Transfer of Human Myocarditis Into Severe Combined Immunodeficiency Mice

Peter L. Schwimmebeck, Cornel Badorff, Heinz-Peter Schultheiss, Bodo E. Strauer

Abstract Severe combined immunodeficiency (SCID) mice possess neither T nor B lymphocytes and are thus suitable recipients for lymphocytes of different species. Because autoimmune mechanisms are suspected in the pathogenesis of myocarditis (MC), we attempted to determine whether peripheral blood lymphocytes (PBLs) from patients with MC could be transferred into SCID mice and whether they had an autoimmune effect. Groups of three mice each were injected intraperitoneally with up to 50 million PBLs from five MC patients with autoantibodies against the adenine nucleotide translocator (ANT), a myocardial autoantigen. The PBLs from three healthy blood donors were used as controls. After 60 days, human PBLs could be demonstrated in the peripheral blood of the SCID mice transfused with the PBLs of MC patients, representing up to 9.9% of the peripheral blood mononuclear cells. The transfused SCID mice sera showed human immunoglobulin levels of up to 3 mg/mL, both IgG and IgM. Autoantibodies against ANT were present in the mice receiving PBLs from MC patients but not from the control subjects. In addition, infiltrating human lymphocytes were present in the hearts of the SCID mice transfused with PBLs from MC patients. The presence of an ongoing autoimmune process in the SCID mice transfused with PBLs from MC patients is suggested by increased levels of soluble interleukin-2 receptor in the serum in contrast to SCID mice transfused with PBLs from healthy blood donors. We conclude that the autoimmune reactions seen in human MC can be transferred to SCID mice by the transfer of PBLs from MC patients. These findings stress the significance of autoimmune mechanisms in the pathogenesis of human MC.

Key Words • human myocarditis • cell transfer • severe combined immunodeficiency mice

The pathogenesis of human myocarditis (MC) and dilated cardiomyopathy (DCM) is still poorly understood. Enteroviruses, especially serotype B coxsackieviruses, are thought to be the etiologic agents of human MC. This idea is supported both by epidemiologic studies and by the recent demonstration of enteroviral RNA in the myocardium of patients with MC and DCM. In the animal model, MC can be initiated in susceptible mice by infecting them with coxsackievirus B3, strain Nancy. Depending on the genetic background of the animals, the viral MC can develop into an autoimmune-mediated disease. The autoimmune nature of the disease is stressed by the observation that immune-compromised animals do not develop severe MC. Also, in the human disease, several lines of evidence suggest an ongoing autoimmune reaction both on the cellular and humoral levels as the basic pathomechanism in MC and DCM. This evidence includes the demonstration of an increased expression of major histocompatibility complex class I and II antigens within the myocardium of patients with MC and patients with DCM. An increased activation of peripheral blood lymphocytes (PBLs) was demonstrated by measuring the lymphocyte reactivity to the myocardium and the expression of activation markers. On the humoral level, autoantibodies directed against myosin, actin, laminin, vascular endothelium, the β-adrenergic receptor, and the adenine nucleotide translocator (ANT) of the inner mitochondrial membrane were demonstrated. The autoantibodies against ANT were shown to have pathophysiological significance both in vitro and in the animal model. Guinea pigs immunized with ANT exhibit a disturbed energy metabolism in vivo and a diminished cardiac performance, thus suggesting a direct inhibition of energy metabolism by the autoantibodies.

However, until now no experimental model has been available to examine the in vivo significance of the human autoimmune response occurring in patients with MC and DCM. Severe combined immunodeficiency (SCID) mice lack both functional T and B lymphocytes because of a defective differentiation of both T- and B-lymphocyte progenitors. The SCID mutation, which maps to chromosome 16, leads to a lack of functional antigen receptors and allows the engraftment of allogeneic murine cells and even of xenogenic human immune system cells. Thus, it is possible to establish a functional human immune system in the SCID mouse by the intraperitoneal injection of human PBLs. By use of this approach, lymphocytes from patients with a variety of autoimmune diseases, such as systemic lupus erythematosus, primary biliary cirrhosis, rheumatoid arthritis, and Grave's disease, were transferred successfully. The preliminary results derived from these studies are quite promising and indicate that the SCID mouse perfused with human PBLs (SCID-hu mouse) serves as a valuable animal model for the evaluation of human autoimmune diseases.

In the present study, we attempted to transfer the autoimmune phenomena seen in patients with MC into SCID mice and to create a new animal model to evaluate the pathophysiological in vivo significance of the human autoimmune response present in patients...
with MC. Therefore, PBLs from patients with MC were transferred into SCID mice, and the resulting immune and autoimmune phenomena in the mice were monitored at 60 days after transfer.

Materials and Methods

MC Patients and Control Subjects

Five patients with the clinical and immunhistological diagnosis of persistent MC according to the Dallas criteria were included in our study.19 All patients underwent left ventricular catheterization and endomyocardial biopsy. The patients were selected on the basis of the presence of autoantibodies against ANT and a negative Epstein-Barr virus (EBV) serology. Patients with a positive EBV serology were excluded, because a lymphoproliferative disease might occur in the SCID mice after transfer.20 The coronary angiograms of all patients showed no signs of coronary heart disease. All of the patients had a dilated left ventricle with impaired left ventricular function. For control subjects, three healthy blood donors with negative EBV serology and no history of heart disease were used. All patients and control subjects gave their informed consent before blood donation.

Mice

Homozygous male CB-17 SCID mice were purchased from BOM-Mice, Copenhagen, Denmark. All animals were kept in sterile cages under specific pathogen-free conditions at the animal facilities of the Heinrich-Heine University, Dusseldorf, Germany. The animals received sterile mouse chow and acidified water ad libitum. The mice received neither prophylactic antibiotic treatment nor irradiation before transfer. All mice were serologically screened for “leakiness” before transfer, and any mice with murine IgG levels >0.5 μg/mL were excluded because the leaky phenotype is associated with an increased rejection rate of allografts.21 The mice were 6 to 10 weeks of age when the lymphocytes were transferred. Sixty days after transfer, the animals were bled by cardiac puncture under light ether anesthesia and killed for the preparation of heart, skeletal muscle, and spleen.

Cell Transfer

Human blood samples were collected in heparinized tubes, and PBLs were isolated by a Ficoll density gradient (Sera-med). After additional washing, the cells were resuspended in a volume of 0.5 mL of PBS, and 15 or 50 million PBLs were injected intraperitoneally into at least two mice each. Additionally, lymphocyte subpopulations were isolated from 50 million complete PBLs using magnetic beads coated with a monoclonal antibody specific for the CD4 antigen (Dynal). CD4-positive cells were recovered after separation from the beads using Detach-a-Beads (Dynal). Purity of the lymphocyte subpopulations was checked by fluorescence-activated cell scanner analysis (FACS) and was >99%. CD4-depleted PBLs and CD4-positive cells were separately injected intraperitoneally into additional mice.

Cell Culture

Hearts and spleens removed from the SCID mice were gently squeezed with sterile needles and transferred to microtiter plates. The cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 80 mmol/L L-glutamine, 10 mmol/L pyruvate, 10% penicillin/streptomycin (SeroMed), and 20 U/mL human recombinant interleukin-2 (Boehringer Mannheim). The cultures were kept in an incubator at 37°C in 5% CO2.

FACS Analysis

The following monoclonal antibodies conjugated either with fluorescein isothiocyanate or phycoerythrin were used: anti-mouse CD3 (Boehringer Mannheim), anti-human CD45, anti-human CD3, anti-human CD19, anti-human CD4, and anti-human CD8 (all from Dianova). One nonlabeled primary monoclonal antibody from rat specific for mouse CD45 (Boehringer Ingelheim) with a polyvalent phycoerythrin-conjugated secondary antibody was included. All monoclonal antibodies were titrated to an optimal concentration, and the monoclonal antibodies specific for human antigens were checked for cross-reactivity with murine cells. Only direct-conjugated murine monoclonal antibodies were used, because the secondary antibody (anti-mouse IgG) was shown to cross-react with murine cells. Discrimination of nonviable cells was achieved by adding propidium iodide (Sigma) at a concentration of 1 μg/mL. After immune fluorescence staining, red blood cells were lysed with lyzing buffer (Becton Dickinson). The cells were analyzed on an FACS (Becton Dickinson).

Detection of Human Immunoglobulins

Radial immunodiffusion kits (Behring) specific for human IgG and IgM were used. The sensitivity of the tests was 8 μg of human IgG per milliliter and 10 μg of human IgM per milliliter. A standard serum (Behring) was used for the generation of a standard curve. The kits were handled according to the manufacturer’s instructions.

IgG Subclass Determination

As capture antibodies, the following monoclonal antibodies were used: anti-IgG 1 (HP 6012), anti-IgG 2 (HP 6014), anti-IgG 3 (HP 6010), and anti-IgG 4 (HP 6023). The monoclonal antibodies were shown to exhibit good affinity and subclass specificity and were checked for cross-reactivity to mouse IgG.22 Briefly, polystyrene flat-bottom 96-well microtiter plates (Falcon) were coated with the monoclonal antibodies at a dilution of 1:300 in phosphate-buffered saline and incubated at 37°C for 24 hours by use of a previously described method of cross-link fixation.23 After blocking the unoccupied binding sites with bovine serum albumin in a concentration of 1 g/L for 1 hour at 37°C, the sera were added in a suitable dilution and allowed to react for 2 hours at 37°C. Horseradish peroxidase (POD)-conjugated Fc-specific anti-human IgG (Dianova), without cross-reactivity to mouse IgG, was added and allowed to react for 1 hour at 37°C. The substrate solution (0.05 mg/mL O-phenylenediamine [Sigma] and 0.1% hydrogen peroxide [Sigma] in citrate buffer, pH 5.0) was added, and after 30 minutes, the reaction was stopped with 4 mol/L H2SO4. The plates were read on an automated enzyme-linked immunosorbent assay (ELISA) reader at 492 nm. For the generation of a standard curve, a subclass standard calibrated to the World Health Organization 67-97 standard (The Binding Site) was used. Sensitivity was 10 ng/mL. The quantitative lambda-tot kappa ratio assays were performed analogously by use of anti-lambda and anti-kappa light chain antibodies (Boehringer Mannheim). All values were performed in duplicate and showed a good intra-assay correlation.

Determination of Autoantibodies Against ANT

Autoantibody levels against ANT were determined by ELISA. ANT was isolated from beef heart as described previously.24 Before coating the microtiter plates (Falcon), the final Triton X-100 concentration of the antigen solution was adjusted to 0.1% by use of biobeads SM-2 (BioRad) to remove abundant Triton X-100. The plates were coated overnight at 4°C. Free binding sites were blocked with 2% skimmed milk (Merck), and the sera were added in suitable dilutions. After an incubation period of 18 hours at 4°C, the plates were washed, and the secondary antibody, an Fc-specific goat anti-human IgG or IgM POD-conjugated antibody (Dianova), was added. After an additional washing, the substrate solution (0.4 mg/mL O-phenylenediamine [Sigma] and 0.01% H2O2 in citrate buffer, pH 5) was added. The reaction was stopped after 30 minutes with 4 mol/L H2SO4. The plates were evaluated at 492 nm with an automated ELISA reader. All samples
were simultaneously run without antigen to measure nonspecific binding of the antibodies. Nonspecific mouse immunoglobulins did not influence the assay and gave no unspecific background. In additional control samples, a murine Ig-specific POD-conjugated antibody was used.

Detection of Mouse IgG

A commercially available ELISA kit (Boehringer Mannheim) based on the sandwich principle was used. The assay was performed according to the manufacturer’s instructions and showed a detection limit of ≈10 ng mouse IgG per milliliter.

Soluble Human Interleukin-2 Receptor Assay

The commercially available assay (Boehringer Mannheim) is based on the quantitative sandwich enzyme immunoassay principle using monoclonal antibodies directed against two different epitopes of human soluble interleukin-2 receptor. The sensitivity of the assay was ≈4 pmol/L. All samples were tested in duplicate. No cross-reactivity with mouse serum was observed.

Human Tumor Necrosis Factor-α Assay

A commercially available assay (Boehringer Mannheim) was used. In this assay, only the biologically active tumor necrosis factor-α is measured on the basis of the sandwich ELISA principle using monoclonal antibodies. The assay was performed according to the manufacturer’s instructions and yielded a sensitivity of ≈10 pg/mL. No cross-reactivity with mouse serum was observed.

Immunhistology

Frozen sections of 5 μm were prepared from excised organs after snap freezing in liquid nitrogen. All sections were fixed in acetone at 4°C for 10 minutes. The following monoclonal human antibodies were used at an optimal concentration: anti-human CD45, anti-human CD2, anti-human CD3 (all from Dacopats), anti-human CD4, and anti-human CD8 (Coulter). All monoclonal antibodies were checked for cross-reactivity to murine antigens and showed no unspecific binding. Secondary antibody used was a POD-conjugated rabbit anti-mouse IgG antibody. The slides were counterstained with Mayer’s hemalum (Merck) and evaluated by light microscopy.

Statistical Analysis

For statistical analysis, the Wilcoxon test was used.

Results

All SCID-hu mice survived 60 days after transfer without any clinical signs of graft-versus-host disease. In particular, we did not observe any wasting disease, ruffled fur, hunched backs, or necrosis of the tips of the toes.

Human IgG and IgM Levels in the Sera of SCID-hu Mice

All mice reconstituted with 15 or 50 million PBLs developed measurable levels of human IgG and IgM. As shown in Fig 1, the levels of human IgG and IgM were donor dependent, and there was considerable variability even within the groups of mice transfused with PBLs from the same donor. Furthermore, the amount of human IgG and IgM is governed by the number of PBLs transferred. By transferring 50 million PBLs, a higher level of human IgG and IgM can be achieved (P<.001). There were no significant differences concerning the levels of IgG and IgM between the PBLs from the patients and from healthy control subjects.

Polyclonality of the Immunoglobulins in the Sera of SCID-hu Mice

As shown in Table 1, the quantitative IgG subclass distribution in SCID-hu mouse sera corresponds to the pattern of the donor sera, revealing an IgG1 predominance and the presence of all four IgG subclasses. There were no differences due to the source of PBLs (patients versus control subjects) or the number of PBLs transferred to the mice.

The lambda-to-kappa ratios were mostly within the normal interval (ie, 0.33 to 0.66), thus indicating polyclonality of the light chains. The incidence of polyclonality is influenced by the number of cells transferred. Comparing 15 million PBLs with 50 million PBLs, the percentage of polyclonal sera increases from 50% to 75%, as shown in Table 1.

It seems notable that in the group of mice transfused with 50 million PBLs, two of the sera from the mice receiving PBLs from one patient with borderline EBV serology (patient 4) exhibited a skewed lambda-to-kappa ratio. This stresses the importance of the selection of donors with a negative EBV serology.

Presence of Human ANT-Specific Autoantibodies in the Sera of SCID-hu Mice

Sera showing an optical density of >0.1 in the ELISA were considered to be positive. As shown in Table 1, all sera from mice transfused with PBLs from healthy donors showed no significant binding to the isolated ANT. In contrast, the sera from the mice transfused with PBLs from patients with MC contained both human IgG and IgM autoantibodies that bound to the native ANT. Again, the incidence of positive sera and the degree of binding was influenced by the number of PBLs transferred. Comparing sera from mice injected with 50 million PBLs from MC patients with sera from mice receiving 15 million PBLs, the mice having received the higher cell numbers clearly showed higher binding to the isolated ANT. All the antibodies against ANT were of human origin, because tests of the sera with a mouse immunoglobulin-specific second antibody were completely negative.

Levels of Soluble Interleukin-2 Receptor and Human Tumor Necrosis Factor in the Sera of SCID-hu Mice

After the transfer of 15 million complete PBLs, human soluble interleukin-2 receptor could be detected in 63% of the SCID-hu mouse sera with a mean value of 20.6 pmol/L in the positive sera and with a range of 5 to 42 pmol/L. After transfusion with 50 million PBLs, all the sera contained measurable levels of soluble human interleukin-2 receptor, indicating T-cell activation in all animals. The mean values were 81.25 pmol/L with a range of 28 to 180 pmol/L. As shown in Table 1, sera of mice having received PBLs from patients with MC contained markedly higher levels of soluble interleukin-2 receptor compared with mice receiving the same number of PBLs from healthy donors (P<.05).

In addition, the sera were also tested for the presence of human tumor necrosis factor-α. In the animals receiving 50 million PBLs, measurable levels of human tumor necrosis factor were present in seven of eight animals (see Table 1). However, a significant difference
between the levels of human tumor necrosis factor-α between the animals receiving PBLs from patients with MC and from healthy control subjects was not found. Nevertheless, these experiments show the presence of activated antigen-presenting cells, particularly macrophages in the mice reconstituted with PBLs from patients with MC.

**Demonstration of Human Lymphocytes in the Peripheral Blood of SCID-hu Mice**

After the transfer of 15 million human PBLs, human T and B lymphocytes could be detected in 5 of 11 mice, representing 1% to 5% of the total white blood cells. After transfusion with 50 million PBLs, human T and B lymphocytes were present in 8 of 8 mice, representing up to 10% of the total white blood cells. Fig 2 shows a representative FACS profile of cells stained with a mouse and a human leukocyte marker. Approximately half of the human leukocytes in the peripheral blood of the SCID mice were T lymphocytes, with a predominance of CD8-positive cells (see Fig 3). There were no differences concerning the total number or the distribution of lymphocyte subpopulations of human leukocytes in the peripheral blood between the groups of mice that were transfused either with PBLs from patients with MC or from healthy donors.

**Demonstration of Human T Lymphocytes in the Spleens of SCID-hu Mice**

The extent of T-cell migration from the peritoneal cavity (injection site) to the spleen is variable and depends on the number of PBLs transferred. After the transfer of 15 million PBLs, we found human T lymphocytes in the spleens of 63% of the mice, whereas all of the mice had human T lymphocytes in the spleens after the transfer of 50 million PBLs. These cells proliferated after polyclonal stimulation with phytohemagglutinin, showing their proliferative capacity and intactness in the spleen of the SCID-hu mice. These cells could be kept in culture for >60 days. The quantitative evaluation of the immunohistologically stained spleens exhibited a large variability in the amount of human cells, ranging from 1% to 40% of the total number of cells.

**Demonstration of Human T Cells in the Myocardium of SCID-hu Mice**

No infiltrating lymphocytes were detected in the myocardium of the mice after the transfer of 15 million human PBLs. In contrast, after the transfer of 50 million human PBLs, human T lymphocytes were present in the myocardium of all but one mouse. The human lymphocytes could be demonstrated in mice transfused both with PBLs from patients with MC and from healthy control subjects. However, the number of infiltrating lymphocytes was relatively low.
TABLE 1. Human IgG Subtypes, Lambda-to-Kappa Ratio, Presence of Human Adenine Nucleotide Translocator–specific Autoantibodies, and Levels of Human Soluble Interleukin-2 Receptor and of Human Tumor Necrosis Factor-α in the Donor Sera and the Sera of Severe Combined Immunodeficiency Mice at 60 Days After Transfer of Peripheral Blood Lymphocytes From Normal Control Subjects or Patients with Myocarditis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transferred Lymphocytes</th>
<th>Human IgG1, % of IgG total</th>
<th>Human IgG2, % of IgG total</th>
<th>Human IgG3, % of IgG total</th>
<th>Human IgG4, % of IgG total</th>
<th>Lambda/Kappa Ratio</th>
<th>Human IgG-α-ANT, OD</th>
<th>Human IgM-α-ANT, OD</th>
<th>Soluble Human IL-2 Receptor, pmol/L</th>
<th>Human TNF-α, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor sera (n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse sera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>15×10⁶</td>
<td>51.6</td>
<td>32.8</td>
<td>14.1</td>
<td>1.5</td>
<td>0.68</td>
<td>0.150</td>
<td>0.100</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Patient 2</td>
<td>15×10⁶</td>
<td>32.4</td>
<td>55.7</td>
<td>11.3</td>
<td>0.4</td>
<td>0.75</td>
<td>0.131</td>
<td>0.156</td>
<td>21</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Normal 1</td>
<td>15×10⁶</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.060</td>
<td>0.045</td>
<td>ND</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Normal 2</td>
<td>15×10⁶</td>
<td>59.2</td>
<td>25.6</td>
<td>12.8</td>
<td>1.9</td>
<td>0.42</td>
<td>0.038</td>
<td>0.008</td>
<td>ND</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Patient 3</td>
<td>15×10⁶</td>
<td>53.7</td>
<td>35.5</td>
<td>8.8</td>
<td>1.8</td>
<td>0.79</td>
<td>0.031</td>
<td>0.061</td>
<td>22</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Patient 4</td>
<td>15×10⁶</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.053</td>
<td>0.049</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Patient 5</td>
<td>50×10⁶</td>
<td>91.7</td>
<td>5.9</td>
<td>2.3</td>
<td>0.2</td>
<td>0.12</td>
<td>0.188</td>
<td>0.131</td>
<td>180</td>
<td>21</td>
</tr>
<tr>
<td>Normal 3</td>
<td>50×10⁶</td>
<td>53.3</td>
<td>31.4</td>
<td>14.6</td>
<td>0.78</td>
<td>0.63</td>
<td>0.170</td>
<td>0.094</td>
<td>81</td>
<td>22</td>
</tr>
</tbody>
</table>

| #1 | 50×10⁶ | 82.3 | 11.3 | 3.4 | 2.5 | 0.59 | 0.075 | 0.289 | 95 | 16 |
| #2 | 50×10⁶ | 82.5 | 10.1 | 5.1 | 2.1 | 1.89 | 0.211 | 0.126 | 102 | 32 |
| #1 | 50×10⁶ | 66.5 | 22.9 | 8.7 | 1.6 | 0.48 | 0.271 | 0.243 | 80 | 15 |
| #2 | 50×10⁶ | 62.4 | 23.6 | 13.0 | 1.2 | 0.50 | 0.140 | 0.210 | 52 | 13 |
| #1 | 50×10⁶ | 57.3 | 27.9 | 10.8 | 4.0 | 0.41 | 0.038 | 0.041 | 32 | <10 |
| #2 | 50×10⁶ | 50.6 | 42.3 | 3.5 | 2.8 | 0.45 | 0.071 | 0.078 | 28 | 74 |

ANT indicates adenine nucleotide translocator; OD, optical density; IL, interleukin; TNF-α, tumor necrosis factor-α; and ND, not done because of lack of sera.

The left column gives the number of lymphocytes transferred. For the determination of human anti-ANT autoantibodies, an OD of >0.1 at a 1:10 dilution was considered positive.

human lymphocytes was considerably higher in the mice receiving PBLs from MC patients, and only in the mice receiving PBLs from MC patients were focal accumulations of human lymphocytes observed, which is a characteristic sign of inflammation of the heart (see Table 2). There were no differences between the numbers of CD2- and CD3-positive cells, indicating that all human T cells in the hearts were mature. Among the human T lymphocytes, both CD4- and CD8-positive cells could be demonstrated, and in all cases, the CD4-positive cells were predominant compared with the numbers of CD8-positive cells. However, the mice receiving 50 million PBLs from patient 5 (Table 1) showed markedly increased levels of lymphocytic infiltrates compared with the mice transfused with PBLs from healthy control subjects. In addition, mouse 2 reconstituted with peripheral blood lymphocytes from patient 5 showed several foci of infiltrating lymphocytes. A representative example of such a focus is shown in Fig 4.

The organ specificity of these lymphocytic infiltrations was proven by the examination of the skeletal muscle of the SCID-hu mice by immunohistology, which failed to demonstrate a reproducible number of human lymphocytes (<0.1 CD2-positive cells per 10 high-power fields in all specimens evaluated).
Transfer of Human Myocarditis Into SCID Mice

Effects of Depletion of CD4-Positive Cells on the Transfer of Autoimmune Phenomena

We included two patients (patients 4 and 5) and one healthy control subject (control 3) for the additional experiments in which we investigated the influence of CD4-positive cells on the various immune phenomena as described above. In two mice each, we transferred either 50 million complete PBLs or the same number of CD4-depleted PBLs or only CD4-positive cells derived from 50 million cells. After the depletion of CD4-positive cells, we saw a decrease of the human IgG levels by ~90%. In the SCID-hu mice receiving only CD4-positive cells, we saw no detectable IgG levels. Similarly, we found no detectable levels of IgM in the

Fig 2. Demonstration of human leukocytes in the peripheral blood of severe combined immunodeficiency mice injected with human peripheral blood lymphocytes. The figure shows the results of the fluorescence-activated cell scanner analysis after double staining of the cells. The x and y axes give the relative fluorescence of the cells.

Fig 3. Demonstration of human lymphocytes in the peripheral blood of severe combined immunodeficiency mice injected with human peripheral blood lymphocytes. The fluorescence-activated cell scanner analysis after double staining with monoclonal antibodies is shown. The relative fluorescence of the cells is given on the x and y axes.
TABLE 2. Infiltrating Human Lymphocytes in the Hearts of Severe Combined Immunodeficiency Mice After the Transfer of Human Peripheral Blood Lymphocytes

<table>
<thead>
<tr>
<th>Source of Transferred PBLs</th>
<th>Positive Cells, per 10 high-power fields</th>
<th>Focal Infiltrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>0.2</td>
<td>No</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td>Patient 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>0.5</td>
<td>No</td>
</tr>
<tr>
<td>Mouse 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>0.8</td>
<td>No</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>0.9</td>
<td>No</td>
</tr>
<tr>
<td>Patient 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>2.3</td>
<td>No</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>6.1</td>
<td>Yes</td>
</tr>
</tbody>
</table>

PBL indicates peripheral blood lymphocyte.
The sections were stained with a monoclonal antibody against CD3 (pan-T cell marker). The values give the average number of cells positive per 10 high-power light microscopy fields. All evaluations were done in a blinded fashion.

mice receiving either CD4-positive cells or PBLs without CD4-positive cells. These data indicate that in the SCID-hu mice the production of human IgG and IgM is helper T-cell dependent. However, the titers of ANT-specific human IgG autoantibodies were hardly reduced in the animals receiving CD4-depleted PBLs, suggesting that the ANT autoantibodies of the IgG class are obviously produced by spontaneously secreting autoreactive plasma cells (results not shown). After the transfer of CD4-depleted or CD4-positive PBLs into SCID-hu mice, we did not find human leukocytes in the peripheral blood of the animals at 60 days after transfer. In addition, no infiltrating lymphocytes of the myocardium could be seen.

Discussion

In our experiments, we demonstrated that it is possible to transfer the autoimmune phenomena present in human myocarditis to SCID mice by the transfer of human peripheral blood lymphocytes. In the peripheral blood of the SCID mice receiving the human PBLs of patients with MC, we were able to demonstrate at 60 days after transfer both human autoantibodies against ANT and circulating human T lymphocytes. A pathogenic significance of the transferred lymphocytes is suggested by the focal accumulation of infiltrating lymphocytes in the myocardium of the mice receiving PBLs from a patient with MC.

The SCID mouse model was used for transfer experiments in several autoimmune diseases like systemic lupus erythematosus, primary biliary cirrhosis, and rheumatoid arthritis. In these diseases, it was possible to transfer at least the serological findings of the disease into the animals by the transfer of human PBLs. Although experimental data from animal models of MC support a T-cell-mediated mechanism, in human disease several groups suggest a humoral immune response as the pathogenic mechanism. The major advantage of the transfer of human PBLs into SCID mice is that with this model the pathogenic significance of the human T and B lymphocytes can be tested directly. Thus, the human disease can be studied without using an animal model of the disease that may be induced by a different pathomechanism. In addition, it seems feasible to enhance the effect of the autoimmune attack in the SCID mouse by an immunization with the suspected autoantigen, thus demonstrating the significance (or insignificance) of an autoantigen.

The etiology of human myocarditis is unknown, and although an autoimmune etiology initiated by a viral infection is suspected, so far no proof of this hypothesis has been provided. This is due to the problems of transferring immunoglobulins or leukocytes from patients into immunocompetent animals in which the xenogenic transplants have been eliminated by the host’s immune system. In contrast, the SCID mouse
model allows the transfer of human leukocytes that survive in the mouse host for an extended period of time and establish a human immune system in the host. Thus, the immune and autoimmune functions of the transferred leukocytes can be studied. However, as in our experiments, proper control experiments must be performed to exclude possible interactions of human lymphocytes with murine xenoantigens.

In our experiments, both CD4- and CD8-positive lymphocytes were required to obtain measurable levels of autoantibodies in the mice transferred with human PBLs and infiltrating human lymphocytes in the myocardium. Transferring only CD4-positive human cells or CD4-depleted human lymphocytes did not result in a measurable human immune response in the recipients. This suggests that both cellular and humoral immune mechanisms are involved in the pathogenesis of human MC.

Whereas the target of the cellular immune response in the SCID mice is not defined, our own findings suggest, at least in some cases, the ANT to be the target of autoreactive T lymphocytes in humans. However, other autoantigens may also be the target of infiltrating lymphocytes in the myocardium. This issue may possibly be addressed by the transfer of antigen-specific T lymphocytes, thus demonstrating the pathogenic significance of autoantibodies in question. In addition, findings of our own group and others have demonstrated ANT to be one of the main autoantigens recognized by the autoantibodies present in a majority of sera from patients with MC. Also, in the experiments described here, we were able to demonstrate autoantibodies against ANT in the sera of patients with MC, and these human autoantibodies were produced in the mice receiving the human PBLs from patients with MC. Because these autoantibodies were absent in the control subjects and also absent in the mice receiving PBLs from the control subjects, an ongoing immune response leading to the continuous production of autoantibodies against the ANT has to be surmised. These findings suggest the significance of ANT as an autoantigen in the pathogenesis of human MC and DCM. The notion of an ongoing immune response against ANT in the mice receiving PBLs from patients with MC is also supported by the demonstration of increased levels of human soluble interleukin-2 receptor in the sera of the animals. Such increased levels of the interleukin-2 receptor are not only seen in MC but also in other chronic autoimmune diseases. However, the interleukin-2 receptor levels were normal in the mice receiving PBLs from human control subjects, thus excluding a chronic graft-versus-host reaction as the origin of the increased interleukin-2 receptor levels.

However, the exact mechanism of the supposed development of the disease is unknown. It seems quite conceivable that the human lymphocytes demonstrated in the SCID mouse hearts after transfer of PBLs from patients with MC are guided to the heart by the expression of adhesion molecules in the endothelium of blood vessels. However, this issue was not addressed in the present study but is the topic of ongoing experiments.

In our experiments, we found a positive correlation between the number of leukocytes transferred and the presence of human immunoglobulins and leukocytes in the blood of the mice. However, between animals receiving equal numbers of PBLs from the same patients, there also were considerable differences in the engraftment of the human immune system in the mouse. Since there were only minor differences of the site of the injection of the PBLs into the mouse, most of the difference in the effectiveness of the engraftment is probably due to rejection of the engrafted human PBLs by the remaining immune system of the SCID mouse. This was especially obvious in “leaky” SCID mice that did not show any human PBLs at 60 days after transfer of human PBLs. This is also supported by recent studies demonstrating a major enhancement of engraftment efficiency after immunosuppression of the recipient mice by natural killer cell depletion.29

We found infiltrating human lymphocytes in the myocardium of mice receiving PBLs from patients with MC but not in mice receiving PBLs from healthy control subjects. These infiltrating lymphocytes seem to feature quite similar characteristics compared with the lymphocytes seen to occur during the inflammation of the human heart typical for myocarditis. They were also CD3-positive pan-T lymphocytes that were either CD4- or CD8-positive, and they tended to form foci of inflammation.

The antigen specificity of the human lymphocytic infiltrates in the hearts of the SCID-hu mice is unknown to date. However, there are several lines of evidence suggesting that these infiltrating lymphocytes in the myocardium of SCID mice after the transfer of PBLs derived from patients with MC were myocarditis related and not due to an ongoing xenogenic graft-versus-host reaction: First, all mice reconstituted with human PBLs survived 60 days after engraftment without showing any clinical signs of xenogenic graft-versus-host reaction (such as running, ruffled fur, diarrhea, or necrosis of the tips of the toes), whereas an acute xenogenic graft-versus-host disease in the SCID-hu mice is associated with a high morbidity (60% to 100%) and mortality (50% to 100%). Second, the human lymphocytic infiltrates were only observed in the heart and were absent in the skeletal muscle, thus suggesting an organ specificity, whereas a chronic graft-versus-host disease usually runs a multisystemic course. Finally, we exclusively transfused nonleaky animals with human PBLs, whereas a graft-versus-host reaction in the SCID-hu mice seems to be largely confined to engrafted leaky animals.

The mechanism by which human T cells can recognize murine antigens (eg, in the myocardium of SCID-hu mice) is only poorly understood. One possible mechanism could be that human antigen-presenting cells (eg, macrophages), transferred with the T cells, present xenogenic antigens to the autologous lymphocytes. Alternatively, Bach’s group reports that human T cells can respond directly and in an antigen-specific manner to murine antigens in vitro in the presence of human interleukin-1 and interleukin-2 and in the absence of autologous antigen-presenting cells. The authors conclude that the human T-cell repertoire does include the ability to recognize very widely disparate, ie, murine, antigens and that direct recognition can be obtained, provided that human cytokines are available in the system. As in our experiments, the presence of human infiltrating lymphocytes correlated with the presence of human cytokines in the serum of the SCID mice.

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thus, a direct recognition of murine myocardial antigens by human T cells could be possible.

In conclusion, the SCID mouse model appears to be an appropriate in vivo tool to study the significance of the immune reactions present in human MC. Moreover, we demonstrated for the first time that human MC can be transferred by PBLs, clearly providing proof for the autoimmune origin of the disease. This supports the use of immunosuppression as a therapeutic regimen in MC and DCM to suppress the pathogenic autoimmune response in these patients. However, further experiments are necessary to establish the relative significance of different lymphocyte subsets for the induction of the disease.

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