"Differential Interactions between Th1/Th2, Th1/Th3, and Th2/Th3 Cytokines in the Regulation of Thyroperoxidase and Dual Oxidase Expression, and of Thyroglobulin Secretion in Thyrocytes in Vitro"

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Abstract
Hypothyroidism, together with glandular atrophy, is the usual outcome of destructive autoimmune thyroiditis. The impairment in the thyroid function results either from cell destruction or from Th1 cytokine-induced alteration in hormonogenesis. Here, we investigated the impact of the local immune context on the thyroid function. We used two rat thyroid cell lines (PCCL3 and FRTL-5) and human thyrocytes incubated with IL-1alpha/interferon (IFN) gamma together with IL-4, a Th2 cytokine, or with TGF-beta, or IL-10, two Th3 cytokines. We first observed that IL-4 totally blocked IL-1alpha/interferon gamma-induced alteration in dual oxidase and thyroperoxidase expression, and in thyroglobulin secretion. By contrast, TGF-beta and IL-10 had no such effect. They rather repressed thyrocyte function as do Th1 cytokines. In addition, IL-4 blocked IL-10-induced repression of thyrocyte function, but not that induced by TGF-beta. In conclusion, Th1 cytokine- and IL-10-induced local inhibitory action...

Document type: Article de périodique (Journal article)

Référence bibliographique

DOI: 10.1210/en.2007-1316
Differential Interactions between Th1/Th2, Th1/Th3, and Th2/Th3 Cytokines in the Regulation of Thyroperoxidase and Dual Oxidase Expression, and of Thyroglobulin Secretion in Thyrocytes in Vitro

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Hypothyroidism, together with glandular atrophy, is the usual outcome of destructive autoimmune thyroiditis. The impairment in the thyroid function results either from cell death or from Th1 cytokine-induced alteration in hormoneogenesis. Here, we investigated the impact of the local immune context on the thyroid function. We used two rat thyroid cell lines (PCCL3 and FRTL-5) and human thyrocytes incubated with IL-1α/interferon (IFN) γ together with IL-4, a Th2 cytokine, or with TGF-β, or IL-10, two Th3 cytokines. We first observed that IL-4 totally blocked IL-1α/IFN-γ-induced alteration in dual oxidase and thyroperoxidase expression, and in thyroglobulin secretion. By contrast, TGF-β and IL-10 had no such effect. They rather repressed thyrocyte function as do Th1 cytokines. In addition, IL-4 blocked IL-10-induced repression of thyrocyte function, but not that induced by TGF-β. In conclusion, Th1 cytokine- and IL-10-induced local inhibitory actions on thyroid function can be totally overturned by Th2 cytokines. These data provide new clues about the influence of the immune context on thyrocyte function. (Endocrinology 149: 1534–1542, 2008)

HASHIMOTO’S THYROIDITIS (HT) is a destructive autoimmune disease characterized by the infiltration of the parenchyma by inflammatory cells. The immune response is mostly cell mediated and is accompanied by a gradual loss of function, eventually leading to permanent hypothyroidism (1).

Cytokines that act as mediators of immune responses are classified into different groups. Th1 cytokines, usually termed pro-inflammatory, are involved in the regulation of cell-mediated immune responses and play a leading role in HT (2–4). Th2 cytokines are involved in the control of antibody production, in disease remission, and the suppression of immune responses. Actions resulting from Th1 and Th2 cells are mutually suppressive (5–8). More recently, a third group composed of Th3 cytokines was described, including IL-10 and TGF-β (8, 9). Both TGF-β and IL-10 are immunosuppressive and play a role in the resolution of inflammatory responses in a large variety of organ-specific autoimmune diseases (9, 10). They are also involved in various biological processes, including tumorigenesis and fibrosis (10–15).

Th1 cytokines exert direct local effects in the thyroid through receptors located on thyrocytes (16–21). They affect the expression of thyroid-specific proteins, including thyroglobulin (Tg), thyroperoxidase (TPO), Na⁺/I⁻ symporter, and the H₂O₂ generating enzymes [dual activity oxidase (DUOX)], which are all down-regulated (22–26). Thus, hypothyroidism may accompany HT not only because of irreversible apoptotic cell death (27, 28), but also because of impairment of the thyroid function without cellular destruction (26). This may explain the observation made by clinicians in their daily medical practice about the highly variable clinical outcome of HT patients. Some of them have a glandular atrophy along with pronounced hypothyroidism, whereas others remain euthyroid or subclinical hypothyroid for a while. Some patients can even sometimes recover from hypothyroidism toward euthyroidism, suggesting a possible reversibility of the immunological injury (29–31). Mechanisms responsible for changes in the thyroid function are not yet quite explained. Our previous paper (26) provided some clues on how IL-1α/IFN (Th1 cytokines)-induced inhibition of TPO and DUOX protein expression without affecting cell survival. For instance, their effects are partially mediated by nitric oxide (NO) and can be overshadowed by increased concentrations of TSH, suggesting a mutual antagonism between Th1 cytokines and TSH-activated intracellular pathways. In the present paper, we propose that the local immune context and interactions between cytokines of different nature could also deeply influence the functional outcome of inflammatory thyroid glands. We looked at whether IL-4, a Th2 cytokine, and TGF-β or IL-10, two Th3 cytokines, may reverse IL-1α/IFNy (Th1 cytokines)-induced inhibitory effects on thyroid function. To answer this question, we used three different in vitro models of thyrocytes [two rat thyroid cell lines (PCCL3 and FRTL-5) and one in the human (thyrocytes in primary cultures)], in which, in the presence of IL-1α/IFNy, thyroid function is hampered without cell death (26).

First Published Online January 10, 2008

Abbreviations: DUOX, Dual oxidase; FRTL-5, Fischer Rat Thyroid Low Serum 5%; HT, Hashimoto’s thyroiditis; IFN, interferon; L-N′-nitroso-L-arginine methyl ester; NCS, newborn calf serum; NO, nitric oxide; PCCL3, pancreatic cancer cell line 3; STAT, signal transducer and activator of transcription; Tg, thyroglobulin; TPO, thyroperoxidase.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
**Materials and Methods**

**Cell cultures**

FRTL-5 cells (32) were a gift from Dr. P. Kopp (Northwestern University, Chicago, IL), and PCCL3 cells (5 or 3 for human thyrocytes, and n = 6 for nitrite and Tg ELISA) were purchased from the American Type Culture Collection (Manassas, VA). TSH, 1 mU/ml, was used in the recommended buffer containing 1:4000. Membranes were incubated at room temperature in PBS (pH 7.4), 5% non fat dry milk, and 5% μg/ml transferrin. Recombinant rat IL-1α (2 ng/ml; R&D Systems) and IL-4 (0.2, 2, or 20 ng/ml; a gift from Dr. Vanderbruggen, Université catholique de Louvain, Institute of Cellular Biology, Brussels, Belgium) Western blotting were performed as previously described (26). Briefly, proteins (30 μg/lane) were heated at 95°C for 5 min in the loading buffer (Laemmli buffer containing 100 mm diethiothreitol and 0.1% bromophenol blue), separated by 8% SDS-PAGE, and transferred onto a nitrocellulose membrane (Hybond ECL; Amer sham Biosciences, Rosendaal, The Netherlands). Membranes were blocked for 1 h at room temperature with EnVision (2:1) peroxidase-labeled secondary antibody, and visualized with enhanced chemiluminescence (SuperSignal West Pico; Pierce) on CL-Xposure TM films (Pierce). Only DUOX expression was analyzed in FRTL-5 and PCCL3 cells.

**RT-PCR**

Cells were suspended in TriPure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany), and total RNA was purified according to the manufacturer's protocol. RT was performed by incubating 2 μg RNA with 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen, Merelbeke, Belgium) in the recommended buffer containing 1 ml Rnasin (Promega), 0.5 mm each deoxynucleotide triphosphate (Promega), 2 μM oligo-deoxynucleotide (Sigma), and 10 mm diethiothreitol (20 μl final volume) overnight at 42°C. The end products were used for PCR amplifications. cDNA (2.5 μl) was mixed with 0.65 U TaKaRa Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 0.2 mm deoxynucleotide triphosphate, and 0.5 μM specific primers. Amplification cycles were as follows: 94°C for 1 min, annealing temperature/2 min, and 72°C/1 min, followed by 10 min/72°C. Specific primers used were: 5'-GGGCTGCGCCACCAT-3' (sense human DUOX-1/2), 5'-GGGGACTGGTAGGAAAG-3' (antisense human DUOX-1/2); 5'-CACGTTGCAAGAATCC-3' (sense human TPO), 5'-ATAGACTGGAGGGGACCAT-3' (antisense human TPO), 5'-GGGGCTGCGGCACCAT-3' (sense rat DUOX1/2), 5'-CCGGTAAACCTCCTTGT-3' (antisense rat DUOX1/2), 5'-CAGGCTTTCTGAGGAGAG-3' (sense rat TPO), 5'-CTGGCACTTCAATCATC-3' (antisense rat TPO), 5'-CATCTCGCTGGACGCT-3' (sense human and rat β-actin), and 5'-AGGAGGAGCAATGTCTT-3' (antisense human and rat β-actin). All primers were intron spanning to avoid genomic DNA amplification. The annealing temperatures were 62°C for β-actin, 60°C for human TPO and rat DUOX, 56°C for human DUOX, and 58°C for rat TPO. PCR products were separated by agarose gel (1 or 1.5%) electrophoresis.

**Data analysis and statistics**

Data were expressed as mean ± SEM (n = 6 for nitrite and Tg ELISA). Statistical analyses were performed using ANOVA, followed by the Tukey-Kramer Multiple Comparison Test (GraphPad InStat, San Diego, CA), and P < 0.05 was considered statistically significant.

Western blots were scanned and quantified by densitometry using the National Institutes of Health (NIH) Scion Image Analysis Software (NIH, Bethesda, MD). Values were expressed as mean ± SEM of one representative experiment (n = 5 or 3 for human thyrocytes, and n = 6 for FRTL-5 and PCCL3 cells). Digital images of ethidium bromide-stained agarose gels were quantified by densitometry using the NIH Scion Image Analysis Software. Values were normalized by reporting the signal intensity to β-actin expression. Values were expressed as mean ± SEM of one representative experiment performed in triplicate for both FRTL-5 cells and human thyrocytes, and n = 6 for PCCL3 cells. The statistical analysis was performed using the unpaired t test. P < 0.05 was considered statistically significant.
Results
IL-4 counters IL-1α/IFNγ-induced effects in PCCL3, FRTL-5, and human thyrocytes

DUOX protein was detected in cells incubated with TSH (1 mU/ml) as a single band of 179 kDa in control FRTL-5 and PCCL3 cells. Its expression was down-regulated by IL-1α/IFNγ. Coincubation with IL-4 blocked the Th1-induced inhibitory effect on DUOX protein expression in PCCL3 cells and in a dose-dependent manner (Fig. 1A). Cells treated with IL-4 alone showed no alteration in DUOX protein expression (data not shown). No effect on cell viability was observed whatever the dose used (data not shown). IL-4 at a dose of 2 ng/ml was then used for the following experiments.

DUOX protein expression was significantly down-regulated by IL-1α/IFNγ, an effect blocked by IL-4 in both PCCL3 and FRTL-5 cell lines (Fig. 1B). Identical results were obtained when analyzing DUOX mRNA expression by RT-PCR. TPO protein expression was not investigated because of a lack of antirat TPO antibody. Its mRNA expression was detected when analyzing DUOX mRNA expression by RT-PCR. TPO protein expression was not investigated because of a lack of antirat TPO antibody. Its mRNA expression was significantly decreased by IL-1α/IFNγ, an effect completely reversed by IL-4 in both PCCL3 and FRTL-5 cells. A slight increase was even observed compared with control FRTL-5 cells (Fig. 1B).

Identical results were obtained in primary cultures of human thyrocytes. DUOX and TPO proteins were detected as a single band of 179 and 110 kDa, respectively, in control thyrocytes incubated with TSH (1 mU/ml). Without TSH, TPO and, to a lesser extent, DUOX expression was low, as expected (data not shown) (26). IL-1α/IFNγ significantly decreased DUOX and TPO protein expression (40 and 38% decrease, respectively; P < 0.05), an effect prevented by IL-4 cotreatment. Similarly, DUOX and TPO mRNA expression was significantly reduced after IL-1α/IFNγ treatment (47 and 63% decrease, respectively; P < 0.05). In the presence of IL-4, DUOX and TPO mRNA expression was restored (Fig. 2, A and B). IL-1α/IFNγ significantly decreased Tg production (51% decrease; P < 0.05; Fig. 2C), as previously reported (22, 24, 35). In cells coincubated with IL-4, Tg release was restored.

In human thyrocytes, nitrite levels, the stable end product of NO generation, were low in media from control cells. By contrast, they were greatly induced by IL-1α/IFNγ (P < 0.05) and strongly reduced after coincubation with L-NAME (86%; P < 0.05), as previously reported (26, 28). In Th1-treated cells coincubated with IL-4, nitrite levels were reduced by half (P < 0.05), compared with IL-1α/IFNγ-incubated cells. Nevertheless, they remained significantly higher as compared with the L-NAME group (Fig. 2D), indicating that IL-4 acts through other pathways than solely NO.

IL-1α/IFNγ-induced DUOX and TPO down-regulation is not affected by Th3 cytokines in PCCL3 and FRTL-5 cells

In PCCL3 cells, TGF-β alone induced a dose-dependent down-regulation of DUOX protein expression (data not shown). In contrast with IL-4, the inhibitory effects of IL-1α/IFNγ on DUOX protein expression were not counteracted when cells were coincubated with TGF-β (Fig. 3A). No effect on cell viability was observed (data not shown). The dose of 2 ng/ml was used for the following experiments.

Western blot quantification showed that cotreatment with TGF-β had no further effect on IL-1α/IFNγ-induced DUOX down-regulation, both in PCCL3 and FRTL-5 cells. Th1-induced inhibitory effect on DUOX mRNA was moderately reinforced in Th1-treated FRTL-5 cells coincubated with

**Table 1.** IL-4 counteracts the inhibitory effects of IL-1α/IFNγ in PCCL3 and FRTL-5 cells. A, Total protein extracts of PCCL3 cells were analyzed by Western blotting using a DUOX antibody. IL-4 was added at a concentration of 2 ng/ml. *, P < 0.05 vs. control cells. +, P < 0.05 vs. IL-1α/IFNγ-treated cells.
**FIG. 2.** IL-4 counteracts the inhibitory effects of IL-1α/IFNγ in human thyrocytes. A, DUOX protein (left panel) and mRNA (right panel) expression in human normal thyrocytes. B, TPO protein (left panel) and mRNA (right panel) expression in human normal thyrocytes. Densitometric values of Western blot results are expressed as mean ± SEM of one representative experiment performed in triplicate. Densitometric values of RT-PCR, adjusted to the β-actin signal, are expressed as mean ± SEM of one representative experiment performed in triplicate. IL-4 was added at a concentration of 2 ng/ml. C, Tg production by human thyrocytes. Tg accumulation in the supernatant was assayed by ELISA. Results are expressed as means ± SEM of six individual wells of one representative experiment. IL-4 was added at a concentration of 2 ng/ml. D, Nitrite accumulation in the culture medium of human thyrocytes. Results are expressed as means ± SEM of six individual wells of one representative experiment. IL-4 was added at a concentration of 2 ng/ml. *, P < 0.05 vs. control cells. †, P < 0.05 vs. IL-1α/IFNγ-treated cells.
TGF-β, although the difference was not significant. Th1-induced inhibitory effect on TPO mRNA expression was not affected by TGF-β in both cell lines (Fig. 3B).

To verify if the well-known inhibitory action of TGF-β (37) was specific or inherent to all Th3 cytokines, we tested a second Th3 cytokine, IL-10. In PCCL3 cells, IL-10 alone also inhibited DUOX protein expression in a dose-dependent manner (data not shown). In contrast with IL-4, the inhibitory effects of IL-1α/IFNγ on DUOX protein expression were not counteracted when cells were coincubated with IL-10 (Fig. 4A). No effect on cell viability was observed (data not shown). The concentration of 2 ng/ml was used for the following experiments.

Western blot quantification confirmed that the cotreatment with IL-10 did not restore DUOX protein expression down-regulated by IL-1α/IFNγ. Identical results were obtained when analyzing DUOX and TPO mRNA expression (Fig. 4B).

IL-1α/IFNγ significantly decreased DUOX and TPO protein expression (45 and 51% decrease, respectively; \( P < 0.05 \)) in primary cultures of human thyrocytes. IL-10 cotreatment did not prevent this effect. Similar results were obtained when analyzing DUOX and TPO mRNA expression by RT-PCR. The expression of the two mRNAs was significantly reduced after IL-1α/IFNγ treatment (46 and 73% decrease, respectively; \( P < 0.05 \)), but not restored by IL-10 (Fig. 5, A and B). The inhibition of Tg release induced by IL-1α/IFNγ was not prevented by IL-10 (Fig. 5C).

The induction of nitrite levels by IL-1α/IFNγ (\( P < 0.05 \)) was slightly reduced after coincubation with IL-10 (24%; \( P < 0.05 \)), remaining significantly higher as compared with the L-NAME group (Fig. 5D).

**IL-4 counters IL-10- but not TGF-β-induced DUOX and TPO down-regulation in PCCL3 cells**

In PCCL3 cells, the inhibition of DUOX protein expression induced by IL-10, but not that of TGF-β (another Th3 cytokine), was reversed after coincubation with IL-4 (Fig. 6A). Identical results were obtained when DUOX and TPO mRNA expression was analyzed (Fig. 6, B and C).

**Discussion**

Our data provide evidence that IL-1α/IFNγ-induced inhibitory effects on the thyroid function can be overturned by IL-4, a Th2 cytokine, but not by TGF-β and IL-10, two Th3 cytokines. In addition, IL-4 was able to reverse the inhibitory effect of IL-10, but not that of TGF-β.

IL-4 reversed IL-1α/IFNγ effects in the two rat thyroid cell lines, as well as in human thyrocytes. It blocked the decrease in DUOX and TPO expression, both at transcriptional and translational levels, and provoked a complete recovery of Tg secretion. These effects are evocative of those reported in pancreatic islet cells. Therefore, after a 12-h treatment with Th1 cytokines, whereas cell survival is not affected, IL-4 is able to reverse the decrease in insulin secretion. After a treatment of 72 h, IL-4 has no more effect on insulin secretion, but blocks cell apoptosis (5). In our model there was no cell death. Thus, changes in protein expression or secretion resulted from a direct effect of IL-4 on thyroid cell function,
IL-4 effects were not mediated by NO, even though its production was partially blunted. Thus, whereas IL-4 fully restored DUOX and TPO expression, L-NAME, a potent NO synthase inhibitor, was unable of such an effect (26). Likewise, Tg secretion was restored by IL-4, but not L-NAME (35). Of note, IL-4 effects were observed in FRTL-5 and PCCL3 cells, two cell models that are known for their inability to release NO. Thus, IL-4 may directly interfere with IL-1α/IFNγ transduction pathways in thyrocytes.

TGF-β did not influence IL-1α/IFNγ-induced down-regulation of DUOX and TPO expression in PCCL3 and FRTL-5 cells. These results are in contrast with those observed in pancreatic β-cells, in which TGF-β can prevent IL-1β-induced inhibition of insulin secretion. In addition, TGF-β is able to suppress the immune reaction in experimental autoimmune encephalomyelitis and during allergy (10, 38–40). These discrepancies are not really surprising. Our results should actually be interpreted having in mind previously reported TGF-β effects in the thyroid gland. Therefore, accumulated evidence indicates that TGF-β alters several important steps of hormonogenesis as well as the thyroid cell proliferation (37, 41–43). We also found that DUOX protein expression was reduced in cells incubated with TGF-β alone and that pretreatment with TGF-β made Th1 cytokine-induced effects even worse instead of switching them off (data not shown). This suggests that in contrast with IL-4, TGF-β is a potent inhibitor of thyroid function and cannot reverse Th1 effects. Nevertheless, cytokine-induced effects should be interpreted differently because they are investigated in vivo or in vitro. An in vivo paper (44) lately reported a favorable role for TGF-β in the outcome of thyroiditis. Thus, TGF-β may overturn Th1 cytokine-induced inhibitory effects by acting directly on Th1 lymphocytes. By contrast, when it acts directly on thyrocytes, its inhibitory effects become predominant.

To verify whether findings with TGF-β were specific for this molecule, but not necessarily for other Th3 cytokines, we looked at the effects of IL-10. They were similar. Thus, IL-10 inhibited DUOX and TPO expression, without affecting Th1 inhibitory effects. It is the first time that a direct inhibitory effect of IL-10 on thyrocyte function is reported. In fact, little is known about how IL-10 is affecting the outcome of autoimmune thyroiditis. In vivo reports indicate a rather beneficial role in experimental autoimmune thyroiditis (45–47). Additional investigations are required to figure out what IL-10 really does in the thyroid, and should be performed in vitro and in vivo to sort out direct (on thyrocytes) and indirect (on infiltrating cells) effects of this cytokine. At this point, it seems reasonable to propose that, as for TGF-β, IL-10 inhibits the thyroid cell function when it acts directly on thyrocytes, but plays instead a rather favorable role in reducing autoimmunity when it is systemically administered.

In addition to its ability to reverse inhibitory effects of Th1 cytokines, IL-4 was also able to switch off inhibitory effects of IL-10, but not those of TGF-β. These results suggest that instead of an eventual recovery from IL-1α/IFNγ-induced cell death (26). This obviously does not exclude the involvement of other mechanisms of protection activated in vivo, such as the likelihood for IL-4 to interact directly with inflammatory cells.
both Th3 cytokines actually act through different pathways and that IL-4 may interfere with transduction pathways activated by ILs but not by TGF-β. Although appropriate tools were not used in this study to sort out this question, literature data suggest that, indeed, intracellular signaling pathways are most likely different. Therefore, ILs act mainly through the Janus kinases (JAK)/signal transducer and activator of transcription (STAT) signal pathway, involving various
STAT molecules (STAT6 for IL-4, STAT1 for IFNγ, and STAT1, 3, and 5 for IL-10), and nuclear factor κB for IL-1 (12, 48, 49), whereas TGF-β acts through Smads (50, 51). Unfortunately, nothing is known yet about the signaling pathways activated by IL-10 and IL-4 in the thyroid cells.

Despite the obvious limitation of in vitro models, our data bring new insights on how cytokines functionally influence the thyrocyte, and why the thyroid function of HT patients may vary between hypothyroidism and euthyroidism. Although thyroiditis is usually considered as predominantly of Th1 type (4), different cytokines may actually coexist (2, 3, 27). Depending on their nature and/or local content, Th1 cytokines may either be lethal or impair thyroid function by altering hormone synthesis. In the presence of Th2 cytokines (e.g. IL-4), the intraglandular conditions change because they can block thyrocyte-specific Th1 cytokine-induced effects, notwithstanding parallel effects on immune cells. These results are in line with those previously reported in which Th1 cytokine effects faded away in the presence of TSH. This could explain why hashitoxicosis patients with TSH receptor autoantibodies may switch from hypothyroidism to euthyroidism and even hyperthyroidism.

In conclusion, when thyrocytes are treated with IL-1α/IFNγ, their viability is not affected, but DUOX, TPO, and Tg expression or production is hindered. This can be overturned by IL-4, a Th2 cytokine. Therefore, the ability of Th1 cytokines to affect the thyroid hormonal status is influenced by the local immune context. By contrast, Th3 cytokines, TGF-β and IL-10, failed to switch off Th1 effects but, rather, exerted inhibitory effects as Th1 cytokines. IL-10- but not TGF-β-induced inhibition was reversed by IL-4, indicating that various intracellular signaling pathways are differentially regulated according to the cytokine predominant in the scene. Further experiments are required to sort out what intracellular signaling pathways are activated by these cytokines.

Acknowledgments

We thank Dr Y. Nizet (Unité d’Immunologie Expérimentrale, Université catholique de Louvain) for his technical support.

Received September 24, 2007. Accepted December 28, 2007.

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The work is supported by a Fonds spécial de recherche grant from Université catholique de Louvain, Belgium. A.-C.G. is the recipient of a grant from the Belgian National Fund for Scientific Research.

Disclosure Statement: The authors have nothing to disclose.

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