Aspirin (5 mmol/L) Inhibits Leukocyte Attack and Triggered Reactive Cell Proliferation in a 3D Human Coronary In Vitro Model

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Background—Leukocyte attack (LA) and the triggered reactive proliferation of smooth muscle cells (SMCs) are key events for the development of early atherosclerosis and restenosis. In the present study, we used a 3D human coronary in vitro model of LA (3DLA model) to examine the effect of high-dose aspirin on the adhesion and chemotaxis of leukocytes and the reactive proliferative response of SMCs.

Methods and Results—for dose-finding, the effect of aspirin (1, 2, 5, and 10 mmol/L) on the tumor necrosis factor-α–induced upregulation of intercellular adhesion molecule-1 was analyzed in monocultures of human coronary endothelial cells (HCAEC) and the SMCs of the human coronary media (HCMSMC). In cytoflow and Northern blot experiments, the expression of intercellular adhesion molecule-1 was slightly reduced after incubation with 5 mmol/L aspirin, and strong inhibition was found after incubation with 10 mmol/L. In 3DLA models, HCAECs and HCMSMCs were cultured on both sides of a porous filter. For LA, human monocytes or CD4+ lymphocytes were seeded on the HCAEC side of the 3DLA unit. A dose of 5 mmol/L aspirin inhibited the adherence of monocytes or CD4+ lymphocytes by 50% (P<0.01) and the chemotaxis of monocytes by 90% (P<0.01). The reactive proliferative response of cocultured HCMSMCs after LA, as measured by the uptake of bromodeoxyuridine, was significantly reduced by 83% after selective monocyte attack (P<0.001) and by 42% after selective CD4+ lymphocyte attack (P<0.05).

Conclusions—A local concentration of 5 mmol/L aspirin should be accepted as the lowest rational concentration for the beneficial in vitro effects of high-dose aspirin to be reproduced in clinical studies. (Circulation. 2001;103:1688-1694.)

Key Words: aspirin ■ leukocytes ■ atherosclerosis ■ restenosis

Aspirin and its analogs are among the most widely used drugs worldwide. Therapeutic doses of aspirin exhibit 2 types of actions, depending on the dose of the drug.1 At low therapeutic doses, aspirin is an effective inhibitor of the cyclooxygenase pathway and, hence, prostaglandin-mediated signaling. At higher therapeutic doses, aspirin has antiinflammatory effects that are independent of the inhibition of prostaglandin synthesis.1

The infiltration of inflammatory leukocytes, accumulation of cholesterol-laden macrophages, proliferation of smooth muscle cells (SMC), deposition of extracellular matrix, and thrombosis are crucial events that occur in early atherosclerosis and restenosis. Among these, the localized accumulation of monocytes/macrophages and T lymphocytes in the arterial intima after prior expression of adhesion molecules2,3 and triggered cellular and subcellular events are of outstanding importance. Studies have demonstrated that coronary atherosclerotic lesions express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1.4,5 Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) upregulate ICAM-1, vascular cell adhesion molecule-1, and E-selectin, as well as the production of chemokines (eg, monocyte chemoattractant protein-1), at the level of gene transcription involving the binding of nuclear factor-κB (NF-κB).6–9 Recently, Kopp and Sankar10 reported that aspirin inhibits the activation of NF-κB through the stabilization of inhibitor protein–κB.

Three-dimensional human coronary in vitro models of leukocyte attack (3DLA models) focus on leukocyte adhesion and chemotaxis and the triggered proliferative response of cocultured SMCs. These models are a further development of the transfilter coculture model that was first described by Betz and colleagues.11 However, in contrast to the mechanical injury of the endothelium applied in the transfilter coculture technique,11 only an indirect injury is performed by leukocyte attack (LA) with human monocytes or human CD4+ lymphocytes in the 3DLA model.

Before the effect of high-dose aspirin was studied in the 3DLA model, dose finding was performed in monocultures of...
human coronary endothelial cells (HCAEC) and HCMSMCs. As reported earlier by our laboratory,\textsuperscript{12} the surface expression of ICAM-1 in HCAECs and HCMSMCs is strongly upregulated by TNF-\(\alpha\). In part I of the study, the effect of aspirin (1, 2, 5, and 10 mmol/L) on the TNF-\(\alpha\)-induced surface expression of ICAM-1 was characterized. In part II of the study, the 3DLA model was used to investigate the effect of high-dose aspirin (5 mmol/L) on the adhesion and chemotaxis of monocytes and CD\(^4\) lymphocytes and on the reactive proliferation of HCMSMCs.

**Methods**

**Cell Culture**

HCMSMCs (Clonetics) were cultured in Smooth Muscle Cell Basal Medium (Clonetics). For the identification of SMCs, monoclonal antibodies against smooth muscle \(\alpha\)-actin were used (Renner). HCAECs (Clonetics) were grown in Endothelial Basal Medium (Clonetics). For the identification of endothelial cells, antibodies against von Willebrand factor (Dakopatts) were used.

Human CD\(^4\) lymphocytes and monocytes were isolated from the residual leukocytes of single donors using the MACS cell-isolation kit (Miltenyi Biotec GmbH).

**Aspirin**

A total of 500 mg of aspirin (Sigma) was dissolved in 70% ethanol (Merck). In all investigations, aspirin was added for a period of 18 hours; during the last 6 hours of incubation, an additional stimulus with TNF-\(\alpha\) (20 ng/mL, Sigma) was administered.

**Flow Cytometry**

For flow cytometry analysis of the expression of ICAM-1 in HCAECs and HCMSMCs, \(5\times10^5\) cells were seeded into petri dishes (75 cm\(^2\)). Aspirin (1, 2, 5, 10 mmol/L) was added to the cultures for a period of 18 hours. During the last 6 hours of aspirin incubation, the expression of adhesion molecules was stimulated by adding TNF-\(\alpha\).

After aspirin/TNF-\(\alpha\) treatment, cells were washed twice with phosphate-buffered saline, detached by trypsin treatment, and suspended in phosphate-buffered saline (pH 7.2) containing 1% bovine serum albumin and 0.2% sodium azide at 4°C. Cells were resuspended in 100 \(\mu\)L of a FITC-conjugated monoclonal antibody directed against ICAM-1 (clone 84H10, Dianova Immunotech; final concentration 10 \(\mu\)g/mL) and incubated for 20 minutes at 4°C. A total of \(5\times10^5\) cells (100% gated) were analyzed immediately with a fluorescence-activated cell sorter (FACScan, Becton Dickinson; Macintosh System Software 7.1.0).

**RNA Extraction and Northern Blot Analysis**

For Northern blot studies of the effect of aspirin/TNF-\(\alpha\) treatment on the expression of ICAM-1, monocultures of HCAECs and HCMSMCs were incubated with aspirin (1, 2, 5, 10 mmol/L) for a period of 18 hours. During the last 6 hours of aspirin incubation, an additional stimulus with TNF-\(\alpha\) (20 ng/mL) was performed. Total RNA (3\(\times10^5\) cells) was isolated with RNEasy Mini Kit (Quiagen), and 10 \(\mu\)g of RNA was used in standard Northern blot analysis with a ICAM-1 probe.

Phosphorimaging was used to detect the relative band density of ICAM-1 mRNA in comparison with TNF-\(\alpha\)-stimulated cells. GAPDH was used as a control. Experiments were performed in triplicate.

**3DLA Model**

The central part of the 3DLA model is a polycarbonate filter (diameter, 50 mm; thickness, 10 \(\mu\)m; pore size, 5 \(\mu\)m; Costar) that separates a layer of endothelial cells from a layer of SMCs.\textsuperscript{11} The use of HCAECs and HCMSMCs adapts the model as closely as possible to the human situation.\textsuperscript{13} HCAECs and HCMSMCs were seeded in a density of \(2.5\times10^6\) cells/cm\(^2\) and were supplied with the appropriate culture medium.\textsuperscript{13}

At day 14, the 3DLA units were incubated with aspirin (5 mmol/L) for 18 hours. During the last 6 hours of aspirin incubation, the models were treated with TNF-\(\alpha\) (20 ng/mL). For LA, the required number of monocytes and CD\(^4\) lymphocytes was calculated in relation to the relative concentration of monocytes or CD\(^4\) lymphocytes in the full human blood. For selective LA, \(3\times10^5\) monocytes or \(8\times10^5\) CD\(^4\) lymphocytes were seeded on the HCAEC side of the 3DLA units. Selective LA with monocytes and CD\(^4\) lymphocytes was studied for a period of 30 minutes. At 1, 2, 3, 4, 6, and 24 hours, controls were performed without aspirin treatment and without TNF-\(\alpha\) stimulus.

**Adhesion and Chemotaxis Assays**

All membrane filters were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 3 hours at room temperature; each filter was divided in 4 segments. One segment of the filter was used to identify monocytes, CD\(^4\) lymphocytes, and ICAM-1 using indirect immunofluorescence techniques. Monocytes were identified with primary antibodies directed against CD68 (Dakopatts), and CD\(^4\) lymphocytes were identified with primary antibodies against CD4 (Dianova). To identify ICAM-1, primary antibodies against ICAM-1 were used (Dianova). TRITC-labeled antibodies (goat anti-mouse, Dianova) were used as secondary antibodies. The number of cells with positive staining against CD68 (monocytes) or CD4 (CD\(^4\)) was detected horizontally after adhesion on the surface of HCAECs and after transmigration on the HCMSMC side of the 3DLA units. The expression of ICAM-1 was only analyzed in HCAECs according the criterion of positive or negative upregulation after TNF-\(\alpha\) stimulus. For each investigation, 10 microscopic fields of 3 different filters were analyzed (magnification, 40X).

**Triggered Cell Proliferation After LA**

Proliferation of HCMSMCs was calculated 24 hours after LA. Eighteen hours before fixation, 5-bromo-2\'-deoxyuridine (BrdU, 20 \(\mu\)mol/L, Serva) and 2\'-deoxy-ctydine (20 \(\mu\)mol/L, Serva) were added to the culture medium of 3DLA units. Proliferation was subsequently detected with monoclonal antibodies directed against BrdU (Dakopatts). For second antibodies, biotinylated goat anti-mouse antibodies were used.

One segment was embedded in Epon (Araldite, Serva,) and was polymerized at 60°C for 3 days. For histological examination, semithin sections were mounted on poly-l-lysine–coated cover slips. One section was stained with toluidine blue (Merck). In another section, araldite was dissolved from the sections using potassium hydroxide dissolved in methyl and propylene oxide immediately before immunological staining. HCAECs were identified by a positive reaction with polyclonal primary antibodies directed against anti–von Willebrand factor (Dakopatts). In each of these groups, 10 sections of 3 filters were analyzed completely (magnification, 40X), and the percentage of BrdU-positive HCMSMCs was calculated in relation to the total cell number.

**Statistical Analysis**

The Mann-Whitney rank-sum test was used to investigate the significance of differences in the adhesion and chemotaxis assays of monocytes and CD\(^4\) lymphocytes and in the proliferation assays of HCMSMCs. Results are expressed as mean±SD. Differences were considered significant at \(P < 0.05\).

**Results**

**Identification of Cells**

In monocultures of HCAECs, cells were identified by a positive reaction with antibodies directed against von Willebrand factor and by the typical “cobblestone” growth pattern in culture. Monocultures of HCMSMCs exhibited the “hill
and valley” growth pattern and reacted positively with antibodies against smooth muscle α-actin.

Effect of Aspirin on Expression of ICAM-1: Flow Cytometry Studies

The effects of aspirin (1, 2, 5, and 10 mmol/L) on the TNF-α–induced expression of ICAM-1 are demonstrated in Figure 1. After treatment with TNF-α, a 5.6-fold increase in the mean fluorescence levels of ICAM-1 expression in HCAECs was found; mean fluorescence levels increased from 47.50 to 266.09. The incubation of HCAECs with aspirin in concentrations of 1 and 2 mmol/L did not attenuate the expression of ICAM-1 in HCAEC; mean fluorescence levels in these groups were 330.25 and 328.02, respectively. After incubation of HCAECs with aspirin at concentrations of 5 and 10 mmol/L, a 37% and ≈80% decline in the expression of ICAM-1 was seen, respectively; mean fluorescence levels were 167.40 and 53.99, respectively. As controls, HCAECs were incubated with actinomycin (1 mg/mL). Incubation with 70% ethanol or adding aspirin without prior TNF-α stimulus exhibited no effect on the expression of ICAM-1.

In HCMSMCs, the baseline expression of mean fluorescence levels was 38% more than that of HCAECs (Figure 1). After treatment with TNF-α, a 2.5-fold increase in the mean fluorescence levels of ICAM-1 expression in HCMSMCs was found; mean fluorescence levels increased from 17.93 to 45.38. Treatment of HCMSMCs with aspirin at concentrations of 1, 2, and 5 mmol/L only slightly reduced the expression of ICAM-1 to 22%, 15%, and 29% of normal, which corresponded to mean fluorescence levels of 35.26, 38.66, and 32.17, respectively. A >70% decrease in ICAM-1 expression was observed after treatment of HCMSMCs with aspirin in a concentration of 10 mmol/L. As controls, HCMSMCs were incubated with actinomycin (1 mg/mL). Incubation with 70% ethanol or adding aspirin without prior TNF-α stimulus did not affect the expression of ICAM-1.

Effect of Aspirin on ICAM-1 mRNA Levels:
Northern Blot Studies

After TNF-α stimulus, the band density of mRNA ICAM-1 was increased 6.6-fold in HCAECs and 3-fold in HCMSMCs, which corresponded to a relative band density of 100% (Figure 2).

In HCAECs, the TNF-α–induced expression of ICAM-1 was inhibited in a dose-dependent manner after incubation with aspirin in a concentration of 5 and 10 mmol/L. Relative mRNA ICAM-1 band density was reduced by 15% after incubation with 5 mmol/L aspirin (85±21.3% of normal) and by 42% after incubation with 10 mmol/L aspirin (58.3±7.7% of normal). No effect was found after incubation with 1 or 2 mmol/L aspirin (100±6.5% and 98±24.9% of normal, respectively).

In HCMSMCs, a dose-dependent inhibition of the TNF-α–induced expression of ICAM-1 was detected after incubation with 2, 5, and 10 mmol/L aspirin. Relative ICAM-1 RNA band density was inhibited by ≈11% after incubation with 2 mmol/L aspirin (89±7.9% of normal), by 23% after incubation with 5 mmol/L aspirin (76.5±16.3% of normal), and by 37% after 10 mmol/L aspirin (62.6±30% of normal). No inhibitory effect was found after incubation with 1 mmol/L aspirin (96.8±0.8% of normal).

3DLA Model

The 3DLA units with HCAECs and HCMSMCs were successfully established (Figure 3A). On the HCAEC side of the units, 1 to 2 layers of cells were found; the superficial layer of these cells was composed of HCAECs, as identified by a positive reaction with antibodies directed against von Willebrand factor (Figure 3D). On the HCMSMC side of the units, 3 to 5 cell layers with the typical hill and valley growth pattern were observed.

Human monocytes and CD4+ lymphocytes were isolated from the residual leukocytes of single donors and identified by positive reaction with antibodies directed against CD68 and CD4+ lymphocytes, respectively. The purity of monocyte and CD4+ lymphocyte preparations was determined by flow cytometry. Monocyte preparations had a purity of 93%, and CD4+ lymphocyte preparations exhibited a purity of 89%.

3DLA Model: Effect of Aspirin on Leukocyte Adhesion

In 3DLA units, the effect of aspirin (5 mmol/L) on TNF-α–stimulated leukocyte adhesion, chemotaxis, ICAM-1 expression, and the proliferation of HCMSMC was studied. The effects of aspirin on TNF-α–stimulated leukocyte adhesion...
are depicted in Figure 4A. No significant decrease of the number of attached monocytes was found 30 minutes or 1 or 2 hours after adding aspirin. Three hours after adding aspirin, the number of adherent monocytes was significantly decreased by almost 40% ($P<0.001$); 4 and 6 hours after adding aspirin, a significant 50% ($P=0.001$) and 66% ($P<0.001$) decrease was found, respectively. At 24 hours after adding aspirin, no effect on the adhesion of monocytes was found.

In comparison with the number of attached monocytes, the number of adherent CD4$^+$ lymphocytes was decreased whether aspirin was added or not (Figure 4B). At 30 minutes after adding aspirin, a 36% decline of the number of adherent monocytes was found; however, significant differences were not reached. At 1 and 2 hours after adding aspirin, the number of adherent CD4$^+$ lymphocytes was reduced by 36% ($P<0.01$) and 39% ($P<0.01$), respectively; 3, 4, and 6 hours after adding aspirin, 39%, 73%, and 69% reductions, respectively, of the number of adherent CD4$^+$ lymphocytes were noticed ($P<0.001$). By 24 hours after adding aspirin, no inhibitory effect on the adhesion of CD4$^+$ lymphocytes was found.

In comparison with monocytes, the number of adherent CD4$^+$ lymphocytes was always reduced, although the differences between monocytes and CD4$^+$ lymphocytes were smaller with increasing time after adding aspirin. At 30 minutes and 1 and 2 hours after seeding, the number of adherent CD4$^+$ lymphocytes had decreased by 56%, 55%, and 64%, respectively, in relation to the corresponding numbers of adherent monocytes. At 3, 4, and 6 hours after adding aspirin, the differences between monocytes and CD4$^+$ lymphocytes were getting smaller and the

Figure 2. Northern blots and relative band densities of expression of ICAM-1 mRNA after incubation of HCAECs and HCMSMCs with 1, 2, 5, and 10 mmol/L aspirin.
The number of adherent CD4⁺ lymphocytes was decreased in comparison with the corresponding number of adherent monocytes by 44%, 38%, and 24%, respectively. By 24 hours after adding aspirin, the number of adherent CD4⁺ lymphocytes was decreased to 19% in comparison with monocytes.

**Effect of Aspirin on Leukocyte Chemotaxis in 3DLA Units**

Chemotaxis on the HCMSMC side of the 3DLA units was found in only 10% to 20% of the adherent monocytes on that side (Figure 5A). No chemotaxis was detected after CD4⁺ lymphocyte attack (Figure 5B). The effect of aspirin (5 mmol/L) on the chemotaxis of leukocytes from the HCAEC side to the HCMSMC side of the 3DLA units is depicted in Figure 5.

TNF-α stimulus increased the number of transmigrated monocytes in comparison with the group without prior stimulus; however, significant differences were only seen 6 hours after monocyte attack ($P<0.01$). An 8-fold and 6-fold increase of the number of monocytes was found 2 and 3 hours after seeding, respectively, and 4 and 24 hours after seeding, the increase was 1.8- and 4-fold, respectively ($P=NS$ for all). CD4⁺ lymphocytes were never detected on the HCMSMC side of the units.

**Effect of Aspirin on Proliferative Activity of HCMSMCs in 3DLA Units**

Proliferation of HCMSMCs was analyzed in TNF-α–stimulated 3DLA units after selective monocyte and selective CD4⁺ lymphocyte attack. The data are presented in Figures 3B and 6.
selective monocyte or CD4+ lymphocytes attack significantly increased proliferation (6.6 ± 3.6 cell divisions, P < 0.001, and 4.3 ± 3.2 cell divisions, P < 0.001, respectively). Proliferation of HCMSMCs after selective monocyte attack was significantly increased (P < 0.001) in comparison with the proliferation of HCMSMCs after selective CD4+ lymphocyte attack.

Incubation with 5 mmol/L aspirin significantly inhibited the proliferation of HCMSMCs in 3DLA units after both selective monocyte or CD4+ lymphocyte attack. A 83% decrease (P < 0.001) was detected after selective monocyte attack (1.1 ± 1.6 cell divisions), and a 42% decrease (P < 0.05) was detected after selective CD4+ lymphocyte attack (2.5 ± 2.2 cell divisions).

**Discussion**

LA and triggered cellular and subcellular reactions are pivotal events of early atherosclerosis and restenosis. We came to 3 basic conclusions after completing the present study. First, in monocultures of HCAECs and HCMSMCs, the surface expression of ICAM-1 was reduced in a dose-dependent manner after incubation with 5 and 10 mmol/L aspirin, but no effect was detected after incubation with 1 and 2 mmol/L aspirin. Second, in the 3DLA model, 5 mmol/L aspirin significantly inhibited the adherence of monocytes and CD4+ lymphocytes and the chemotaxis of monocytes. Third, 5 mmol/L aspirin significantly reduced the reactive proliferative response of cocultured HCMSMCs after selective monocyte or CD4+ lymphocyte attack in the 3DLA model.

A dose-dependent inhibitory effect of aspirin on NF-κB–mediated signal cascades agrees with the prior reports of Weber et al14 and Amberger et al,15 who studied endothelial cells from human umbilical veins. The exact mechanism of aspirin on TNF-α–induced and NF-κB–mediated events is not entirely clear. NF-κB comprises a family of cellular transcription factors that are involved in the inducible expression of a variety of cellular genes that regulate the inflammatory response.16,17

In 3DLA units, 5 mmol/L aspirin significantly inhibited adhesion after selective monocyte or CD4+ lymphocyte attack. These data match the preceding reports of Gerli et al18 and Amberger et al,15 which describe an inhibitory effect of 5 mmol/L aspirin on the adhesion of human T lymphocytes in human umbilical vein endothelial cells. A dose of 5 mmol/L aspirin significantly inhibited the triggered reactive proliferation of HCMSMCs after both selective monocyte and CD4+ lymphocyte attack. Moreover, aspirin (5 mmol/L) significantly inhibited the TNF-α–induced proliferation of HCMSMCs without selective LA. These data are in conformity with a study by Osnes and colleagues,19 who reported a 50% inhibition of TNF-α in the supernatant of human monocytes after adding aspirin in a concentration of 5 mmol/L.

Plasma levels of aspirin in vitro and in vivo are complex to calculate because aspirin is rapidly hydrolyzed to salicylic acid by nonspecific esterases. Hydrolysis occurs in the liver and, to a lesser extent, the stomach, so that only 68% of the dose reaches the systemic circulation as aspirin.20 Recently, Cerek et al21 reported that aspirin (100 mg · kg−1 · d−1) significantly inhibited reactive cell proliferation after angioplasty in male rats. Data on rat plasma levels after the administration of 100 mg · kg−1 · d−1 aspirin are not available (to our knowledge); however, it has been reported that a dosage of 200 mg · kg−1 · d−1 results in peak plasma concentrations of 5 μg/mL,22 which corresponds to ~0.03 mmol/L. In humans, the peak plasma levels of aspirin rise 20 minutes after the oral administration of 650 mg/d to a concentration of 25 μg/mL (which corresponds to ~0.15 mmol/L).23 These levels decline to ＜5 μg/mL after 2 hours. Plasma salicylate concentrations raise equally rapidly, reaching a peak of ~45 μg/mL by 1 hour after administration.23

Despite these low plasma levels after oral administration, aspirin has positive effects on carotid plaque growth (at doses of 50 mg and 900 mg per day),24 endothelial dysfunction (at 1000 mg per day),25 and the reduction of proinflammatory cytokines (at 300 mg per day).26 With reference to the fact that, in humans, peak concentrations of ~0.15 mmol/L can be obtained after the oral administration of 650 mg of aspirin,23 it becomes evident that orders of magnitudes exist in comparison with the concentration of 5 mmol/L aspirin that was proven effective in the present study. Thus, the beneficial effects of aspirin in the clinical trials cited24–26 have not been caused by the inhibitory effect of aspirin on leukocyte
adhesion and chemotaxis or the triggered proliferative response of HCMSMCs.

In accordance with the data presented here, it was previously demonstrated in large clinical trials that the daily administration of 330 mg of aspirin (which corresponds to \(\approx 0.1 \text{ mmol/L} \)) or 1000 mg of aspirin (which corresponds to \(\approx 0.2 \text{ mmol/L} \)) are not successful to prevent restenosis after coronary peripheral angioplasty. The discrepancy between the positive effect of a concentration of 0.03 mmol/L aspirin to inhibit restenosis in rats\(^2\) and the negative effect of an increased concentration in clinical trials\(^2\) underlines the need for complex in vitro models that more closely mimic the clinical situation.

The authors suggest accepting the fact that a permanent local concentration of aspirin of 5 mmol/L is the lowest rational concentration to obtain the reported beneficial in vitro effects of high-dose aspirin in clinical studies. Recently, Laham and colleagues\(^9\) opened the door to the use of the pericardium as an perivascular drug depot. Further studies are mandatory to investigate whether the perivascular administration of high-dose aspirin (5 mmol/L) opens the field for a new cardiovascular indication for one of the oldest and most widely used drugs in the world.

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