal of the Y chromosome until PTD 239 and 407.

Biopsy specimens (15 from 7 patients; PTD 1-626) without evidence of rejection or infectious disease showed sparse interstitial infiltrates of lymphocytes of recipient origin with distribution in the alveolar septa, peribronchially, perivascularly, without forming cuffs, and in the pleura. Two biopsy specimens on PTD 407 and 629 showed a few scattered aggregates of recipient lymphocytes.

During episodes of acute and chronic rejection and infectious disease, the infiltrating lymphocytes showed the sex-specific centromere signals of the male or female recipient.

**Macrophages**

We evaluated interstitial and intra-alveolar macrophages separately.

**Interstitial Macrophages**

In 22 biopsy specimens, it was possible to count and distinguish recipient and donor interstitial macrophages. Eighteen biopsy specimens from 5 female and 3 male recipients contained 1% to 20% (9.8% ± 5.4%) interstitial macrophages of donor sex, from PTD 1 to 480 (117 ± 158). One month after transplantation, the interstitial macrophages of the donor had decreased to very rare residual cells. The remaining biopsy specimens (n = 4) from 2 male recipients obtained from PTD 101 to 620 showed no interstitial macrophages of the donor.
Intra-alveolar Macrophages

AMs of donor sex could be detected during the entire period under study (PTD 1-781). Nine biopsy specimens were not evaluable owing to technical problems or insufficient content of macrophages in the lung tissue.

Calculated from the total amount of AMs, 44 biopsy specimens from 9 female and 3 male recipients contained 0% to 70% (17.8% ± 16.2%) AMs of the donor, from PTD 1 to 781 (217 ± 239).

Biopsy specimens from cases with and without a histologically determined increase in content of intra-alveolar macrophages were evaluated after application of both the X and Y probes by NISH (Table 3). Because the half-life of macrophages is about 40 days,9 the biopsy specimens were differentiated according to days after transplantation.

During the time after transplantation, the portion of donor AMs decreased and the portion of recipient AMs increased, but a small percentage of donor AMs were detected during the entire period under study (Table 4).

Follow-up Analysis

We compared the clinical course of the patients (Table 1) with the content of donor-derived lymphocytes and AMs within the first 200 days after transplantation. Within this interval after transplantation, the median number of donor lymphocytes was 0% to 20% (7.6% ± 5.0%), and the median number of donor macrophages was 7% to 67% (21.9% ± 11.5%). Our results indicate that patients with high numbers of donor macrophages (<20%) within the first 200 days after transplantation (P < .05) Table 5. On the other hand, a low content of donor lymphocytes (<10%) during the first year after transplantation in the allografted lung seems to be correlated with worse graft function (P < .05) (Table 5).

Patients who died and/or in whom BOS developed within 1 year after transplantation had more acute rejection episodes (1-3 episodes; mean, 2) and more severe rejection episodes than patients with a better clinical course (0-3 rejection episodes; mean, 1). Patients with a low portion of donor lymphocytes and a high portion of donor AMs had more acute rejection episodes than the patients with a high portion of donor lymphocytes and a low portion of donor AMs.

Discussion

In 1965, Medawar1 hypothesized that the vascular endothelium can be replaced after transplantation by endothelial cells of host origin. He suggested that this phenomenon might result in tolerance of the transplanted organ.1 The present study started from the question of whether Medawar’s hypothesis proves true. Therefore, the origin of vascular endothelium, of the epithelial cells but also of lymphocytes and macrophages within the transplanted lung, was studied.

Frequently, endothelial cells are damaged during rejection,10 and replacement, therefore, could be imaginable. Recent studies of kidney, heart, liver, and aorta11-14 described reendothelialization after transplantation. The detection was

### Table 3

<table>
<thead>
<tr>
<th>Posttransplantation Day</th>
<th>No. of Biopsy Specimens</th>
<th>Female</th>
<th>Male</th>
<th>Range</th>
<th>Mean ± SEM</th>
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<tbody>
<tr>
<td>1-39</td>
<td>20</td>
<td>9</td>
<td>2</td>
<td>0-30</td>
<td>10 ± 12</td>
</tr>
<tr>
<td>51-407</td>
<td>5</td>
<td>2</td>
<td>—</td>
<td>Small lymphocytic aggregates</td>
<td>—</td>
</tr>
<tr>
<td>42-781</td>
<td>21</td>
<td>7</td>
<td>4</td>
<td>—</td>
<td>—</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Posttransplantation Day</th>
<th>No. of Biopsy Specimens</th>
<th>Female</th>
<th>Male</th>
<th>Range</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-39</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>8-39</td>
<td>18 ± 13</td>
</tr>
<tr>
<td>58-781</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td>0-26</td>
<td>9 ± 9</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Posttransplantation Day</th>
<th>No. of Biopsy Specimens</th>
<th>Female</th>
<th>Male</th>
<th>Range</th>
<th>Mean ± SEM</th>
</tr>
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<tr>
<td>6-39</td>
<td>12</td>
<td>5</td>
<td>1</td>
<td>0-70</td>
<td>27 ± 21</td>
</tr>
<tr>
<td>45-728</td>
<td>11</td>
<td>7</td>
<td>2</td>
<td>4-41</td>
<td>16 ± 11</td>
</tr>
</tbody>
</table>

1 Without histologically increased content of AM.

2 With histologically increased content of AM.
made immunohistochemically by means of antibodies against blood group antigens12,13 or the determination of the sex chromatin.11,14

NISH by means of nonisotopic probes of the human X and Y chromosomes permits the determination of the genotype of cells involved in transplant reaction. In previous studies, fluorescenated dyes such as acranil and quina-crine15-19 were used to stain the distal part of the long arm of the Y chromosome (Y body) and the Barr body (X chromatin). In these studies, the sensitivity in frozen sections for the Y body ranged from 47% to 89%;17 and for the Barr body, from 19% to 30%.18 The sensitivity decreased further when formalin-fixed, paraffin-embedded material was used. Another limitation of these techniques was the difficulty of identifying morphologic characteristics of cells and tissue. Compared with the aforementioned studies, NISH yields a high sensitivity and has the advantage of clear attribution of the hybridization signal to an individual cell type in a preserved morphologic structure.20,21 NISH with a biotinylated DNA probe for the Y chromosome has been used primarily in bone marrow transplantation, where fluorescent probes allow discrimination of residual leukemic cells in male recipients of marrow grafts from female donors.22,23

Comparison of NISH signals in cell suspension and paraffin sections revealed that in the latter, fewer signals were detectable than in cell suspension. This phenomenon was a result of truncation of the nuclei and correlated with the nuclear diameter.6,7 In the setting of these findings, using only 1 probe, the identification of the donor or recipient origin of single cells was sometimes impossible.

In our study, we therefore used probes specific for the human Y and X chromosomes in serial sections to evaluate the cellular changes after lung transplantation. In addition, using a combination of immunohistochemical study and NISH improved the identification of different cell types.

Our study clearly revealed that there is no reepithelialization or reendothelialization of the lung by recipient cells...
after transplantation. Similar results were obtained by Yousem et al,4 who used only a probe for the Y chromosome to analyze the cellular changes after lung transplantation. A previous study of transplanted kidneys with chronic allograft reaction using DNA probes of the X and Y chromosomes also showed no reepithelialization or reendothelialization of the donor organ by recipient cells.24

Another aim of our investigation was to study the origin of lymphocytes and macrophages with an important role in rejection of transplanted tissue. Lymphoid tissue is distributed throughout almost the entire lung. Encapsulated lymph nodes are seen adjacent to the trachea and major bronchi to the level of the third-order airway bifurcation. Distal intraparenchymal lymph nodes have been identified in approximately 17% of autopsy specimens. The bronchial-associated lymphoid tissue may have an important role in pulmonary antigen presentation to immune-competent cells. In human beings, the bronchial-associated lymphoid tissue is poorly developed and consists of diffuse collections of subepithelial lymphoid tissue.25 Moreover, there are large numbers of T cells normally present in the human lung, but only a small percentage of B cells.26 Furthermore, Richter et al27 demonstrated that donor lungs contain an increased load of activated mononuclear cells.

We could almost always detect lymphocytes of the donor until PTD 39 in lung biopsy specimens. With exception of 5 biopsy specimens, all remaining specimens obtained after PTD 39 showed only lymphocytes of the recipient sex. These 5 biopsy specimens from 2 female recipients contained small aggregates of donor lymphocytes until PTD 239 and 407.

Biopsy specimens without evidence of infection or rejection showed sparse infiltrates of recipient lymphocytes. Two biopsy specimens additionally contained a few scattered aggregates of bland lymphocytes of the recipient. These results indicate a repopulation of the lung by recipient lymphocytes.

Macrophages exist throughout the lung. They are not confined to the alveoli. Macrophages have been described not only in alveolar ducts and spaces but also in airways, connective tissue, pulmonary capillaries, and the pleural space.28 The AMs previously were thought to be derived exclusively from bone marrow cells circulating in the blood before migrating into the lung.29 However, other studies revealed evidence that in addition to monocyctic migration from the blood, a proportion of AMs arise from proliferation of the interstitial macrophage population.30 Shellito and coworkers31 postulated that within the normal rat, AMs are maintained mainly by cellular proliferation in situ, rather than by monocyte influx from the blood compartment. During acute inflammatory responses, there is an increase in the number of AMs, which is partly due to an influx of blood monocytes and partly to cellular division within pulmonary interstitium.30,32

Our study indicates that the population of lung macrophages consists both of maturing recipient monocytes derived from bone marrow and from resident donor lung macrophages. Whether an interstitial stem cell of macrophages exists or local AMs can proliferate must be evaluated in further studies. Interstitial donor macrophages decreased over time, but few donor macrophages were found until PTD 480.

During the entire study period, ie, up to 781 days after transplantation, AMs were mainly of recipient origin. However, in events of acute rejection or alveolar damage, the number of donor AMs increases, so that resident macrophages and monocytes of the donor lung seem to be capable of proliferation.

O’Connell et al8 demonstrated that good graft function after lung transplantation was associated with a higher number of donor leukocytes, especially AMs in the bronchoalveolar lavage fluid, compared with patients with failed grafts owing to acute rejection or BOS. Our own preliminary results demonstrate that the clinical course of patients with a low portion of donor lymphocytes and a high portion of donor AMs was worse than that of patients with a high portion of donor lymphocytes and a low portion of donor AMs. Our findings fit the theory of Starzl et al33 who hypothesized a graft-vs-host reaction of passenger lymphocytes, which neutralized the host-vs-graft response in a 2-way interaction leading to graft acceptance. Bishop et al34 supported and expanded this hypothesis and proposed a massive, host-derived T-helper 1 response stimulated by donor-derived antigen-presenting cells in the regional lymph nodes of spontaneously tolerant liver allograft recipients, which may lead to clonal exhaustion in low-responder strain combinations.

Our observation that a higher portion of donor AMs seems to be correlated with a worse clinical course is not inconsistent with the theory of the 2-way recipient-donor balance proposed by Starzl et al.33 Our findings reflect the observation that the portion of donor AMs increases during episodes of acute rejection and diffuse alveolar damage. Both alterations are known as possible prognostic indicators for development of BOS.35

Further studies with a greater number of patients need to be done to substantiate our results and to evaluate, if it is possible to obtain data, which factors possibly can predict the clinical course of patients after lung transplantation.

**Conclusion**

After transplantation, no replacement of endothelial or epithelial cells by recipient cells was observed. The lymphocytes of the donor lung were replaced almost completely by
recipient cells about 1 month after transplantation. Small aggregates of donor lymphocytes could be detected until 207 and 403 days after transplantation. AMs of the donor were present during the entire study period, but the greater portion of macrophages was derived from recipient bone marrow cells. This result suggests that AMs of the lung arise partly from a proliferation of resident lung macrophages and monocytes and that a chimeric status of the macrophage population of the lung exists. Low numbers of donor lymphocytes and high numbers of donor AMs in the allografted lung seem to correlate with a worse clinical course and with earlier development of BOS.

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References


