Efficacy of Pig-to-Rhesus Lamellar Corneal Xenotransplantation

Hyuk Jin Choi,1,2,3,4 Mee Kum Kim,2,3,4 Hyun Ju Lee,5 Jung Hwa Ko,5 So Hee Jeong,5 Jae-Il Lee,5 Byoung-Chol Oh,5 Hee Jung Kang,6 and Won Ryang Wee2,3

PURPOSE. To solve the shortage of donor corneas, a decellularizing method based on hypertonic saline treatment was introduced, and a favorable outcome was observed in pig-to-rabbit lamellar corneal transplantation. This study was an investigation of the efficacy of pig-to-nonhuman primate lamellar corneal transplantation, using both decellularized and fresh porcine corneas to assess feasibility as a substitute for human corneas.

METHODS. Nine Chinese rhesus macaques underwent lamellar corneal transplantation using both decellularized (n = 5) and fresh (n = 4) porcine corneas. Clinically acceptable graft size (7.5 mm in diameter) and minimal immunosuppression based on topical and systemic corticosteroids were applied. Rejection signs, histology of porcine grafts, and serial changes in recipients’ blood profile, including memory T-cell subset, anti-α-Gal and donor pig-specific antibodies, and complement were evaluated. Changes in aqueous complement concentration were also assessed at 4 weeks after transplantation.

RESULTS. Of the decellularized porcine lamellar grafts, 80% remained transparent for more than 6 months, whereas half of the fresh porcine lamellar grafts developed chronic rejection. Rejected grafts showed extensive cellular infiltration, predominantly CDS’ T lymphocytes and macrophages. Immunologic profiles of the recipients with rejected grafts showed a significant increase in the concentration of aqueous complement, an enhancement of memory T cells, and an abrupt increase in donor pig-specific antibodies.

CONCLUSIONS. The findings suggested that decellularized porcine cornea could be a promising substitute for human corneal allograft. Fresh porcine cornea may be a feasible option for a substitute if combined with more potent immunosuppression or if obtained from transgenic pigs with complement-regulatory proteins. (Invest Ophthalmol Vis Sci. 2011;52: 6643–6650) DOI:10.1167/iovs.11-7273

Many Asian countries, including South Korea, are facing a growing problem regarding the shortage of donor corneas. This issue is now beginning to emerge as a worldwide problem, as populations age, as the prevalences of transmissible diseases such as HIV increase, and as refractive and cataract surgeries become more commonplace.

Porcine cornea may be a suitable substitute for the human cornea, because refractive properties and size are comparable with those of the human cornea.1–5 Moreover, pigs are commercially and ethically appropriate donors and have been extensively investigated with respect to the xenografting of various organs.4–12

The most important problems faced in xenotransplantation are how antigenic differences between species can be overcome.4,5,15 Recently, to overcome this antigenic difference, the transplantation of xenogenic substrates lacking cellular components was introduced.14–20 However, for functionally acceptable corneal xenografts, a special decellularizing strategy is necessary, to maintain the transparency of corneal substrates for vision. In an effort to solve this problem, we introduced partial-thickness corneal transplantation using hypertonic saline–treated decellularized porcine corneas and reported a favorable outcome in the pig-to-rabbit corneal transplantation model.18 From a different point of view, since the eye is an immune-privileged organ,21 it seems possible that tolerance may develop to xenocorneal tissues in primates, as has been previously reported.22

Therefore, in the present study, we investigated the efficacy of pig-to-nonhuman primate lamellar corneal transplantation using both decellularized and fresh porcine corneas.

METHODS

All procedures used in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In addition, the primate study protocol was approved by the Research Ethics Committee at Seoul National University Hospital.

Preparation of Donor Porcine Corneas and Study Groups

Porcine corneas were obtained from adult inbred miniature pigs (>3 years of age), which were kindly donated by Yoon Berrm Kim and were bred in the Xenotransplantation Research Center at Seoul National University Hospital. The fresh (n = 4; Fresh group) and decellularized (n = 5; Decell group) corneas were preserved in corneal storage media (Optisol; Chiron Ophthalmics, Irvine, CA) for 2 to 3 days and 5 to 9 days, respectively, before transplantation. Decellularization was performed as described in our previous report.18

From the 1Department of Ophthalmology, Healthcare System Gangnam Center and the 2Xenotransplantation Research Center, Seoul National University Hospital, Seoul, Republic of Korea; the 2Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Republic of Korea; the 3Laboratory of Corneal Regenerative Medicine and Ocular Immunology, Seoul Artificial Eye Center, Seoul National University Hospital Biomedical Research Institute, Seoul, Republic of Korea; and the 6Department of Laboratory Medicine, Hallym University College of Medicine, Anyang, Republic of Korea.

*These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Corresponding author: Won Ryang Wee, Department of Ophthalmology, Seoul National University College of Medicine, 101 Daehangro, Jongno-gu, Seoul 110-744, Republic of Korea; wrwee@snu.ac.kr.

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Orthotopic Lamellar Corneal Transplantation

Nine Chinese rhesus macaques (Orient Bio, Seongnam, Republic of Korea), aged 3 to 4 years and weighing from 4.38 to 5.48 kg were the recipients. Anterior lamellae (n = 9) were incised 312.5- to 375-μm-thick with a 7.5-mm-diameter Barron vacuum trephine (Katena Products, Denville, NJ) and manually dissected from the recipient’s right eye with a crescent knife (Alcon Surgical, Fort Worth, TX). Porcine corneas, which were maintained in Barron artificial anterior chambers (Katena Products), were manually dissected with the same knife to the same thickness and size as the recipient corneas. A lamellar graft was placed in each recipient bed and secured with 16 to 24 interrupted 10-0 nylon sutures (Ethicon, Somerville, NJ). At the end of the surgical procedure, a contact lens (Acuvue Oasys; Johnson & Johnson, Jacksonville, FL) was inserted, and the eyelids were closed with 6-0 black silk for 1 day.

Postoperative Management

Monkeys received levofloxacin 0.5% (Gravit; Santen Pharmaceutical, Osaka, Japan) and prednisolone acetate 1% (Pred forte; Allergan, Irvine, CA) topically once a day. Dexamethasone 1.5 mg/0.5 mL (JW Pharmaceutical, Seoul, Republic of Korea) was injected subconjunctivally weekly for 6 months, and methylprednisolone (Solu-medrol, Pfizer, New York, NY) was injected intramuscularly at an initial dose of 2 mg/kg/d, tapered over 5 weeks, and discontinued at a final dose of 0.25 mg/kg. All sutures were removed within 5 weeks.

Central corneal thickness (CCT) and intraocular pressure (IOP) were measured using an ultrasonic pachymeter (Quantel Medical, Clermont-Ferrand, France) and a handheld tonometer (Tono-Pen; Medtronic Solan, Jacksonville, FL), respectively. Results are the mean of five measurements.

Histopathology

Corneas from the killed recipients were divided into two equal parts, and each portion of the cornea was subjected to hematoxylin and eosin (H&E) or immunohistochemical staining.

To evaluate for the presence of T lymphocytes, B lymphocytes, and macrophages in the corneas, tissue was sliced at a thickness of 4 μm, and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 30 minutes at 97°C in retrieval solution (pH 9.0; Thermo Scientific, Waltham, MA). For the conventional staining procedure, the slides were incubated at room temperature with protein block solution (Thermo Scientific) for 10 minutes and then with the following primary antibodies for 20 minutes: mouse anti-human CD4 (1:20; Thermo Scientific), rabbit anti-human CD8 (1:20; Thermo Scientific), and rabbit anti-human CD68 (1:20; Thermo Scientific).

Postoperative Clinical Evaluations

Grafts were evaluated by slit lamp biomicroscopy. The periods required for complete graft epithelialization were noted, and graft clarity, edema, and neovascularization were evaluated. Clarity was graded as described previously.23 Grafts that showed grade 4 opacity for a week or more were considered to have been completely rejected.

Central corneal thickness (CCT) and intraocular pressure (IOP) were measured using an ultrasonic pachymeter (Quantel Medical, Clermont-Ferrand, France) and a handheld tonometer (Tono-Pen; Medtronic Solan, Jacksonville, FL), respectively. Results are the mean of five measurements.

FIGURE 1. Representative serial photographs and changes in CCT after pig-to-rhesus lamellar corneal transplantation. (A) In the Fresh group (n = 4), two recipients showed graft edema with peripheral neovascularization (red arrows) at ~4 weeks after transplantation, resulting in rejection at 112 days (Fresh 2) and 69 days (Fresh 3) after transplantation, respectively. The other two recipients showed no signs of rejection. In the Decell group (n = 5), all five recipients showed a near-total epithelial defect (yellow arrows) on the day after transplantation, but these defects were completely re-epithelialized by day 5 after transplantation. Four recipients showed no signs of rejection during follow-up, but the remaining recipient (Decell 5), which received decellularized posterior porcine stroma, showed a persistent epithelial defect for more than 3 weeks (yellow arrow head), and eventually the graft was rejected with severe edema and new vessels (red arrows). (B) Changes in CCT. In the Fresh group, all recipients showed the lowest CCT at 1 week after transplantation. Two recipients in the Fresh group (Fresh 2 and 3) showed an abrupt increase in CCT (arrows) at around 3 to 4 weeks after transplantation, and CCT gradually increased as clinical rejection progressed. There was little change in the CCTs of the two grafts that survived long-term (Fresh 1 and 4). Recipients in the Decell group showed graft edema to various degrees on the day after transplantation. CCT then slowly decreased over several weeks (except in Decell 5, in which the graft was rejected). In Decell 5, CCT increased abruptly twice during the course of rejection (arrows). The subsequent reduction in CCT was presumably related to resorption of the matrix. Fresh group received untreated fresh porcine corneal lamellar grafts. Decell group received hypertonic saline–treated porcine corneal lamellar grafts.
(1:100; Thermo Scientific), mouse anti-human CD20 (1:300; Thermo Scientific), and mouse anti-human CD68 (1:200; Cell Marque, Free- mont, CA). The immune complexes were then detected with dextran polymer reagent (UltraVision labeled polymer system; Thermo Scientific) and a slide processing system (Autostainer 720; Thermo scientific).

To confirm the deposition of complement C3c in the corneal tissues, rabbit anti-human C3c antibody (1:100; Dako, Glostrup, Denmark) was used as a primary antibody, and FITC goat anti-rabbit IgG antibody (1:500; Southern Biotech, Birmingham, AL) was used as a secondary antibody. The nuclei of cells were stained with Hoechst 33342 and the distribution of complement C3c was investigated with fluorescence microscopy (Venoz AHBT3/Q; Olympus, Tokyo, Japan).

**Flow Cytometry–Based Blood Immune Cell Assay**

For extracellular surface staining of memory T cells, cell suspensions were incubated for 20 minutes at 4°C with fluorescein-conjugated mouse anti-human antibodies (eBioscience, San Diego, CA) as follows: CD4-FITC (1:200), CD8-PerCp-Cy5.5 (1:200), CD28-APC (1:200), and CD95-PE (1:200). Data were acquired with a flow cytometer (FACScanto; BD Biosciences, Mountain View, CA) and data analysis was performed (FlowJo software; Tree Star, Ashland, OR). All data are presented as the absolute number of cell per unit volume.

**Anti-α-Gal Antibody Assay**

Plasma concentrations of anti-α-Gal IgG/IgM antibodies were determined using enzyme-linked immunosorbent assays (ELISA). In brief, each well was coated with 100 μL of Galα1-3Galβ-APE-HAS (α-Gal-HAS, 5 μg/mL; GlycoTech, Gaithersburg, MD) and incubated overnight at 4°C. Mixtures of recipient plasma (100 μL) diluted in PBS and serial dilutions of selected rhesus monkey plasma containing a high titer of IgG or IgM antibodies against α-Gal (as a calibrator) were incubated at 37°C for 30 minutes. Peroxidase-conjugated anti-human IgG (1:20,000) and IgM (1:2,000) antibodies (Sigma-Aldrich, St. Louis, MO) were used as secondary antibodies. The color reaction was then developed and absorbances were measured at 450 nm. Mean absorbances of the samples were compared with those of the negative control (PBS) and the calibrator. Concentrations of binding antibody were expressed as artificial units (AU)/ milliliter versus the mean absorbance of the undiluted calibrator, which was designated as 1,000 AU/mL.

**Donor Pig–Specific Antibody Assay**

Plasma concentrations of donor pig–specific IgG/IgM antibodies were determined by flow cytometry (FACSCalibur; BD Biosciences). Collected peripheral blood mononuclear cells (PBMCs; 10⁶/100 μL)
were mixed with 50 μL of recipient rhesus plasma and incubated at room temperature for 30 minutes. Antibody-bound PBMC was then detected by incubation with FITC-conjugated F(ab)’2 fragments of rabbit anti-human IgG or IgM antibodies (Dako). Concentrations of donor-specific antibody were semiquantitatively expressed as mean fluorescence intensities (MFIs). MFIs of donor serum were used as negative controls and net MFIs (nMFIs) were calculated by subtracting donor MFIs from respective sample MFIs. Serial postoperative nMFIs of IgG and IgM antibodies were compared with preoperative nMFIs.

**Complement Assay**

Plasma samples were taken at 1, 2 or 3 days and 1 week after transplantation, and aqueous humor was obtained at 4 weeks (n = 7; three of the Fresh group, four of the Decell group). C3a concentrations were evaluated with ELISA kits (OptEIA Human C3a ELISA Kit; BD Biosciences), which use anti-human C3a-desArg monoclonal antibody as a capture antibody and biotinylated anti-human C3a polyclonal antibody as a detector antibody. A standard curve was prepared by adding serial dilutions of a standard stock solution: a high standard of 5 ng/mL. Absorbance was measured at 450 nm after color development. All experiments were performed in duplicate.

**RESULTS**

**Clinical Course**

In the Fresh group, two of the four recipients showed no evidence of rejection during follow-up (>398 and >194 days). However, the other two recipients showed acute graft edema with peripheral neovascularization at 3 to 4 weeks after transplantation. In these two, neovascularization slowly increased and led to graft opacity, which progressed to chronic rejection (Fig. 1A, Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7273/-/DCSupplemental).

In the Decell group, all grafts showed total epithelial defects on day 1, which were confirmed by fluorescein stain and were completely re-epithelialized on days 5 to 8. All four recipients that received porcine anterior stroma showed no sign of rejection during follow-up (>391, >265, >208, and >195 days), whereas the single recipient of porcine posterior stroma had a persistent epithelial defect for more than 3 weeks after surgery, which triggered an inflammatory reaction and eventually led to rejection at 7 weeks (Fig. 1A, Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7273/-/DCSupplemental).

**FIGURE 3.** Immunofluorescence staining for CD4⁺ and CD8⁺ T lymphocytes in rejected versus surviving porcine corneal xenografts. (A, B) CD4⁺ T lymphocytes. (C, D) CD8⁺ T lymphocytes. A rejected graft (Fresh 3; 102 days after transplantation) showed extensive infiltration of CD8⁺ T lymphocytes, although a few CD4⁺ T cells also infiltrated the graft (A, C). On the contrary, a surviving graft (Decell 3; 208 days after transplantation) showed no infiltration of CD4⁺ and CD8⁺ T lymphocytes (B, D). Arrows: positively stained cells. Top images in each panel: ×200 magnification; bottom images: ×400 magnification.
In recipients with rejected grafts, CCT increased in parallel with graft rejection, whereas in recipients with surviving grafts, CCT slowly decreased over several weeks after transplantation to achieve the lowest value (Fig. 1B). IOP was not increased in any recipient, and no systemic or local infection occurred.

**Histopathology**

With H&E staining, rejected grafts showed severe edema, inflammatory cellular infiltration, and complete distortion of stromal matrix, including the recipient’s posterior corneal bed, whereas accepted grafts showed well-organized porcine stromal matrix and indwelling recipient’s keratocytes without any inflammatory cellular infiltration (Fig. 2).

Immunohistochemical staining for inflammatory cells revealed that cells infiltrating the rejected graft were mainly CD8+ T lymphocytes and CD68+ macrophages. CD4+ T lymphocytes and CD20+ B lymphocytes were also found in the rejected graft (Figs. 3, 4). Moreover, the rejected fresh porcine lamellar graft showed extensive deposition of complement C3c, whereas deposition in the surviving decellularized porcine lamellar graft was sparse (Figs. 5A, 5B).

**Immunologic Profiles Associated with Corneal Lamellar Xenograft Rejection**

In the aqueous humor, C3a concentrations were elevated in all tested recipients at 4 weeks after transplantation (Fig. 5C). Furthermore, aqueous C3a concentrations were significantly higher in recipients with rejected grafts (Fig. 5D).

Figure 6 and Supplementary Figures S2 and S3 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7275/-/DCSupplemental) show immunologic profiles of three recipients that had chronic rejection. These recipients shared common immunologic patterns—a very early rise in plasma C3a concentration, a high aqueous C3a concentration, even at 4 weeks after transplantation, increases in memory T-cell populations (especially effector CD8+ T cells) and donor pig-specific antibodies (especially IgG antibody) which closely correlated with the progression in the clinical signs of rejection.

**DISCUSSION**

In the present study, which is the first report to be issued on the immunologic profile changes of decellularized corneal grafts in nonhuman primates, 80% of decellularized porcine lamellar grafts remained transparent without any rejection sign

**Figure 4.** Immunofluorescence staining for CD20+ B lymphocytes and CD68+ macrophages in rejected versus surviving porcine corneal xenografts. (A, B) CD20+ B lymphocytes. (C, D) CD68+ macrophages. A rejected graft (Fresh 3; 102 days after transplantation) showed extensive infiltration of macrophages (C). Of note, many B lymphocytes were also found in the graft (A). On the contrary, a surviving graft (Decell 3; 208 days after transplantation) showed no infiltration of B lymphocytes and macrophages (B, D). Arrows: positively stained cells. Top images in each panel: ×200 magnification; bottom images: ×400 magnification.
for more than 6 months, whereas half of the fresh porcine lamellar grafts developed chronic rejection. However, hyperacute rejection did not occur in either group. These findings suggest that decellularized grafts are more feasible for clinical xenocorneal transplantation than fresh grafts. Histologically, rejected grafts showed extensive cellular infiltration, predominantly CD8+ T lymphocytes and macrophages. Immunologic profiles showed memory T-cell enhancement, complement activation, and variable increases in anti-α-Gal/donor pig-specific antibodies in both groups and recipients that underwent chronic rejection shared common immunologic patterns. These findings provide comprehensive understanding of the mechanism of xenocorneal graft rejection, and suggest that decellularized grafts are not completely exempt from immunologic reactions.

FIGURE 5. (A, B) Immunofluorescence staining for complement C3c. A rejected fresh porcine corneal grafts showed extensive deposition of C3c along with marked infiltration of inflammatory cells (A, Fresh 3; 102 days after transplantation), whereas a surviving decellularized porcine corneal graft showed no deposition of C3c (B, Decell 3; 208 days after transplantation). Cellular nuclei were counterstained with Hoechst 33342. (C, D) Change in aqueous complement C3a concentration after transplantation. Compared to preoperative values, C3a concentrations were significantly increased at 4 weeks after transplantation in both groups (C). At 4 weeks after transplantation, C3a concentrations were significantly higher in the recipients that underwent rejection than in those that did not (D); n = 3 in the Fresh and Rejection+ groups, and n = 4 in the Decell and Rejection− groups; *P = 0.021, **P = 0.05, ***P = 0.034; Mann-Whitney U test. All data represent the mean ± SD. Fresh, received untreated fresh porcine corneal lamellar grafts. Decell, received hypertonic saline–treated porcine corneal lamellar grafts.

FIGURE 6. Changes in anti-α-Gal and donor pig-specific antibodies in three recipients that underwent chronic rejection. There were minimal changes in anti-α-Gal antibodies, while Fresh 2 and 3 showed abrupt increases in donor pig-specific IgG antibodies that correlated with the initiation of clinical features of rejection. Fresh 2, rejection at 112 days after transplantation; Fresh 3, rejection at 69 days after transplantation; Decell 5, rejection at 49 days after transplantation. Fresh, received untreated fresh porcine corneal lamellar grafts. Decell, received hypertonic saline–treated porcine corneal lamellar grafts.
Our outcomes were encouraging compared with other reports issued on corneal xenotransplantation using the pig as a donor.\textsuperscript{22,24,25} Especially, two porcine grafts survived >12 months. In addition the duration of fresh pig corneal graft survival in primates was much longer than that of pig-to-rabbit lamellar corneal grafts in a previous study,\textsuperscript{25} and was comparable to that of pig-to-rhesus lamellar keratoplasty reported by others.\textsuperscript{27} The fact that decellularization procedure increased graft survival supports the notion that keratocytes are important targets during xenograft rejection.\textsuperscript{25,26} Furthermore, the outcomes from the present study are worthy of note in that we simulated the clinical setting by using a normal graft size (7.5 mm in diameter) and minimal immunosuppressive therapy.

Under the histologic evaluation, we directly revealed that the main inflammatory cellular components infiltrating into the rejected graft were CD8\textsuperscript{+} T lymphocytes and macrophages, and complements were extensively deposited in the rejected graft. From a different point of view, we also suggested meaningful immunologic profiles in aqueous humor or blood, by which we could imply posttransplantation immune responses in the local xenocorneal graft and regional lymph nodes indirectly. That is, the Fresh group had a tendency to show more prolonged cellular responses. Although cellular responses were variable case-by-case, most of these responses occurred during the first 3 months. Aqueous C3a concentrations were increased at 4 weeks after transplantation in all recipients, especially recipients that rejected showed significant higher complement activation in aqueous humor. These results suggest that the complement system is also involved in xenograft rejection in primates, which concurs with a previous report on pig-to-mouse corneal transplantation\textsuperscript{27} and support the need to decellularize the porcine corneas. In particular, three recipients that underwent chronic rejection shared common immunologic patterns that correlated with the progression of rejection signs—an increase in memory T cell populations (predominantly CD8\textsuperscript{+} effector memory T cells), an abrupt increase in donor pig-specific (especially IgG) antibodies, and an activation of plasma and aqueous complement. These observations contribute to our understanding of the mechanism of xenocorneal rejection, which includes both cellular and humoral responses, with T cells being a central feature, and provide indications of how the xenogeneic immunologic response may be overcome. Potent immunosuppression targeting T-cell responses during the early postoperative period may be crucial for longstanding graft survival, although systemic therapy may not be optimal in patients with corneal transplants. Alternatively, the use of α1,3-galactosyltransferase knockout (GTKO) pigs\textsuperscript{29,30} or pigs transgenic for one or more human complement-regulatory proteins\textsuperscript{27-31} is likely to enhance xenocorneal graft survival.

The observed increases in cellular and humoral immunity, even in the Decell group, raise questions regarding the nature of the antigen(s) to which these responses were directed. (However, the immunologic responses to α-Gal epitope were much weaker than those in nonhuman primate recipients of pig islet xenografts at our center; Supplementary Fig. S4, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11-7273;/DCSupplemental.) The porcine extracellular matrix may still contain α-Gal, even after decellularization, and the matrix alone may stimulate the host immune responses. However, despite these responses, decellularized grafts showed good survival, suggesting that a partial reduction in the antigen load is beneficial.

Our study has some limitations. First, the sample was small. In particular, in view of the variable immunologic reactions observed in cases, a larger sample with a long-term follow-up is necessary before solid conclusions can be draw. Second, the grafts were of variable thickness. In fact, two thicker fresh grafts, which had a larger immunologic burden, were rejected by rhesus recipients, whereas the other two thinner fresh grafts showed better survival. Accordingly, the determination of an optimal graft thickness appears to be mandatory in future studies. Third, serial immunohistochemical evaluations of xenocorneal grafts and regional lymph nodes are needed to observe more accurately the course of the progression of local rejection.

In summary, our findings suggest that the hypertonic saline–treated decellularized porcine cornea is a promising substitute for a human corneal allograft. In addition, the use of more potent immunosuppression and/or of GTKO pig grafts expressing a human complement-regulatory protein is likely to increase the survival of fresh porcine corneal grafts.

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