Complement-mediated Phagocytosis of Herpes Simplex Virus by Granulocytes

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Abstract

The role of complement receptors in phagocytosis of herpes simplex virus (HSV) by PMN was examined. Complement components were deposited on the surface of the virus particle in the presence or absence of specific anti-HSV antibodies. Flow cytometry was used to analyze the phagocytosis of fluorescence-labeled viruses and demonstrated that although a virion is able to associate with PMN in the presence of complement alone, the granulocyte is not triggered to mount a metabolic burst. Efficient stimulation of PMN occurs when complexes are formed consisting of virus, specific antibodies, and complement. To address the question whether the viruses were inside or outside the cell, a combined enhancement/quenching method was developed using ammonium chloride as a lysosomotropic agent and trypan blue as a quenching dye. The data indicate that Fc receptor-mediated phagocytosis by PMN results in the ingestion of all cell-associated herpes virions. Interactions of virions through PMN-complement receptors CR1 and CR3 results solely in binding to the PMN but not in internalization. Interactions via both complement and Fc receptors cause synergistic stimulation of the PMN and result in very efficient association of viruses, > 80% of which were inside the cell.

Introduction

Little attention has been paid to the role of phagocytes in antiviral defense. Recently it has been shown that phagocytes can ingest and kill virions and methods have been developed to measure the uptake of these virions using either radiolabeled techniques (1–3) or chemiluminescence (4–6). The role of this phenomenon in vivo is shown by experiments with influenza virus and foot and mouth disease virus in a murine model (1, 5, 7). PMN are often found abundantly in viral lesions (8, 9) and especially in herpes simplex virus (HSV) keratitis the role of phagocytosis by PMN seems to be established (2, 4).

How these small particles are ingested and which receptors are used in the process of internalization by the PMN are both largely unknown. The role of the different PMN receptors in the process of phagocytosis is best illustrated in experiments performed with red blood cells and microspheres coated with isolated complement components. After the binding of IgG opsonized particles through Fc receptors present on the surface of PMN, the PMN is triggered to internalize the particles and to mount a respiratory burst (10, 11). Complement receptors are present in low amounts on the surface of PMN and can be upregulated from internal stores by different mechanisms: by fluid-phase C3b (12), by chemotactic factors, and during purification steps (13, 14). Complement receptor 1 (CR1), CR3 and CR4 preferentially bind C3b, C3bi, and C3dg, respectively. There is a marked increase in the uptake by PMN or macrophages of erythrocytes coated with both antibodies and complement as compared with red blood cells coated with antibodies alone (15). Particles coated with C3 fragments alone are bound to the PMN, whereas internalization through complement receptors requires an extra stimulus. Furthermore, the trigger to a metabolic burst is absent (16–19). Microorganisms, by contrast, are often efficiently ingested in the presence of complement alone. In the case of unencapsulated bacteria or yeast particles coated with complement alone, cell wall components of these microorganisms provide the extra trigger needed for ingestion and metabolic burst (Yeast β glucan, LPS) (20–23). This phenomenon is not completely explained, because it is often difficult to determine whether microorganisms are inside or outside the PMN.

We studied the interaction of herpes simplex virions with human granulocytes, the main invading leukocytes in herpes lesions (9, 24). In earlier work (25), we reported that complement activation by HSV-infected fibroblasts in the absence of specific antibodies resulted in adherence of human granulocytes to these fibroblasts. Here we describe the analysis of the deposited serum factors on purified herpes simplex virions after exposure to sera containing complement, specific antibodies, or both. We also studied the interaction of human granulocytes with purified herpes simplex virions. Using a phagocytosis assay based on flow cytometric measurements of fluorescent virions (26), we describe how to discriminate between adherent virions and internalized particles.

Methods

Chemicals. The following chemicals were used: RPMI 1640, medium 199 (m199), FCS, and gentamicin (Gibco Laboratories, Paisley, UK); Hepes (BDH Chemicals Ltd., Poole, UK); dextran T-70 and T-10, protein A-Sepharose and Ficol-Hypaque (Pharmacia Fine Chemicals AB, Uppsala, Sweden); and FITC and isomer I (Sigma Chemical Co., St. Louis, MO). Monoclonal anti-human C3b (αC3b) (32) was purchased from Cytochrome (San Diego, CA), peroxidase-conjugated goat anti-human C3 (αC3-PO) from Cappel Laboratories (Organon Teknica, The Netherlands), peroxidase-conjugated goat anti-mouse
IgG(H+L) (aMlgG-PO) from Nordic Immunology (Tilburg, The Netherlands).

Cells and viruses. Human embryonic fibroblasts were isolated and cultured in our own laboratory. Raji and HeP-2 cell lines were obtained from Flow Laboratories (Irvine, CA). The cells were cultured in RPMI 1640/199 (1:1, vol/vol), supplemented with 10% FCS, 5 mM HEPES, 2 g/liter sodium bicarbonate, and 10 μg/ml gentamicin. HSV-1, strain F, was a gift from Dr. B. Roizman (University of Chicago, Chicago, IL).

For determination of virus titer, serial 10-fold dilutions were made and each dilution was combined with 106 fibroblasts per well in microtiter plates. After 6 d each well was scored for the presence of cytopathic effect and the 50% tissue infective dose was calculated.

Sera. Sera from healthy volunteers were screened for HSV-specific antibodies using an ELISA and an antibody-dependent killer cell-mediated cytoxicity assay, both have been described previously (25). Sera with no detectable amount of anti-HSV antibodies as well as sera with high levels of specific anti-HSV antibodies were pooled and then stored at −70°C (referred to as negative and positive sera, respectively). All serums were tested in a 50% hemolytic complement-activity assay and only those with normal complement levels were used. To deplete sera of complement activity they were either heated for 30 min at 56°C or treated with 10 mM EDTA.

Isolation of human PMN. PMN were isolated according to the method of Böyum (27) and briefly described here. Heparinized human blood was mixed with dextran T-70 to a final concentration of 1.2% and sedimented for 60 min. Leukocyte-rich plasma was withdrawn and leukocytes were separated on Ficoll-Hypaque. Residual erythrocytes in the PMN fraction were lysed by hypotonic shock. Purity of the PMN preparation was >98%; viability as determined by trypan blue exclusion was 99%.

Virus purification and FITC labeling. HeP-2 cells were grown to confluency on 15-cm culture dishes (Greiner, Nurtingen, FRG) and infected with HSV-1 (the multiplicity of infection was 20). After 1 h, residual virus was removed, fresh medium was added, and cells were incubated for an additional 22 h. Cells were harvested by scraping and then washed and then lysed in a homogenizer (Dounce; Wheaton Scientific, Millville, NJ) by 20 strokes with each pestle. Cell fragments and nuclei were pelleted at 500 g and 1 ml was layered on top of a block-gradient consisting of 7 ml 2.5% and 1 ml 25% dextran-10, a modification of the technique described by Cassai (28). Gradients were centrifuged at 100,000 g for 2 h at 10°C. Virus bands were aspirated and dextran was removed by dialysis against PBS in dialysis membranes with a molecular weight cutoff of 50,000 (Spectrum Medical Industries, Inc., Los Angeles, CA). Purity was checked by electron microscopy showing that >95% were enveloped virions. Virus was stored at −70°C. For use in phagocytosis assays, purified virus was mixed 10:1 (vol/vol) with FITC (1 mg/ml in 1 M sodium carbonate buffer, pH 9.6) and incubated at room temperature for 1 h under constant rotation. After dialysis against PBS for 18 h at 4°C fluorescein-labeled virus was stored at −70°C in small aliquots until use.

Fluorometric phagocytosis assay. A total of 60 μl PMN (3 × 107/ml) in HBSS with 1% FCS (HBSSF) was mixed with 30 μl purified FITC-labeled virus and 30 μl human serum (diluted in HBSSF). After incubation for various time periods in a shaking water bath (150 rpm) the reaction was stopped by adding ice-cold PBS. PMN were separated from free virus by centrifugation at 160 g, washed twice, suspended in 0.1 ml PBS, and fixed by adding 0.02% paraformaldehyde. Cells were analyzed in a flow cytometer (FACStar; Becton-Dickinson & Co., Mountain View, CA) with an excitation wavelength of 488 nm. The FITC emission was measured through a 530/30 nm filter and represented the relative uptake of labeled virions. Phagocytosis was expressed either as the percentage relative to the maximum observed phagocytosis using 20% human anti-HSV serum or simply as the mean fluorescence channel calculated from the observed frequency distribution as measured in the FACS. The fluorescence was calibrated each day using standardized calibration beads (Becton-Dickinson & Co.).

Deposition of C3b/C3bi on virions. Virions were incubated with different sera and the different constituents of the immune complexes identified. A total of 100 μl of purified virus was incubated with 100 μl diluted serum for various time periods. Subsequently 10 ml ice-cold PBS was added and the virus was washed twice to remove unbound C3 by centrifugation for 1 h at 150,000 g. The virus was then suspended in 2.5 ml PBS and 50 μl per well was coated on microtiter plates (polystyrene flat bottom, Flow Laboratories) by desiccation overnight at 37°C. Plates were washed five times with 0.05% Tween-20 in water, incubated for 1 h at 37°C with a blocking agent (4% skimmed milk, 0.05% Tween-20 in PBS) and washed again five times. Incubations with aC3-P (1/8,000) were performed for 1 h at 37°C and were followed by washing (five times) using 0.05% Tween in water. For C3bi measurement, wells were first incubated with aC3bi (1/5,000) for 1 h, washed five times and then incubated with aMlgG-PO (1/5,000). Afterwards, 50 μl substrate (0.1% tetramethylbenzidine and 0.03% urea peroxide in 0.1 M sodium acetate-citrate buffer, pH 6.0) was added to each well. After incubation for 10 min at 37°C, the enzyme reaction was stopped by the addition of 50 μl 2 NH4SO4. Absorbance was measured at 450 nm in a spectrophotometer (Titertek Multiscan; Flow Laboratories). As controls, EDTA treated serum, C3-deficient human serum, aMlgG-PO without aC3bi and samples without HSV were assayed.

Fluorescence quenching technique. Trypan blue staining was used as a method for extracellular quenching of fluorochrome emission. This technique was described for use in fluorescence quenching on microscopic slides by Sahlin (29) and more recently for use in flow cytometry applying crystal violet as a quenching dye (30). Raji cells, which are incapable of uptake but can bind immune complexes at their surface, were used as the control (31).

After the binding of viruses was complete, cells were suspended in PBS. Then part of the cells were washed, suspended in 0.02 M sodium acetate buffer (pH 5.8), and measured. The remaining cells were suspended in sodium acetate buffer containing 0.2 mg/ml trypan blue. After 20 s, the cells were washed twice and resuspended in the sodium acetate buffer. It was necessary that the pH of the extracellular environment be 5.8 to optimize the trypan blue quenching, as determined by Sahlin (29).

Raising the intracellular pH. An additional assay to confirm the intracellular location of virions used the lysosomotropic agent ammonium chloride. When a FITC-labeled virion is taken up into a PMN vacuole and phagosome-lysosome fusion occurs, the pH drops and the fluorescence intensity diminishes. By adding a lysosomotropic agent, the pH increases and the enhancement of fluorescence can be calculated. Therefore, after the phagocytosis assay, the cells were washed and resuspended in PBS. After the sample was measured in the FACS the remaining cells were mixed with one-third volume 150 mM ammonium chloride, which resulted in a final concentration of 50 mM ammonium chloride, maintaining isotonicity and remeasured.

Chemiluminescence. To measure PMN metabolic burst, luminol-enhanced chemiluminescence was monitored over time. A total of 50 μl virus suspension and 50 μl diluted serum were mixed and incubated at 37°C for 30 min. Then 300 μl PMN (2 × 107/ml) and 50 μl luminol 6 × 10−5 M were added. Light emission was measured for 6 s, with 2-min intervals during 30 min in a picolite luminometer (Packard Instruments, Brussels, Belgium).

Statistical analysis. Results are expressed as the mean±SE of three or more independent observations.

Results

Deposition of C3b/C3bi on virions. An ELISA was used to measure the C3b and/or C3bi deposition onto virus particles. In Fig. 1A the measured optical density (OD) is plotted against the time of incubation of virions in 10% serum. Using polyclonal anti-C3 an increased deposition of C3-like molecules in time is observed in the presence of both antibodies and com-
The deposition of C3 fragments in 10% serum over time, pos, serum with a high amount of specific anti-HSV antibodies. neg, represents the serum pool without specific antibodies. The antibodies used in this ELISA are either αC3-PO (C3), or αC3bi + αMlgG-PO (C3bi).

4°C is a control incubation at 4°C with positive serum and anti-C3.

(B) The amount of total C3 fragments was assayed after 30 min of incubation with different amounts of serum. C3def, human serum deficient in C3; C3edta, serum in presence of 10 mM EDTA.

When complement alone is present the association of FITC labeled virions to the granulocytes reaches the same level as in the case of antibodies alone. However, it must be taken into account that due to the pH of the environment (either inside a vacuole or outside the PMN) the fluorescence intensity is not the absolute reflection of the bound FITC. Therefore, the next series of experiments were performed.

Fluorescence quenching with trypan blue and vacuolar pH raise using ammonium chloride. The efficacy of the trypan blue–quenching method was controlled with Raji cells. These cells possess large amounts of both Fc and complement receptors but are not able to ingest immune complexes. Cells, positive serum and different amounts of purified FITC-labeled virions were mixed and incubated under circumstances comparable to those described for PMN. Cells were washed and suspended in buffer (pH 5.8). Fluorescence intensity was measured before and after addition of trypan blue. The effect of ammonium chloride on the surface fluorescence was determined in an identical manner except that the buffer control had a pH of 7.4. Table I shows that trypan blue always lowered the fluorescence to background levels, independent of the total amount of fluorescence. Ammonium chloride did not affect the surface fluorescence. Viability of the cells remained 95% throughout the whole procedure as determined by trypan blue exclusion.

Inside or outside. After incubation of PMN with virions in different sera, the amount of fluorescence was measured under four different conditions: (a) at pH 7.4, (b) at pH 7.4 in the presence of ammonium chloride to raise the intravacuolar pH and thereby the intensity of fluorescence, (c) at pH 5.8, similar to the intravacuolar pH, and (d) at pH 5.8 after incubation of the PMN with trypan blue. In Table II the increase of fluorescence after the addition of ammonium chloride compared with the pH 7.4 control, and the decrease of fluorescence after the addition of trypan blue compared with the pH 5.8 control, are shown.

No quenching by trypan blue and a strong increase after ammonium chloride addition are observed when only antibodies are present. The absence of enhancement by ammonium chloride in the case of negative serum (only complement) is accompanied by total quenching by trypan blue.

Calculation of percentage viruses inside the PMN. The percentage of particles located inside the PMN can be calculated from both above described experimental approaches. Measurements at pH 7.4 = a, with ammonium chloride = b, at pH

<table>
<thead>
<tr>
<th>Added virus</th>
<th>Control</th>
<th>NH4Cl Control</th>
<th>Trypan blue</th>
</tr>
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<tr>
<td>pH 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>49±5</td>
<td>48±4</td>
<td>41±4</td>
</tr>
<tr>
<td>5</td>
<td>301±12</td>
<td>310±16</td>
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<td>340±25</td>
<td>330±17</td>
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<td>465±23</td>
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<td>25</td>
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<td>487±12</td>
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<td>30</td>
<td>496±34</td>
<td>484±43</td>
<td>362±36</td>
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</table>

Table 1. Effect of Trypan Blue and Ammonium Chloride Addition on FITC-labeled Virions Adherent to Raji Cells
Table II. Fluorescence Intensities after Quenching or Vascular pH Rise after Phagocytosis of FITC-labeled Herpes Virions by Human PMN

<table>
<thead>
<tr>
<th>Serum</th>
<th>pH 7.4 Control</th>
<th>+NH₄Cl</th>
<th>pH 5.8 Control</th>
<th>Trypan blue</th>
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<tr>
<td>Pos</td>
<td>439±32</td>
<td>950±69</td>
<td>401±34</td>
<td>300±31</td>
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<tr>
<td>Pos (heated)</td>
<td>144±17</td>
<td>360±41</td>
<td>142±21</td>
<td>140±25</td>
</tr>
<tr>
<td>Neg</td>
<td>105±20</td>
<td>107±31</td>
<td>102±12</td>
<td>26±5</td>
</tr>
<tr>
<td>Neg (heated)</td>
<td>26±4</td>
<td>30±3</td>
<td>32±3</td>
<td>20±4</td>
</tr>
<tr>
<td>FCS (heated)</td>
<td>21±3</td>
<td>25±2</td>
<td>20±3</td>
<td>16±3</td>
</tr>
<tr>
<td>PMN alone</td>
<td>12±2</td>
<td>13±2</td>
<td>13±2</td>
<td>12±2</td>
</tr>
</tbody>
</table>

Abbreviations: pos, serum pool with high amounts of specific anti-HSV antibodies; neg, pool without these antibodies; heated, incubated for 30 min at 56°C; PMN alone, PMN without the addition of virus.

5.8 = c, and with trypan blue = d. In experiments with trypan blue, after trypan blue addition, no quenching can be observed. All viruses are located inside. The experiments with nonphagocytic Raji cells (Table I) indicate that fluorescence on the outside can be completely quenched by trypan blue, independent of the added amount of fluorochrome. After quenching, only background (bgr) fluorescence remains. The percentage of particles inside thus can be expressed as (d - bgr)/(c - bgr) × 100%. For experiments with ammonium chloride the formula becomes more complicated. When all viruses are located outside, as in the case of Raji cells, no enhancement is observed. That is also true for PMN with HSV and complement alone. The fluorescence observed at pH 7.4 (a) is the sum of particles outside (FL[out]) and the particles inside (FL[in]). Thus,

\[ a = FL[out] + FL[in] \]

The fluorescence after addition of ammonium chloride is the sum of the particles outside (FL[out]) plus enhanced fluorescence of particles inside (N × FL[in]), where N stands for the enhancement factor. Thus

\[ b = FL[out] + (N × FL[in]) \]

The percentage inside can be expressed as

\[ \frac{N × FL[in]}{FL[out] + (N × FL[in])} \times 100% = \frac{N × FL[in]}{b} \times 100% \]

With these equations, we can calculate the percentage of particles inside the PMN in serum with antibodies and complement from Table II. This results in 75% inside the PMN according to the trypan blue method and 83% inside the PMN according to the ammonium chloride method, both with SE of ∼ 5%.

Chemiluminescence using complement and/or antibodies. When purified virions were mixed with antibodies, they stimulated PMN to mount a metabolic burst, as measured by detection of chemiluminescence (Fig. 3 A). When both complement and antibodies were present, chemiluminescence was markedly enhanced (Fig. 3 A and B). However, when complement alone was present, no oxidative burst could be observed (Fig. 3 A).

Discussion

The role of complement receptors in PMN-mediated phagocytosis is commonly determined using red blood cells or agarose beads as assay particles. From such studies, one can conclude that adhesion and enhancement of Fc receptor–mediated phagocytosis are functions of complement receptors. In addition to complement receptor binding, internalization of particles and the triggering of PMN to mount a metabolic burst both require an extra signal (16–19). With pathogenic target organisms, however, these findings have not been confirmed. The PMN is probably triggered by surface components of the microorganism (20–23). Complement receptors on macrophages can be converted into uptake receptors by lymphokines (33), fibronectin, and laminin (34). Endocytosis of small aggregated molecules via complement receptors has been reported with PMN but not with U937 cells (35, 36). It has been proposed that phosphorylation of the complement receptors plays a crucial role in these events (37). Furthermore, this research is complicated by the difficulty of determining the ratio of particles inside to outside. For red blood cells this can be determined by hypotonic shock and for Staphylococcus aureus it can be determined by lysisophathin treatment; both methods use the lysis of extracellular particles. However, for other bacteria and yeast cells, determining the ratio of particles inside to outside is more difficult.

In our model of phagocytosis of FITC-labeled HSV by PMN using flow cyometric analysis of fluorescence intensity per PMN, we evaluated the relative role of the different receptors in binding, uptake, and stimulation.

![Figure 3](image-url)

Figure 3. (A) Luminal-enhanced chemiluminescence of PMN after stimulation with purified herpes virions in the presence of 25% of different sera. pos, neg, and a are as defined in the legend to Fig. 2. (B) Serum with antibodies and complement is shown in different concentrations.
In earlier work (25) we prepared two different pools of human sera that contained either very large or nondetectable amounts of anti-HSV antibodies. In the negative pool, complement activation by HSV-infected cells was still significant, as shown by the deposition of C3 on the surface of infected cells, which was not due to classical pathway activation. To show that the virion itself also activates complement in the absence of antibodies, an ELISA described by Gordon et al. (38) was modified to monitor the C3b and C3bi deposition on virions incubated with human complement. Use of a C3bi neoantigen-specific MAb demonstrated that C3bi was formed on these virions, indicating again actual complement activation and not aspecific sticking of C3b to the virions (32).

Our recently described method of measuring phagocytosis of purified virions by human PMN with flow cytometry demonstrated the association of PMN with virions loaded with complement alone. To determine whether FITC-labeled particles are really taken up or just attached to the PMN, two independent methods were developed. The first was fluorescence quenching, described by Sahlin et al. (29) for use with microscopic slides, and based on the quenching of the fluorochrome in the presence of a dye. FACS measurements of non-phagocytic Raji cells revealed that complete quenching by trypan blue occurs. The second method used intracellular pH as a guideline. Fluorescence intensity of FITC is dependent on pH; in a low-pH environment, the fluorescence of FITC will be much lower than that seen in an environment of physiological pH. As can be concluded from several studies by others, the pH of a PMN phagosome will drop rapidly to > 600 (29, 39), but can be raised by the addition of a lysosomotropic agent (40). Hence, when a virion is present in a phagosome, the fluorescence will increase after the addition of such an agent, e.g., ammonium chloride. To exclude interference with phagosome-lysosome fusion, the lysosomotropic agent must be added after the assay. Using Raji cells as a control, we showed that the addition had no effect on the outer fluorescence. An irreversible effect of PMN on FITC fluorescence has also been described (41). This was due to chlorination of the fluorescein by a myeloperoxidase-dependent pathway. The reconstitution of fluorescence by ammonium chloride in our system must represent a reversible process and thus favors the possibility that acidification is the main cause of lower fluorescence. Although chlorination may play a role, it will not affect the lack of enhancement with complement alone.

The fluorescence detected when measuring phagocytosis using PMN and complement was comparable to that seen when specific anti-HSV antibodies alone were used. However, the two techniques used here allowed us to discern an important difference: viruses loaded with complement alone are all extracellular, whereas when only specific antibodies are present without complement, all are located intracellularly in phagolysosomes. When both antibodies and complement are present, virions are located both intra- and extracellularly, although far more particles are associated than in either of the former cases. We calculated, using both quenching with trypan blue and the ammonium chloride assay, that 80% of the viruses are inside the cell. This complement enhancement of antibody-dependent phagocytosis was also reported by Smith et al. (2), and may be due to the formation of larger immune complexes or a more efficient adhesion to the PMN, because more receptors are involved. This enhanced adhesion will lead to enhanced internalization because the PMN is triggered by Fc receptors.

We measured PMN activation under circumstances comparable to those occurring in the phagocytosis assay using luminol-enhanced chemiluminescence. It was found that virions plus antibodies stimulated the PMN and that this signal could be enhanced markedly by adding complement. Complement alone plus virions could not induce a chemiluminescent response in PMN.

In conclusion, complement receptors present on the PMN in our model are incapable of promoting either an internalization trigger or stimulation of the PMN. This not only confirms the observations of others, using coated red blood cells or agarose beads (16-19), but establishes that this phenomenon also occurs with pathogenic organisms, such as herpes simplex virus, which makes it even more interesting.

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References


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