Plasma citrulline levels and acute cellular rejection early after small bowel transplantation in pigs

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ABSTRACT: Small bowel transplantations (SBT) are increasingly performed to treat patients with irreversible intestinal failure or short-bowel syndrome. Histologic evaluation of small bowel allograft biopsies is important for the diagnosis of acute cellular rejection (ACR). A reliable serological marker of ACR after SBT is still unknown. Recently, citrulline was identified as a potential biomarker of reduced enterocyte mass. The aim of our study was to analyze rejection and plasma citrulline levels early after SBT in pigs. 24 pigs were used and divided into four groups. Group A, autologous SBT (n = 3) as a control group; Group B, allogeneic SBT with tacrolimus monotherapy (n = 7); Group C, allogeneic SBT immunosuppressed with tacrolimus and sirolimus (n = 8); and Group D, without immunosuppresion (n = 6). The observation period was 30 days. Mucosal biopsies were obtained on Days 0, 3, 5, 7, 10, 14, 20, 28 and simultaneously plasma citrulline levels were measured. ACR was classified according to standardized grading schema on a scale of indeterminate, mild, moderate, and severe. There were no significant differences in citrulline plasma levels between cases with mild ACR and indeterminate for ACR. A significant decline in plasma citrulline levels occurred in cases of moderate and severe rejection. Plasma citrulline levels constituted a marker of more advanced injury of small bowel epithelium.

Keywords: citrulline; small bowel; transplantation

Small bowel transplantations (SBT) are being increasingly performed to treat patients with irreversible intestinal failure or short-bowel syndrome. Acute cellular rejection (ACR) is the major cause of intestinal graft failure after transplantation (Braun et al., 2007). The diagnosis of intestinal ACR requires close correlation of clinical, endoscopic, and pathologic findings. Histologic evaluation of small bowel allograft biopsies is very important for the diagnosis of ACR. ACR which is not treated early can rapidly increase in severity and cause graft failure, sepsis, and death. Ileostomy is used to control the small bowel graft condition with regular obtaining of mucosal biopsy samples for several months after SBT. If the post-transplant course is favorable, the ileostomy is closed within one year from SBT, and the monitoring of the graft function becomes much more difficult. Therefore, there has been an intense effort to discover noninvasive markers to monitor the intestinal graft during the last decade. Recently,

plasma citrulline levels were recognized as a promising marker of small bowel mucosal injury (Pappas et al., 2002; Gondolesi et al., 2006). Citrulline is named after *Citrullus vulgaris* (known as the watermelon) from which it was first isolated 70 years ago (Curis et al., 2005). Citrulline represents one of the most potent "catchers" of the hydroxyl radical, and watermelon accumulates citrulline because this plant has no other way to allow the specific decomposition of the free hydroxyl radical. It is assumed that citrulline mainly comes from the conversion of glutamine in the enterocytes located in the middle and upper parts of the intestinal villi. After that it is released from the enterocytes into the circulation and converted to arginine in the epithelial cells of proximal convolute tubules in the kidneys. Because citrulline is neither present in food (except for watermelon) nor in endogenous proteins, we can assume that the plasma citrulline level depends only on "de novo" synthesis in enterocytes (Collins et al., 2007).

Because ACR represents the major cause of mucosal injury and destruction after SBT, we analyzed rejection episodes and plasma citrulline levels early after SBT in pigs.

MATERIAL AND METHODS

Animals

Pigs weighing 35–40 kg were used according to the Czech Law and the standards for handling laboratory animals as stipulated by Act 246/92 Coll (1992). All the animals were kept in quarantine for 14 days. After that 24 pigs were randomly divided into four groups. Group A, autologous SBT (n = 3) as a control group; Group B, allogeneic SBT with tacrolimus monotherapy (n = 7); Group C, allogeneic SBT immunosuppressed with tacrolimus and sirolimus (n = 8); and Group D, without immunosuppression (n = 6).

Anesthesia and operation

Prior to graft harvesting and/or transplantation, the animals were pre-treated (stresnil 7 mg/kg, ketamine 10 mg/kg, atropine 0.001 mg/kg), with subsequent anesthesia induction (hypnomidate 1 mg/kg + fentanyl 5 ml). Throughout the operation, anesthesia was maintained with a mixture of anesthetics (isofluran 1–2% + fentanyl 0.2 ml/kg) used on a standard basis in our laboratory, with ventilation controlled by an SV Siemens 900 system. Together with pretreatment, all the animals were given veterinary amoxicillin-clavulanate at a dose of 0.05 mg/kg. Another two doses were administred at 24 h intervals after the first dose. Pain in the postoperative course was controlled by analgesia (butorphanol 0.2 mg/kg). Technical aspects of the operation procedure have been described in more detail elsewhere (Oliverius et al., 2009).

Postoperative monitoring and immunosuppression

The observation period was 30 days. Postoperative care included daily monitoring of general condition (appearance of the stoma, weight, temperature, output and nature of stool). All the animals were given 2 l lactate Ringer's and 500 ml 20% glucose solution daily during the three postoperative days. Subsequently, they were given liquid food, and from the 4th or the 5th day they resumed a normal diet. No induction therapy or steroids were used. Monotherapy with tacrolimus was administered in Group B to maintain a serum level of 15 ± 5 ng/ml. Double immunosuppressive therapy with tacrolimus and sirolimus was administered in Group C to maintain a serum level of 7–10 ng/ml for tacrolimus, and 5–10 ng/ml for sirolimus.

Histopathological examination

Mucosal biopsies were obtained on Days 0, 3, 5, 7, 10, 14, 20, and 28. Biopsy samples were fixed in 10% formalin, paraffin embedded, cut at 3–4 μm and stained with hematoxylin-eosin (H&E) and PAS.

The histologic criteria for grading of acute cellular rejection included combination of infiltration by a mixture of mononuclear inflammatory cells, extent of crypt injury, increase in the number of crypt apoptotic bodies and distortion of villous and crypt architecture. ACR was classified according to standardized grading schema on a scale of indeterminate, mild, moderate and severe (Wu et al., 2003;

Table 1. Histological criteria for grading of small bowel allograft acute rejection

Grade	Major histological findings
Indeterminate for ACR (0)	minimal localized inflammatory infiltrate, minimal crypt epithelial injury, increased crypt epithelial apoptosis, none to minimal architectural distortion
Mild ACR (1)	mild localized inflammatory infiltrate with activated lymphocytes, mild crypt epithelial injury, increased crypt epithelial apoptosis, mild architectural distortion
Moderate ACR (2)	widely dispersed inflammatory infiltrate in *lamina propria*, diffuse crypt epithelial injury, increased crypt apoptosis with focal confluent apoptosis, more prominent architectural distortion, no mucosal ulceration
Severe ACR (3)	features of moderate ACR plus mucosal ulceration, possible severe intimal arteritis or transmural arteritis may be seen

Table 2. Survival time and immunosuppression

Group	IS after SBT	Level of Tac (ng/ml)	Level of Sir (ng/ml)	Aproximate survival time (days)
A ($n = 3$)	No IS	–	–	30
B ($n = 7$)	Tac	15 ± 5	–	20
C ($n = 8$)	Tac + Sir	7–10	5–10	25
D ($n = 6$)	No IS	–	–	7

SBT = small bowel transplantation; IS = immunosuppression; Tac = tacrolimus; Sir = sirolimus

Ruiz et al., 2004). The criteria and grading score of rejection are summarized in Table 1.

Citrulline analysis and measurement of the level of immunosuppression

Citrulline levels in blood samples available at the time of the biopsies were measured. Analysis based on ion-exchange chromatography with ninhydrin for citrulline levels was used (Beckman Amino Acid Analyzer, Beckman Coulter, Fullerton, CA, USA). Serum citrulline was determined as described by Gondolesi et al. (2002). Tacrolimus and sirolimus were analyzed from the same blood samples. Tacrolimus levels were measured by Emit® 2000 Tacrolimus Assay and sirolimus by Chemiluminescent Microparticle Immunoassay (CMIA).

Statistics

Variables are presented as means and SEM. Differences among groups were analyzed by Kruskal-Wallis one-way ANOVA followed by the multiple comparison method. The relationship of the plasma citrulline levels to the ACR score was evaluated by the linear trend test in ANOVA. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Survival

Survival time and immunosuppression are summarized in Table 2.

The best survival was attained in Group A, and all animals died within nine days in Group D. Statistically significant differences were demonstrated among Groups A, B, and C in contrast to Group D ($P < 0.01$, $P < 0.05$, resp. $P < 0.01$).

Histology

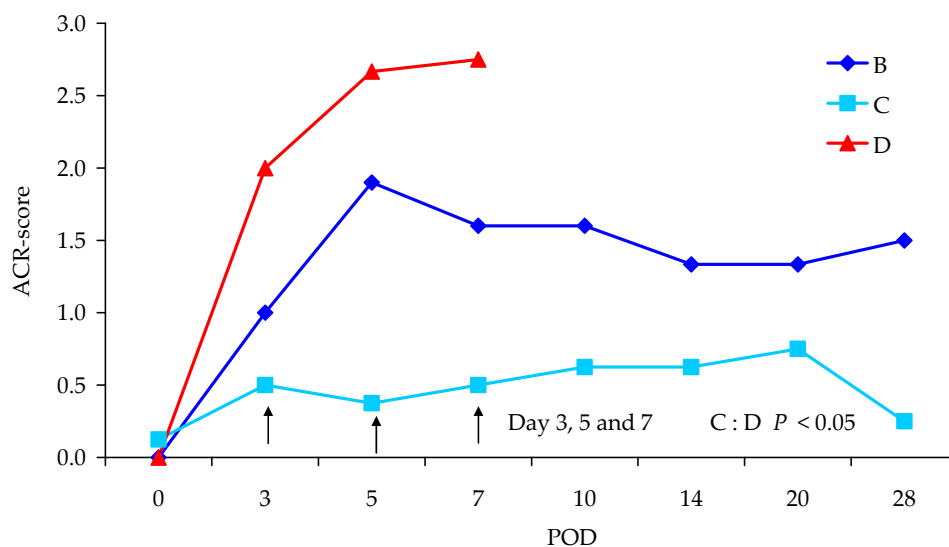153 biopsy samples of small bowel mucosa were evaluated. The results are summarized in Figure 1.

Figure 1. ACR-score in different groups of animals within the observation period

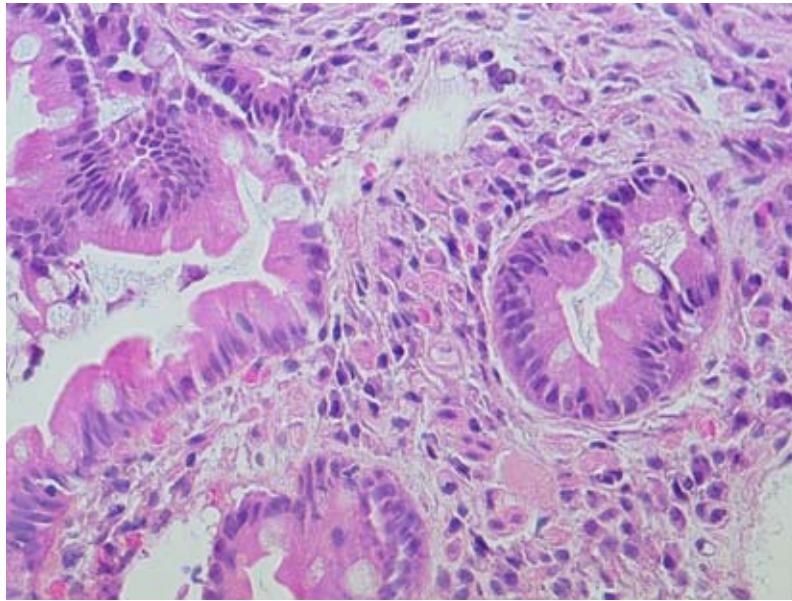ACR = acute cellular rejection; POD = postoperative day

Figure 2. Mild acute rejection is characterized by a mild and localized inflammatory infiltrate in the *lamina propria*. The crypt epithelial cells display evidence of mild injury (H&E, original magnification × 200)

The histopathological evaluation revealed no relevant histopathological changes in the control animals. Mild nonspecific inflammatory infiltrate was present in the mucosa, but no increase in crypt epithelial apoptosis was evident, and morphological features of ACR were absent. There were a lower number of animals with a milder score of episodes of ACR in Group C than in Groups B and D (Figure 2 and 3). Statistically significant differences were observed on the 3, 5, and 7 postoperative day ($P < 0.05$). All animals in Group D suffered from rejection from Day 3 (Figure 4). The episodes of ACR in this group rapidly progressed into severe grade, which was followed by sepsis and the death of the animals.

Plasma citrulline levels

The mean preoperative plasma citrulline level was 40 ± 10 µmol/l, without any significant difference among the groups. The dynamics of plasma citrulline levels is shown in Figure 5. There was a strong decrease in POD 1 seen in all groups, which were followed by a progressive increase in Groups A, B, and C. In Group A, the plasma citrulline level extended as far as the preoperative value on Day 4, and remained stable during the entire observation period. On the other hand, there was a progressive decline in plasma citrulline levels in Group D. Statistically significant differences between Groups
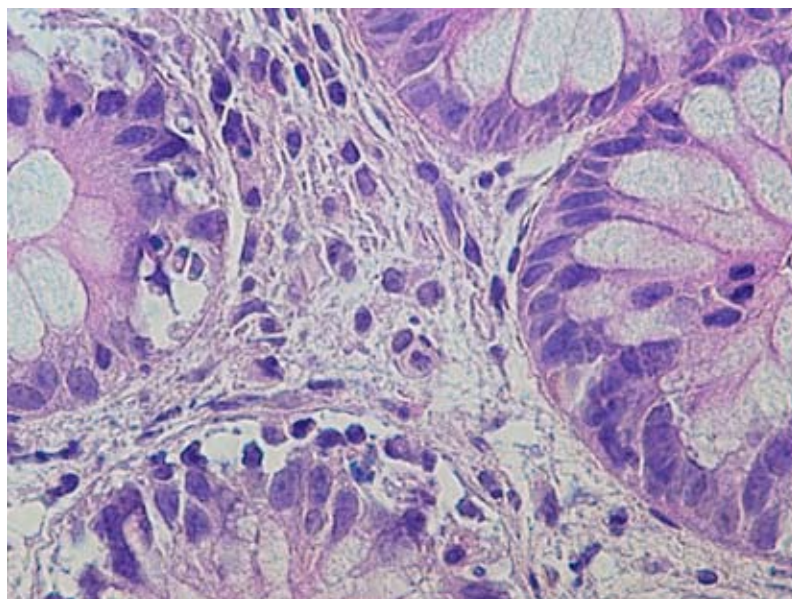

Figure 3. Moderate acute rejection. Crypt damage and apoptosis are distributed more diffusely than in mild acute rejection. The number of apoptotic bodies is increasing, with focal confluent apoptosis (H&E, original magnification × 400)
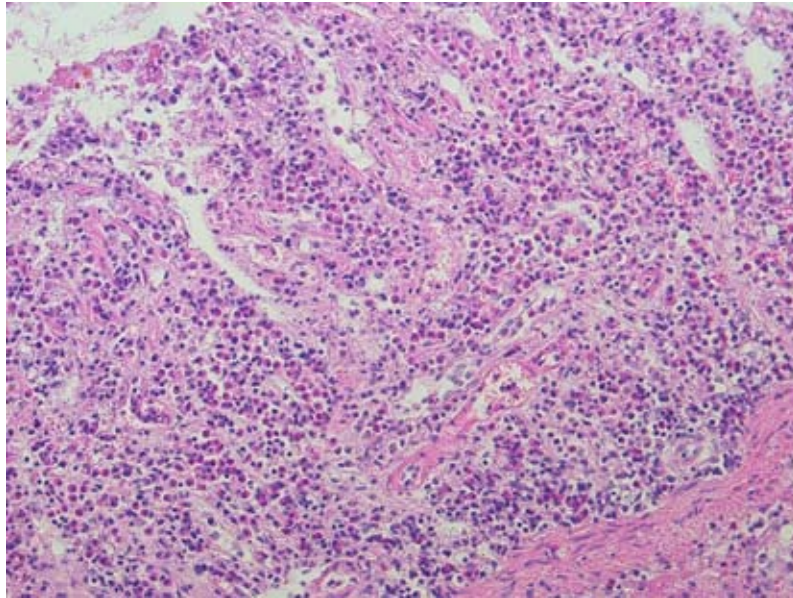
Figure 4. Severe acute rejection is distinguished by marked crypt damage with mucosal destruction (H&E, original magnification × 100)

A and B were observed as early as Day 3 ($P < 0.05$). Between Groups B and D a statistically significant difference was evident on Days 5 and 7 ($P < 0.05$); and between Groups C and D on Day 7 ($P < 0.05$). Group D demonstrated a permanent decrease in plasma citrulline levels to a very low concentration (lower than 10 µml/l) due to the progressive course of rejection. The relationship of the plasma citrulline level to the grade of rejection is shown in Figure 6. A linear trend in the decrease of the average citrulline level ($P = 0.009$) was demonstrated in connection with the grade of ACR (0–3).

DISCUSSION

Small bowel transplantation has become a life-saving therapy in patients with irreversible loss of intestinal function and complications of total parenteral nutrition. Acute cellular rejection is the major cause of intestinal graft failure after transplantation. Because intestinal rejection, if not treated promptly, can rapidly increase in severity, is regularly followed by sepsis, and can result in graft loss, early detection and treatment are essential. In a clinical setting, ACR and infectious
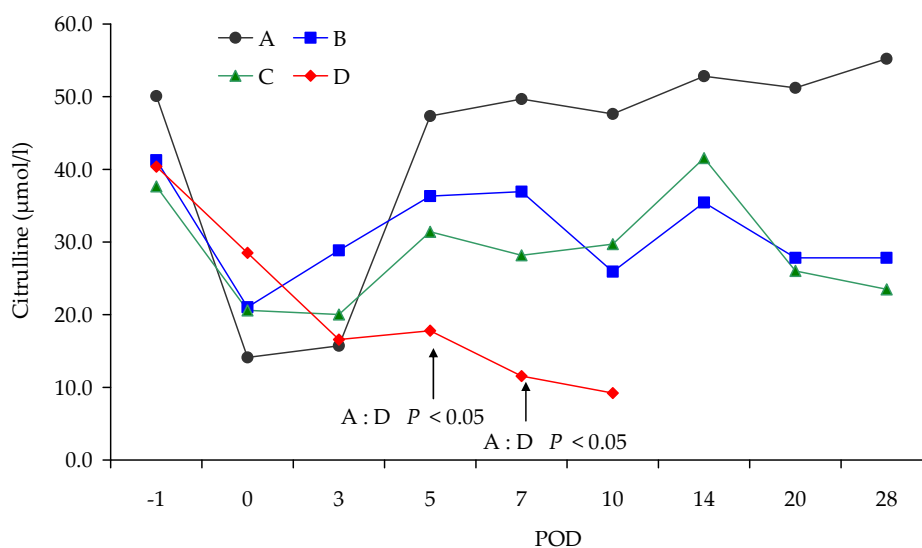

Figure 5. The dynamics of plasma citrulline levels in the groups of animals after SBT
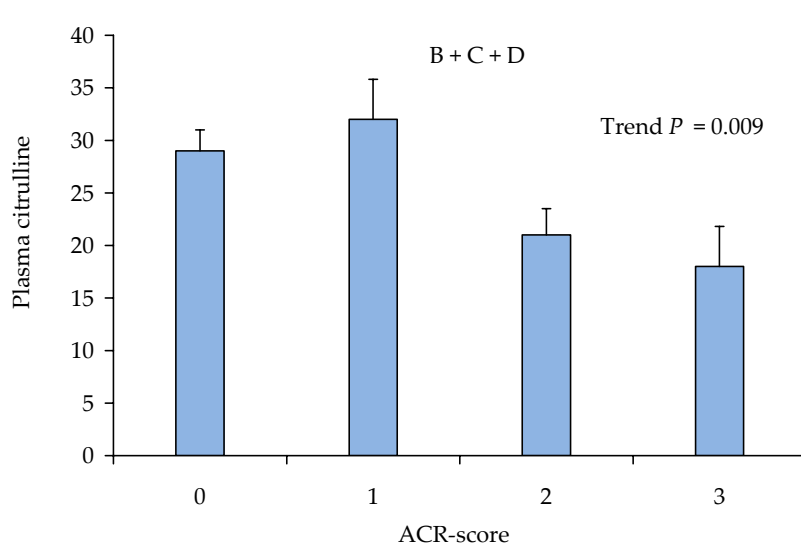
POD = postoperative day

Figure 6. Plasma citrulline levels and grade of rejection

complications with ensuing sepsis are connected, and infectious episodes are often associated with ACR. The cause is the loss of the mucosal barrier during rejection, which makes the bowel susceptible to bacterial translocation with endotoxemia (Cicalese et al., 2001).

Histological evaluation of small bowel biopsies represents the gold standard of diagnosis of rejection. Rejection changes are often inconsistent, and thus multiple biopsy samples are required. Early after transplantation the ileostomy makes it possible to obtain repeated endoscopic biopsies. Within the first year after SBT, ileostomy is closed in cases with good graft function, and the regular monitoring of the graft becomes more complicated. At present there is no powerful noninvasive indicator of acute dysfunction of intestinal grafts. Recently, citrulline, a nonprotein amino acid which is produced from glutamine almost exclusively by enterocytes, especially at the proximal jejunum, was proposed as a very promising biomarker of small bowel mucosal injury. The reason for the use of citrulline lies in its metabolic features: citrulline is incorporated neither into enteral food nor endogenous proteins, and it is not released by proteolysis as are most other amino acids (Curis et al., 2007). In humans, metabolic processes of citrulline have been studied in patients with short bowel syndrome, Crohn's disease, after radiation-induced bowel injury and also after small bowel transplantation (Jianfeng et al., 2005; Luo et al., 2007; Lutgens and Lambin, 2007; Papadia et al., 2007; Crenn et al., 2008). It is assumed that the concentration of citrulline in plasma can be a marker of the number of functioning epithelial cells of the small bowel mucosa.

Recently, in Nadalin's experimental study, the results suggested that measurement of the plasma citrulline level could be useful as a marker of rejection (Nadalin et al., 2007). However, the plasma level of citrulline was not sufficient for identifying a mild form of ACR in this experiment. During rejection, the mononuclear inflammatory cells increased, and the damage to enterocytes was focal and mild in the lower part of the small bowel mucosa at the beginning of rejection. Therefore, it seems logical that mild and focal damage to enterocytes, above all in the lower part of the mucosa, where the synthesis of citrulline is low-level, will not translate into a dramatic change in the plasma level of citrulline. Moreover, in the period shortlyafter SBT, when this experiment was performed, also I/R injury was differentially diagnosed, which at first involved the epithelium at the upper part of the mucosa (where citrulline is predominantly produced). This is why a transitory decrease of the plasma citrulline level is frequent during the first days after SBT.

Also in our experiment, we identified a decrease in the plasma citrulline level in all animals operated on due to I/R injury in the first three days after SBT. During the following two days, the control group with autotransplantation (A), and also both groups treated by immunosuppression (B, C), showed a progressive increase in the plasma citrulline level. On the other hand, the group without immunosuppression demonstrated a permanent decrease in the plasma citrulline level to a very low concentration (lower than 10 μml/l) due to the progressive course of rejection. In our experiment, we were able to chart a linear trend in the decrease of the average citrulline level ($P = 0.009$)

in connection with the grade of ACR (0–3). Our results are very similar to those of Nadalin et al. (2007); we carried out a very similar experimental study at approximately the same time. The main difference was various types of IS therapy. In their experiment there was only one type of immunosuppresive therapy (tacrolimus with steroids), whereas we performed the experiment with two different groups, one with monotherapy (tacrolimus), and the second with a combination of tacrolimus and sirolimus. Both studies showed the plasma citrulline level to decrease differently according to the grade of ACR demonstrated by the subjects: no significant changes in the plasma citruline level during mild episodes of ACR, but significant decreases in the plasma citrulline level during moderate and severe ACR. These data suggest that a decline in the plasma citrulline level correlates more with the extent of mucosal injury during rejection than to rejection per se.

CONCLUSION

Our data demonstrate that the plasma citrulline level reflects the functional integrity of the intestinal mucosa. A decrease in citrulline levels is a characteristic of I/R injury. Changes in the plasma citrulline level did not detect indeterminate or mild ACR in the early phase after SBT, whereas a correlation was established during moderate to severe ACR. Although the diagnostic interpretation of a mild decrease in the plasma citrulline level is difficult early after SBT, at a later stage the same analysis can contribute significantly to diagnostics.

Acknowledgments

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