Histomorphometric Evaluation of Natural Mineral Combined with a Synthetic Cell-binding Peptide (P-15) in Critical-size Defects in the Rat Calvaria

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Purpose: *The objective of this study was to histomorphometrically evaluate the synthetic peptide analog P-15 bound to anorganic bovine mineral (Pepgen/P15) in critical-size defects in the rat calvaria.* ***Materials and Methods:*** *A 5-mm-diameter critical-size defect was prepared in 48 rat skulls and divided into 4 equal groups: Pepgen/P15 particles covered by a membrane, Pepgen/P15 particles uncovered, nongrafted membrane-protected sites, and nongrafted uncovered control sites. At 12 weeks, histomorphometric measurements were made of the percentage area of newly formed bone and residual particles, the length of internal and external bone bridging, and linearly, the regenerated marginal and central total tissue augmentation height.* ***Results:*** *Nongrafted, membrane-protected sites gained 60.6% of newly formed bone, followed by 50.6% and 44.2% (P < .05 versus membrane only) at the grafted covered and uncovered sites, respectively. All experimental sites contained significantly (P < .005) more bone than did control sites (19.9%). In both types of grafted sites, the percentage area of Pepgen/P15 particles was similar. Mean internal and external length of bone bridging at nongrafted membrane-protected sites (76.7% and 71.2%, respectively) was significantly greater (P < .005) than that of the grafted covered (43.95% and 51.8%, respectively), grafted uncovered (28.7% and 23.9%, respectively), and control (28% and 25.5%, respectively) groups, except for internal bone bridging in the grafted covered sites. Regenerated marginal and central augmentation heights (0.92 mm and 1.02 mm, respectively) were greatest in the grafted covered group, followed by the nongrafted membrane-protected (0.88 mm and 0.51 mm, respectively), and grafted uncovered (0.89 mm and 0.12 mm, respectively) groups, all of which were significantly greater (P < .001) than the control group (0.63 mm and 0.04 mm, respectively).* ***Conclusion:*** *While anorganic bovine mineral/cell-binding peptide contributes in volume, membrane application significantly increases the amount of bone regeneration.* INT J ORAL MAXILLOFAC IMPLANT 2008;23:1063–1070

Key words: *bovine bone mineral, cell-binding peptide, critical-size defect, guided bone regeneration, histomorphometry, PepGen/P-15*

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Extensive research has been conducted to find the ideal material to enhance bone repair or regeneration. Alloplasts and/or xenografts used in craniomaxillofacial and orthopedic surgery for bone augmentation and reconstruction offer considerable advantages. Once incorporated, regenerated bone behaves functionally as native bone.[1–4]

In the present study, the osteogenic capacity of a biomaterial—a combination of an organic component reproduction, P-15, and an anorganic bovine bone mineral (PepGen P-15, Dentsply Friadent Ceramed, Lakewood, CO, USA)—was tested. This combined biomaterial (Pepgen/P15) has been used in various bone augmentation procedures. P-15, a synthetic peptide analog of a 15-amino acid sequence within type I collagen, is involved in cell attachment and plays a significant role in bone regeneration.[5–8] In bone augmentation procedures, the addition of a

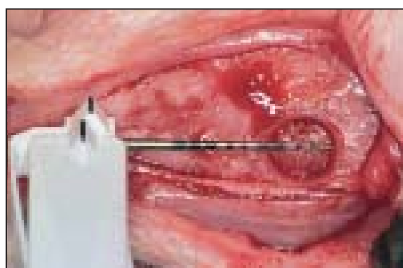


Fig 1a Circular defect, 5 mm in diameter, made in the parietal bone of the rat calvaria.

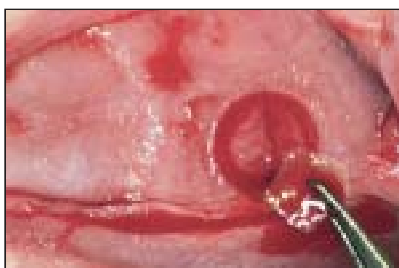


Fig 1b The bottom bone layer of the defect was removed manually using surgical forceps to avoid damage to the dura mater.

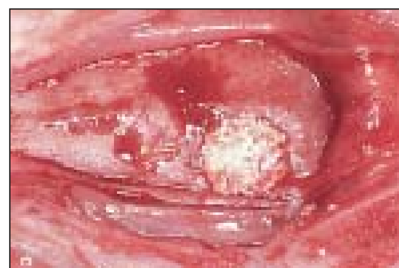
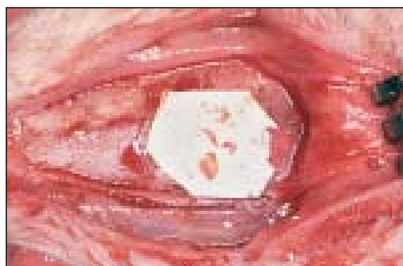


Fig 1c Experimental defect filled with Pepgen/P15 particles.



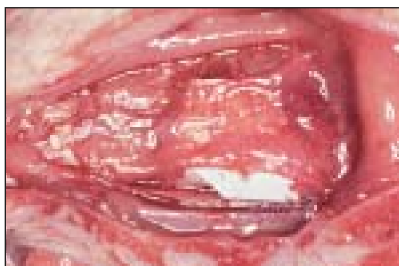


Fig 1d *(Left)* Bilayer collagen membrane used to completely cover the grafted site (Pepgen/P15-mem group).

Fig 1e *(Right)* Periosteum repositioned over the experimental site.

collagen mimetic to an established osteoconductive bone replacement material could upgrade its properties. Although this material fulfills its tasks in clinical use,[9–24] only a few controlled histomorphometric studies[25–27] have validated the efficacy of this filler.

A critical-size defect is the smallest bone wound that is not spontaneously healed by bone formation during the animal's life.[28] Since the control defect shows incomplete healing, it is an appropriate model to test biomaterials.[29] A 5-mm-diameter defect in the rat calvaria fulfills the criteria for a true critical-size defect.[30–40]

The aim of this study was to histomorphometrically evaluate osseous tissue healing in a critical-size defect in rat skulls grafted by Pepgen/P15 with and without a covering of a guided tissue regeneration (GTR) membrane.

MATERIALS AND METHODS

The Ethics and Institutional Animal Care and Use Committees of Tel Aviv University approved the study protocol. The study included 48 albino Wistar rats, between 4 and 5 months old, weighing approximately 300 g. Surgery was successfully completed, and healing was uneventful in 48 of the 50 rats, which were randomly allocated to 4 groups of 12 animals each. Animals were housed in plastic cages at a temperature of 22°C, with 12-hour light/darkness cycles and free access to tap water and a standard laboratory diet.

Animals were anesthetized using an intraperitoneal injection of ketamine chlorhydrate (Rhône Merieux, Lyon, France) at 90 mg/1 kg body weight and 2% xylazine (Vitamed, Bat-Yam, Israel) at 10 mg/1 kg body weight. The dorsal part of the skin covering the scalp was shaved and aseptically prepared for surgery. A no. 15 Bard Parker knife was used to make a U-shaped incision in the scalp between the eyebrows caudally connecting 2 sagittal incisions that extended posteriorly over the parietal bone. This enabled elevation of a full-thickness flap exposing the parietal bones. Soft tissues and periosteum were raised in 2 layers. In the midportion of one of the randomly selected parietal bones, a 5-mm-diameter critical-size defect was prepared with a water-cooled high-speed diamond wheel (Strauss, Ra'anana, Israel). Final dimensions of the defect were measured clinically with a periodontal probe (Fig 1a). At the bottom of the defect, bone was retrieved with special surgical forceps to avoid damage to the dura mater (Fig 1b). Thus, a standardized "through-and-through" defect was made, exposing the entire dura mater. During surgery, care was taken not to injure the midsagittal suture and superior sagittal sinus.

One of the following 4 modalities was used to treat each site: *(1)* application of 0.5-mm^3 Pepgen/P15 particles (one half of the vial) suspended in injectable hyaluronate hydrogels (PepGen P-15 Putty, Dentsply Friadent Ceramed) (Fig 1c) followed by coverage with a resorbable collagen membrane (BioGide, Geistlich Biomaterials, Wolhusen, Switzerland) (Pepgen-mem) (Fig 1d); *(2)* application of Pepgen/P15 putty without

membrane coverage (Pepgen); *(3)* a nongrafted site filled with blood clot and membrane coverage (Mem); and *(4)* a nongrafted, uncovered site filled with blood clot to serve as a control. Subsequently, the periosteum was carefully repositioned without suturing (Fig 1e), followed by suturing of the scalp tissues with 4-0 polyglactin resorbable interrupted sutures (Vicryl, Ethicon, Johnson & Johnson, Somerville, NJ, USA).

Twelve weeks after surgery, rats were euthanized and the calvariae were removed for histologic processing.

Histologic Processing

The calvariae were fixed in 10% neutral buffered formalin for 7 days. Subsequently, specimens were dehydrated with a graded series of ethanol for 9 days, infiltrated with a light-polymerized embedding resin (Technovit 7200 VLC, Heraeus Kulzer, KG, Hanau, Germany) for 20 days with constant shaking at a normal atmospheric pressure, and polymerized using 450-nm light at 40°C. Blocks were sectioned using the EXAKT cutting/grinding method (EXAKT Technologies, Oklahoma City, OK, USA) as described by Donath and Breuner.[41] Briefly, 150-μm-thick sections were polished to a 40-μm thickness using the EXAKT microgrinding system followed by alumina polishing paste, and then stained with Stevenel's blue and Van Gieson's picro fuchsin. Three sections were removed from each specimen, and the most central and complete section was selected for histomorphometry using a 5-mm ruler superimposed on the image.

Histomorphometric Analysis

Sections were examined and photographed (× 25 magnification) with a Zeiss Axiolab photomicroscope (Carl Zeiss Microimaging, Thornwood, NJ, USA).

For each section, the borders of the defect were identified by an abrupt cessation of the original lamellar bone and the continuation of new, less organized bone. The inferior border of the defect was defined as a continuation of the inferior borders of the original bone surrounding it.

Histomorphometric measurements were performed with the Bioquant Nova Image Analysis System (Bioquant Image Analysis Corp, R&M Biometrics, Nashville, TN, USA). The following parameters were measured:

1. Percentage area of newly formed bone matrix.
2. Percentage area of the residual filling material (%Pepgen/P15).
3. Length of the internal (inferior) and external (superior) bone bridging formation as percentage of the defect width (diameter) (Fig 2a).
4. Regenerated augmentation height as measured at 4 points: 2 points 1 mm internally from the defect margins and 2 points 1 mm apart in the center of the defect. Each pair of measurements was averaged to yield the mean central and marginal heights. These heights were composed of calcified osseous tissue, grafted biomaterial, and/or the interface soft tissue (Fig 2b).

Statistical Analysis

Data are presented as mean ± SE. Differences between group means were evaluated using 1-way analysis of variance or a nonparametric Kruskal-Wallis test, followed by multiple comparisons using either the Tukey honestly significant difference or Dunnett T3 tests.

RESULTS

Newly formed bone was evident primarily in the membrane-protected sites regardless of grafting (Figs 2b and 2c). Newly formed bone was also evident to a lesser degree at the Pepgen/P15 uncovered sites (Fig 2d), and only minimally at the control sites (Fig 2e).

At the Pepgen/P15-mem sites (Fig 2b), new bone was abundant, whether in direct contact with the grafted particles or in proximity to soft connective tissue. Direct bone-particle contact was more evident within the deep layers close to the dura mater rather than within the outer (external) layers of the defect. However, newly formed bone was frequently observed at the most superior aspect of the defect, under the resorbable membrane. In fact, grafted particles and regenerated bone established complete hard tissue augmentation.

At the Pepgen/P15 uncovered sites (Fig 2c), newly formed bone and incomplete bridging were observed primarily at the defect margins. Only a few of the grafted particles were surrounded by new bone in direct contact, and most were surrounded by soft tissue. Thus, only partial bone bridging was obtained primarily at the deep layer. In both types of grafted particle sites, there were no multinucleated cells (osteoclasts).

At the membrane-covered, nongrafted sites (Fig 2d), newly formed bone was abundant, along with remarkable internal and external bone bridging. However, the regenerated hard tissue volume of the healed parietal bone was smaller than that of grafted-covered sites, primarily in the defect center.

At the control sites (Fig 2e), no complete osseous healing was observed in any of the examined sections. Newly formed bone was noticeable only at the

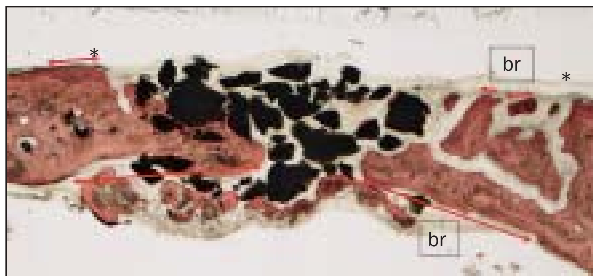


Fig 2a Partial internal and external bone bridging (br) and, remarkably, no external bridging in the Pepgen/P15 uncovered (*) site (Stevenel's blue and Van Gieson's picro fuchsin; × 25 original magnification).

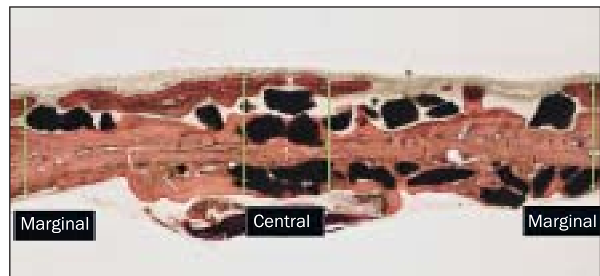


Fig 2b Nondecalcified section of the Pepgen/P15-mem group. Newly formed bone surrounds Pepgen/P15 particles. Arrows illustrate vertical bone height at the marginal and central regions of the regenerated site (Stevenel's blue and Van Gieson's picro fuchsin; × 25 original magnification).

Fig 2c At the Pepgen/P15 uncovered sites, only a few particles, mainly proximal to the original osseous margins, surrounded by newly formed bone (Stevenel's blue and Van Gieson's picro fuchsin; × 25 original magnification).

Fig 2d Complete bone bridging evident at the Mem sites (Stevenel's blue and Van Gieson's picro fuchsin; × 25 original magnification).

Fig 2e Lack of bone bridging evident at the healing phase of the control group (Stevenel's blue and Van Gieson's picro fuchsin × 25 original magnification).

margins, with no significant internal and external bone bridging.

Morphometrically, the amount of new bone was significantly affected by the treatment modes. Membrane-only (Mem) sites presented the greatest amount of new bone percentage area (60.6% ± 4.5; Table 1, Fig 3), followed by the Pepgen/P15-mem sites (50.6% ± 4.4) and Pepgen/P15 only sites (44.2% ± 5.5). Control sites contained the least amount of new bone (19.9% ± 3.4). Differences were significant between the 3 treated sites and control sites. The percentage area of new bone at the Mem-type defect was significantly ($P < .05$) greater than that at the Pepgen/P15 type defect, but not compared with the Pepgen/P15-mem defects. New bone was slightly but not significantly greater in the Pepgen/P15-mem sites than in Pepgen/P15 sites.

The percentage area of residual grafted biomaterial was similar in both types of grafted sites: 28.4% ± 2.6 at the Pepgen/P15-mem sites and 29.7% ± 4.4 at the Pepgen/P15 sites (Table 1, Fig 3).

Bone bridging across the critical-size defect behaved in a similar manner to the percentage area of new bone within the defect: Mem sites showed the greatest amount (76.7% ± 4.1 and 71.2% ± 5.1 for internal and external bridging, respectively), followed by Pepgen/P15-mem sites (43.9 ± 7.8 and 51.8 ± 8.6) and Pepgen/P15 sites (28.7 ± 5.3 and 23.9 ± 9.8). The latter resembled the control sites (28 ± 6.5 and 25.5 ± 4.3). Both types of bridging were significantly greater in the Mem sites compared with all other defect types, except for the mean external bridging of the Pepgen/P15-mem sites (Fig 3). The finding that bridging at Pepgen/P15 sites was not significantly greater than that in the control sites is noteworthy.

Table 2 shows the vertical augmentation height of the various defects. At the margins of the defect, all types of treatment resulted in significantly greater augmentation height (0.92 mm ± 0.03 for Pepgen/P15-mem, 0.89 mm ± 0.03 for Pepgen/P15, and 0.88 mm ± 0.05 for Mem) compared with control sites (0.63 mm ± 0.04). Differences between the 3

Table 1 Percentage Area (Mean ± SE) of Bone and Grafted Particles (Pepgen/P15) and Internal and External Bone Bridging in Critical-Size Defects in Rat Skulls*

	Pepgen/ P15	Pepgen/ P15-mem	Mem	Control
New bone	44.2 ± 5.5[a,c]	50.6 ± 4.4[d]	60.6 ± 4.5[b,c]	19.9 ± 3.4[a,b,d]
Pepgen/P15	29.7 ± 4.4	28.4 ± 2.6	NA	NA
Internal bone bridging	28.7 ± 5.2[b]	43.9 ± 7.8[a]	76.7 ± 4.1[a,b,d]	28.1 ± 6.5[d]
External bone bridging	23.9 ± 9.8[d]	51.8 ± 8.6	71.2 ± 5.1[b,d]	25.5 ± 4.3[b]

*Values with identical letters are significantly different: a = $P < .005$; b and d = $P < .001$; c = $P < .05$.
NA = not applicable.

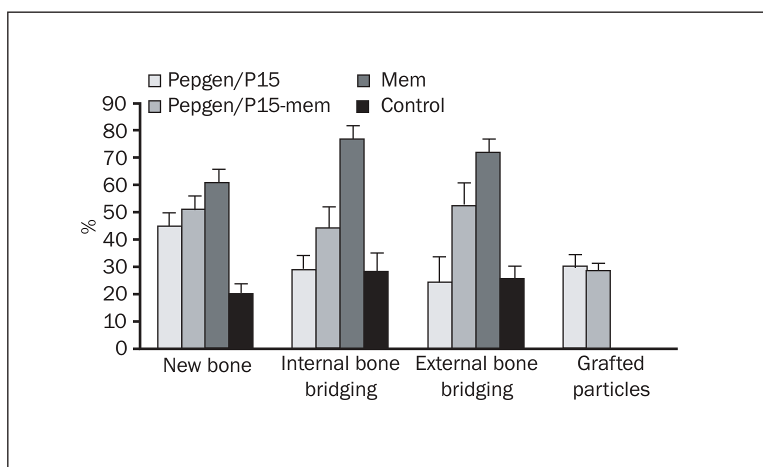


Fig 3 Percentage of new bone, internal and external bone bridging, and grafted particle percentage areas at the different sites. Values of significance are described in Table 1.

Table 2 Mean (± SE) Vertical Augmentation Height (mm) in the Marginal and Central Zones of Critical-Size Defects in Rat Skulls*

Group	Marginal	Central
Pepgen/P15	0.89 ± 0.03[a]	0.12 ± 0.04[a]
Pepgen/P15-mem	0.92 ± 0.03[b]	1.02 ± 0.09[a,b,c]
Mem	0.88 ± 0.05[d]	0.51 ± 0.07[b,d]
Control	0.63 ± 0.04[a,b,d]	0.04 ± 0.01[c,d]

*Values with identical letters are significantly different ($P < .001$).

treated sites were not significant. In contrast, remarkable differences were found in the central zone of the defect.

The central regenerated height was greatest in the Pepgen/P15-mem group (mean 1.02 mm ± 0.09; Table 2), followed by the Mem group (0.51 mm ± 0.06) and the Pepgen/P15 group (0.12 ± 0.04), which resembled the control group (0.04 ± 0.01). The central regenerated tissue height in the Pepgen/P15-mem group was significantly greater than in all other groups ($P < .001$), and that of the Mem group was significantly greater than that of the Pepgen/P15 and control groups.

DISCUSSION

In all control sites, complete bone healing was not observed. Thus, a 5-mm calvarial defect in adult rats fulfills the criteria for a critical-size bone defect,[30–40] and has been used to examine numerous types of bone graft substitutes.[28,29,34,38,42–44] In the present study, this model demonstrated the capacity of Pepgen/P15 to regenerate bone, primarily in a membrane-protected environment.

Bone replacement materials were examined in a critical-size defect in the rat calvaria for different healing periods, ranging from 4 to 18 weeks.[38,45–50] Complete bone bridging was observed at the extended experimental periods.[47,49] There was a significant difference in the amount of bone regeneration at the 4- and 12-week observation periods.[49] Furthermore, when Pepgen/P15 was evaluated after 6 weeks of healing, the osseous repair was not significant.[51]

Since bovine bone mineral particles in the canine show a slow rate of biodegradation,[27,52] it was decided that 12 weeks would be an appropriate healing period to evaluate Pepgen/P15 in a critical-size defect and to compare the results with other studies.[38,49]

In this study, all morphometric data suggested that the mode of the applied technique affected treatment outcome. Despite the application of an osteoconductive biomaterial, ie, Pepgen/P15, the nongrafted, membrane-protected type defects (Mem) showed the greatest amount of newly formed bone. Previous data indicate that the application of a biologic selective barrier significantly increases the regenerative capability[36,38,44,53] beyond the presence or absence of a guided-rail osteoconductive material. In the present study, the contribution of the membrane was evident in the finding that bone formation was greater in the membrane-protected grafted sites (Pepgen/P15-mem) than in the unprotected grafted sites (Pepgen/P15, 50.6% versus 44.2%); however, this difference was not statistically significant.

Although the membrane greatly affected the amount of newly formed bone within the critical-size defect, it did not have an impact on the biodegradation of the grafted particles. Thus, particle area percentages were similar in both grafted defect types.

The main advantage of the GTR membrane was shown in the formation of the external and internal parietal bone bridging. In both parameters, the Mem group approached complete bridging, which was significantly better than the results of all other groups. The fact that both types of bridging were significantly greater in the Mem group than in those of the Pepgen/P15-mem group suggest that the physical presence of the grafted particles interferes with and/or inhibits this process relative to a well-protected blood clot, at least when observed at 3 months.

In a similar study,[38] anorganic bovine bone was evaluated with enamel matrix protein after 4 months of healing. This study also confirmed the critical role of the GTR membrane in the regeneration process.

The osteoconductive properties of Pepgen/P15 were evident primarily in the Pepgen/P15-mem group, in which most of the particles were surrounded by newly formed bone. However, this was not evident at the Pepgen/P15 uncovered sites. Furthermore, except for the total new bone area, there was no difference between this particle-grafted site and the controls. In light of these findings, it appears that this biomaterial is osteoconductive in nature.[6–8] Its presence adds to the thickness of new bone formation, as long as it is accompanied by an over-latticed membrane. However, the present study did not measure the osteoconductivity level[4] recently reported for this biomaterial in dogs.[27]

An additional and remarkable contribution of Pepgen/P15 in membrane-protected sites is related to the resulting volume of regenerated osseous tissue. While the marginal vertical regenerated tissue height near the calvarial bone edges was similar in all treated sites (except the control group), there were marked differences in the vertical height of the central zone between the different groups, in favor of the Pepgen/P15-mem group. The additive contribution of an osteoconductive material and an osteo-promotive membrane is evident particularly in an area furthest from the nourishment of the osseous rim. The central bone height correlated well with the total calcified volume (sum of new bone and grafted particles) in that maximal calcified tissue volume resulted in maximal tissue regeneration height, as shown in the Pepgen/P15-mem group. However, it should be noted that these marginal and central regenerated bone heights actually represent the total augmentation height, which could harbor grafted particles and/or soft tissue marrow.

In clinical practice, the main advantage of applying bone graft material is to obtain the desirable augmented volume, while the selective barrier membrane is responsible for its quality. In each defect type, the vertical heights measured indicated the amount of established bone augmentation to be obtained. Consequently, the justification for using this bone graft material is determined by the hard tissue volume desired for future implant site preparation, since this would be the osseous housing for osseointegrated implants in load-bearing function.

This clinical volumetric outcome has high stiffness with an excellent micromechanical elastic property.[54] Thus, the application of Pepgen/P15 putty in a membrane-protected site is indicated to establish adequate volumetric bone augmentation. However, it does not enhance bone regeneration.

CONCLUSION

The findings in this study demonstrated a combined contribution of Pepgen/P15 as the biomaterial filler and the GTR membrane in achieving nearly complete regeneration in volume and content in a critical-sized calvarial defect. While the membrane is probably the major contributor to the newly formed bone, Pepgen/P15 increases the total tissue augmentation volume, thus enhancing the size of the regenerated site. However, bridging the defect and attaining a full defect thickness depend on the presence of the membrane.

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