Functional Changes in Aging Polymorphonuclear Leukocytes

Kayoko Tanji-Matsuba, MSc; Stephan F. van Eeden, MD, PhD; Yuji Saito, MD; Mitsushi Okazawa, MD; Maria E. Klut, PhD; Shizu Hayashi, PhD; James C. Hogg, MD, PhD

Background—Previous studies from our laboratory have shown that the expression of L-selectin on polymorphonuclear neutrophils (PMN) decreases as the cell ages in the circulation and that these older PMN have more fragmented DNA and show morphological features of apoptosis.

Methods and Results—The present study was designed to compare the functional capabilities of PMN expressing low levels of L-selectin (L-selectinlow) and the total population of PMN they were isolated from (L-selectinmixed). The results show no difference of the baseline filamentous actin (F-actin) content between PMN expressing low and high levels of L-selectin. However, the ability of L-selectinlow PMN to assemble F-actin was impaired after stimulation by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (1 mmol/L fMLP: P<.02, 10 mmol/L fMLP: P<.01). The ability of L-selectinlow PMN to change shape when stimulated (10 mmol/L fMLP) was also decreased (P<.05). Filtration studies showed no difference in baseline deformability between L-selectinlow and L-selectinmixed leukocytes, but the L-selectinlow cells showed a decreased ability to stiffen after fMLP stimulation (P<.05). L-selectinlow cells demonstrated a decreased ability to migrate toward a chemoattractant (1, 3, and 10 mmol/L fMLP) (P<.004) but have an enhanced ability to upregulate CD18 (P<.00002) and produce hydrogen peroxide (P<.00004).

Conclusions—We conclude that PMN undergo substantial functional changes as they age in the circulation. (Circulation. 1998;97:91-98.)

Key Words: neutrophils • cells • chemotaxis • adhesion molecules • free radicals

Polymorphonuclear neutrophils marginate along vessel walls, adhere firmly to endothelium, and migrate toward a chemotactic stimulus as they are recruited to an inflammatory site.1-4 Their primary role on reaching the site of inflammation is to engulf and destroy pathogenic organisms by generating reactive oxygen species and releasing hydrolytic enzymes into the phagocytic vacuoles containing the engulfed bacteria. The hypothesis that PMN become progressively activated during this process and that host tissue injury occurs when activation is either abnormal or excessive has been the subject of many reports.5,6 Whyte et al7 have shown that PMN cultured in vitro lose functions and undergo apoptosis. A recent report from our laboratory has shown that the aging members of the population of circulating PMN expressing low levels of L-selectin and have morphological features of apoptosis with high levels of fragmented DNA.8 These findings suggest that these cells started undergoing programmed cell death in the circulation, and the purpose of this study was to compare the function of this group of older cells with the general PMN population. To test the hypothesis that the functional capabilities of the older cells were different, we examined their abilities to assemble and reorganize F-actin from G-actin, change their deformability and shape, migrate toward a chemotactic stimulus, degranulate, and generate oxygen radicals.

Animals
The study was approved by the Animal Experimentation Committee of the University of British Columbia. Four female New Zealand White rabbits were used in each of these studies except in the F-actin study, in which 5 rabbits were used.

Baseline F-actin Contents
F-actin content of PMN in whole blood was determined with the use of a modification of a previously described method.9 PMN in whole blood were stained for both L-selectin with DREG-200 (mouse monoclonal antibody against rabbit L-selectin, kind gift from Dr E.C. Butcher) and F-actin with fluorescein phalloidin (Molecular Probes, Inc) Exitation (Ex.) 495 Emission (Em.) 520) (375 μg/mL FITC phalloidin). Phycoerythrin-conjugated goat anti-mouse-IgG (PE) (Ex.495 Em. 576) (DAKO) was used as a secondary antibody for L-selectin. A negative control for L-selectin, nonimmune mouse IgG followed by PE conjugated goat anti-mouse IgG was used. F-actin–negative control was processed by adding phosphate-buffered saline (PBS) instead of FITC phalloidin. The influence of the double labeling procedure was evaluated by comparing results from cells labeled for a single antigen (either L-selectin or F-actin) with results from double-labeled cells. Briefly, 100 μL of whole blood was added to 200 μL of PBS and 20 μL of 20 μg/mL DREG-200, incubated for 10 minutes, washed with 5 mL of PBS by centrifugation at 200g for 7 minutes, resuspended in 25 μL of 15 μg/mL PE in PBS and incubated for 10 minutes in the dark. Red blood cells were lysed by IMMUNO-LYSE (Coulter Immuno) for 2 minutes and fixed by 200
μL of formaldehyde, EM-Grade 16% solution (Electron Microscopy Sciences) for 30 minutes in the dark. After washing, cells were mixed with 200 μL of FITC phallolidin permeabilizing solution (2.5 μL of 200 U/ml FITC phallolidin in methanol evaporated and mixed with of 200 μL of 0.2 μmol/L L-a-lysophosphatidylcholine palmitoyl (Sigma) in PBS) and incubated for 30 minutes at 37°C in the dark. This solution permeabilizes the cell membrane and stains F-actin. Cells were washed and analyzed by flow cytometry, EPICS XL-MCL cell analysis system (Coulter). Analysis gates for PMN were established with distinctive forward and side scatter profiles and expressed as MFI on a log-scale analyzing 3000 to 6000 cells per specimen. The expression of F-actin on 40% of PMN expressing the lowest and 40% expressing the highest levels of L-selectin were determined (Fig 1). The 40% cutoff line was selected because the average purity of the in vitro separation of L-selectin deficient cells was ~40% of the total population (see next section).

**In Vitro Separation of Cells Expressing Low Levels of L-selectin**

The selection of cells expressing low levels of L-selectin was achieved by binding the monoclonal antibody DREG-200 to magnetic beads as follows. An aliquot of 1 mL of DYNABEADS M-450 (Dynal) at a concentration of 4×10¹⁰ beads/mL with surface conjugated sheep anti-mouse IgG was washed in 10 mL of 0.01 mol/L HEPES, 0.09% sodium chloride irrigation, USP (Baxter) buffer (HEPES normal saline, HNS). A magnet, MPC (DYNAL), was then applied to the surface of the tube for 2 minutes and the wash fluid was decanted off. This process was repeated three times. The washed beads were then resuspended in 500 μL of HNS and 65 μL of DREG-200 at 100 μg/mL was added. This mixture of 0.2 μg of DREG-200 and 1 mg of beads was incubated overnight at 4°C and the beads were kept in suspension by bidirectional rotation. Unbound DREG-200 antibody was removed from the magnetic beads by washing the mixture in 10 mL HNS/0.1% BSA, pH 7.4, at 4°C using bidirectional rotation for 30 minutes, applying the magnet, and decanting the wash fluid. This washing procedure was repeated four times. After resuspending in 500 μL of HNS/0.1% BSA buffer, DREG-200–coated magnetic beads were stored at 4°C and kept in suspension until used as described below.

Leukocyte-rich-plasma (LRP) prepared from peripheral blood collected from the central ear artery of each rabbit using acid citrate dextrose (ACD) (Fenwal, Baxter) was used as starting material. Red blood cells were sedimented with 4% dextran (average molecular weight of 162 000) (Sigma) in 1.38 mmol/L NaCl, 27 mmol/L KCl, 8.1 mmol/L Na₂HPO₄, 7H₂O, 1.5 mmol/L KH₂PO₄, and 5.5 mmol/L glucose pH 7.4 buffer (PMN buffer) for 25 minutes. Endotoxin-free solutions were used throughout all experiments. The leukocyte-rich surface layer that contains the LRP was removed and centrifuged at 300g for 7 minutes. The pellet of cells was resuspended in 600 μL of HNS/0.1% BSA and divided in two aliquots. One aliquot was incubated at 4°C for 1 hour with bidirectional rotation with either DREG-200–coated magnetic beads at a ratio of approximately 20 to 1 (beads to leukocyte) and the other aliquot incubated in buffer using the same experimental conditions (control cells). Leukocytes that adhered to the magnetic beads by virtue of the binding of L-selectin on their surface to DREG-200 were removed by the magnet and referred to as L-selectinlow leukocytes. Those that remained in solution were referred to as L-selectinhigh leukocytes. The starting solution (LRP) was composed of 70±5.4% PMN and the rest were mononuclear cells (lymphocytes and monocytes), and this composition did not change significantly after the selection procedure with the magnetic beads (65±6.6% PMN). The functional capabilities of the L-selectinlow were compared with the total population of leukocytes that they were isolated from (control cells or L-selectinhigh cells).

These L-selectinlow and L-selectinhigh leukocytes were used in each experiment below because the magnetic beads bound to L-selectinhigh leukocytes interfere with the flow cytometry analysis as well as in the filtration studies. In preliminary experiments, incubating LRP with either magnetic beads alone, IgG coated beads, or DREG-200 followed by sheep anti-mouse IgG did not change the surface expression of the cell activation marker CD18 or the expression of L-selectin on PMN. The purity of the selection process was determined by measuring L-selectin expression on the L-selectinlow population of PMN by using flow cytometry with DREG-200 as a primary antibody and goat anti-mouse FITC as a secondary antibody. The average MFI of L-selectinlow PMN was ~40% of that of the mixed population of PMN they were isolated from (L-selectinmixed PMN). All
Pelouge et al. 23 Briefly, L-selectinlow and L-selectinmixed leukocytes were resuspended in 100 µL of 1/10 dilution of 10X Hanks’ salt (Stem Cell Technologies Inc) (Hanks’ buffer) with the final concentration to be 4X10^6 cells/mL, incubated at 37°C for 3 minutes, and stimulated with 25 µL of fMLP in PBS (final concentration, 1 and 10 nmol/L). Leukocytes were fixed by 3% paraformaldehyde (PFA) for 30 minutes at 0, 5, 10, 20, and 80 seconds after stimulation, washed with 5 mL of PBS (centrifugation at 200g for 7 minutes). Leukocytes were incubated with 10 µL of 200/µL N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD phallacidin. Molecular Probes, Inc) (Ex.405 Em.530) in methanol was evaporated and mixed with 200 µL of 0.2 µmol/L PC in PBS and further processed as described above. The concentrations of fMLP were (1 and 10 nmol/L) were in the linear range of the dose-response curve of cells stimulated by fMLP in concentrations between 0.01 and 300 nmol/L (data not shown).

Cell Shape Changes

The changes in PMN shape were measured according to the method described by Holden et al. 24 Briefly, L-selectinlow and L-selectinmixed leukocytes were resuspended in 100 µL of Hanks’ buffer with the final concentration to be 4X10^6 cells/mL, prewarmed at 37°C for 3 minutes, stimulated by 10 nmol/L fMLP, and 0, 0.25, 0.5, 1, 2, 5, and 10 minutes afterward fixed by the same volume of 5% glutaraldehyde (1/5 dilution of 25% solution Glutaraldehyde EM Grade. Electron Microscopy Sciences). The cells were cytospun onto precoated slides (Fisher Scientific), stained by the hematoxylin and eosin method, 25 mounted in a permanent medium (Entellan, New BDH) and analyzed by light microscopy (Nikon Labphoto-2). One hundred PMN were counted in random fields of view to determine the fraction of PMN that had changed shape. 10 The interobserver variability was determined by two observers counting 14 randomly selected coded slides (100 cells/slide) and the intraobserver variability by the same observer counting these slides 2 weeks apart. Both the interobserver variability and the intraobserver variability were within the standard deviation.

PMN Deformability

Leukocytes were filtered in vitro according to the filtration method described by Lowe and Lennie. 26 Briefly, L-selectinlow and L-selectinmixed leukocytes were resuspended to 1X10^6 cells/mL in 30 mL of PBS containing 0.5% human albumin (albumin [human] 5%, USP, Plasmin-5, Miles) (PBS/albumin). A 35-µL polypropylene syringe (Sherwood, Monaject Plastic) and three-way stopcock (Medxinc) were mounted onto a pump (Harvard apparatus model 55 to 27 mmol/L KCl, 8.1 mmol/L Na2HPO4, and 250 to 6000 cells per specimen.

Chemoattractant Ability

L-selectinlow and L-selectinmixed leukocytes were resuspended in Hanks’ buffer with the final concentration to be 1X10^6 cells/mL. A 48-well modified Boyden chamber (Neuro Probe, Inc) and 5-µm pore polycarbonate membrane filters (Poretics) were used following the manufacturer’s instructions. FiveX10^6 cells were loaded into the top chamber with fMLP solution (final concentration to be 1, 3, and 10 nmol/L) in the lower chamber. Chambers were incubated for 45 minutes at 37°C. Nonchemotaxed cells left on the loaded surface of the membrane were removed. The filters were fixed by methanol for 20 seconds and stained by Diff-Quik (Dade Diagnostics of P.R. Inc) following the manufacturer’s instruction. The cells that had passed through the filters were counted in random fields of view under the light microscope, counting at least 100 cells.

Hydrogen Peroxide Production

The respiratory burst activity of PMN was determined by measuring PMN hydrogen peroxide (H2O2) production at baseline and after fMLP stimulation according to the method previously described by Bass et al. 14 Briefly, L-selectinlow and L-selectinmixed leukocytes were resuspended in Hanks’ buffer with the final concentration to be 4X10^6 cells/mL, prewarmed at 37°C for 3 minutes, and stimulated with 25 µL of fMLP in PBS (final concentration, 1 nmol/L). Measurements were performed at 0, 2, 4, 8, 16, and 32 minutes after the addition of fMLP. Cells were fixed with 0.025% (final concentration) glutaraldehyde for 10 minutes, cell solution was washed, 25 µL of 75 µg/mL anti-CD18 antibody (Beta chain, clone MHM23, DAKO) in PBS was added for a final concentration of 5 µg/mL. Nonimmune mouse IgG in the same final concentration was used as a negative control. After 15 minutes of incubation, cells were washed, incubated in 25 µL of 1/25 diluted anti-mouse IgG (whole molecule) FITC conjugated (Sigma, Ex.495 Em.520) in the dark, washed, and fixed by 1% PFA. Analysis gates for PMN were established using distinctive forward and side scatter profiles, and results were expressed as MFI on a log-scale analyzing 3000 to 6000 cells per specimen.

Statistical Analysis

Data were analyzed with a two-way ANOVA for repeated measurements with blocking on subjects in F-actin (Fig 2), cell shape changes (Fig 3), chemoattractant ability (Fig 6), CD18 expression (Fig 7), and hydrogen peroxide production (Fig 8) studies. The sequential rejective Bonferroni procedure was used to correct for multiple comparisons. The methods described by Ratkowsky 27 was used to analyze the deformability of PMN (Figs 4, and 5). Each curve was fit to the equation \( y=a/[1+exp(-b-x)] \) to determine an estimate for the plateau, represented here by a. 27 The mean plateau for each subject (taken over two to four trials per subject) in both groups was calculated according to Mood et al. 27 For each subject, the difference in mean plateau between the two groups to be compared was then calculated by using the method above. 27 An overall mean difference was computed, and a t statistic was calculated to determine significance.
P.05). The Bonferroni correction was considered in the calculation of the \( t \) statistic to correct for multiple comparisons.

**Results**

**F-actin Content of PMN**

Figs 1A and 1B show the purity of L-selectin\(^{low}\) PMN as measured by flow cytometry. In this representative example, 40% of the population of PMN was selected as L-selectin\(^{low}\) cells. Figs 1C and 1D show the baseline F-actin content of L-selectin\(^{high}\) and L-selectin\(^{low}\) PMN populations. There was no difference in baseline F-actin content between the 40% of PMN expressing high levels of L-selectin and 40% of PMN expressing low levels of L-selectin. Fig 2 shows F-actin assembly in a L-selectin\(^{low}\) and L-selectin\(^{mixed}\) population of PMN after they were stimulated by 1 and 10 nmol/L fMLP. Baseline F-actin was similar \((P>.05)\); however, at 10 seconds after stimulation with both 1 nmol/L \((P<.02)\) and 10 nmol/L fMLP \((P<.01)\), L-selectin\(^{mixed}\) PMN contained more F-actin than L-selectin\(^{low}\) PMN. This difference was not seen at the later time points.

**Cell Shape Changes in PMN**

At baseline, \( \approx \)20% of PMN in both of L-selectin\(^{low}\) and L-selectin\(^{mixed}\) cell populations had a nonspherical shape. Fig 3 also shows the fraction of PMN that changed shape after stimulation by 10 nmol/L fMLP. At 2, 5, and 10 minutes after stimulation, more PMN in the mixed population changed shape \((P<.05)\) compared with the L-selectin\(^{low}\) population.

**PMN Deformability**

The baseline pressures recorded over 8 minutes by filtration of L-selectin\(^{low}\) and L-selectin\(^{mixed}\) populations of leukocytes through polycarbonate filters with a 5-\(\mu\)m pore size are shown in Fig 4 (n=7). Under baseline conditions, these curves were similar. Filtration of fMLP-stimulated (1 nmol/L) cells showed a significant increase in the plateau pressure during filtration of the mixed population with little change in the L-selectin\(^{low}\) cells \((P<.05)\). The fraction of PMN in the cell suspension before and after filtration in both populations were not significantly different \((P>.05)\) (data not shown).
Chemotactic Ability of PMN
The chemotactic ability of L-selectin<sup>low</sup> and L-selectin<sup>mixed</sup> populations of PMN after they were stimulated by 1, 3, and 10 nmol/L fMLP are shown in Fig 6. More PMN migrated through the filters with 1, 3, and 10 nmol/L fMLP gradient in the mixed population of PMN compared with the L-selectin<sup>low</sup> population of PMN (P<.0004).

CD18 Expression by PMN
Fig 7 shows CD18 expression on a population of L-selectin<sup>low</sup> and L-selectin<sup>mixed</sup> PMN after stimulation by 1 nmol/L fMLP. Baseline expression of CD18 was similar in L-selectin<sup>low</sup> and L-selectin<sup>mixed</sup> PMN (P>.05). However, after fMLP stimulation (1 nmol/L), L-selectin<sup>low</sup> PMN expressed more CD18 on the surface than a mixed population of PMN (P<.00002).

Hydrogen Peroxide Production by PMN
Fig 8 shows hydrogen peroxide production in a population of L-selectin<sup>low</sup> and L-selectin<sup>mixed</sup> PMN after they were stimulated by 1 nmol/L fMLP. Baseline production of hydrogen peroxide was similar in the two populations of PMN (P>.05), but with stimulation L-selectin<sup>low</sup> PMN produced more hydrogen peroxide than a population of mixed PMN (P<.00004).

Discussion
This report concerns the functional changes that occur in older PMN in the circulation. Previous studies from our laboratory have shown that PMN lose their L-selectin as they age in the circulation, and we have used this marker of cell age to select a population of older cells from the circulating population of PMN. Several studies have shown that PMN aged in vitro lose functions such as chemotaxis, adhesion, spreading, and phagocytosis and then undergo apoptosis. In this study we compared the functional properties of an older circulating population of PMN and found that they have a decreased ability to convert G-actin to F-actin, change shape, and migrate toward a chemotactic stimulus. Although L-selectin<sup>low</sup> PMN baseline deformability was similar to that of L-selectin<sup>mixed</sup> PMN, their ability to stiffen after stimulation was impaired. Furthermore, these older PMN appear to degranulate more readily and produce higher levels of oxygen radicals than a mixed population of circulating PMN.

The peripheral blood PMN consists of a heterogeneous population of cells with respect to surface expression of Fc receptors, fMLP receptors, and responses to chemotactic factors. Heterogeneity in the maturation of circulating PMN maturity has been recognized since the 1920s and is thought to account for the variability in PMN locomotion and alkaline phosphate content. Studies from our laboratory have shown that PMN newly released from the bone marrow express higher levels of L-selectin than their circulating counterparts and that they lose their L-selectin as they age in the circulation. The role of these different populations of circulating PMN in the pathogenesis of inflammatory diseases is still unclear.

The coordinated conversion of G-actin to F-actin is essential for migration of PMN out of the vascular space toward pathogens. Actin constitutes 10% of the total protein of PMN, and ~50% of this actin is not polymerized in the resting cell. Stimulation by chemotactic factors causes a rapid and transient increase in the polymerization of actin with the assembly of F-actin. Our results show no difference in
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baseline F-actin content between L-selectin<sub>low</sub> and L-selectin<sub>high</sub> PMN (Figs 1C and 1D); however, the ability of older PMN to rapidly polymerize actin (Fig 2), change shape (Fig 3), and migrate in a chemotactic gradient (Fig 6) is impaired after stimulation with fMLP. These defects in older PMN could occur at any of the numerous control sites of this well-regulated process, including the interaction of the chemottractant with its receptor on the cell surface, subsequent signal transduction, intracellular calcium mobilization, and changes in the content and regulation of the actin binding proteins. The normal to above normal response of these older PMN to degranulate and produce hydrogen peroxide after fMLP stimulation suggests that this defect is not at the fMLP receptor level. Therefore the reduced ability of older PMN to assemble F-actin as well as their decreased ability to change shape and migrate into the tissues by moving toward a chemotactic gradient most probably represents defective intracellular events.

The defective regulation of actin assembly was supported by experiments showing that the ability of these older PMN to stiffen after stimulation was impaired (Fig 5). Cell deformability was measured as the pressure developed during cell filtration through micropore membranes where the mean diameter (5 μm) is similar to the average diameter of the human and rabbit pulmonary capillary segments. Selby et al.<sup>48</sup> have shown a relationship between PMN deformability in vitro and sequestration of PMN in the pulmonary capillaries by using a similar system. In our experiments, baseline measurements showed no difference of the deformability between older and younger PMN, but on stimulation the ability of older PMN to increase their stiffness was impaired (Fig 5). This result is consistent with the defective F-actin assembly in these older PMN. Changes in the deformability of PMN have been shown to be the major factor resulting in sequestration of PMN in pulmonary capillaries. This functional defect of older PMN to stiffen together with the results of a decreased ability to change shape and chemotaxis suggested that these PMN are less likely to be trapped in microvessels. This sequestration of PMN in the lung has been shown to be an important initial step in PMN-mediated lung injury in several models of acute and chronic lung inflammation.<sup>5,6,47,48,50</sup>

In the systemic circulation, the low levels of L-selectin on older PMN could contribute to a decreased margination of these cells along capillary walls by reducing their ability to roll on activated endothelium in postcapillary venules. Our studies showed that older PMN, expressing low levels of L-selectin, had a reduced ability to migrate toward a chemotactic stimulus (Fig 6). Interestingly, this is similar to the defect described by Lichtman and Weed<sup>51</sup> in immature granulocytes in the bone marrow suggesting that chemotactic ability is one of the last functions that PMN attained during maturation but also one of the first functions to deteriorate during aging. These defects in the early functional responses of older PMN could preclude them from an inflammatory site both in the systemic circulation and the pulmonary circulation.

The retention and migration of PMN through an endothelial barrier are due, at least in part, to an increase in PMN adhesiveness.<sup>52</sup> Several studies have shown that recruitment of PMN into tissues can be attenuated by blocking surface PMN adhesion molecules.<sup>53–56</sup> The CD18 antigen is required for firm adhesion and migration of PMN, and cell activation causes degranulation of specific granules that increases CD18 on the cell surface.<sup>7,58</sup> Measurements at baseline showed that the expression of CD18 was similar in older and younger PMN (P > .05), but cell activation with fMLP increased CD18 expression significantly more in older PMN (Fig 7). The enhanced degranulation response of older PMN as well as their increased ability to produce oxygen radicals (Fig 8) suggested that these older PMN are primed. This priming of PMN may occur during their life in the circulation, where they encounter mildly activated vascular beds such as in the bladder, gums, and upper respiratory tract. Removal of these older primed PMN from the circulation may be beneficial in that it would protect the vascular bed from inappropriate oxygen radical damage when these cells encounter an intravascular stimulus generated from the complement, coagulation, or kinin-generating system of the plasma. Several studies have shown that activated PMN expressing higher levels of CD18 are removed from the circulation.<sup>1–3</sup> We speculate that this enhanced ability of older PMN expressing low levels of L-selectin to recruit CD18 to the cell surface could augment their permanent removal from the circulation.

In summary, the results presented here support the hypothesis that older PMN have different functional responses from a mixed population of circulating PMN. These defects are mainly in their early response functions, such as F-actin assembly, changes in deformability, and chemotaxis. These functions are important for PMN to facilitate their movement into an inflammatory sites and migration toward an activating stimulus. As these older PMN are defective in their ability to migrate but degranulate more readily and produce more oxygen radicals when stimulated, they are potentially harmful in situations of diffuse intravascular cell activation. These defects could make older PMN less likely to be recruited to a site of inflammation and may mark them for permanent removal from the circulation.

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