Surface Analysis and Effects on Interfacial Bone Microhardness of Collagen-Coated Titanium Implants: A Rabbit Model

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Purpose: *The aim of this study was to evaluate the surface chemistry and the microhardness at the implant-bone interface using a recently developed collagen-coated titanium implant in a short-term rabbit model.* ***Materials and Methods:*** *Surface chemistry was evaluated by x-ray photoelectron spectroscopy (XPS), while in vivo studies involved 4-week implants mid-diaphysis in the lateral femurs of adult male rabbits. After conventional embedding and evaluation of histologic sections, the resin-embedded blocks containing the implanted screws were used to measure bone hardness by means of an indentation test.* ***Results:*** *Decomposition of the C1s peak obtained by XPS analysis confirmed that surface-immobilized collagen retained all the molecular features of the control, nonimmobilized reference. As to microhardness measurement, newly formed bone at the collagen-coated-implant/bone interface was significantly harder than bone at the interface of the uncoated control implant and bone.* ***Discussion:*** *These results suggested that collagen coating significantly improves bone maturation and mineralization at the interface in comparison with uncoated commercially pure titanium. Surface modification of titanium implants by collagen coating has recently been discussed as a promising approach to the biochemical modification of implant surfaces. The present results support previous histologic findings and demonstrated that the biomolecular layer linked over the titanium implant can increase the bone healing rate, at least in this animal model.* ***Conclusions:*** *The present microhardness measurement at the bone-implant interface showed that collagen coating can significantly improve bone maturation and mineralization at the interface in comparison with uncoated commercially pure titanium, confirming and substantiating previous findings by histomorphometric measurements from the same model.* Int J Oral Maxillofac Implants 2005;20:23–30

Key words: *collagen, dental implants, microhardness, surface analysis, surface modification*

Modification of the surface properties of titanium dental and orthopedic implants remains an active area of research.[1] Most of the current approaches to surface modification focus on control of the surface morphology: plasma spraying, sandblasting, acid etching, and electrochemical modification (anodization) are well established and widely used in clinical practice.[2–8] However, at least at the research level, more and more interest has arisen concerning biochemical approaches to surface modification.[9–18] As underlined by Puleo and Nanci,[9] biochemical methods of surface modification offer an alternative or adjunct to morphologic approaches. Biochemical methods are aimed at control of the tissue-implant interface by the immobilization and/or delivery of proteins, enzymes, or peptides for the purpose of inducing specific cell and tissue responses.[9] They rely on current understanding of the biology and biochemistry of cellular function and differentiation and on suitable surface modification techniques. The recent literature contains several interesting examples of biochemical approaches to surface modification.[10–18]

In a recent article,[18] the present authors demonstrated that immobilization of a thin collagen layer on titanium implant surfaces, deposited by a process

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Fig 1 The experimental samples after toluidine blue staining. The collagen-coated titanium (colTi) screw *(left)* absorbed the dye and shows homogenous staining.

involving deposition from plasma followed by grafting, can increase the healing rate of bone in a rabbit model 4 weeks postplacement as compared to an uncoated control (this short-term animal model was chosen to evaluate the effect on the bone healing rate at a fairly early phase[17,18]). Thus, this biochemical modification of titanium implant surfaces could lead to an accelerated stable fixation between bone and implant, and it would allow early or immediate loading, with significant implications in terms of decreased patient morbidity, patient psychology, and health-care costs.

In this report, the previous work was extended to yield a more complete description of the surface chemistry of the implant and of its effects on interfacial bone. In particular, high resolution peaks obtained by x-ray photoelectron spectroscopy (XPS) analysis are presented and discussed, elaborating on the molecular details of surface-linked collagen.

Then, mechanical properties of bone, as gathered from microhardness measurements, are presented by comparing collagen-coated implants to uncoated control implants. Interfacial mechanical properties of bone surrounding implants have been reported to be useful in understanding the healing response and adaptation of the bone adjacent to endosseous implants.[19] The quality of the bone-biomaterial interface is a fundamental element of the success of a prosthesis, and microhardness and nanoindentation tests have been used to obtain information on mechanical characteristics of bone, not only at the bone-material interface, but over different distances from it.[19–29] Microhardness measurement results must be considered as an expression of the grade of bone qualities such as mineralization or calcification degree, arrangement and number of collagen fibers, ratio between collagen fibers and ground substances, the mineral quantity per unit volume, and osteocyte number.[19–29] Moreover, good correlation between microhardness and elastic modulus has been demonstrated.[19,30] Thus, microhardness could give information on bone maturation and mineralization degree during bone-reparative processes at the bone-biomaterial interface, microhardness increases, and the maturation and mineralization of the newly formed bone.[25]

MATERIALS AND METHODS

The specimens used in this study were obtained from a larger project currently in progress on the evaluation of a collagen coating on commercially pure titanium (cpTi).[18] The materials used, together with the surface modification process and extensive surface characterization data, were presented in the aforementioned publication and will not be detailed here. Figure 1 shows the experimental implants used in the studies and animal trials. Both samples in Fig 1 were stained using toluidine blue. The collagen-coated one, on the left, absorbed the dye and resulted in a blue color, as extensively described previously.[18] Staining tests were used to show the homogeneity of the coating. The implant surfaces were machined without further roughening treatments. As described previously,[18] the surface roughness induced by the collagen coating is, in aqueous, of the order of a few nanometers, well below the intrinsic roughness of machined surfaces, as evaluated by atomic force microscope analysis.

Surface Characterization by XPS

XPS analysis was performed with a PHI 5500 ESCA system (Areva, Chanhassen, MN). The instrument is equipped with a monochromatic x-ray source (A1 Kα anode) operating at 14 kV and 300 W. The diameter of the analyzed spot is approximately 400 μm; the base pressure is approximately 10^{-7} Pa. The angle between the electron analyzer and the sample surface was 45 degrees. Quantification of elements was accomplished using the software and sensitivity factors supplied by the manufacturer.

High-resolution peaks (C1s, N1s, O1s) were obtained by using a pass energy of 2.95 eV in a range of 20 eV around the peaks. The decomposition procedure was carried out using PC-ACCESS PHI software (Areva). As a first trial, the energy peak positions were fixed at 285.0 eV (C-C, C-H); 286.3 eV (C-N, C-O); and 288.6 eV (N-C=O). A Gaussian shape was chosen and other parameters (full width at half maximum [FWHM] and areas) were left as adjusting ones.

XPS analysis was performed on an experimental collagen-coated sample (coded colTi subsequently in this report) and, as a reference, on the collagen pow-

der used as a raw material in the coating process (KNC Semed S collagen powder; Kensey Nash, Exton, PA).

Animal Studies and Microhardness Measurement

Briefly, 2-mm-diameter screws (Fig 1) were placed mid-diaphysis in the lateral femurs of 6 adult male rabbits (mean body weight ± SD 3.250 ± .350 kg). The epiphyseal plates were radiographed to confirm closure before the experiments were started. The femurs mid-diaphyses were exposed, and 2 defects with a 1.9-mm diameter were drilled at low speed and under continuous saline irrigation in the cortical bone of the right and left femurs. Uncoated cpTi screws (n = 6) were transversely implanted in the left femurs of all rabbits, while colTi screws (n = 6) were positioned in the right femurs. The bone was allowed to heal for 4 weeks.

After the animals had been pharmacologically euthanized, femurs were removed, cleaned of soft tissues, and prepared for resin embedding. The bone specimens containing the implants were fixed in 4% buffered paraformaldehyde for 24 hours, dehydrated in a graded series of alcohols for 24 hours at each concentration, and then included in polymethylmethacrylate resin until solidification, which usually occurred after 7 days. All processing was carried out at a temperature of 22 ± 1°C and humidity rate of 48%.

The resin-embedded blocks containing the implanted screws were used to measure bone hardness by means of an indentation test (Microhardness VMHT 30, Leica, Wien, Austria), as described in previous reports.[20,21,26,27] A rotary wheel set at 150 rpm was used with silicon carbide paper and water lubrication. The resin-embedded blocks containing the specimen were then polished using diamond pastes with progressively finer grain sizes. The smooth surface obtained was observed under the microscope and clearly showed the bone-material interface and the other areas to be examined.

The microhardness measurements were taken tangentially to the interface with a Vickers indenter (4-sided pyramid with square base and an apex angle between opposite sides of 136 ± 15 degrees applied to the bone at a load of 0.05 kg of force and dwell time of 5 seconds. The Vickers hardness degree (HV) was calculated by dividing the indentation force by the surface of the imprint (4 pyramid surfaces) observed at the microscope.

The average value for each sample was calculated from a mean of 10 values for each examined area at 2 sites: *(a)* in the regrown bone, within 200 μm from the interface and in the inner area in which the threads of the screw engage ($HV_{200\ \mu m}$) or *(b)* outside the threads in the pre-existing host bone, at 1,000 μm from a line connecting the top of the threads ($HV_{1,000\ \mu m}$). Finally, the bone maturation index (BMI) was calculated by dividing the microhardness HV of the bone regrown at the interface by the HV of the pre-existing bone multiplied by 100.

The in vivo portion of the study was performed in accordance with European and Italian law on animal experimentation and according to the principles stated in "Animal Welfare Assurance" (no. A5424-01), published by the U.S. National Institutes of Health. The experimental protocol was submitted to the Italian Ministry of Health.

Table 1 Atomic Concentration of Surface Chemical Elements, as Obtained from XPS Analysis

Material	C 1s	O 1s	N 1s	Additional contaminants
Pure collagen	69.1	17.5	11.7	NA, Cl
colTi	68.0	17.4	14.6	Na, Ca

Statistical Analysis

Statistical analysis was performed using SPSS v. 10.1 software (SPSS, Chicago, IL). Data are reported as mean ± SD at a significance level of $P < .05$. Paired Student *t* test was used to compare microhardness data between materials.

RESULTS

XPS Analysis

The surface composition of the experimental sample colTi, as detected by XPS analysis, is reported in Table 1 and compared to that of collagen (as obtained from XPS analysis of the raw material). As extensively discussed in the quoted paper,[18] the overall surface composition of these implants is significantly different from that of conventional implant surfaces.[31] Because of the organic overlayer on the titanium implant, no titanium is detected, and the chemical composition is similar to that of collagen, as shown in Table 1.

While general surface stoichiometry has been previously discussed,[18] the aim of this report was to describe in some detail the molecular aspects of the modified surface, as detected by XPS analysis. In this respect, peak decomposition was performed only for carbon peaks, which had structured shapes and well known components, corresponding to the main chemical environments of carbon atoms expected in collagen: C-C, C-O/C-N, and O=C-N (amide) bonds, as shown in Figs 2a and 2b. From the data in Table 2, the similarity between the 2 cases can be seen. Actually,

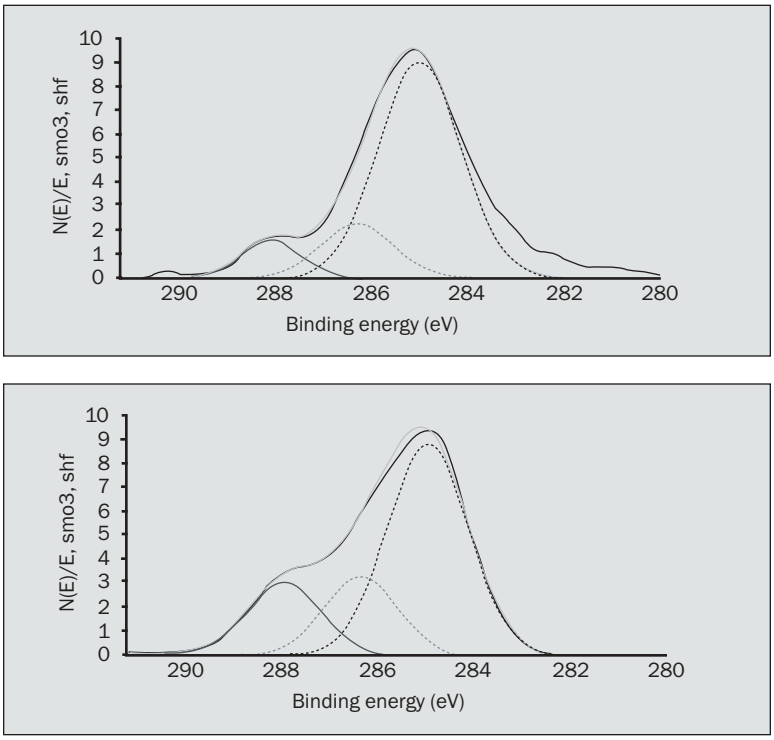

Fig 2 Decomposition of the C1s peak, obtained by XPS measurement, of *(top)* collagen powder and *(bottom)* ColTi.

Table 2 High-Resolution C 1s Component Parameters

Compound	BE (eV)	FWHM (eV)	Area (%)
Pure collagen			
C-C, C-H	285.0 ± 0.1	2.0	74
C-N, C-O	286.3	1.8	16
N-C=O	288.1	1.5	10
Sample screw			
C-C, C-H	284.9 ± 0.1	2.0	61
C-N, C-O	286.3	1.8	20
N-C=O	287.9	1.8	19

data from the collagen-coated screw showed that the N-C=O component was broader and more intense than that from pure collagen, at the expense of the C-C/C-H component. This fact confirms that there was only a thin layer of collagen: the former is dominated by external ligands, while the latter is dominated by the bulk composition. Moreover, an uncompensated charging tail, which appeared only for collagen toward lower binding energies (BEs), confirmed different thicknesses. N1s and O1s peaks did not have structured shapes and were very broad for pure collagen and quite narrow for the thin layer covering the uncoated screw. This broadening was induced by charging effects.

Microhardness Measurement

The measurements of bone hardness were performed within 200 μm from the screw interface and at 1,000 μm because of the need to compare newly formed bone with normal healthy bone. In previous studies it was observed that the influence of the implant and of the implantation surgery was avoided at a distance of 1,000 μm from the biomaterial interface.[20]

In Fig 3, examples of hardness measurements at the bone-screw interface and in pre-existing bone are shown. Microhardness data are reported in Table 3. As expected, no differences existed in bone of implanted rabbits far from the bone-biomaterial interface ($HV_{1,000\ \mu m}$ cpTi = 90.00 ± 2.94; $HV_{1,000\ \mu m}$ colTi = 92.91 ± 4.37). At the bone-screw interface, there was a significant decrease in bone hardness for both materials in comparison with pre-existing bone unaffected by the implantation surgery ($HV_{200\ \mu m}$ cpTi −24.5%, $P < .001$; $HV_{200\ \mu m}$ colTi −19.8%, $P < .005$). When comparing bone hardness in the interfacial area, presence of the collagen coating significantly improved (ie, by nearly 10%) the hardness at the bone-screw interface of colTi screws in comparison with uncoated cpTi ($P < .05$). Measured BMI values were 67.9 HV and 74.5 HV for cpTi and colTi, respectively.

DISCUSSION

Biochemical modifcation of dental implant surfaces is a highly promising route for stimulating bone healing mechanisms. Among the molecules of direct relevance to the biochemical modification of bone-contacting surfaces, collagen is of definite interest.

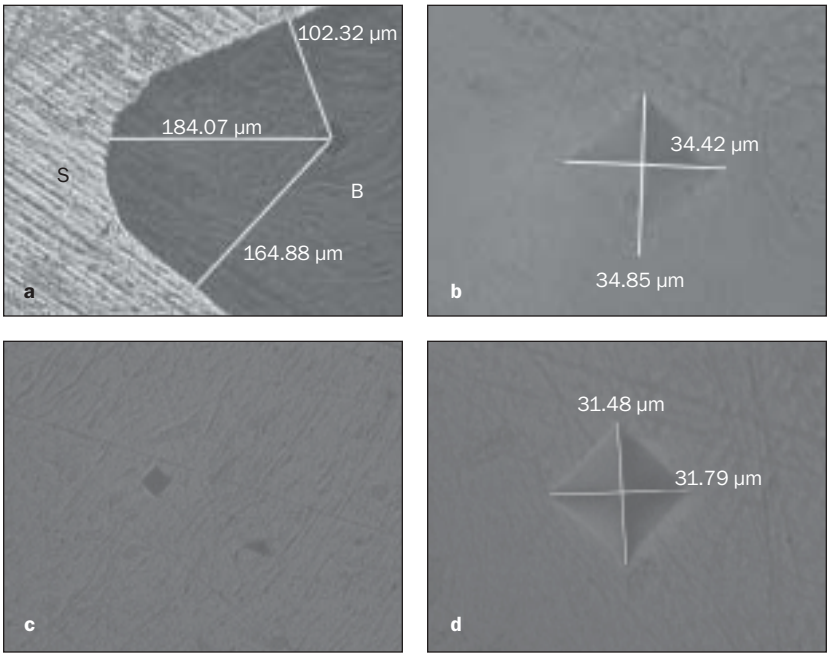

Fig 3 Examples of microhardness measurements. *(a)* Rhomboidal imprint at the bone-screw interface (within 200 µm of the interface) (original magnification ×10). *(b)* Rhomboidal imprint at the bone-screw interface (within 200 µm of the interface) (original magnification ×40). *(c)* Rhomboidal imprint at 1,000 µm from the interface (original magnification ×10). *(d)* Rhomboidal imprint at 1,000 µm from the interface (original magnification ×40). The rhomboidal imprint at the bone-biomaterial interface is wider than that of bone far from the implant (ie, 1,000 µm away) because of the higher HV value in pre-existing normal cortical bone (92.65) in comparison with bone at the interface (77.36). S = screw, B = bone.

Table 3 Microhardness Results of cpTi and colTi at 4 Weeks

	Control material (cpTi)		Experimental material (colTi)	
	$HV_{200\ \mu m}$	$HV_{1,000\ \mu m}$	$HV_{200\ \mu m}$	$HV_{1,000\ \mu m}$
1	63.10	91.34	60.15	96.14
2	72.26	86.01	79.31	93.57
3	77.00	92.89	95.46	97.14
4	59.00	87.01	71.23	85.18
5	55.94	89.84	60.71	94.16
6	80.15	92.89	80.21	91.00
Mean ± SD	67.91 ± 9.97	90.00 ± 2.94*	74.51 ± 13.43†	92.91 ± 4.37‡

$HV_{200\ \mu m}$ = the microhardness at 200 µm from the interface and in the inner area in which the threads of the screw engage; $HV_{1,000\ \mu m}$ = the hardness outside the threads in the pre-existing host bone, at 1,000 µm from a line connecting the top of the threads.
*For $HV_{1,000\ \mu m}$ cpTi versus $HV_{200\ \mu m}$ cpTi, $P < .001$ (paired Student t test).
†For $HV_{200\ \mu m}$ cpTi versus $HV_{200\ \mu m}$ colTi, $P < .05$ (paired Student t test).
‡For $HV_{1,000\ \mu m}$ colTi versus $HV_{200\ \mu m}$ colTi, $P < .005$ (paired Student t test).

Together with other extracellular matrix proteins, it controls adhesion of cells of direct relevance to orthopedic applications,[32] through the amino acid sequence Arg-Gly-Asp (RGD) it contains. It is commonly used in dental surgery as an osteogenic and bone filling material,[33,34] and it has been shown that heterologous type I collagen provides a more rapid regeneration of bone defects.[34] It exerts a strong pro-coagulant (hemostatic) activity and stimulates platelets in a unique way,[35] promoting the release of growth factors from activated platelets.[36,37] Biochemical modification of implant surfaces allows direct application to the implant site of this interesting biomolecule.[9]

The present characterization by XPS analysis showed that the chemistry of surface-immobilized collagen is the same as for bulk collagen. In particular, the 3 typical components (C-C/C-H, C-N/C-O, and N-C=O) were evident for both cases, with similar relative ratios; the only difference was a relative increase of the more external component (N-C=O) in the thin layer of the uncoated screw, as expected by passing from a bulk sample to a thin layer. Preservation of the native chemistry of the biomolecule, together with the conformational freedom at the implant-host tissue interface imparted by the specific coupling procedure,[18] likely plays a pivotal role in determining the performances of the present device. In this respect, an important finding arising from a recent study of the present authors was that the biochemical modification of the cpTi surface by collagen affects the in vivo response of bone and increases

the healing rate at a fairly early healing phase, at least in the present animal model.[18] The collagen coating on titanium significantly accelerates bone-to-implant contact and bone ingrowth. In fact, the histomorphometric investigation of the implanted materials showed direct apposition of bone to both Ti and colTi screw surfaces, even if in some cases where uncoated Ti was used, the osseointegration process was still in progress at 4 weeks. A significant increase in bone-to-implant contact and bone ingrowth was observed for colTi versus uncoated cpTi (+23.8%, $P < .01$; +7.6%, $P < .01$, respectively) in the cortical bone of rabbits and 4 weeks after the implantation surgery.[18]

However, morphologic methods such as histomorphometry can only provide approximate indices of bone maturity and mechanical resistance,[29] and a mismatch between histomorphometric results and bone hardness has been observed in other studies where both histomorphometry and microhardness were used to morphologically and microstructurally evaluate the bone-biomaterial interface.[24,29] Moroni and coworkers[24] and Stea and colleagues[29] studied hydroxyapatite (HA) -coated and uncoated pins in the cortical bone of sheep and observed that the more intensive bone formation favored by the ceramic material was not always accompanied by proper mineralization at 6 weeks.[24] The HA coating significantly improved bone ingrowth and mechanical attachment as evidenced by histomorphometry and biomechanics, but no differences in microhardness (when studying coated and uncoated pins at the bone-interface) were found at 6 weeks.[29] The present authors have used microhardness techniques after the implantation of pins and screws made of different materials and with different surfaces.[21,26,27] Also at 16 weeks and also in the case of a high osseointegration rate, a decrease in bone hardness at the bone-biomaterial interface in comparison with pre-existing normal bone was observed. Only with the adjuvant use of electromagnetic stimulation was it possible to obtain a mineralization rate at the bone-HA interface superposable to the one of the normal pre-existing bone far from the implant 6 weeks postimplantation.[20]

These results are not contradictory because histomorphometric parameters give information on the amount and architecture of bone, while microhardness provides microstructural data, and it is known that bone remodeling, mineralization, and maturation around implants is a long process that has been reported to continue many months after implantation surgery.[19]

Concerning quantitative aspects of the microhardness data, many in vitro factors affect the mechanical properties of bone, and among them histologic processing may be carefully evaluated.[38] How such processing affects microhardness is not clear, but it is known to cause cross-linking of collagen.[22] Some authors have reported no effect on hardness after brief formalin and formaldehyde fixation, while other authors found an increase of about 20% in bone hardness after formalin fixation for 24 hours.[22,39] Regarding infiltration in polymethylmethacrylate resin, it has been reported that it increases microhardness by 30% to 40%.[22] These observations support the concept that it would be preferable to use nonfixed and noninfiltrated specimens for microhardness testing. However, many authors have used fixed and infiltrated specimens when using the microhardness technique for measurement of bone and the interface around an implanted biomaterial.[20,21,23–27,29,40] This practice stems from the convenience of measuring bone hardness around implants by using the same resin-embedded specimens investigated with the histomorphometric technique. This procedure avoids the need to double the number of animals used in the research and, more importantly, permits biomechanical and histomorphometric analyses to be performed on the same interfaces. In fact, it is important to characterize bone ingrowth around implants with both techniques because, as previously highlighted, bone ingrowth does not mean that bone has remodeled and mineralized in the physiologic manner. To avoid differences between specimens, standardized conditions were adopted during the fixation and infiltration phases of the specimens.

The present results show that collagen coating can significantly improve bone maturation and mineralization at the interface in comparison with uncoated cpTi, even if, as in the case of the collagen coating, bone hardness at the interface was significantly lower than unaffected pre-existing bone. However, the values for bone hardness were obtained just after 4 weeks from surgery. Thus, they are very encouraging because they suggest an actual faster maturation of bone around colTi screws. Further investigations at longer experimental times are needed.

Focusing the attention on osseointegration, the shortcoming of the present report is that a mechanical test measuring implant fixation was not performed. However, the principal aim of the present initial phase of the in vivo study was the characterization of the bone tissue quality at the bone-biomaterial interface to ascertain whether a collagen coating could have a biologic effect on the endogenous bone ingrowth toward the implant surface.

CONCLUSIONS

The present data substantiate previous findings from the same authors on the nature and effect of collagen immobilization on titanium dental implants, using a previously described surface-modification approach.[18] From the analytic perspective, the present data show that the immobilization procedure does not significantly affect the molecular details of collagen, opening the way to the exploitation of its biochemical properties at the interface between the coated titanium implant and healing bone. From the point of view of microhardness measurement, it was concluded that a faster maturation of bone occurred around colTi screws compared to conventional ones, supporting and complementing previous histomorphometric findings.

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