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Bordetella pertussis Infection in 2-Month-Old Infants Promotes Type 1 T Cell Responses

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Neonatal immaturity of the immune system is currently believed to generally limit the induction of immune responses to vaccine Ags and to skew them toward type 2 responses. We demonstrated here that Bordetella pertussis infection in very young infants (median, 2 mo old) as well as the first administration of whole-cell pertussis vaccine induces B. pertussis Ag-specific IFN-γ secretion by the PBMC of these infants. IFN-γ was secreted by both CD4+ and CD8+ T lymphocytes, and the levels of Ag-induced IFN-γ secretion did not correlate with the age of the infants. Appearance of the specific Th-1 cell-mediated immunity was accompanied by a general shift of the cytokine secretion profile of these infants toward a stronger Th1 profile, as evidenced by the response to a polyclonal stimulation. We conclude that the immune system of 2-mo-old infants is developmentally mature enough to develop Th1 responses in vivo upon infection by B. pertussis or vaccination with whole-cell pertussis vaccines. The Journal of Immunology, 2003, 170: 1504–1509.

Early immunization is required to protect infants from infectious diseases to which they are exposed during their first month of life. However, neonatal immune immaturity is known to limit the induction of immune responses to vaccine Ags. Numerous studies, both in mice and humans, have assessed the functional capacity of neonatal T and B cells in models of polyclonal activation in vitro and demonstrated a significant degree of immaturity of T and B cells (1, 2). However, studies in mice have demonstrated that significant vaccine-specific responses can be induced by neonatal immunization, but that these responses differ qualitatively from those obtained in adults, with a predominance of a Th2 pattern of T cell response in the neonates (3). Such a bias toward Th2 responses could reduce the efficacy of vaccines, especially against intracellular pathogens.

In human newborns, reduced IFN-γ secretion has been reported in response to polyclonal stimulation (4–6) which remained significantly lower than that of older children or adults until 12 mo of age (7). This decreased IFN-γ production is in large part due to an intrinsic T cell defect determined mostly by a deficiency of memory T cells in infants (2, 5, 8, 9). It has also been related partially to the immature function of neonatal APC, such as macrophages (9) and dendritic cells (10), deficient in their ability to produce IL-12. Adult-like levels of IFN-γ by neonatal CD4+ and/or CD8+ T lymphocytes have so far only been reported in special settings, such as upon strong in vitro costimulation of CD28 (11) or after neonatal bacillus Calmette-Guérin (BCG) vaccination (12). The ability of neonatal BCG vaccination to induce IFN-γ may be related to the potent APC-activating properties of BCG (13, 14). However, generally neonates more often develop Th2-dominant responses. The induction of Ag-specific IL-5 and IL-13 responses has been reported in 2- to 12-mo-old infants after administration of the diphtheria-tetanus-acellular pertussis vaccine (15). Only low and transient IFN-γ responses were detected at 6 mo. In contrast, significant measles virus-specific IFN-γ responses were demonstrated in 9-mo-old infants after anti-measles vaccination at 6 mo of age (16), suggesting that 6-mo-old infants are able to mount an Ag-specific Th1-specific response.

Since information about Ag-specific cellular immune responses developed in infants before 6 mo of age is scarce, the present study was undertaken to characterize the T cell cytokine secretions induced in 2-mo-old infants by a Bordetella pertussis infection or vaccination with whole cell pertussis vaccine. The recent resurgence of B. pertussis infections, especially in very young infants, has been attributed to the lack of specific cellular immunity in these children. Recovery from whooping cough in older infants is associated with selective induction of Th1 cells (17), and studies in mice have suggested an important role of Th1-type immune responses in the protection against infection (18).

Materials and Methods

Patients

Blood samples were collected from 27 one- to 4-mo-old (median, 2 mo) children suffering from an acute B. pertussis infection, from 46 two-mo-old infants who had not yet received any vaccine, and from 17 infants who had been injected with combined whole-cell pertussis vaccine (Tétracocq, Aven-tis Pasteur, Lyon, France). The vaccinated children received the vaccine injection at 21/2 mo and were bled before (at 2 mo) and 15 days after vaccination (at 3 mo). The B. pertussis-infected children were not vaccinated before the onset of the disease. Diagnosis of B. pertussis infection

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was based on typical whooping cough attacks, high peripheral lymphocytosis, and in 21 of 27 infants, a nasopharyngeal culture positive for *B. pertussis*. For the remaining six infants, PCR (19) was positive for *B. pertussis*. The median duration of illness, as estimated by the median duration of cough, when the blood samples were taken was 13 days (range, 4–35 days). The ethical committee of the Université Libre de Bruxelles has approved this study. For ethical reasons, samples from nonimmune children as well as from vaccinated children were collected from HIV-negative children born from HIV-infected mothers, as blood samples are routinely collected from these children at 2 and 3 mo. The socioeconomic status of the two groups of infants was comparable, as a consequence of the Belgian social security system. The medians of the infant’s weight at the time of blood sampling were 5.000 kg (range, 3.200–6.270 kg) in the group of infants born from HIV-infected mothers and 5.080 kg (range, 3.600–6.300 kg) in the group of *B. pertussis*-infected infants. None of the *B. pertussis*-infected children were born prematurely, and three of them were breast-fed. Among the 46 nonvaccinated 2-mo-old infants, one was born at 33 gestational wk and three were born at 36 gestational wk. None of them was breast-fed. All of the children were enrolled in the study after their parent’s consent. Blood samples from 23 healthy blood donors were used as controls.

**Cell isolation and culture conditions**

PBMC were isolated from heparinized venous blood by density gradient centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). The cell-containing interface was collected, washed three times with HBSS (Life Technologies, Grand Island, NY), and resuspended in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS, 2 mM t-glutamine, 50 μM 2-ME, and 40 μg/ml gentamicin.

Isolated PBMC were phenotyped by flow cytometry and were assayed for their capacity to secrete cytokines after antigenic or polyclonal stimulation with two different complemented reagents. The ELISPOT was used to evaluate the numbers of IFN-γ or IL-4–secreting cells (SC), whereas the ELISA was used to measure the IFN-γ or IL-13 concentrations in cell culture supernatants. However, because of limitations in the numbers of PBMC harvested, not all assays could be performed for all infants.

**Circulating lymphocyte phenotyping**

Lymphocyte subpopulations were analyzed by four-color flow cytometry using a panel of monoclonal-specific Abs as follows: FITC-, RPE-, PerCP-, or PE-labeled anti-CD3 mAb, allophycocyanin-labeled or PerCP-labeled anti-CD4 mAb, PE-, PerCP-, or allophycocyanin-labeled anti-CD8 mAb, PE-labeled anti-CD16 mAb, PE-labeled anti-CD56 mAb, FITC-labeled anti-γ-δ receptor mAb, allophycocyanin-labeled anti-CD45RO mAb, allophycocyanin- or FITC-labeled anti-CD62 ligand (CD62L) mAb (BD Pharmingen, San Jose, CA).

The data were acquired and analyzed with the FACSCalibur flow cytometer using CellQuest software (BD Pharmingen). The results are expressed as percentages of CD3⁺ and CD16⁺ 56⁺ cells among lymphocytes and of CD4⁺, CD8⁺, γδ⁺ cells among CD3⁺ T lymphocytes. Within the CD4⁺ and CD8⁺ lymphocytes, the percentages of CD45RO⁺ and of CD62L⁺ cells were analyzed.

**B. pertussis Ags used for in vitro stimulation**

Purified pertussis toxin (PTX-R), provided by the Pasteur Institute (Lille, France) (20), and filamentous hemagglutinin (FHA),1 provided by Chiron Biocine (Sienna, Italy), were used for in vitro stimulation. The enzymatically inactive form PTX-R was used instead of natural pertussis toxin to avoid potential interference by the toxin-expressed ADP ribosylation of signal-transducing proteins in the target cells. FHA was dialyzed before use to avoid interference in cell culture of thiomersal added as conservative to the Ag. The LPS content of the Ags was determined by the Limulus amebocyte test and was 67 pg LPS/μg PTX-R and 37 pg LPS/μg FHA. A series of preliminary experiments indicated that LPS concentrations up to 2500 pg had no significant effect on IFN-γ secretion. The optimal concentrations of *B. pertussis* Ags to be used for in vitro stimulation had been evaluated in preliminary studies by comparing the IFN-γ secretions induced in samples from acutely infected children (positive controls) to those obtained in cord blood samples (negative controls) (data not shown).

**Enumeration of the numbers of IFN-γ and IL-4-SC by ELISPOT**

Details of the ELISPOT technique have been reported previously (21). Briefly, 2 × 10⁶ PBMC/ml were preincubated in the presence of one of the following agonists: 2 μg/ml FHA (Abbott Laboratories, Abbott Park, IL), 1 μg/ml FHA, or 1 μg/ml PTX-R in RPMI 1640 supplemented with 5% FCS. Preincubation times were 4 h for IL-4-SC and 48 h for IFN-γ-SC. The cells were then transferred into nitrocellulose-bottom microtiter plates (Mahan 4550; Millipore) coated with anti-IL-4 mAb (clone 82–2; Mabtech, Stockholm, Sweden) or with anti-IFN-γ mAb (clone 1/D1K; Chromo- genix, Stockholm, Sweden) and incubated in a humidified 5% CO₂ atmosphere at 37°C during 48 h for the enumeration of IL-4-SC and during 24 h for the enumeration of IFN-γ-SC. After washing the plates, the secreted cytokines were detected by the addition of either biotinylated anti-IL-4 mAb (clone 12.1; Mabtech) or biotinylated anti-IFN-γ mAb (clone 7B7/61; Chromogenix).

The numbers of spots were counted with an image analyzer (Carl Zeiss Vision Imaging Systems; Zeiss, Jena, Germany). The coefficient of variation of spot counts with this analyzer was 2%.

**ELISA for detection of cytokine proteins in cell culture supernatants**

IFN-γ and IL-13 concentrations were measured by ELISA in the supernatants from PBMC cultured for 72 h at 37°C under 5% CO₂ in the presence of 1 μg/ml FHA, 1 μg/ml PTX-R, or 2 μg/ml PHA. Optimal kinetics for the collection of the supernatants had been determined by preliminary experiments (data not shown).

The capture Abs for the ELISA were, respectively, anti-human IFN-γ IgG1 (clone 350B 10G6; BioSource International, Camarillo, CA) and polyclonal anti-human IL-13 Abs (Endogen, Woburn, MA). The detection Abs were biotin-labeled anti-human IFN-γ IgG1 (clone 67F 12A8; BioSource International) and anti-human IL-13 IgG1 (clone 5A2; Endogen). The standard curves were generated using serial dilutions of recombinant human IFN-γ (BioSource International) and IL-13 (Endogen), respectively. The sensitivity limits of the assays were 4 pg/ml for IFN-γ and 30 pg/ml for IL-13.

**Phenotyping of Ag-specific IFN-γ-producing cells**

The Ag-specific IFN-γ-producing cells were identified by four-color immunofluorescent staining of intracellular IFN-γ and three different surface Ags, followed by flow cytometry analysis, following the manufacturer’s instructions (BD Biosciences, Mountain View, CA). Briefly, 2 × 10⁶ PBMC/ml suspended in supplemented RPMI 1640 were incubated overnight at 37°C under 5% CO₂, either nonstimulated or stimulated with 10 μg/ml FHA or with 10 μg/ml PTX-R in the presence of a mixture of anti-CD28/CD49D Abs (2 μg/ml, L293/1L235; BD Biosciences). Brefeldin A (10 μg/ml) was then added for 4 h, followed by EDTA (1 mM final concentration) for 15 min to remove adherent cells. After lysis of the erythrocytes and leukocyte fixation (lysing solution; BD Biosciences), the cells were permeabilized (FACS-permeabilizing solution), washed, and then incubated in the dark for 30 min in the presence of FITC-labeled anti-IFN-γ mAb, allophycocyanin-labeled anti-CD3 mAb, PerCP-labeled anti-CD4 mAb, and PE-labeled anti-CD8 mAb (BD Biosciences).

The samples were acquired and analyzed within 24 h of staining using a FACSCalibur flow cytometer and CellQuest software (both from BD Biosciences). Lymphocytes were first gated based on the light-scattering characteristics (forward- and side-scattered light signals) of the cells. The percentages of IFN-γ–producing cells were analyzed, first in the lymphocyte gate, then in the CD4⁺ gate, and finally in the CD8⁺ gate. The use of three different surface markers allowed us to avoid misidentification of the cell cultures.

**Statistical analysis**

The data are depicted as the median and 25th to 75th percentile values. The significance of differences between the two groups was determined using the Mann-Whitney U test and between three groups using the Kruskal-Wallis test followed by Dunn’s post tests (GraphPad Prism program; GraphPad, San Diego, CA). The significance of correlations was analyzed by the nonparametric Spearman test. Values of p < 0.05 were considered to be significant.

**Results**

*T lymphocyte phenotypes in 2-mo-old infants*

Whereas the percentages of CD3⁺ and CD4⁺ T lymphocytes were similar in infants and adults, the percentages of CD8⁺ lymphocytes were significantly lower (26% in infants compared with 34% in adults; ranges, 11–37 and 25–60; p < 0.05). The percentages of γδ⁺-bearing CD3⁺ lymphocytes were not different between the two groups, but infants displayed lower proportions of CD16⁺CD56⁺...
NK cells (3.8% compared with 4.6% in adults; ranges, 1.8–11.8 and 3.4–11.1; \( p < 0.001 \)). Within both CD4\(^+\) and CD8\(^+\) T lymphocyte subsets, the proportions of memory CD45RO\(^+\) cells, as well as those of the effector cells identified by the loss of CD62L expression, were lower in infants than in adults (\( p < 0.01 \)).

Acute \( B. \) pertussis infection did not significantly modify the proportions of these circulating lymphocyte subpopulations.

Ag-induced Th1 and Th2 cytokine secretions during acute \( B. \) pertussis infection

Cytokine secretions by PBMC stimulated in vitro with FHA or PTX-R were analyzed by two complementary techniques, ELISPOT and ELISA, as the numbers of cytokine-SC (ELISPOT) may not always faithfully reflect the total amount of cytokines secreted (ELISA). The results obtained for 2-mo-old (median, 25th and 75th percentiles: 1–2.7 mo) acutely \( B. \) pertussis-infected children were compared with those of 2-mo-old nonimmune control children.

Both the numbers of IFN-\( \gamma \)-SC induced by FHA or PTX-R and the concentrations of IFN-\( \gamma \) released in culture supernatants by FHA- or PTX-R-stimulated cells were significantly higher in acutely infected children compared with the nonimmune children (\( p < 0.005 \) for FHA and \( p < 0.05 \) for PTX-R) (Fig. 1). In the two groups of infants, the numbers of the Ag-specific IFN-\( \gamma \)-SC correlated well with the IFN-\( \gamma \) concentrations (\( r = 0.7544, p < 0.0001 \)). Ag-induced IFN-\( \gamma \) secretion did not correlate with the age of the infected children (\( r = 0.108, p = 0.60 \)) (Fig. 2). It also did not correlate with the duration of the cough (\( r = 0.29, p = 0.19 \)).

Primary cytokine responses of 2-mo-old infants are difficult to compare to those of older, immunologically more mature children, or adults, since older children have generally been vaccinated and only nonvaccinated infants display a primary immune response to \( B. \) pertussis Ags upon infection. Incidentally, the PBMC from three older \( B. \) pertussis-infected children (>5 years old, not included in this study) actually produced somewhat lower amounts of IFN-\( \gamma \) upon stimulation by FHA or PTX-R (FHA, 58, 112, 367 pg/ml; PTX-R, 354, 320, 3310 pg/ml). These children had not completed their full vaccination program. However, they may have been partially protected and, although they may therefore not have been exposed to as much antigenic load as the younger, nonvaccinated infected infants, their immune response should be regarded as a secondary response, which was nevertheless not higher than the primary response of the 2-mo-old infants. These results suggest that \( B. \) pertussis-induced IFN-\( \gamma \) secretion may be as high in 2-mo-old children as it is in older children.

**Phenotype of the Ag-specific IFN-\( \gamma \)-producing cells during acute \( B. \) pertussis infection**

The phenotype of the Ag-specific IFN-\( \gamma \)-producing cells from an infant producing high amounts of IFN-\( \gamma \) in response to FHA and PTX-R was characterized by flow cytometry. The percentages of lymphocytes containing Ag-induced IFN-\( \gamma \) were 2.17% for the PTX-R induction and 0.96% for the FHA induction after subtracting the background obtained on nonstimulated cells using the specific Abs (Fig. 3). For both Ags, IFN-\( \gamma \) was produced by CD4\(^+\) and by CD8\(^+\) lymphocytes. In this particular infant, PTX-R induced more IFN-\( \gamma \)-containing CD8\(^+\) lymphocytes, whereas FHA induced more IFN-\( \gamma \)-containing CD4\(^+\) lymphocytes.
PHA-induced cytokine secretion

Since defective production of cytokines in response to mitogens has been reported for up to 12 mo of age, we have compared IFN-γ/H9253 and IL-13 secretions of PHA-stimulated PBMC from 2-mo-old children to those of adults. The results illustrated in Fig. 4 confirm the significantly lower IFN-γ/H9253 secretion in response to mitogens in 2-mo-old infants compared with adults. However, these differences disappeared during acute B. pertussis infection of the infants.

The numbers of PHA-induced IFN-γ/H9253-SC in B. pertussis-infected children were significantly higher than those obtained for nonimmune 2-mo-old infants (p < 0.05) but were not different from those obtained for adults (Fig. 4).

The differences in PHA-induced cytokine secretions between the nonimmune and the B. pertussis-infected 2 mo-old infants were further analyzed by comparing the concentrations of both IFN-γ and IL-13 released in PHA-stimulated cell culture supernatants. Whereas the IFN-γ concentrations were higher for the infected children than for the noninfected infants (p < 0.05), there was a trend to lower PHA-induced IL-13 release (Fig. 4). This resulted in a significant increase of the IFN-γ:IL-13 ratio in infected children compared with nonimmune 2-mo-old infants (p < 0.01).

FHA- and PTX-R-specific cytokine secretions in infants in response to the first injection of whole-cell pertussis vaccine

To investigate the primary immune response to whole-cell pertussis vaccine in the very young children, we measured the numbers of FHA- or PTX-R-induced IFN-γ- and IL-4-SC in PBMC from 3-mo-old infants who had been injected 2 wk earlier with one dose of whole-cell pertussis vaccine. The numbers of IFN-γ-SC induced by FHA or PTX-R were significantly higher in postimmune samples compared with preimmune samples (p < 0.05) (Fig. 5). In contrast, no IL-4-SC could be detected among FHA- or PTX-R-stimulated PBMC, indicating that 2- to 3-mo-old infants are also able to mount a significant Th1 response to vaccination with whole-cell pertussis vaccine.
Discussion
The neonatal cellular immune system is currently considered to be relatively immature. Although neonatal and infant T cells are as efficient as adult T cells in secreting IL-2 and in proliferating in response to various stimuli (23–25), they produce on average 10-fold less IFN-γ than adult T cells in response to polyclonal activation (4–6). As these differences in the production of IFN-γ between adult and neonatal T cells parallel differences in mRNA abundance, they probably reflect reduced transcription of the IFN-γ gene in infants in response to T cell activation (26). IFN-γ secretion in response to PHA has been shown to be barely detectable in infants <2 mo old and to be still significantly lower in 2- to 12-mo-old infants than in older children (7, 15). Based on these in vitro findings and on results obtained with cloned naive neonatal CD4+ T cells (27), it has been concluded that newborns are unable to develop efficient Th1-mediated responses in vivo and are instead heavily biased toward Th2 responses. Information on the in vivo development of Ag-specific Th function in infants is limited and virtually nonexistent for very young infants (around 2 mo). Measles-specific IFN-γ secretion in 9-mo-old vaccinated infants has been shown to be similar to the secretion obtained in adults (16), whereas a polarized Th2 response to tetanus toxoid has been reported in 4- to 12-mo-old infants vaccinated with diphtheria-tetanus-acellular pertussis vaccine (15). Considered as an exception to the concept of neonatal T cell immaturity, the immune response to mycobacteria, induced either after in utero mycobacterial exposure (28) or after BCG vaccination of newborns (29), has been reported to be of the Th1 type. This exception has been attributed to the unique capacity of mycobacteria, including BCG, to infect dendritic cells and to markedly increase their ability to present Ags to T cells (13, 14). However, neonates infected with HSV also develop a cellular immune response and produce HSV-specific IFN-γ at 6 wk of age. These responses are similar to those of their mothers 6 wk after infection (30), indicating that neonates are able to mount a Th1 response to certain viral infections.

In accordance with the notion of defective cellular immunity in infants, the recent resurgence of B. pertussis infections in very young infants and the often severe outcome of these infections has been attributed to the lack of specific cellular immunity of these children. Recovery from whooping cough in older infants (2 years or older) is indeed associated with a selective induction of T cells secreting Th1 type cytokines (17). Similarly, PBMC isolated from 7-mo-old infants who had received three doses of whole-cell pertussis vaccine also produced IFN-γ and IL-2 in response to a specific antigenic stimulation (31). However, these levels of Th1 cytokines have been considered possible because of the increased maturity of the immune system of these children compared with younger infants.

We report here that 2-mo-old (1- to 4-mo) infants are able to mount a specific primary cellular immune response to B. pertussis Ags, which is similar to that elicited by these Ags in older children. Actually, Ag-specific IFN-γ secretion in the infected 2-mo-old children was at the same order of magnitude as that induced by PHA in nonimmune children of the same age. Although B. pertussis has traditionally been viewed as an extracellular pathogen, data accumulated over the last decade indicate that it is also able to survive intracellularly within macrophages and epithelial cells (32, 33). However, intracellular survival of B. pertussis is not required for the induction of a strong Th1 cellular immune response, as similar responses were induced by the s.c. administration of whole-cell pertussis vaccine not containing any viable microorganism. The IFN-γ secretion was the result of a selective induction of IFN-γ synthesis in T cells as evidenced by flow cytometry analysis of intracellular IFN-γ synthesis. The reduced ability of neonatal T cells to secrete IFN-γ has been proposed to be partially a consequence of the absence of memory T cells in infants (8). We confirmed the low numbers of circulating memory T cells in infants.

The absence of an intrinsic defect of neonatal T cells has been demonstrated in vitro, as adult levels of cytokines are produced when exogenous IL-12 is added (34) or in the presence of anti-CD3/B7.1 Abs or allogeneic dendritic cells (11). The observations reported here provide new evidence that T cells from very young infants are able to produce adult-like amounts of IFN-γ upon in vivo infection with B. pertussis or vaccination with whole-cell vaccine. It is possible that B. pertussis infection or whole-cell pertussis vaccination provides optimal conditions for Ag presentation at priming, leading to the development of a mature Th1 immune response in infants. Macrophages of mice infected with B. pertussis or immunized with whole-cell vaccine produce IL-12 in vivo and B. pertussis LPS is able to induce IL-12 secretion by mouse macrophages in vitro (35). In humans, pertussis toxin synergizes with LPS to induce IL-12 secretion by dendritic cells (36). B. pertussis infection or vaccination with whole-cell vaccine could therefore boost IL-12 secretion which has been reported to be deficient in newborns (10). In line with this notion, we have observed p70 IL-12 secretion by FHA-or PTX-R-stimulated PBMC from a B. pertussis-infected, 1.5-mo-old infant (data not shown).

The Ag-specific Th1 cellular immune response appearing during acute B. pertussis infection or after a single dose of whole-cell pertussis vaccine is associated with a general shift of the cytokine secretion profile of these infants toward a stronger Th1 profile as indicated by the response to polyclonal stimulation of the PBMC by PHA, which is in contrast to the Th1 response obtained after diphtheria-tetanus-acellular pertussis vaccine administration (15). The orientation of naive T cells toward a Th1 profile could result from the induction of transcription factors responsible for the global generation of Th1 cells and/or from the suppression of transcription factors needed for the differentiation of Th2 cells as has been hypothesized by Erb (37).

In conclusion, we found that 2-mo-old infants are able to mount fully mature Th1 responses to B. pertussis infection or whole-cell vaccine administration and that the ability of the very young children to skew their immune responses toward a Th1 profile is therefore not restricted to the context of mycobacterial infection. These findings indicate that infants are developmentally more mature than previously assumed in their capacity to develop Th1 responses in vivo, as long as they have encountered the appropriate stimuli in very early childhood.

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