Rapid Publication

Transplantation of Thymic Autoimmune Microenvironment to Severe Combined Immunodeficiency Mice
A New Model of Myasthenia Gravis

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Abstract

To study the role of the thymus in the cellular pathogenesis of myasthenia gravis (MG) we transplanted thymus tissue fragments from MG thymuses beneath the kidney capsule of severe combined immunodeficiency (SCID) mice. Immunocytochemical studies documented that the human thymus tissues are accepted as long-term grafts in the host SCID mice, with human lymphocytes, thymic stroma, and thymic myoid cells demonstrable in transplanted thymus for at least 15 weeks after transplantation. Human anti–acetylcholine receptor antibodies became detectable 1 to 2 weeks after transplantation, and in most chimeras the titers increased over at least 11 weeks to reach levels typically found in severe human MG. Human Ig deposits were detected at skeletal muscle end-plates, demonstrating that the human (auto)antibodies bound to murine acetylcholine receptor. In contrast, transfers of dissociated thymus cells only lead to a transient increase of anti–acetylcholine receptor antibodies. Our data prove that myasthenia gravis thymus is able to induce and maintain autoantibody production in immunodeprived host animals, and that this tissue contains all cellular components required for autoantibody production. Transplantation of solid thymus tissue seems to transfer an autoimmune microenvironment, which will allow direct studies of the mechanism of autosensitization inside the thymus. (*J. Clin. Invest.* 1992. 90:245–250.) Key words: autoimmunity • thymus • autosensitization • acetylcholine receptor

Introduction

Several lines of evidence suggest that the thymus is profoundly involved in the pathogenesis of myasthenia gravis (MG).[1]

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This work is part of F. Padberg's medical thesis at the Ludwig-Maximilians-University, Munich.

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1. *Abbreviations used in this paper:* Abs, antibodies; AchR, acetylcholine receptor; α-BGT, α-bungarotoxin; E, eosin; H, hematoxylin; MG, myasthenia gravis; SCID, severe combined immunodeficiency.

More than 70% of MG patients have a thymus with lymphofollicular hyperplasia, and at least 10% have thymic epithelial neoplasia (1, 2). Patients with recent onset of disease and with hyperplastic thymic changes benefit best from thymectomy (3). In addition to these clinical observations, strong experimental evidence implies the thymus as the primary site of a pathogenic autoimmune response against the neuromuscular acetylcholine receptor (AchR). Thymic myogenic precursor cells are inducible to differentiate in vitro into AchR-expressing myotubes (4–7). In fact, AchR is expressed on thymic "myoid cells" (8), rare muscle-like, HLA-DR–negative cells concentrated at the cortico–medullary junction and in the thymic medulla (9, 10). In MG, but not in the normal thymus, HLA-DR–positive dendritic cells were found in close proximity to myoid cells (8–10). The MG thymus also contains AchR-specific B lymphocytes and plasma cells, that spontaneously produce anti-AchR antibodies (Abs) in vitro (11–13). Finally, AchR-specific T lymphocytes can be readily isolated from thymus tissue of most MG patients investigated (14, 15). Hence, in principle, all the cellular elements required for the initiation of a myasthenogenic autoimmune reaction, i.e., antigen, antigen-presenting cells, autoreactive T and B cells, are present in the MG thymus. The details of the complex interactions between these elements remain, however, largely obscure.

We implanted thymus tissue of MG patients into severe combined immunodeficient (SCID) mice that lack a functional immune system (16) and hence tolerate xenografts (17, 18). We demonstrate that intact MG thymus tissue engrafted in SCID mice contains all elements necessary to produce and sustain high titers of AChR-specific human auto-Abs.

Methods

Patients. All patients had the typical clinical and electrophysiological signs of generalized MG and elevated anti-AchR serum Abs. Therapeutic thymectomy was performed at the Department of Surgery, Martha Maria Hospital, Munich, Germany; the Department of Surgery, Grosshadern Medical Center, Gross-hadern, Munich; and the Department of Pediatric Surgery, Kinderkrankenhaus an der Bult, Hannover, Germany. All MG thymuses used for transplantation showed histological lymphofollicular hyperplasia. Control thymuses were obtained from patients undergoing corrective heart surgery (Professor Sebening, German Heart Center, Munich, Germany).

Animals. C.B-17 scid/scid mice were kindly supplied by Professor St. Thierfelder (Gesellschaft für Strahlenforschung, Munich, Germany). The mice were kept under specific pathogen-free conditions, and used between 5 and 8 wk of age. "Leaky" mice with serum titers of murine Ig > 50 ng/ml were excluded from the experiments (19).

Preparation of thymus and operation. The thymuses transplanted from MG patients showed lymphofollicular hyperplasia without evi-

dence of thymoma. The thymuses from control donors showed no abnormality. 5 to 10 mice were transplanted from each thymus. 6 MG thymuses and 2 normal thymuses were engrafted.

All tissue was processed immediately and always transplanted within 6 h after thymectomy. For engraftment, SCID mice were anesthetized with ketamine, 0.05 mg/g intraperitoneally (i.p.) (Ketanest®; Parke-Davis, Freiburg, Germany) and ether. The kidney was exteriorized and small pieces of thymus (1 mm^3) were placed beneath the renal capsule. The wound was closed with 5-0 vicryl sutures.

To obtain single cell suspensions the thymus was carefully teased manually and then further dissociated using a loosely fitting tissue homogenizer. 1×10^8 thymocytes were injected i.p. in 200 μl PBS.

All procedures were performed under sterile conditions. Blood was sampled from the lateral tail veins.

Reagents. For immunofluorescence the following anti–human mAbs were used: OKT 4 (anti-CD4), L243 (anti-HLA-DR) (both from the American Type Culture Collection, Rockville, MD), T8 (anti-CD8; Coulter Electronics, Krefeld, Germany), Leu 14 (anti-CD22; Becton Dickinson, Heidelberg, Germany), antidesmin (Boehringer Mannheim GmbH, Mannheim, Germany), and KS 1A3 (anti–cytokeratin peptide 13 [20], Sigma Chemical Co., Deisenhofen, Germany). TE3 (anti–human cortical thymic epithelium and fibroblasts) (21) and TE4 (anti–medullary thymic epithelium) (22) were a generous gift from Barton F. Haynes, Department of Medicine, Durham, NC.

Unlabeled and FITC-labeled control mouse IgG1 and mouse IgG 2a/b were purchased from Becton Dickinson, Heidelberg, Germany. Polyclonal goat anti–mouse, goat anti–human, and mouse anti–goat Abs (unlabeled, FITC-labeled, or rhodamine-labeled) were obtained from Dianova, Hamburg, Germany. FITC-labeled α-bungarotoxin (α-BGT) was a product from Sigma Chemical Co., Deisenhofen, Germany.

Immunofluorescence staining. The tissue was embedded in O.C.T. Compound Tissue-TEK (Miles Laboratories Inc., Elkhart, IN) and cryosectioned into 10-μm/sections using a System Dittes-Duspiva Kryostat (Dittes, Heidelberg, Germany). The sections were either used immediately or stored at −80°C until use and fixed in acetone for 5 min at −20°C. For hematoxylin (H) and eosin (E) stains the tissue was fixed in paraformaldehyde for 10 min. The engrafted kidney was serially cut. H & E stains were performed every 150 μm. Fixed sections were blocked with a solution containing PBS, ph 7.2, 2% BSA, 10% heat-inactivated serum from AB+ donors, and 5% heat-inactivated horse serum. Subsequently, mAbs were added at the optimal concentration in the same solution for 30 min. After extensive washing with PBS the fluorochrome-labeled secondary Ab was added. The sections were finally mounted in a glycerol-based mounting medium containing 1 mg/ml p-phenylenediamine. The preparations were examined in a Zeiss photomicroscope Photo III®.

For staining of muscle tissue, the sections were first blocked with a solution containing PBS, ph 7.2, and BSA 2%, and goat anti–human IgG was then added in the same solution. Subsequently, the rhodamine-labeled secondary Ab and FITC-labeled α-BGT were added together in PBS, ph 7.2, containing 2% BSA and 2% mouse serum.

Detection of human anti-AchR Abs. Abs against human AchR were measured with a radioimmunoprecipitation assay (23). Briefly, human AchR was labeled with ^{125}I-α-BGT and incubated with appropriate serum dilutions. Ab were precipitated with anti–human IgG, and the concentration of anti-AchR Abs was calculated from the amount of radioactivity precipitated. Human and mouse IgM and IgG levels were measured using a two-site ELISA. The plates were read at OD 405 using a Titertek Multiskan® (Flow Laboratories, Meckenheim, Germany). Ig standards were obtained from Dianova (ChromPure™). The assay was sensitive to an Ig concentration of 10 ng/ml. Mice were considered leaky if murine Ig concentration was > 50 ng/ml and were excluded from experiments. However, when mice became leaky after transplantation they were observed further. Approximately 30% of the transplanted mice became leaky as indicated in Results.

Results

Histological and immunocytochemical characterization of MG thymus grafts in SCID mice. The thymic grafts were examined after three days and then every two weeks after transplantation for the presence of human lymphocytes, epithelial cells, and myoid cells. In addition, murine spleen, liver, lungs, and the contralateral kidney were stained for human cells. Human cells could not be detected in the peripheral blood or organs of transplanted SCID mice. The grafts were stained with mAbs against MHC Class I (HLA-A, B, C), MHC Class II (HLA-DR), T cells (CD4, CD8), B cells (CD22), epithelial cells (cytokeratin peptide 13), thymic medullary epithelium (TE4), thymic cortical epithelium (TE3), and myoid cells (desmin). Control Abs (IgG1, IgG2a, IgG2b isotype controls, unlabeled or FITC-labeled) gave either no or trace staining in the transplanted thymus. The IgG2a isotype control Ab stained tubular structures in the murine kidney.

Fig. 1, *A–C,* shows an overview of the implants. In the H & E stain (Fig. 1 *A*), the graft can be clearly distinguished from the surrounding kidney without evidence of an inflammatory reaction. Two weeks after transplantation the borders of the grafts were usually organized with evidence of neovascularization. Complementary structures of thymic cortex and thymic medulla were both present in the graft (Fig. 1, *B* and *C*). No areas of TE3-TE4 double positive cells were found (24).

Fig. 2, *A, B,* and *C,* shows a more detailed analysis of the transplants in serially cut sections four weeks after transplantation. The H & E stain shows a germinal center in the grafted thymus. Cytokeratin peptide 13, which was present in all transplants, was demonstrated around the germinal center. The interior of the germinal centers did not stain for epithelial cells. The cells inside the germinal center shown in Fig. 2 were positive for HLA-DR (Fig. 2 *C*). Some of the epithelial cells outside the germinal center also stained with HLA-DR. Among the cells inside the germinal center were many CD22-positive B cells located preferentially at the margin of the follicle. CD4+ T cells were also present at this site (not shown).

We searched for thymic myoid cells using an antidesmin Ab. Thymic myoid cells were sparsely distributed in some transplants. These desmin-positive cells were HLA-DR–negative and did not stain with mAb 35 that binds the α-subunit of human AchR (not shown).

Human anti-AchR Abs are detectable in the serum of transplanted SCID mice. Fig. 3, *A–D,* shows the time kinetics of Ab titers against human AchR after i.p. injection of 1×10^8 thymocytes (Fig. 3, *A* and *C*) and after transplantation of pieces of MG thymus (Fig. 3, *B* and *D*) into SCID mice. The 1-mm^3 pieces of thymus contained ~ 1–2 × 10^6 cells. The upper panel (Fig. 3, *A* and *B*) represents thymus from two patients with low anti-AchR Ab titers (4.5 nmol/liter) and the lower panel (Fig. 3, *C* and *D*) a thymus obtained from a patient with a high preoperative anti-AchR Ab titer (32 nmol/liter). In the mice grafted with thymic cells or tissue from the patient with low anti-AchR Ab titer, anti-AchR Abs were barely detectable after transfer of thymocyte suspensions (Fig. 3 *A*), but rose markedly after transplantation of intact thymic fragments (Fig. 3 *B*). In contrast, in mice grafted with thymic cells or tissue from the patient with high anti-AchR Ab titer, relatively high concentrations of anti-AchR Abs could be detected both in the mice that had received thymocyte suspensions (Fig. 3 *C*) and
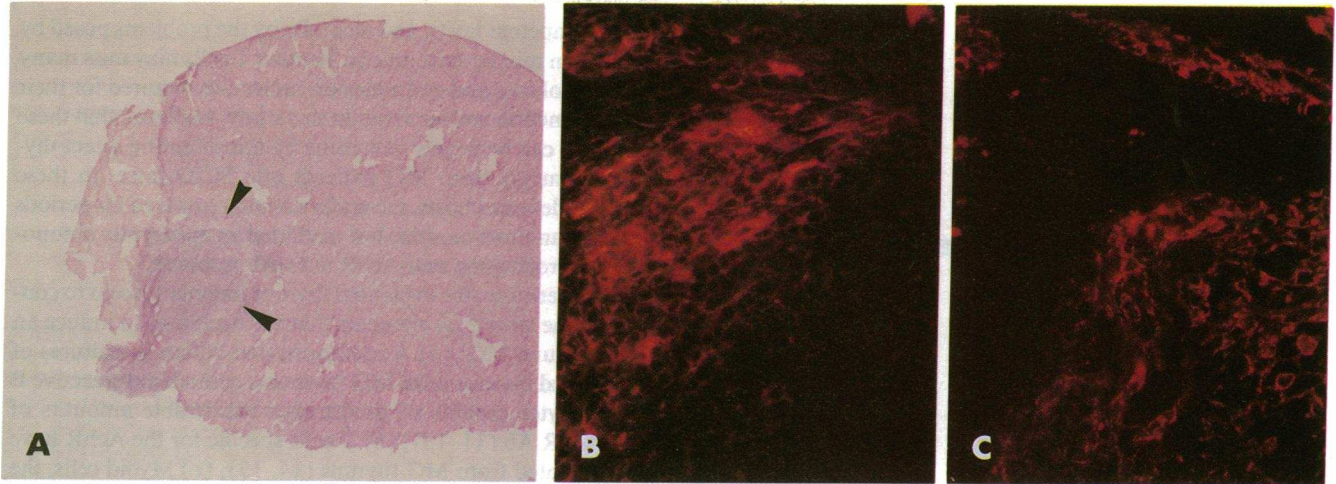
Figure 1. Overview of a kidney with an MG thymus graft placed under the kidney capsule of SCID mice. All stainings in Fig. 1 were performed on sequential sections from the same kidney. (*A*) H & E stain 5 wk after transplantation (×7.5). (*B*) Anti-TE3 (Ab against thymic cortex); indirect immunofluorescence staining (×60). (*C*) Anti-TE4 (Ab against thymic medulla); indirect immunofluorescence staining (×60). (*B*) and (*C*) represent the same field. Complementary structures of thymic medulla and thymic cortex are demonstrated without double positive areas.

in the mice that had received intact thymic microenvironment (Fig. 3 *D*). The peak levels of Ab titers were similar in both groups (Fig. 3, *C* and *D*). Because the mice injected with thymocyte suspensions received 100 times as many cells as did the mice transplanted with intact thymus, the intact thymus was 100 times more effective than the transfer of dissociated thymocyte suspensions. Further, the time course of Ab production differed between the two groups in that the Ab response appeared more slowly in the animals engrafted with thymic tissue (Fig. 3 *D*). In the mice injected with thymocyte suspension, titers began to fall after 3–7 weeks (Fig. 3 *C*). In the majority of

mice engrafted with intact thymus, titers began to fall after 11–15 weeks (Fig. 3 *D*). The long-term fate of engrafted thymus will be examined in future experiments.

Only a small fraction (~ 0.1 to 0.01%) of the human IgG detected in mouse serum was directed against AchR, indicating that only some of the B cells present in the transplanted thymus were specific for AchR. Two to four weeks after transplantation the titers of total human IgG in the mice ranged from 2 to 13 μg/ml.

In the SCID mice engrafted with normal human thymus, human IgG could also be detected, but the titers were generally
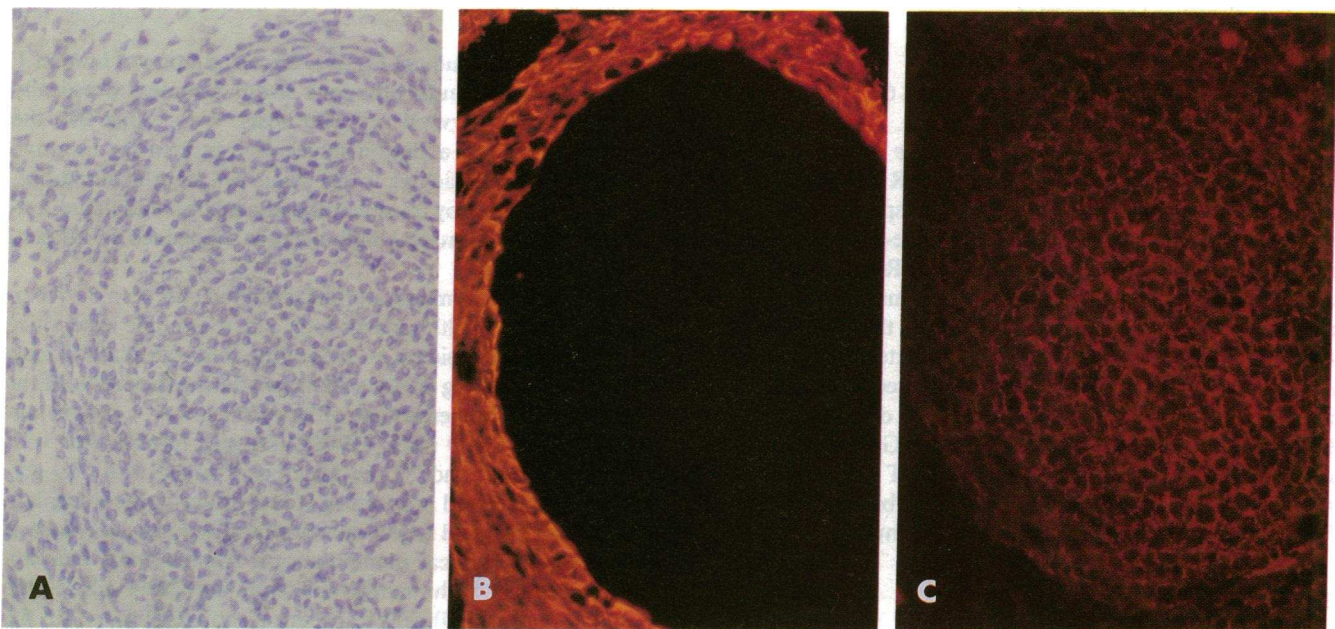

Figure 2. More detailed analysis of an MG thymus graft transplanted under the kidney capsule of SCID mice 6 wk after transplantation. All stainings in Fig. 2 were performed on sequential sections from the same kidney and represent the same field. (*A*) H & E stain of a germinal center, a typical finding in lymphofollicular hyperplasia of the thymus in MG (×75). (*B*) Anti–cytokeratin peptide 13; indirect immunofluorescence staining (×75). Bright staining is seen around the germinal center but not inside. (*C*) Anti-HLA-DR; indirect immunofluorescence staining (×75). The germinal center and some of the surrounding epithelial cells stain brightly for HLA-DR.

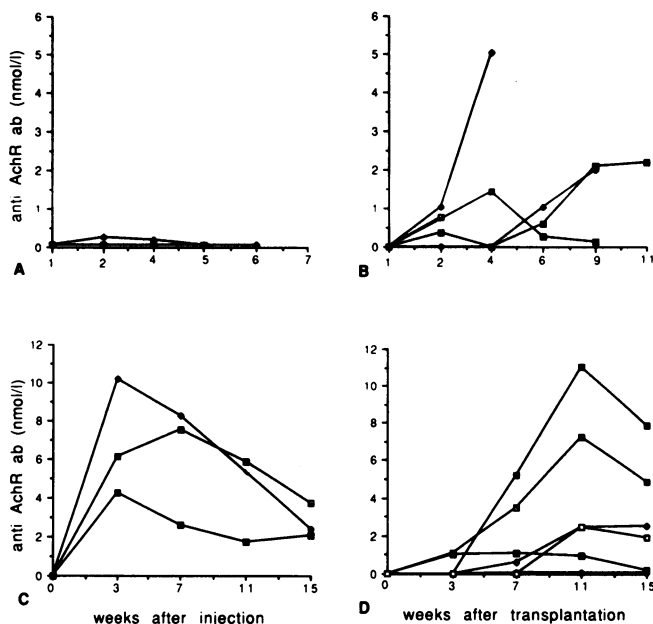*Figure 3.* Kinetics of anti-AchR Ab titers after i.p. injection of 1×10^8 thymocytes from MG thymus into SCID mice (A and C), and transplantation of a 1-mm^3 piece of MG thymus tissue under the kidney capsule (B and D). Each graph represents a group of mice that had received pieces from the same thymus. The material for (C) and (D) was obtained from the same thymus. In the upper panel (A and B), pieces of MG thymus were injected (A) or transplanted (B) from a patient with a low preoperative anti-AchR Ab titer. In the lower panel (C and D) a group of nine mice is shown which received single cell injections (C) or MG thymus transplants (D) from one patient with a high preoperative anti-AchR Ab titer. In (C) anti-AchR Abs were not measured at week 11 in the mouse represented by the black diamond. Anti-AchR Abs were measured by the α-BGT binding assay and given in nmol/liter. On the horizontal axis the weeks after injection (transplantation) are indicated.

lower (0.25–3 μg/ml between weeks 3 and 6). Abs against the AchR were never detected in these mice.

Human anti-AchR Abs bind to mouse muscle endplates. We next asked whether human anti-AchR Abs could be detected at neuromuscular junctions of transplanted mice. Gluteal muscle of transplanted mice was double-stained for human IgG (red), and for the α-subunit of AchR (green). In mice transplanted with myasthenic thymus human Ig could be detected at neuromuscular junctions. Note that the pattern of staining for IgG is very similar to the pattern of staining for AchR (Fig. 4). In control mice transplanted with normal human thymus, muscle endplates could be demonstrated with FITC-α-BGT, but no binding of human IgG was detected. The intensity of fluorescence staining for α-BGT was lower in SCID mice with circulating anti-AchR auto-Abs than in control SCID mice transplanted with normal thymus.

Discussion

The pathogenic role of anti-AChR auto-Abs was established by direct transfer into appropriate recipient mice (25). The cellular mechanisms leading to the generation of pathogenic autoAbs have been less readily accessible to study. Transfer strategies to demonstrate the autoimmune potential of T cells are complicated first by graft rejection responses by (partly) im-

munocompetent hosts, and secondly by the problems posed by the foreign microenvironment. Human T cells may miss many of the cytokines and cell adhesion molecules required for their proper function and survival. In this study, we found that these problems can be largely overcome by transplanting intact thymus fragments from MG patients into SCID mice. In these immunodeficient hosts, the grafts not only survived for periods longer than three months, but mediated an apparently autonomously progressing anti-AchR auto-Ab response.

Furthermore, the explanted thymus fragments seem to contain all the structural elements that are necessary to induce an autoimmune MG: (*a*) As demonstrated before in cultures of dissociated thymus cells, MG thymuses contain autoreactive B lymphocytes capable of producing considerable amounts of anti-AchR Ab (11–13); (*b*) T cells specific for the AchR have been isolated from MG thymus (14, 15); (*c*) Myoid cells, the supposed intrathymic source of AchR, were demonstrated in thymus, often in contact with thymic antigen-presenting cells (8–10).

At least in theory, SCID mice are ideal recipients for human immune cells. They are immunodeficient on the B and T cell level (19), owing to their inability to rearrange lymphocyte receptor genes (26). Indeed, several reports indicated that dissociated human immune cells survived for considerable periods in SCID recipient mice (18), and that these cells continued to produce their (auto-)Ab for some time (27, 28).

In a first set of experiments we injected single cell suspensions of thymocytes obtained from MG (or normal) thymus i.p. into SCID mice. Although human Ig and anti-AchR auto-Abs could be detected within a few days after cell transfer, the transfer of suspensions of dissociated thymocytes was at least 100 times less effective than the transplantion of intact thymic microenvironment. Further, human lymphocytes could not be detected in murine lymphoid organs, indicating that they could not efficiently recirculate and home in the xenogeneic organism.

Like in other studies (17, 29), transplantation of human T cells within their autochthonous stromal context drastically improved their survival in SCID hosts. We assume that the cytokines and cell adhesion molecules required for immune cell survival were lacking in the single cell transfer situations, but were provided by transplanting intact lymphoid tissue containing (auto)immune lymphoid cells embedded in autochthonous stroma.

The human thymus grafts had profound effects on the recipient SCID mice. Although we took care to exclude leaky mice from the graft recipients, ~ 30% of the xenotransplanted mice developed mouse Ig levels > 50 ng/ml during the course of the experiment. The percentage of leaky mice was < 10% in untransplanted SCID mice, 10–20% in mice transplanted with normal thymus, and 70% in mice transplanted with MG thymus. The increased proportion of leaky mice in the transplantation groups could reflect infections acquired in the course of the multiple surgical manipulations. Alternatively, lymphokines produced by human lymphocytes may have induced the immature murine B cells to produce immunoglobulin. In the MG thymus groups the increased proportion of leakiness could be related to the unique properties of the thymic microenvironment in MG (1, 8–10).

Leakiness did not interfere with the success of graft take. All engrafted kidneys contained human tissue at the time of autopsy as shown by the immunocytochemical demonstration of
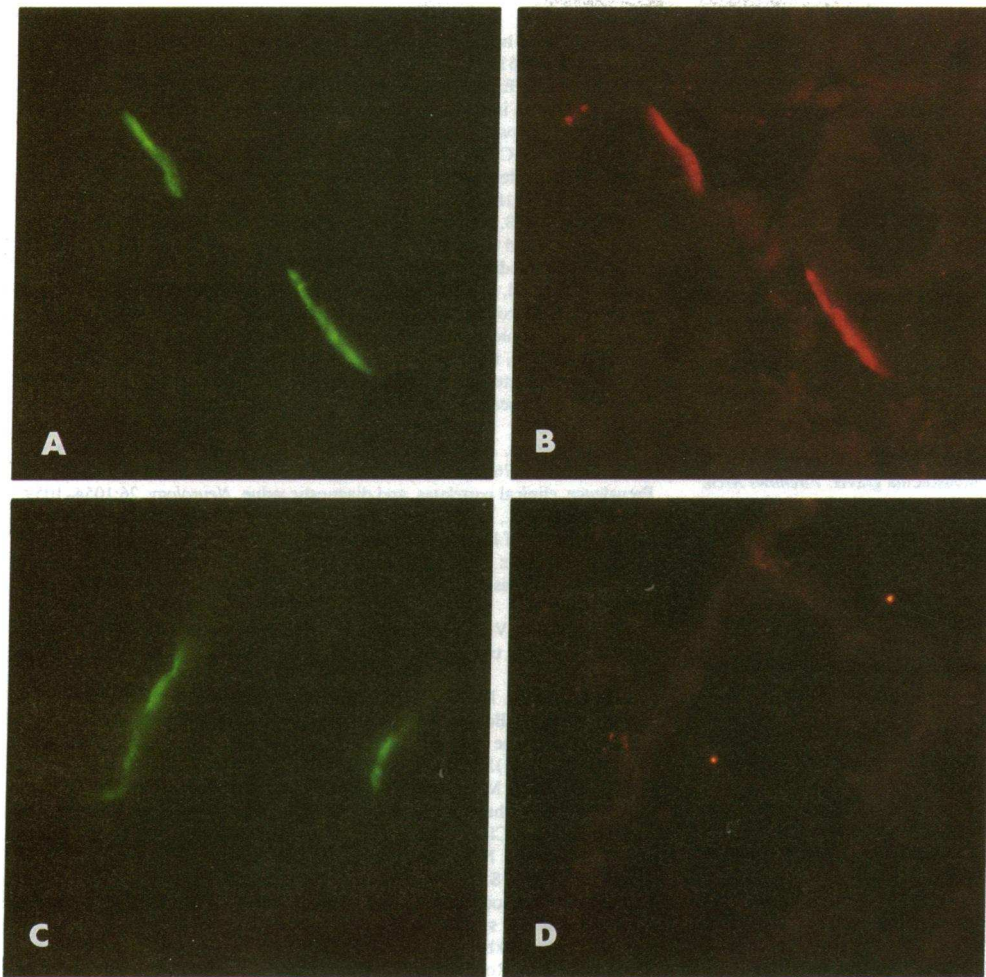
Figure 4. Double staining of muscle endplates of SCID mice with FITC-α-BGT (*A*) and rhodamine-labeled (*B*) anti–human IgG seven weeks after transplantation of human MG thymus (×560). Mice that had received a transplant taken from a control thymus showed staining with FITC-α-BGT (*C*) but not with anti–human IgG (*D*).

cytokeratin and human HLA-ABC. This is in some contrast to data reported by Barry et al. (24). Using a similar experimental protocol, but postnatal normal human thymus, they showed that only 73% of engrafted thymuses could be detected in the kidney at the time of autopsy. Apart from technical differences, the high proportion of "peripheralized" lymphoid tissue typically seen in MG thymuses (8) could account for the discrepancy.

The MG thymus fragments continued to produce human anti-AchR auto-Ab for at least three months. The serum titers reached levels typically seen in human MG patients. It is not surprising, however, that the level of human Ig exceeds the level of human anti-AChR auto-Ab. It is known that MG thymuses not only contain B cells producing anti-AChR Abs, but seem to attract any B cells activated in the course of a recent peripheral Ab response (12).

We found a very notable decoration of neuromuscular endplates with human immunoglobulin. This proves that the human auto-Ab cross-reacted with murine AchR. The fact that the transplanted mice did not show clinical myasthenia is not too surprising, considering the amazing resistance of mice to manipulations of endplate AChR (30). However, in future studies the potential pathogenic effects of the anti-AchR Abs could be analyzed by quantitating endplate specific α-BGT binding, determining C3 and C9 localization, and by examination of the endplate ultrastructure. Further, it will be interesting to see whether at any time endplates are invaded by macro-

phages, as happens in some species after immunization with AchR (2).

This new MG thymus/SCID mouse transplantation model described here provides firm support for the key role of the thymus in spontaneous human MG (4, 5, 14). It should be useful to study the cellular interplay between antigen-producing, -presenting, and -recognizing thymus cells. We anticipate that it will also help to develop strategies for immune intervention at early (afferent) stages of the pathogenesis.

Acknowledgments

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