THE ROLE OF MONOCYTES IN SERUM SICKNESS NEPHRITIS*

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Despite extensive study of the immunological events in acute serum sickness in rabbits (1–3), the mechanism of glomerular inflammation and injury seen in this model has remained obscure (3). It has generally been thought that the glomerular inflammation, like the vasculitis, results from the activation of humoral mediator systems by deposited immune complexes, but no such mediator system has been identified (3). Specifically, complement and neutrophils, important in the pathogenesis of the vasculitis of serum sickness (3), and in the glomerular lesions of anti-glomerular basement membrane nephritis (2), are not critical in the pathogenesis of the glomerular lesions in this model (3), and neutrophil infiltration is not a consistent finding in the glomerulus. In addition, the source of cells responsible for glomerular hypercellularity has not been definitively established. It has usually been assumed that hypercellularity results from an increased division of intrinsic glomerular cells, recently shown to be present in this model (4). But other authors (5, 6) have asserted on morphological grounds that glomerular hypercellularity results principally from infiltration by blood monocytes.

The possibility that glomerular hypercellularity results from infiltration by monocytes suggests that cellular, rather than humoral, inflammatory mechanisms might be responsible for glomerular inflammation and injury in this model. For this reason we have studied the relative contributions of intrinsic glomerular cell division and monocyte infiltration to glomerular hypercellularity, using cell kinetic techniques, electron microscopy, and enzyme histochemistry. Our results demonstrate that glomerular hypercellularity is, indeed, principally a result of monocyte infiltration.

Materials and Methods

Model. Acute serum sickness was induced in 2-3 kg New Zealand white rabbits by immunization on day 0 with 6 mg of alum-precipitated bovine serum albumin (BSA) (Reheis Chemical Co., Chicago, Ill.) in 0.25 ml killed Bordetella pertussis vaccine (Connaught Medical Research Laboratory, Ontario, Canada) given into the rear foot pads, followed on day 4 with a single intravenous dose of BSA, 125 mg/kg. The adjuvant was used to maximize the consistency of the model (7). Because the half-life of BSA in the rabbit before onset of immune clearance is 4 d, the dose of 125 mg/kg was chosen to be compatible with the classical model.

* Supported by the Medical Research Service of the Veterans Administration, and by grants from the Kidney Foundation of Iowa and the American Heart Association Iowa Affiliate (grant 78-G-19).

1 Abbreviations used in this paper: BSA, bovine serum albumin; IF, immunofluorescence; PAS, periodic acid-Schiff; TLI, tritium-labeling index.

THE JOURNAL OF EXPERIMENTAL MEDICINE • VOLUME 150, 1979

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Serum BSA levels were measured daily by an end-point nephelometric precipitin assay (8) modified for manual performance, using monospecific rabbit antiserum to BSA which had been purified to homogeneity by DEAE cellulose chromatography. Daily urine protein excretion was measured, using rabbit serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) as a standard, with the Bio-Rad Laboratories (Richmond, Calif.) protein assay kit which is based on Coomassie Blue dye binding (9). As in the classical model, serum BSA levels declined in three phases: an initial phase of equilibration generally complete in 24 h, a slow exponential (nonimmune) clearance phase with a biological half-life of 4 d, and a rapid phase of immune clearance. 46 of 48 animals followed at least to day 10 exhibited a phase of immune clearance as evidenced by clear deviation of the BSA curve from the prior exponential decay. Onset of immune clearance was most frequently seen on day 9 or 10 (34/46 animals) but ranged from day 8 to day 15. Proteinuria was defined as a 24-h protein excretion  150 mg or a concentration in bladder urine at sacrifice  500 ~tg/ml. Normal animals, and experimental animals before onset of immune clearance, never exceeded these values. Proteinuria occurred in 30 of 38 rabbits, followed at least 48 h after immune clearance of BSA; 8 rabbits never developed proteinuria despite clearcut curves of immune clearance. Proteinuria lasted an average of 2 d. Proteinuria almost always began the day of or immediately after the drop of the serum BSA level below 0.1 mg/ml (estimated 95% clearance of injected protein); therefore, this level was taken as complete immune clearance. In most cases, immune clearance was complete by the day after the first drop of the BSA level below the prior exponential decay curve.

Using this model, animals were studied at varying times to determine the rate of intrinsic glomerular proliferation and the ultrastructural and histochemical characteristics of glomerular cells. Because of the varying intervals to onset and completion of immune clearance and onset of proteinuria, experimental animals were grouped, not by time from immunization, but rather, according to whether they were studied (a) before the onset of immune clearance, (b) after partial, but not complete, immune clearance (serum BSA  0.1 mg/ml), (c) after complete immune clearance (BSA < 0.1 mg/ml), but before the onset of proteinuria, or (d) at varying times after onset of proteinuria. Five normal animals were studied in identical fashion.

In Vivo Labeling of Dividing Cells. Under ketamine and promazine anesthesia, animals underwent laparotomy with retrograde cannulation of the left suprarenal lumbar artery (a branch of the left renal artery). The left renal artery was clamped with an aneurysm clip just proximal to the cannula and the left kidney flushed with saline to be certain of uniform perfusion. Then, 0.2 mCi[3H]thymidine (Amersham Corp., Arlington Heights, Ill., 50-65 Ci/mM) in 2 ml of heparinized autologous blood was infused into the left kidney. The clamp was left on the renal artery for 5 min to permit pulse labeling, then was removed. 30 min later, the animal was sacrificed. Biopsies of the left kidney were removed for immunofluorescence microscopy and routine paraffin embedding. The left kidney was then perfused with saline and perfusion fixed with 2% glutaraldehyde in 0.02-M phosphate-buffered saline, pH 7.4. Sections of perfusion-fixed kidney were further processed for electron microscopy and nonspecific esterase stain as described below.

Histological Methods. Immunoglobulin fractions of mono-specific rabbit anti-BSA, produced as noted above, goat anti-rabbit immunoglobulin (kindly provided by Dr. Shaun Ruddy, Medical College of Virginia, Richmond, Va.) and goat anti-rabbit albumin (kindly provided by Dr. John Butler, University of Iowa) were fluoresceinated by the dialysis bag technique (10) and fractionated as described in the reference (11) to minimize nonspecific binding that results from overlabeled molecules. Fresh tissue for immunofluorescence was embedded in Tissue-Tek II O.C.T. Compound (Lab-Tek Products, Div. Miles Laboratories, Inc., Naperville, Ill.) on a cork disk and snap frozen in isopentane at −70°C. Approximately 3-μm sections were cut in a cryostat, stained with the fluorescent reagents described above, and examined by fluorescence microscopy as described in (11). Biopsies were graded on an arbitrary scale of 0−4 +, based on the distribution of the stains, and the predominant pattern (mesangial or peripheral capillary) noted. In each case, there was no staining with goat anti-rabbit serum albumin. The results of staining with anti-BSA and anti-rabbit immunoglobulin were generally quite similar, and therefore, only results with anti-BSA will be discussed.

Other fresh sections were fixed in Duboseq-Brasil's fixative, washed repeatedly with 70% ethanol, dehydrated, and paraffin embedded. 2-μm sections were then stained with the periodic
acid-Schiff stain (Harleco, American Hospital Supply Corp., Gibbstown, N. J.) with hematoxylin counterstain or were prepared for light microscopic autoradiography. For the latter, deparaffinized sections were periodic acid treated, washed in water, and dried, then dipped in Kodak NTB emulsion (Eastman Kodak Co., Rochester, N. Y.) diluted 1:1 with distilled water. Dried slides were exposed at 4°C for 1 wk, developed in Dektol (Eastman Kodak Co.) diluted 1:1 with distilled water, then stained with Schiff reagent, and counterstained with hematoxylin. This method for combining the periodic acid-Schiff (PAS) stain with autoradiography was described by Sawicki and Rowinski (12) and has been shown to lead neither to loss of label nor to positive or negative chemography. With each batch of slides, autoradiographed controls were included for positive and negative chemography (a section of unlabeled normal kidney and a light exposed slide, respectively). Neither type of chemography was seen. Using this method the background was very low with only an occasional grain seen over nonlabeled tissue. No more than one background grain per nucleus was ever seen. Labeled nuclei, on the other hand, were generally heavily labeled with 40-50 grains per nucleus.

Perfusion-fixed tissue for electron microscopy was postfixed in 1% OsO4 in 0.1 M cacodylate buffer pH 7.4, dehydrated in alcohol followed by propylene oxide, and then embedded in Epon 812 (Shell Chemical Corp., N. Y.). In selected cases sections were rapidly dehydrated with 2,2-dimethoxypropane (DMP, J. T. Baker Chemical Co., Phillipsburg, N. J.) (13) instead of ethanol. This technique accentuates cell outlines because of dehydration artifact. 0.5-μm thick sections were cut, stained with toluidine blue and examined by light microscopy. Selected glomeruli were then thin sectioned at 500-600 Å, mounted on copper mesh or carbon-coated Formvar slot grids (Electron Microscopy Sciences, Fort Washington, Pa.), stained with 1% uranyl acetate in methanol and lead citrate and examined in a Siemens Elmskop 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.).

Other portions of perfusion-fixed tissues were washed in sucrose-gum acacia buffer (14), and frozen sectioned as described above. Sections were then stained for nonspecific esterase as in (15). Proximal tubules stain strongly for nonspecific esterase and the degree of staining can be judged grossly by development of a red color in the tissue. Generally, sections were properly stained in 4–5 min. Because nonspecific esterase gives monocytes a diffuse cytoplasmic stain, it was not possible to count accurately the number of nuclei associated with the stain. Therefore, biopsies were blindly graded for esterase activity on an arbitrary scale of 0-4+, based on the impression of the number of esterase-positive cells.

To determine the average number of cells per glomerular section and the fraction in DNA synthesis (tritium-labeling index = TLI), 20 random glomeruli from each animal were photographed using Kodak Ektachrome-160 35-mm film. As glomeruli were photographed the number of labeled cells (≥5 grains per nucleus) were counted. The slides were then projected onto a viewer screen overlaid with a piece of clear plastic film, the nuclei were counted, and marked off with a wax pencil. The TLI (TLI = labeled cells per total cells) and average glomerular cellularity (= total cells per total glomeruli counted) were calculated.

Results

Intrinsic Glomerular Proliferation. The results of glomerular cell counts and TLI are given in Fig. 1. The normal number of cells per glomerular section averages 43 (range 39-49). Fig. 1 is plotted with 35 on the bottom line so that the open bars represent approximately the increase in cellularity over normal. There is a slight increase in glomerular cellularity before the onset of immune clearance, and a sharp rise almost to peak levels with first onset of immune clearance. There is a slight further increase after complete immune clearance and onset of proteinuria, the peak cellularity representing a 55% increase over normal. Cellularity diminishes after the 1st d of proteinuria, as the proteinuria is resolving.

There is no significant increase in the normally very low TLI until after peak cellularity has developed. The average TLI in normals (0.2%, range 0–1.1%) is equal to that of any group before onset of proteinuria. There is a burst of tritium
incorporation at the onset of proteinuria, which declines substantially on the 2nd d of proteinuria and is over by the 3rd d.

These data show that the increased rate of division of glomerular cells occurs too late to explain the observed glomerular hypercellularity. In addition, the intrinsic rate of cell division is too low to explain the observed rate of increase in glomerular cellularity. The fraction of cells entering S-phase (the phase of DNA synthesis) per hour is equal to the TLI divided by the duration of S-phase in hours. Most of the dividing cells in the glomerulus are endothelial cells (16, 17) and the duration of S-phase for regenerating mouse endothelial cells has been estimated to be 7 h (18). The animals studied on the 1st d of proteinuria were, on average, in their 2nd d of immune clearance. That is, 48 h earlier they would not yet have reached immune clearance, and it can be assumed that their glomerular cellularity would have been ≈51.4 cells per glomerular section. To explain the rise from 51.4 to 67.3 cells per glomerular section in 48 h, given an S-phase duration of 7 h, one would need a TLI of 4.5%, which is 11 times the rate seen at any time before the onset of proteinuria, the period when most of the increase in glomerular cellularity occurs. It is 2.4 times the peak TLI observed after onset of proteinuria. Even if one assumes an S-phase duration of 4 h, the shortest S-phase described for any mammalian cell type (19), a TLI of 2.6% would be required. This is 6.5 times the rate seen before onset of proteinuria and 1.4 times the peak rate seen after onset of proteinuria.

Electron Microscopy. Because the observed rate of division of intrinsic glomerular cells was insufficient to explain the observed glomerular hypercellularity, we sought evidence of infiltration by blood mononuclear cells. In early lesions, large numbers of intraluminal monocytes could be identified by electron microscopy (Fig. 2) by their ruffled cytoplasm, evidences of phagocytosis, and luminal location. In favorable sections, monocytes invading mesangial areas could also be identified (Fig. 3). Monocytes, which lack peripheral filamentous zones and have ruffled cytoplasm, could be distinguished from mesangial cells which possess peripheral filamentous zones and have broad cytoplasmic processes. The identity of one of the monocytes as
a blood-born cell is confirmed by noting a process still protruding into the adjacent capillary lumen. In severe lesions large clusters of monocytes, suggestive of granuloma formation, could be identified by the ruffled cytoplasm of the cells. The glomerular intralobular architecture was often obscured. Although these observations confirmed invasion of the glomeruli by mononuclear cells, the difficulty in severe or later lesions of distinguishing monocytes from intrinsic glomerular cells, and the severe sampling limitation imposed by electron microscopy, precluded a satisfactory quantitative assessment of the role of monocytes.

**Nonspecific Esterase Stain.** We therefore used the nonspecific esterase stain, which gives monocytes a diffuse red cytoplasmic stain, to identify and semiquantitate monocytes in glomeruli. As can be seen in Fig. 4a, the nonspecific esterase stain does not stain normal intrinsic glomerular cells. Proliferated glomeruli from animals with acute serum sickness (Fig. 4b) contain large numbers of esterase-positive cells, some lobules being virtually filled with these cells in a pattern reminiscent of granuloma formation. At high power (Fig. 4c), some of the esterase-positive cells can be seen to be intraluminal cells with reniform nuclei resembling monocytes. Other cells appear to be within mesangial regions. However, even in the presence of severe inflammation, glomerular cells do not stain for nonspecific esterase.

The results of the semiquantitation of esterase-positive cells are given together with the data for glomerular cellularity, in Fig. 5. Only a rare esterase-positive circulating monocyte was seen in normal glomeruli. Before the onset of immune clearance, one
Fig. 3. Mesangial area of glomerulus from rabbit on the first day of acute serum sickness induced proteinuria. The mesangial cell (Me) can be identified by peripheral filamentous zones (arrowheads) and by its broad cytoplasmic process. Monocytes (Mo) lack peripheral filamentous zones and have ruffled cytoplasm. A process of one of the monocytes extends into the adjacent capillary lumen (large white arrow). Bar indicates 1 μm. (× 10,000).
FIG. 4. Nonspecific esterase (NSE) stains of rabbit glomeruli. (a) glomerulus from normal rabbit. Normal glomerular cells do not stain for NSE; proximal tubular cells are stained. (b) glomerulus from rabbit on first day of acute serum sickness induced proteinuria. Large numbers of esterase positive cells fill the glomerular lobules, suggesting granuloma formation. (c) higher power micrograph of another glomerulus from the same rabbit as (b). Large numbers of esterase-positive cells with reniform nuclei can be seen within capillary lumina, and perhaps within a mesangial area. Despite this severe inflammation, intrinsic glomerular cells still do not stain for NSE. (En, endothelial cell; Ep, epithelial cell; Me, mesangial cells.) (a and b, × 600; c, × 1,000).
of two biopsies showed a slightly increased number of esterase-positive cells, but with initiation of immune clearance, each biopsy showed esterase-positive cells, and their numbers increased strikingly. The number of esterase-positive cells peaked at the time of maximum cellularity with onset of proteinuria, and declined thereafter in parallel with glomerular cellularity. Thus, there is an excellent temporal correspondence between glomerular hypercellularity and the number of esterase-positive cells. The number of esterase-positive cells present in the glomerulus can easily explain the observed 50% increase in average glomerular cell count (Figs. 4 b and c).

**Evaluation of Monocyte Infiltration of Glomeruli.** To evaluate the possible role of glomerular immune complexes in trapping monocytes, we compared the humoral immune responses and glomerular changes in proteinuric and nonproteinuric rabbits. Eight rabbits never developed proteinuria despite unequivocal curves of immune clearance. There was no difference between these animals and proteinuric animals in strength of the antibody response as judged by the time to complete immune clearance (nonproteinuric animals; 10.4 d, range 9–11; proteinuric animals; 10.9 d, range 9–15), or in quantity of glomerular immune complexes as estimated by immunofluorescence for BSA (nonproteinuric animals, average immunofluorescence (IF) score 1.8+, range 0–3+; proteinuric animals average IF score 1.9+, range 0–4+). Yet the nonproteinuric animals had only minimally proliferated glomeruli as judged subjectively and had fewer glomerular monocytes compared with proteinuric animals studied at comparable times after complete immune clearance (nonproteinuric animals - average esterase score 1.0+, range 0–2+; proteinuric animals - average esterase score 2.4+; range 2–4+). These findings suggest that monocyte infiltration, and resulting glomerular injury, are the consequence of some factor other than the presence of glomerular immune complexes alone.

Another possibility, that monocytes might be trapped in the glomerulus as a result of release of lymphokines from T lymphocytes, is suggested by our finding by electron microscopy of occasional mononuclear cells resembling lymphocytes adjacent to activated monocytes (Fig. 6).
Discussion

Despite careful and extensive study of the immunological events in acute serum sickness in rabbits, the mechanisms resulting in glomerular proliferation and damage have not been established. Because of the conclusive evidences of immune complex deposition, and in part, because of the previous lack of evidence for glomerular localization of lymphocytes and monocytes, it has been argued (20) that glomerular inflammation results primarily from humoral immune mechanisms, and that cellular immunity plays little or no role. However no humoral mechanism for this glomerular inflammation has been identified, and complement and neutrophils have been conclusively shown (3) not to play a critical role.

Recently substantial evidence has appeared for participation of monocytes in glomerular inflammation (21). First, evidence appeared for a role for monocytes in crescent formation (22, 23). Later, both morphological (22, 24, 17) and cell kinetic (17) evidence derived from study of models of anti-glomerular basement membrane nephritis, demonstrated a contribution of monocytes to hypercellularity of the glomerular tuft itself.

The situation in immune complex-associated glomerulonephritis remains controversial. The common descriptive term proliferative glomerulonephritis implies that hypercellularity is attributable to division of intrinsic glomerular cells. Recently,
Bradfield et al. (4) have confirmed an increased rate of division of glomerular cells in acute serum sickness nephritis in rabbits. Though these authors documented some degree of monocyte and lymphocyte infiltration in the glomerulus, they favored the hypothesis that hypercellularity resulted principally from intrinsic cell division. Mollo et al. (16) have also confirmed a fivefold increase in the rate of intrinsic glomerular cell division in a rat model of passive immune complex nephritis. They did not note a measurable increase in glomerular cell count, however, and concluded that the observed rate of cell division was insufficient to explain glomerular hypercellularity in active models. Sano (5) and Shigematsu and Kobayashi (6) have emphasized on morphological grounds the predominant role of monocyte infiltration in serum sickness nephritis.

Our results (Fig. 1) confirm a significant increase in the rate of division of intrinsic glomerular cells in acute serum sickness nephritis, with a rise from an average TLI of 0.2% in normals to a peak of 1.9% at the onset of proteinuria. The changes are quantitatively comparable to those observed by Bradfield et al. (4) and Mollo et al. (16) in immune complex nephritis, and the results of Schreiner et al. (17) in accelerated anti-glomerular basement membrane nephritis. However, we have confirmed the impression of Mollo et al. (16) that the observed increase in TLI is insufficient to explain observed hypercellularity. The increase in TLI is not seen until the onset of proteinuria, by which time peak cellularity has been achieved, and is, in any case, not quantitatively sufficient even at the peak rate to explain the observed rate of increase in cellularity. The increase in glomerular proliferative rate is short lived, returning to baseline within 48 h.

The fact that the observed rate of cell division before the onset of proteinuria is only 1/10 of that necessary to explain the observed rate of increase in cellularity suggests that no more than 10% of the hypercellularity is attributable to cell division. But even this estimate may be overly generous, for it does not allow for cell death as a result of glomerular injury. The timing and degree of glomerular proliferation suggest, in fact, that this is a reparative response to glomerular injury.

Our observations by electron microscopy confirm those of other observers (5, 17) that large numbers of monocytes can be identified within capillary lumina (Fig. 2) and in mesangial areas (Fig. 3). at the time of increased glomerular cellularity. But we also agree that it can be difficult to distinguish monocytes from intrinsic glomerular cells, especially in more severe and later lesions. Therefore, to evaluate the timing and extent of monocyte infiltration we used enzyme histochemistry.

Using frozen sections of perfusion fixed tissue we were able to show that intrinsic glomerular cells of normal or proliferated glomeruli did not stain for nonspecific esterase (Figs. 4a and c), whereas infiltrating monocytes were strongly stained (Figs. 4b and c). When the results of the esterase stains were semiquantitated and correlated with changes in glomerular cellularity (Fig. 5), a close correspondence was noted. Infiltration by monocytes was both temporally and quantitatively able to explain the observed changes in glomerular cellularity.

Three general explanations can be put forward to explain the monocytic infiltration seen in our model. First, the monocytes may appear as a nonspecific response to tissue injury mediated in some other fashion. Our data would seem to make this unlikely, because monocytic infiltration is seen at the very onset of immune clearance (Fig. 1) before glomerular injury (as indicated by proteinuria) or repair (as indicated by...
intrinsic cell proliferation) is noted. Second, the monocytes may be trapped by interaction with the clustered Fc regions of glomerular immune complexes. Evidence for such a mechanism has recently been presented by Striker et al. (25) who have documented an increased number of mesangial monocytes in glomeruli of mice with a passive immune complex nephritis, presumably attracted and trapped by the immune complexes themselves. This seems not to be the entire explanation for the monocytic infiltration in our model because there were no obvious differences in the strength of the immune response or quantity of glomerular immune complexes in proteinuric and nonproteinuric rabbits. Yet, the nonproteinuric rabbits had fewer monocytes and less hypercellularity. Third, the monocytic infiltration may be a manifestation of T-cell-mediated immunity. Our results are most consistent with this hypothesis. Like Bradfield et al. (4), we have found cells resembling lymphocytes adjacent to activated monocytes (Fig. 6) in proliferative lesions, suggesting a role for T-lymphocyte-mediated immunity in the glomerular lesions of acute serum sickness nephritis, similar to that recently documented in a model of anti-glomerular basement membrane nephritis (26). These are preliminary and indirect evidences, however, and will need to be confirmed in future studies.

Finally, the monocytic glomerular infiltration seen in this model of immune complex-associated nephritis is quite acute and transient. This is also true of the model of accelerated anti-glomerular basement membrane nephritis described by Schreiner et al. (17). These studies do not shed light on the relative roles of monocytic infiltration and intrinsic proliferation in more chronic forms of proliferative nephritis. A recent report by Schiffer and Michael (27) has shown that virtually all the cells in proliferative lesions of recurrent glomerulonephritis in human transplants are of donor, not recipient origin, demonstrating that intrinsic proliferation is important in more chronic and progressive glomerulonephritis. Whether this proliferation is also reparative, and whether monocytes play a role in these diseases, remains to be determined. Use of the nonspecific esterase stain may prove to be of value in further clarifying the role of the monocyte in these forms of glomerulonephritis.

Summary

We have investigated the pathogenesis of glomerular hypercellularity seen in acute serum sickness nephritis induced in rabbits with bovine serum albumin (BSA). The increase in cellularity began with the first stages of immune clearance of BSA, with a peak cellularity occurring at the time of onset of proteinuria. Although there was a significant increase in the fraction of glomerular cells incorporating [³H]thymidine, first seen at the onset of proteinuria, this increase occurred too late and was too small to explain the observed rate of increase in glomerular cellularity. On the other hand, a striking monocytic infiltration of the glomeruli was documented by electron microscopy and by staining for nonspecific esterase. This monocytic infiltration paralleled the observed course of glomerular hypercellularity and was quantitatively sufficient to explain the total increase seen. It appears, therefore, that glomerular hypercellularity seen in this model is principally a result of monocyte infiltration.

The authors wish to thank Dr. P. A. Cancilla for invaluable advice and criticism, and Ms. Karla Wilker for secretarial assistance in preparing the manuscript.

Received for publication 20 February 1979.
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