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**Basic Research**

**EXPERIMENTAL MELANIN-PROTEIN INDUCED UVEITIS (EMIU) IS THE SOLE TYPE OF UVEITIS EVOKE BY A DIVERSITY OF OCULAR MELANIN PREPARATIONS AND MELANIN-DERIVED SOLUBLE POLYPEPTIDES**

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Summary: Experimental melanin-protein induced uveitis (EMIU) is a CD4 T cell-mediated disease involving the choroid and iris, but sparing the retina. The present study was designed to solubilize uveitogenic antigen from melanin granules without enzymatic digestion, and to investigate some of its elements by comparison with different purified melanin preparations. Many melanin surface-derived polypeptides with molecular weights ranging from 1 to >100 kDa were obtained by extractions of the prepurified granules with hot lithium dodecyl sulfate (LDS). The mixture was electrophoretically separated into seven subfractions, each containing many components and capable of evoking the typical features of EMIU after footpad immunization of Lewis rats. The five low-molecular-weight fractions between M, 1 kDa and 30 kDa exhibited most pathogenicity which was evenly distributed among the fractions. Highly uveitogenic material remained in the melanin preparations even after multiple exhaustive extractions with LDS, and represented about 70% of the detectable protein. The uveal pathogen (UP-X) thus proved to be antigenically stable, and the major part of the pathogenic material was strongly bound to the granule surface layer. Concentrated urea solution was also capable of extracting many uveitogenic melanin polypeptides, but in a different composition than LDS did, and less effectively. Human choroidal melanin provided an LDS-soluble fraction with low pathogenicity. A single intraperitoneal injection of bovine melanin polypeptides together with pertussis toxin, but without footpad immunization in Freund's complete adjuvant, evoked EMIU as well. In all experiments, no uveitis except EMIU was observed, indicating that only one type of uveitogenic epitope was present in a wide variety of carrier molecules. An explanation for this phenomenon is discussed.

Key Words: Experimental autoimmune anterior uveitis (EAAU), experimental melanin-protein induced uveitis (EMIU), immunization technique, melanin, uveitogenic melanin polypeptides

**Introduction**

The inflammatory processes of the human uvea have long been studied using experimental autoimmune uveoretinitis induced by retinal photoreceptor antigens in several animal species. The model produced a variety of inflammatory phenomena but retinitis with the elimination of retinal photoreceptor cells was the most striking, consistently observed feature. Two essentially new models for CD4 T cell-mediated autoimmune uveitis have recently been developed: experimental melanin-protein-induced uveitis (EMIU), also called experimental autoimmune anterior uveitis (EAAU); and experimental autoimmune posterior uveitis (EAPU) which is evoked by a retinal pigment epithelial membrane antigen PEP-65, and includes a typical form of uveoretiniti-
tis with extending Dalén-Fuchs nodules.

EMIU can be effectively induced by immunization with purified melanin granules isolated from bovine retinal pigment epithelium (RPE), choroid or iris. CD4 lymphocytes have been shown by immunohistochemical studies to be predominantly infiltrating cells. ICAM-1 and major histocompatibility complex class II appeared to become upregulated just before this infiltration. The most remarkable feature of EMIU is that it includes choroiditis and iridocyclitis but usually spares the retina, as in noninfectious human acute anterior uveitis. In the search for the essential structural, molecular elements responsible for EMIU, we studied the pathogenicity of purified melanin preparations, and detergent- and urea-soluble antigens from bovine and human melanin.

Materials and Methods

Antigens

Melanin granules were prepared from bovine and human choroid (flow chart, Figure 1). The vitreous body and retina were removed from the posterior part of pigmented cow or human eyes. The eyes had been stored for 3 hours, and 24 hours, respectively, at 4°C. The eye cup was rinsed with phosphate-buffered saline (PBS) (pH 7.4), and the choroid was removed with forceps. Collected choroids were rubbed in an unglazed mortar with minimal added PBS; the resulting suspension was filtered through gauze. This process was repeated 5 times on the retained tissue. The combined filtrates were centrifuged at 10,000×g (10 minutes, 4°C), and the sedimented crude melanosomes were washed once with PBS. This material was purified by extraction with 20 volumes (vol/vol) 2% Triton X-100 in distilled water at 37°C for 5 hours. The extraction was repeated 4 times to remove uveitogenic S-antigen and opsin originating from retinal remnants. The efficiency of the purification of the product, Chor(m)TI, was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of lithium dodecyl sulphate (LDS) extracted protein (obtained as described below), followed by immunoblotting with rabbit antiserum to S-antigen, interphotoreceptor retinol binding protein (IRBP) and opsin. These antigens were undetectable in the preparations used.

The purified Triton-insoluble choroidal melanin, Chor(m)TI, was extracted by 1% LDS in distilled water at 75°C for 2 hours. In pilot experiments, it had appeared that the recovery of (unaggregated) protein was optimal at 75°C, while higher LDS concentrations did not extract larger amounts of protein. The extract obtained was dialyzed against distilled water at 4°C for 24~48 hours, and concentrated to a suitable volume by freeze-drying.

This procedure lowered the LDS concentration to less than 0.06%, as was needed to obtain stable adjuvant emulsions in the immunization procedure (described below). The LDS concentration was determined according to Hayashi's method. A small amount of insoluble material was removed by centrifugation, and the supernatant containing the bovine LDS-soluble protein was called Chor(m)TI-LS. The extracted gran-
ules were labeled Chor(m)TI-LI (Figure 1). The LDS-soluble protein obtained from human choroidal melanosomes was labeled HuChor(m)TI-LS. Exhaustively extracted melanin, Chor(m)TI-LI/4, was prepared as the LDS-insoluble bovine melanin by 4 consecutive LDS treatments at 75°C for 24 hours. The volume ratio of detergent solution to melanin sediment was 20:1 in all extractions. A urea-soluble fraction was obtained by extracting Chor(m)TI with 20 volumes 7 mol urea-0.1 mol phosphate buffer (pH 7.0) at 37°C for 2 hours. After centrifugation, the supernatant was dialyzed against 0.005 mol phosphate buffer-0.05% SDS at 4°C for 24 hours. The preparation, Chor(m)TI-LS (Figure 1), was concentrated by freeze-drying to a suitable volume.

Soluble protein and melanin-protein determinations, electrophoretic and immunoblot techniques, antigen and corresponding antiserum preparation and checks for antigen purity have been described previously2,3,6. Melanin samples for analytical gel electrophoresis were kept in 5% SDS-0.1 mol DTT at 22°C for 3 hours, and the 1.3 × 10,000 x g supernatant was added after centrifugation (5 minutes, 22°C). Water soluble proteins (marker proteins) and LDS- or urea-solubilized proteins were treated similarly, without centrifugation. LDS and SDS could be interchanged in the extraction procedures without loss of protein recovery, however LDS was preferred because of its better solubility and dialysis characteristics at low temperature.

Detergent-soluble polypeptides from Chor(m)TI-LS were separated and isolated by prior SDS-PAGE using Tris-Tricine buffer, according to Schägger et al12. We used the 4% and 16.5% acrylamide plus bisacrylamide concentrations for the stacking and separating gel, respectively, while the cross-link concentration was 3% in both gels. No spacer gel was used. The gels were then stained by 3 mol CuCl₂ solution19. The polypeptides were isolated from the excised gel bands in an electro-eluter (Model 422, Bio-Rad, Burlingame, CA, USA) following the manufacturer’s protocol. Protein was precipitated and freed from almost all detergent by 9 volumes cold acetone (0°C). The sediment was dissolved in a suitable volume of distilled water. Protein was determined according to Lees and Paxman29. Molecular weights were estimated by graphic extrapolation, using sets of high and low molecular weight proteins29. LDS was obtained from Serva (Heidelberg, Germany); prestandard molecular weight calibration proteins from Novel Experimental Technology (San Diego, CA, USA); and the markers and other (bio)chemicals from Sigma (St Louis, MO, USA).

**Experimental uveitis**

Female Lewis rats (130~180 g) were immunized with the melanin preparations described (10~15 μg), detergent and urea extracts from melanin (50~150 μg), and the purified polypeptide fractions (75~150 μg), according to the protocol of Broekhuyse et al3. The toxin dose was doubled to 2 μg in order to detect weak pathogenicity more easily. Table 1 shows experimental data. Half the antigen dose emulsified in Freund’s complete adjuvant was injected into the hind footpads (0.1 mL per pad); the rest was mixed with 2 μg purified pertussis toxin (Sigma) in a final volume of 1 mL phosphate-buffered saline, and injected intraperitoneally. Control animals were similarly injected without antigen. In an alternative protocol, 75 μg Chor(m)TI-LS in a 1 mL volume of Freund’s incomplete adjuvant emulsion was intraperitoneally injected with 2 μg pertussis toxin, without footpad immunization.

Pertussis toxin was stored at −80°C in ready-to-use portions, in 50% glycerol-50 mM sodium phosphate - 0.5 M NaCl (pH 7.2). It was tested for adjuvant activity before each experiment by immunizing 5 rats with Chor(m)TI-LI (10 μg per rat), according to the footpad injection protocol, using 1 μg toxin. The same antigen preparation was used throughout all activity tests. The toxin adjuvant was rejected if the group of animals did not develop severe EMIU (score 4) within
Table 1. Experimental uveitis evoked by footpad immunization with melanin-protein preparations or polypeptides solubilized from ocular melanin

<table>
<thead>
<tr>
<th>Injected polypeptides*</th>
<th>Dose (µg)</th>
<th>Incidence</th>
<th>Mild</th>
<th>Severe</th>
<th>Uveitis features</th>
<th>Maximum score†</th>
<th>Day of onset‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>From Chor(m)TI-LS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chor(m)TI-LS</td>
<td>50</td>
<td>4/4</td>
<td>0</td>
<td>4</td>
<td>EMIU &gt;4.0±0.0</td>
<td>11.5±0.3</td>
<td></td>
</tr>
<tr>
<td>40~200 kDa</td>
<td>120</td>
<td>1/3</td>
<td>0</td>
<td>1</td>
<td>EMIU§</td>
<td>4.0</td>
<td>27</td>
</tr>
<tr>
<td>30~40 kDa</td>
<td>120</td>
<td>1/3</td>
<td>0</td>
<td>1</td>
<td>EMIU§</td>
<td>4.0</td>
<td>21</td>
</tr>
<tr>
<td>18~30 kDa</td>
<td>120</td>
<td>3/3</td>
<td>0</td>
<td>3</td>
<td>EMIU§</td>
<td>4.0±0.0</td>
<td>11.5±0.5</td>
</tr>
<tr>
<td>15~18 kDa</td>
<td>80</td>
<td>5/5</td>
<td>0</td>
<td>5</td>
<td>EMIU &gt;4.0±0.0</td>
<td>10.8±0.2</td>
<td></td>
</tr>
<tr>
<td>18~14.5 kDa</td>
<td>75</td>
<td>2/2</td>
<td>0</td>
<td>2</td>
<td>EMIU§</td>
<td>4.0±0.0</td>
<td>15, 18</td>
</tr>
<tr>
<td>6~12 kDa</td>
<td>75</td>
<td>5/5</td>
<td>0</td>
<td>5</td>
<td>EMIU &gt;4.0±0.0</td>
<td>12.5±0.1</td>
<td></td>
</tr>
<tr>
<td>1~6 kDa</td>
<td>110</td>
<td>2/2</td>
<td>0</td>
<td>2</td>
<td>EMIU &gt;4.0±0.0</td>
<td>11, 12</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chor(m)TI-US</td>
<td>110</td>
<td>3/3</td>
<td>0</td>
<td>3</td>
<td>EMIU 4.0±0.0</td>
<td>12.3±0.2</td>
<td></td>
</tr>
<tr>
<td>Chor(m)TI-LI/4</td>
<td>10</td>
<td>4/4</td>
<td>0</td>
<td>4</td>
<td>EMIU 4.0±0.0</td>
<td>12.3±0.3</td>
<td></td>
</tr>
<tr>
<td>HuChor(m)TI-LS</td>
<td>150</td>
<td>2/3</td>
<td>2</td>
<td>0</td>
<td>EMIU 2.0±0.0</td>
<td>12, 27</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Chor(m)TI-LS and Chor(m)TI-US, LDS-soluble and urea-soluble fractions of Triton X-100-insoluble bovine (or human, Hu) choroidal melanin granules. 40~200-KDa etc., polypeptide fractions isolated from Chor(m)TI-LS.
†Mild, score 1–2; severe, score ≥3
‡Mean values ± SEM. Score >4, blood was present in anterior chamber, and prominence of eye was observed in addition to reported features of EMIU.
§Mainly iritis

The development of uveitis was studied by daily slit-lamp examination, and was scored in a masked fashion starting on day 9 after immunization. The severity was recorded using the scoring systems for EMIU (EAAU) on a scale of 0~42. The eyes and pineal glands were fixed in Bouin solution within 4 days after the maximum score was reached, and processed using standard histological techniques. Six-micron paraffin sections were stained with hematoxylin and eosin, and examined by light microscopy. In conducting the research described in this report, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals” (NIH, USA)18.

Results

Polypeptides eliciting EMIU

A single hot LDS extraction of Triton-purified bovine choroidal melanin removed a complex mixture of surface proteins from the granules, Chor(m)TI-LS, with a protein recovery of 20% relative to Chor(m)TI protein. Concentrated urea solution also dissolved a mixture of polypeptides, Chor(m)TI-US, but with a low protein recovery (4%). Figure 2 shows that the polypeptide compositions of the US and the LS extracts differed. Urea did not extract the three components between the 14 and 17 kDa bands of LS. The group of 30~40 kDa components of LS was nearly absent in US. However, in the >40 kDa area, many polypeptides were extracted which were found in only small amounts in LS, around 43 kDa and a ~200 kDa band. The polypeptide pattern of the urea-insoluble protein Chor(m)TI-UI (partially dissolved in SDS at 22°C for gel electrophoresis) is largely complementary to that of the solubilized Chor(m)TI-US. Figure 3 shows that even the sample-SDS (for gel electrophoresis) is barely capable of dissolving protein from Chor(m)TI after 5 Triton X-100 extractions. However, the recovery of the hot (LDS) ex-
traction is much higher as seen in the Chor(m)TI-LS lane. The same is true for HuChor(m)TI-LS. This solubilized human melanin protein exhibited a different polypeptide pattern, but the 14–17 kDa and 55–60 kDa bands were remarkably similar in composition and prominence to the (bovine) Chor(m)TI-LS. Because of the pre-extractions with LDS at 75°C during preparation, no further protein could be dissolved from Chor(m)TI-LI or Chor(m)TI-LI/4 at 22°C with the analytical gel electrophoresis technique described above. Consequently, these fractions are not included in Figures 1–3.

**Uveitogenicity of extracts**

Solubilized choroidal melanin proteins Chor(m)TI-LS and Chor(m)TI-US were used for the immunization of Lewis rats. They proved to be highly pathogenic at the 50 and 110 µg level, respectively, and evoked severe EMIU starting about 12 days after immunization (Table 1). A similar result (not shown in Table 1) was obtained if the whole dose of Chor(m)TI-LS (75 µg) was injected intraperitoneally, but EMIU started at day 14. The solubilized human melanin protein HuChor(m)TI-LS exhibited low uveitogenicity (Table 1).
Uveitogenicity of fractions

Seven polypeptide fractions were isolated from the LDS extract of choroidal melanin granules by prior SDS-PAGE (Figure 4). A brown pigment band (Mr < 1 kDa) running ahead of the polypeptides (not shown) was similarly isolated. The relative recoveries of the fractions were 19% for 40-200 kDa, 13% for 30-40 kDa, 15% for 18-30 kDa, 11% for 15-18 kDa, 14% for 13-14.5 kDa, 11% for 6-12 kDa and 16% for 1-6 kDa. The five low molecular weight Chor(m)-polypeptide fractions between 1-30 kDa (Table 1) most effectively evoked the typical features of EMIU in all animals, clinically as well as histopathologically. The frequency of EMIU was lower for the higher molecular weight fractions (40-200 kDa) and the day of onset was later (Table 1); cell infiltrations were moderate. The pigmented band of the very low molecular weight (<1 kDa) exhibited no uveitogenicity. After 4 lengthy hot LDS extractions of Chor(m)TI, a highly uveitogenic melanin preparation, Chor(m)TI-LI/4, that still contained about 70% of the originally detectable Chor(m)TI-protein, was obtained.

Clinical features

Slit-lamp examination revealed the development of bilateral acute, severe to very severe, anterior uveitis starting between days 10 and 18 after immunization. The clinical features were extensive iris hyperemia accompanied by dense flare and cells obscuring view of the fundus. In very severe uveitis, blood entered the anterior chamber and the eye protruded. The consistently high slit-lamp scores (Table 1) show that the development of EMIU always resulted in severe inflammation although the onset in a few animals could be as late as day 21 or 27 after
around the anterior uvea which was very swollen. The cell infiltrations often formed nodules where the epithelia had been disrupted (Figure 5). Although the retina was usually not affected in the primary process, a mononuclear cell infiltration was occasionally found in the outer retina disturbing or destroying the photoreceptor cells and outer nuclear layer.

Discussion

It has recently been demonstrated that strong uveitogenicity resides at the surface of ocular melanin granules, and that it is not due to pathogenic photoreceptor proteins like S-antigen, IRBP or rhodopsin. Acid digestion or phagocytosis by macrophages destroys this strong pathogenicity while the granules themselves remain intact. Alkaline solutions dissolve the melanin and destroy its pathogenicity, but an apolar detergent like Triton X-100, and polar detergents like LDS (and SDS) leave the granule virtually intact, although dissolving some melanin-associated protein. Previous investigations in our laboratory showed that Triton X-100 (at 37°C) was able to remove the residues of adhering ocular tissue proteins, including traces of photoreceptor proteins originating from the RPE. Because of the abundant presence of rod outer segment membranes in the RPE, traces of opsins could sometimes be detected in Chor(m)TI, but such preparations were excluded for further work. In view of the absence of opsins, and the water-soluble photoreceptor antigens (S-antigen and IRBP) we have assumed that other known soluble pathogens (i.e., phosducin and recoverin) were also lacking in Chor(m)TI. The absence of photoreceptor damage as a major, primary event, (since it is evoked by all photoreceptor proteins) shows that this assumption is a valid one. The Triton X-100 treatment was still mild enough to leave behind the major part of the uveitogenic protein that is more firmly bound to the granules, but that can be dissolved by LDS, SDS, or by 7 mol urea. Pilot experiments also showed that the polar

Histopathology

Histopathological examination of the eyes revealed severe uveitis, generally sparing the retina as is characteristic for EMIU. Pinealitis was absent. The details of the inflammatory pattern have been described previously. The eyes in the present experiments exhibited marked variability in the intensities of posterior and anterior uveitis. Chorioiditis generally varied from mild to moderate (in some cases, severe) while iritis was usually severe to extremely severe. In the latter cases, the ciliary body, especially the iris, was highly dystrophic. Dense, largely mononuclear cell infiltrations were present throughout the entire anterior uvea. A few days after onset, many polymorphonuclear cells were found

Figure 5. EMIU (day 15 after immunization) evoked by immunization of Lewis rats with 15-18 kDa polypeptides from bovine Chor(m)TI-LS. Very severe iridocyclitis with predominantly mononuclear cells in anterior uvea (×360).
detergents exhibited optimal dissolution properties at 75°C for 2 hours. A longer extraction period resulted in increasing aggregation of the polypeptides as revealed by SDS gel electrophoresis (not shown) while shorter extraction lowered the recovery. The determination of the recovery of LDS-soluble and LDS-insoluble protein was based on a previously elaborated melanin-protein assay in which the dissolution of disturbing brown pigment was largely prevented. This method enabled comparison of the applied immunization doses of melanin-bound protein and detergent-solubilized protein. It appeared that up to 20% of the surface melanin-protein could be removed from the granules in the preparation protocol for Chor(m)TI-LS polypeptides; our present results indicate about 30% can be removed by the exhaustive extraction technique. The efficiency of the applied dissolution technique demonstrates that many uveitogenic polypeptides can be obtained in this way without the use of proteolytic enzymes. The smaller fragment obtained by digestion favors structural analysis of the active epitope; the soluble peptides may in addition reveal more extensive molecular structural details.

**Uveitogenicity of melanin preparations**

The results in Table 1 show that 50 μg Chor(m)TI-LS protein is as uveitogenic as 10 μg Chor(m)TI-LI/4. The latter represents a preparation of LDS-treated melanin granules with strongly bound residual protein (70%) that cannot be removed by 4 LDS extractions at 75°C for 24 hours. This highly uveitogenic protein is probably covalently bound to the granule surface and has been reported to be uveitogenic even at doses as low as 1 μg.3

Other laboratories have also recently demonstrated the uveitogenicity of ocular melanin and the unique pattern of inflammatory reactions it produces. Twenty-five micrograms dry-weight purified choroidal melanin granules10, corresponding to 10 μg melanin-protein, according to Broekhuyse et al9, or 100 or 200 μg iris-ciliary body-derived melanin protein1,18 induced severe EMIU.

Differences were reported in the severity of choroiditis in these studies. Whereas we originally reported severe iridocyclitis and mild to moderate choroiditis (as Bora et al2), Chan et al10 reported both marked iridocyclitis and marked choroiditis. Our present results show that if high doses of an appropriate antigen are used, combined with 2 μg high-potency pertussis toxin, very severe iridocyclitis (score >4) is a dominant feature of EMIU (Table 1; Figure 3). The relative severity of anterior inflammation with respect to choroiditis might be ascribed to these dose dependencies of the EMIU characteristics. In addition, we noticed that pertussis toxin stored at −20°C lost appreciable adjuvant activity during a period of 2–3 months. Storing the toxin at ultra-low temperature and avoiding freeze-thawing largely prevented loss of adjuvant activity (Ref 2; unpublished results; present results).

Table 1 also shows that the majority of the pathogenic activity can be ascribed to the low molecular weight polypeptides (1–30 kDa). Only one type of uveitis (EMIU) developed in all experiments after immunization with the great variety of soluble as well as insoluble (granular melanin) antigens. The numerous components suggest that this diversity results from extensive variation in the dimension of the molecule(s) carrying the uveal pathogenic melanin-peptide UP-X. The composition of melanin may encourage structural diversity because it is basically composed of polymeric chromophoric backbones: oxidized derivatives of tyrosine-bearing (presumably) covalently bound protein11,21. The carrier molecules may be, or contain, (partially) homologous melanin-derived structures. The finding that only a relatively small proportion of the pathogenic melanin-protein can be solubilized (Refs 3, 6; present results) indicates that the pathogenicity is primarily represented by the bound protein. The LDS-solubilized fraction exhibits the same type of pathogenicity as the bound protein, and may represent the part that is not (yet) covalently bound. In addition, or alternatively, the
melanin granule surface may associate pathogenic environmental cell components which may also be present, if no (mature) melanin granules are formed. The weak uveitogenicity of isolated bovine amelanotic RPE fractions or other amelanotic preparations seem to exclude this possibility.

Uveitogenicity is not restricted to ocular melanin since partially purified bovine skin melanin also exhibits this activity. The finding that intraperitoneal immunization with solubilized melanin antigen (without the use of adjuvant bacteria) also induces EMIU agrees with a previous study of this technique. However, injection of the granular melanin-bound antigen proved to be much more effective. Only 5 \( \mu \)g melanin-protein was needed to confirm the high uveitogenicity of the granules. The relative efficiency of this immunization route resulted from the effective processing of the granular antigen by macrophages.

In conclusion, our results show that the EMTU-evoking melanin antigen UP-X can be dissolved from pre-purified melanin granules by hot LDS. The major part of the pathogenic activity remains bound. The soluble polypeptides obtained vary widely in molecular weight, and still exhibit uveitogenicity, especially in the 1–30 kDa range. Since no inflammatory pattern other than EMIU was detected, it appears that UP-X is the sole pathogen on the melanin granule surface.

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