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Ultrastructural Evidence for Blood Microvessels Devoid of an Endothelial Cell Lining in Transplanted Pancreatic Islets

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The aim of the present study was to investigate, at the ultrastructural level, the process of revascularization of freshly isolated islets or cultured islets after transplantation under the kidney capsule of syngeneic mice. Native islets in adult pancreases from mice, pigs, and humans contained only capillaries with fenestrated endothelium. However, the endothelial cell lining was disrupted in both freshly isolated and cultured mouse islets. Shortly after transplantation (6 weeks) approximately 80% of graft microvessels contained no endothelial cell lining. Similar data on microvessel morphology were found when fetal porcine islet-like cell clusters were implanted into athymic nude mice. Re-endothelialization was a slow process, with 25% of the microvessels still lacking endothelium 6 months after transplantation of cultured mouse islets or islet-like cell cluster. However, when freshly isolated mouse islets are used only 25% of microvessels within the islet graft lacked endothelium 6 weeks after implantation. We suggest that capillaries damaged during islet isolation may provide a preformed channel, serving as a scaffold for newly formed islet graft blood vessels. The presence of non-endothelialized microvessels, with an associated lack of barrier function, might make transplanted islets more prone to thrombosis or an attack by the immune system. This provides a tentative explanation for the increased vulnerability of islet grafts when compared with whole pancreas transplants. (Am J Pathol 1995, 146:429–435)

Insulin-dependent diabetes mellitus (IDDM) is a disease caused by an autoimmune progressive destruction of the insulin-producing β-cells within the pancreatic islets. Despite meticulous insulin therapy, the appearance of micro- and macroangiopathic complications after 15 to 20 years of disease is difficult to prevent in some patients. Presently, the only option to achieve permanent normoglycemia in diabetic patients is a renewal of the β-cells, either by transplantation of segmental/whole pancreas or isolated islets of Langerhans. Combined kidney and pancreas transplantation is presently an accepted treatment of patients with IDDM and end-stage renal disease.[1] The results of therapeutic islet transplantations, on the other hand, are so far disappointing, and insulin therapy usually has to be reinstated.

The reasons for the differences in success rates when islet implantations are compared with whole pancreas transplantations are unknown. The immunological barrier, the underlying autoimmune disease, as well as the immunosuppressive drugs used are the same in both types of transplantation. Furthermore, islet-transplanted IDDM patients sometimes attain normoglycemia for a limited time before recurrence of hyperglycemia. There are no obvious immunological reasons that could explain the different success rates between the two modes of transplantation, whereas several non-immunological differences may be invoked as an explanation. One important factor may be the engraftment of the islets in the host tissue. The islets in the pancreatic gland have a complex vascular system, with important functional properties based on its three-dimensional arrangement[2] and, in the case of whole pancreas transplantation, present at the time of implantation. By

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Table 1. *Various Types of Blood Capillaries in Transplanted Freshly Isolated or Cultured Mouse Pancreatic Islets at Different Times after Transplantation*

	Recipient	Time after transplantation	No. of Capillaries examined*	Fenestrated capillaries	Continuous capillaries	Vessels without endothelium
Cultured islets	C57BL/6J	6 weeks	90	35 (39%)	4 (4%)	51 (57%)
		6 months	102	82 (81%)	2 (2%)	18 (17%)
	C57BL/6J (nu/nu)	6 weeks	40	20 (50%)	0 (0%)	20 (50%)
		6 months	48	39 (81%)	0 (0%)	9 (19%)
Freshly isolated islets	C57BL/6J	6 weeks	95	71 (75%)	0 (0%)	24 (25%)

*Capillaries from five to six animals were examined in each group.

contrast, transplanted isolated islets need to stimulate an ingrowth of new blood vessels from the surrounding host tissue, with the possibility of an abnormal formation of the new islet vasculature.

Experiments performed in the spontaneously diabetic BB rat, an animal model of human IDDM, show that transplanted isolated islets are prone to recurrent autoimmune destruction, whereas islets in whole pancreatic grafts are partially protected.[3] This difference may be explained by a defective vascular endothelium within isolated islets that permits migration of autoimmune T lymphocytes into the transplanted islets, whereas the endothelium within the islets in a pancreas graft would, at least partially, constrain this migration. In view of these considerations, the present study was performed to evaluate whether the engraftment process, especially the revascularization, may be disturbed in islet transplantations. Both freshly isolated islets and islets maintained in tissue culture were implanted under the kidney capsule of syngeneic mice. The process of revascularization was studied at the ultrastructural level at different times after implantation, with special attention on whether the newly formed microvessels would differentiate into capillaries that are similar to those of intact islets.

Materials and Methods

Islet Isolation and Transplantation

Mouse pancreatic islets were isolated from adult male C57BL/6J mice by a collagenase technique.[4] Fetal porcine islet-like cell clusters (ICC) were produced as previously described.[5] Briefly, pregnant sows were killed by means of a slaughtering mask at approximately day 70 of pregnancy (full term is 115 days). The fetuses (10 to 15 per litter) were immediately harvested from the uterus and the pancreases were dissected, minced, and collagenase digested. The digest was then transferred to culture dishes containing RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with 10% heat-inactivated human serum (Blodcentralen, Huddinge Hospital, Sweden), after which the pancreases were cultured for 4 days. During this culture period there is an outgrowth of a large number of ICC. Isolated mouse islets or porcine ICC were cultured in RPMI 1640 plus 10% newborn calf serum for 2 to 4 days in humidified air with 5% CO_2 at 37 C before transplantation. Normal or athymic, nude (nu/nu) male C57BL/6J mice (Bomholtgaard, Ry, Denmark) were used as recipients. The left kidney was visualized through a flank incision, and either 250 freshly isolated or cultured mouse islets or 500 ICC were implanted under the renal capsule by means of a thin glass pipette. The animals were killed and the islet graft was removed from the kidney at various times after implantation as shown in Tables 1 and 2.

Ultrastructural Examination

The islet grafts and adjacent kidney parenchyma were then prepared for electron microscopic examination, as were pancreases from adult mice, pigs,

Table 2. *Various Types of Blood Capillaries in the Transplanted Fetal Porcine Endocrine Pancreas (ICC) at Different Times after Transplantation*

Time after transplantation	No. of Capillaries examined*	Fenestrated capillaries	Continuous capillaries	Vessels without endothelium
6 weeks	96	28 (29%)	5 (5%)	63 (66%)
6 months	64	47 (73%)	0 (0%)	17 (27%)
>16 months	106	81 (76%)	0 (0%)	25 (24%)

*Capillaries from five to six animals were examined in each group.

and humans. Furthermore, the ultrastructure of freshly isolated or cultured mouse islets and fetal porcine ICC were examined. Immediately after dissection, the tissue specimens were fixed for 6 hours in 2% (w/v) glutaraldehyde in a 0.1 mol/L sodium cacodylate buffer supplemented with 0.1 mol/L sucrose, followed by 1.5 hours post-fixation in 1% (w/v) osmium tetroxide dissolved in the same cacodylate buffer. After dehydration, the specimens were embedded in an epoxy resin (Agar 100, Agar Scientific, Stansted, UK). Ultrathin sections were prepared with an LKB Ultrotome No. V (LKB Products AB, Bromma, Sweden) equipped with a diamond knife. The sections were placed on copper grids covered with a film of polyvinyl formal plastic (Formvar, Agar Scientific) and contrasted with uranyl acetate[6] and lead citrate.[7] Electron micrographs were taken with a Jeol 100 C electron microscope (Japan Electronics Laboratory Co., Tokyo, Japan). Consecutive ultrathin sections were each classified, after examination of at least six cross sections by an examiner unaware of the origin of the sections, as containing continuous or fenestrated endothelium or, as was the case for many microvessels, being devoid of an endothelial cell lining. A microvessel was defined either as a blood vessel delineated by endothelial cells or as a tubular structure completely surrounded by endocrine cells and containing one to four erythrocytes. The presence of pericytes, which were defined as irregularly shaped cells situated between the basement membrane and the endothelial cells, was also studied.

Results

Ultrastructure of Islets in Situ *and Isolated Islets*

The ultrastructure of the different specimens were all normal and displayed well granulated endocrine cells with a normal content of organelles. In intact pancreases from adult mice, pigs, or humans, only fenestrated capillaries were observed within the islets (Figure 1). Both freshly isolated and cultured islets contained well granulated endocrine cells, which contained one of the four major islet hormones. Islet capillaries were regularly seen. However, the endothelial cell lining was damaged, with almost no intact cells present in either freshly isolated or cultured islets. Instead, large numbers of damaged endothelial cells were located within the capillary remnants. During culture these endothelial cells partially disappeared, and a reduction in the width, or even a disappearance, of the basement membrane could be seen (Figure 2). Fetal porcine ICC contained immature endocrine cells of two types distinguishable by their granules as previously described.[8] Between these cells, duct-like structures without any basement membrane were found. Sometimes cytoplasmic pro-

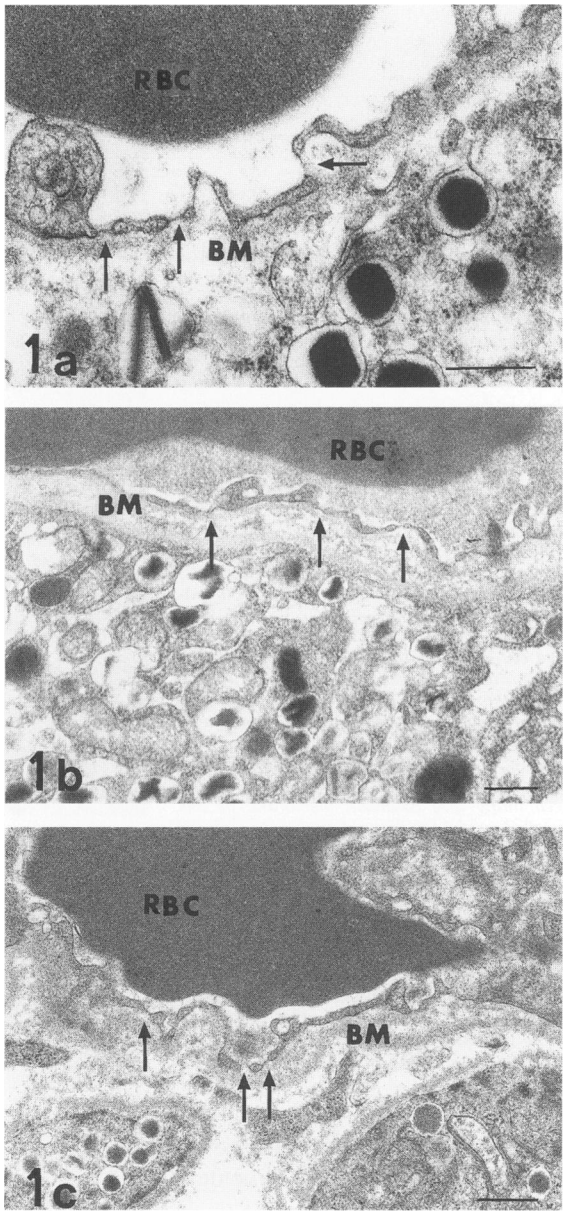

Figure 1. *The capillaries within the intact pancreatic islets of adult C57BL/6J mouse* (a), *pig* (b), *and human* (c) *have a lining of endothelial cells with diaphragm-covered fenestrae* (*arrows*) *and a continuous basement membrane* (*BM*). *Red blood cells* (*RBC*) *are indicated. Magnification,* ×*36,000* (a) *and* ×*18,000* (b *and* c); *bar, 400 nm.*

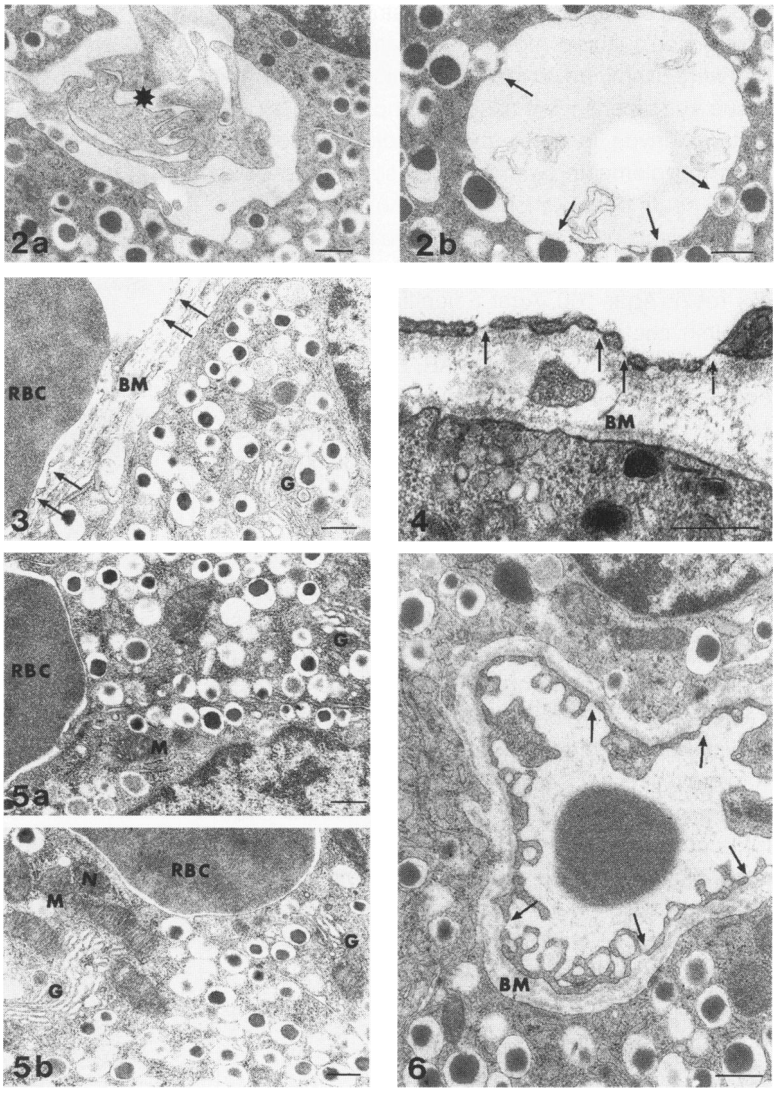

Figure 2. a: *A capillary in an isolated mouse islet after 3 days in culture with a decomposed endothelial cell (*) in the lumen. Morphologically normal β-cells surround the capillary, but no basement membrane is present. Magnification, ×18,000; bar, 400 nm* b: *A capillary, from the same preparation as in* (a), *lacking both endothelium and basement membrane. Protein debris occurs in the lumen. Exocytotic sites from the adjacent β-cells are indicated by arrows. Magnification, ×24,000; bar, 400 nm.*
Figure 3. *Fenestrated endothelium (arrows) and basement membrane (BM) are seen in this microvessel in a mouse islet graft 6 weeks after transplantation. The β-cell has a normal morphological appearance. Golgi apparatus (G) and a red blood cell (RBC) are shown. Magnification, ×18,000; bar, 400 nm.*
Figure 4. *This micrograph shows an endothelial cell with diaphragm-covered fenestrae (arrows) lining a capillary in a porcine ICC graft 1.5 years after implantation into a nude mouse. Note the continuous basement membrane (BM). Magnification, ×54,000; bar, 400 nm.*
Figure 5. *Microvessels devoid of an endothelial cell lining and basement membrane in islet grafts 6 weeks after transplantation of cultured syngeneic islets. The surrounding β-cells seem to have a normal morphological appearance. Golgi apparatus (G), mitochondria (M), nucleus (N), and red blood cells (RBCs) are shown. Magnification, ×18,000; bar, 400 nm.*
Figure 6. *A capillary with a fenestrated diaphragm-covered endothelium (arrows) and a basement membrane (BM) in an islet graft examined 6 weeks after transplantation of freshly isolated islets under the kidney capsule of a syngeneic mouse. Magnification, ×24,000; bar, 400 nm.*

jections from adjacent endocrine cells could be seen within the lumen of these structures.

Ultrastructure of Transplanted Islets

The grafts contained morphologically normal and well granulated endocrine cells with a normal content of organelles in all animals. Islet microvessels could easily be identified in most of the sections. Three different types of microvessels were distinguished within the grafts: 1), capillaries with a continuous layer of endothelial cells; 2), capillaries with a lining of endothelial cells with diaphragm-covered fenestrae (Figures 3 and 4); and 3), erythrocyte-filled cavities surrounded only by endocrine cells and with no endothelial cells or basement membranes present (Figure 5). The diameters of all these vessels were similar (data not shown). Although the ultrastructural technique limits the possibility to obtain a large number of consecutive sections, the impression was that the capillaries did not change ultrastructural appearance along their lengths, ie, no transition from one type of microvessel to another was observed. The frequency of the different types of blood microvessels varied with time after implantation and depending on whether freshly isolated or cultured islets were used (Tables 1 and 2). Shortly after transplantation of freshly isolated islets, 75% of examined capillaries had a lining of fenestrated endothelium (Figure 6). However, when cultured mouse islets (Figure 5) or porcine ICC (Figure 7) were implanted, a large majority of the microvessels were devoid of an endothelial lining, whereas the number of fenestrated capillaries was only 39% of all microvessels. A common finding in the microvessels without endothelium was intraluminal protein deposits and cytoplasmic

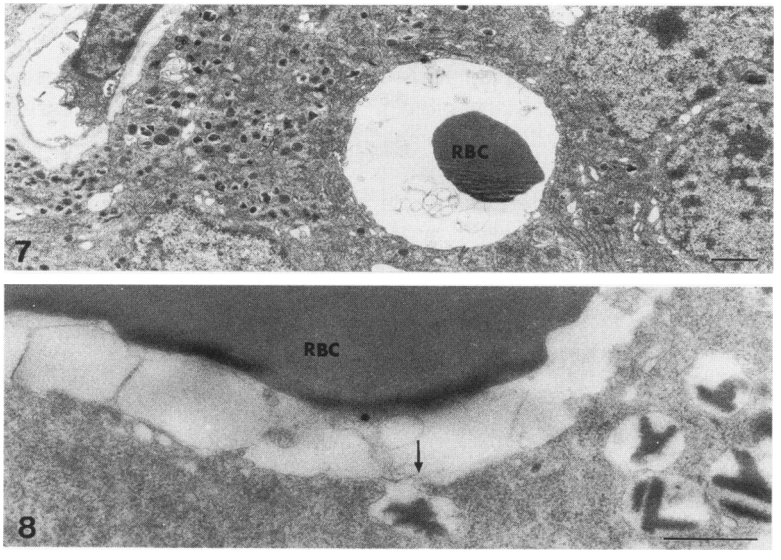

Figure 7. *A fetal porcine ICC graft 6 weeks after implantation under the kidney capsule of a nude mouse. To the left is a capillary with fenestrated endothelium and to the right a red blood cell (RBC) located in a cavity without any endothelial lining to protect and separate the surrounding endocrine cells from the bloodstream. Magnification, ×8400; bar, 1 μ.*
Figure 8. *High magnification of a microvessel in a fetal porcine ICC graft 1.5 years after transplantation to a nude mouse. The erythrocyte-filled (RBC) cavity is surrounded by β-cells, but no endothelial cells or basement membrane are seen. Strings of protein seem to emerge from the β-cell surface. An exocytotic site is indicated (arrow). Magnification, ×54,000; bar, 400 nm.*

processes from the endocrine cells (Figures 7 and 8). With time after transplantation the frequency of blood vessels with fenestrated endothelium increased in these animals, but even 6 months or almost 2 years after transplantation, approximately 20% of the microvessels lacked endothelium (Tables 1 and 2). This was seen both when cultured adult mouse islets or fetal porcine ICC (Figure 8) were transplanted and when normal or athymic nude mice were used as recipients. On the contrary, only capillaries with fenestrated endothelium were observed in the adjacent kidney parenchyma. Only few and scattered pericytes could be seen within islet microvessels, usually in association with continuous endothelium and only in mice examined 6 weeks after transplantation.

Discussion

Pancreatic islets receive their blood supply through a complex system of fenestrated capillaries.[2] These fenestrae are probably induced by the presence of endocrine cells, as capillaries between an islet and an exocrine acinus contain approximately five times as many fenestrae on the endocrine side when compared with the exocrine side.[9] When pseudoislets, which are formed *in vitro* before transplantation and therefore lack both connective tissue and vascular elements, were implanted into the liver, a fenestrated endothelium with a continuous basal lamina, rather than liver sinusoidal blood vessels, was formed within the transplant.[10] These findings suggest that the type of capillaries formed within a graft is determined by the transplanted tissue and not by the type of blood vessels in the implantation organ.

Neovascularization is known to occur in a series of regulated sequential steps.[11,12] These steps include a local breakdown of microvessel basement membranes, a subsequent penetration of endothelial sprouts into the interstitial matrix directed against the angiogenic stimulus, and a final organization of these sprouts into a capillary network. However, an alternative process of revascularization can be seen when intact microvessels are present within the grafted tissue. In this case, connections between the intrinsic capillary system and surrounding host microvessels can be established.[13,14] This mode of revascularization has been suggested to occur after transplantation of freshly isolated islets into the liver.[15]

The findings of blood-filled caverns, with a diameter similar to that of capillaries but lacking an endothelial cell lining, being present up to almost 2 years after implantation is surprising. However, the endothelial cell lining was disrupted during islet isolation, and fragmented endothelial cells could be seen within the lumen of the capillaries. During culture these cells were extruded from the islet. Similar findings have previously been described after islet isolation and culture.[16] Indeed, the method to isolate endothelial cells have marked similarities with the technique of collagenase digestion of the pancreas to obtain isolated islets.[17,18] These capillary remnants within the cultured islets may be connected to ingrowing host capillaries that penetrate into the islet grafts to effect a revascularization. Although this ensures a rapid restoration of the blood supply to the islet graft, the newly formed capillaries lack a continuous endothelium. It may be that this early re-establishment of blood flow diminishes the angiogenic stimulus of the

graft[19,20] to such an extent that the neovascularization/re-endothelialization becomes a very slow process. These microvessels were, however, with time replaced by capillaries with fenestrated endothelium, but even after 6 months 20% of the microvessels within the islet graft lacked endothelium. Transplantation of freshly isolated islets may, however, enable the partially damaged endothelial cells to survive and re-endothelialize the islet capillaries as evidenced by the presence of a majority of capillaries with fenestrated endothelium present already 6 weeks after implantation.

The vascular endothelium expresses minimal major histocompatibility complex class II antigen in a nonactivated state, but shortly after transplantation there is an induction of these surface antigens on the endothelium.[21] Allogeneic endothelial cells are capable of stimulation of resting $CD4^+$ T cells without involvement of antigen-presenting cells.[22] Furthermore, when major histocompatibility complex class II expression was induced in cultured human fibroblasts, smooth muscle cells, epidermal cells, and endothelial cells by the addition of interferon-γ to the culture medium, only the endothelial cells were able to stimulate a proliferative response in normal allogeneic lymphocytes.[23] These findings confirm the notion of the microvasculature as a critical target for the immune response in allograft rejections.[24] These observations, together with the present and previous[16] findings of a persistent loss of vascular endothelium after various times in organ culture provide a morphological explanation for the prolonged survival of islet, thyroid, and ovarian allografts by tissue culture before implantation.[25–27]

The presence of microvessels without an endothelial cell lining within the graft may also cause complications. Important functions for endothelial cells are to promote anticoagulation and to inhibit complement activation. The presence of heparin sulfate proteoglycans was recently demonstrated on microvascular endothelial cells.[28] The enzymes of the coagulation cascade are slowly neutralized by antithrombin III in plasma, a function dramatically enhanced by heparin. In accordance with this, the presence of heparin-like proteoglycans located on the vascular endothelium was postulated to be responsible for the nonthrombogenic properties of blood capillaries. If a large fraction of the islet endocrine cells are in direct contact with the blood circulation, without the protective barrier normally established by the endothelium, their membranes could provide a procoagulant surface prone to induce the formation of microthrombi within the islet graft. Furthermore, microvessels without endothelium provide an easy access route for immune-competent cells and circulating antibodies to antigens expressed by the transplanted β-cells, eg, the glutamic acid decarboxylase protein.[29] The presence of microvessels without endothelium could thus provide an explanation for the increased vulnerability of islet grafts compared with whole pancreas transplants both experimentally[3] and after transplantation to man.[30] Furthermore, the low success rate after islet allotransplantations to IDDM patients compared with transplantation to patients suffering from surgically induced diabetes might also be explained.[31]

Acknowledgments

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