

# Lack of Association Between Duration of Breast-Feeding or Introduction of Cow's Milk and Development of Islet Autoimmunity

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**The hypothesis that early exposure to cow's milk or lack of breast-feeding predisposes to type 1 diabetes remains controversial. We aimed to determine prospectively the relationship of, first, duration of exclusive breast-feeding and total duration of breast-feeding, and second, introduction of cow's milk protein as infant formula, cow's milk, or dairy products, to the development of islet antibodies in early life. Some 317 children with a first-degree relative with type 1 diabetes were followed prospectively from birth for 29 months (4–73). Mothers kept a home diary and answered infant feeding questionnaires at 6-month intervals. No systematic feeding advice was given. Insulin autoantibodies (normal range <5.5%), anti-GAD antibodies (<5.0 U), and anti-IA2 antibodies (<3.0 U) were measured at 6-month intervals. Cox proportional hazards model of survival analysis detected no significant difference between children who did not develop islet antibodies (225 of 317 [71%]), children with one islet antibody raised once (52 of 317 [16.4%]), children with one antibody raised repeatedly (18 of 317 [5.7%]), or children with two or more antibodies raised (22 of 317 [6.9%]), in terms of duration of exclusive breast-feeding, total duration of breast-feeding, or introduction of cow's milk-based infant formulas, cow's milk, or dairy products (relative risk: 0.91–1.09). Four of the children with two or more islet antibodies developed type 1 diabetes. We conclude that there is no prospective association between duration of breast-feeding or introduction of cow's milk and the development of islet autoimmunity in high-risk children. *Diabetes* 48:2145–2149, 1999**

**T**he contribution of an environmental factor or factors to the development of type 1 diabetes can be gauged on the basis of an observed concordance for disease of only 30–40% in identical twins (1). Twin studies, migrant studies, and temporal and geographic

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Ab, antibodies; IAA, insulin autoantibodies; ROC, receiver operator curve.

variation in incidence argue for a critical role of environmental factors in the development of type 1 diabetes (2). Environmental factors may initiate or promote progression of the autoimmune destruction of the pancreatic islets, which precedes the onset of clinical diabetes. Much attention has been directed to the hypothesis that lack of breast-feeding and the early introduction of cow's milk protein are associated with risk of type 1 diabetes.

Borch-Johnsen et al. (3) first drew attention to a possible association between the early introduction of cow's milk and risk of type 1 diabetes. Gerstein (4) performed a meta-analysis on 13 case-control studies and found a small (relative risk of 1.5) but significant association between the early introduction of cow's milk protein and the development of type 1 diabetes. Children with type 1 diabetes were more likely to have been breast-fed for <3 months and to have been exposed to cow's milk protein before 4 months of age (4). These case-control studies are limited by their retrospective design. A further meta-analysis by Norris and Scott (5) suggested that methodological explanations may account for this weak association between early cow's milk introduction and type 1 diabetes, namely recall bias in retrospective studies and disparities in incidence of breast-feeding and incidence of type 1 diabetes in different populations. Furthermore, the development of islet antibodies in early life was not related to early exposure to cow's milk protein in a cross-sectional study of children with a family history of type 1 diabetes in which the recall period was considerably shorter than that in previous studies (6). The other line of evidence presented to support the cow's milk hypothesis is a reported increase in humoral and cellular immunity to cow's milk proteins (bovine serum albumin,  $\beta$ -casein,  $\beta$ -lactoglobulin) in patients at diagnosis of type 1 diabetes (7–10), although some of these observations have been disputed (11).

The Australian Baby DIAB Study began in 1993 and follows infants from birth who have a first-degree relative with type 1 diabetes to determine the relationship of genetic, immunologic, and environmental factors in the development of early islet autoimmunity. The specific aim of the present study was to assess prospectively the effect of both breast-feeding and the introduction of cow's milk protein on the development of islet autoimmunity in infants with a first-degree relative with type 1 diabetes. We examined whether total or exclusive duration of breast-feeding protects against early islet autoimmunity and whether early introduction of cow's milk protein (infant formulas, cow's milk, or other dairy products) is associated with the development of early islet autoimmunity.

TABLE 1  
Breast-feeding and introduction of cow's milk protein in infants with and without islet autoimmunity

	One antibody positive	Two or more antibodies positive	Antibody negative
<i>n</i>	70	22	225
% breast-fed	61	68	75
Duration of exclusive breast-feeding (months)	3.4 ± 0.5 (1.3)	3.9 ± 1.0 (4.5)	4.5 ± 0.3 (3.5)
Duration of total breast-feeding (months)	5.3 ± 0.9 (2.5)	5.8 ± 1.7 (5)	6.1 ± 0.4 (5)
Introduction of infant formula (months)	5.7 ± 0.9 (1.5)	3.5 ± 0.8 (4.5)	7 ± 0.5 (4)
Introduction of cow's milk (months)	10.9 ± 0.4 (12)	10.0 ± 0.9 (10.5)	10.5 ± 0.3 (11)
Introduction of dairy solids (months)	7.6 ± 0.5 (6)	6.5 ± 0.5 (6)	8.0 ± 0.3 (6)

Data are means ± SD (median), unless otherwise indicated.

## RESEARCH DESIGN AND METHODS

**Subjects.** Some 317 newborns (165 male) with a first-degree relative with type 1 diabetes from 268 families were recruited during the pregnancy and followed prospectively from birth for a median of 29 months (range 4–73) in Victoria and South Australia, Australia. The probands included 176 mothers with type 1 diabetes, 98 fathers with type 1 diabetes, and 36 siblings with type 1 diabetes. In seven families, there was more than one member with type 1 diabetes. Venous blood samples were taken at birth (umbilical vein blood), 6 months, 12 months, 18 months, and 24 months and at continuing 6-month intervals. Insulin autoantibodies (IAA) and antibodies (Ab) to tyrosine phosphatase, IA2 (IA2Ab), and GAD65 (GADAb) were measured on serum samples. HLA typing was performed on umbilical venous blood. All infants had normal growth and development, apart from two with congenital hypopituitarism and one with a lingual thyroid and congenital hypothyroidism.

**Questionnaires.** Parents kept a home diary to record infant feeding, illnesses, immunizations, and medications, which was reviewed at each 6-month visit. No systematic infant feeding advice was given. An 18-point questionnaire was administered at each 6-month visit to assess duration of exclusive breast-feeding, total duration of breast-feeding, dairy products consumed by mother while breast-feeding, and the age at which the following were introduced: cow's milk-based infant formulas (including commercial brand), full strength cow's milk, other dairy products (yogurt, custard, cheese), rice, wheat, barley, rye, oat, and mixed cereals, and meat and meat products. Intercurrent illnesses and their symptoms and duration, medications, immunizations, height, and weight were also recorded. The data were analyzed using Cox proportional hazards modeling to determine the longitudinal effect on the development of islet autoimmunity of the total duration of breast-feeding, the duration of exclusive breast-feeding, and the age of introduction of cow's milk protein as either cow's milk protein-based infant formulas, cow's milk, or other dairy products (excluding soy milk products and goat's milk products).

### Laboratory methods

**IAA.** IAA were assayed by a modification of the fluid-phase radiobinding assay reported by Vardi et al. (12). To conserve serum, total insulin binding only was measured using duplicate 150- $\mu$ l aliquots of serum. The normal range (<5.5%) was derived from 190 normal control subjects (mean age 9.7 years [4.9–15.5]). The mean for 80 patients with newly diagnosed type 1 diabetes was 6.8% (1.4–28.5). Children with newly diagnosed type 1 diabetes aged <5 years had insulin binding >5.5% in 11 of 12 (92%) cases.

**GADAb and IA2Ab.** GADAb and IA2Ab were assayed by immunoprecipitation of <sup>35</sup>S-methionine-labeled recombinant human proteins (13). GAD65 and IA2 were synthesized in the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Full-length GAD65 cDNA template cloned into the *Eco*R I site of the pGEM-3 vector and full-length IA2 cDNA cloned into the pSP64 polyA vector, each under control of the SP6 promoter, were generously provided by Dr. Ezio Bonifacio, San Raffaele Hospital, Milan, Italy. mRNA generated direct synthesis of recombinant protein in the presence of methionine-depleted medium containing <sup>35</sup>S-methionine. Routinely, 30% of radioactivity is incorporated into protein as determined by trichloroacetic acid precipitation. Approximately 100,000 counts/min of lysate in 50  $\mu$ l is incubated overnight with 5  $\mu$ l of serum. Immunoglobulin-bound radioactivity is then precipitated by addition of 50  $\mu$ l of protein A-Sepharose. After washing, precipitated radioactivity is measured in a  $\beta$ -scintillation counter (TopCount, Packard, IL). Results are expressed as arbitrary units, calculated as

$$\text{result} = 100 \times (\text{sample} - \text{negative control} / \text{positive control} - \text{negative control}).$$

The normal range for GADAb, derived by receiver operator curve (ROC) analysis of 246 control subjects and 135 patients with newly diagnosed type 1

diabetes, is <5 U; 103 of 135 (76%) patients with newly diagnosed type 1 diabetes had GADAb >5 U. In the International GAD Proficiency Test #2, the assay scored 100% for sensitivity, specificity, validity, and consistency. The normal range for IA2Ab, derived by ROC analysis of 145 control subjects and 94 patients with newly diagnosed type 1 diabetes is <3 U; 72 of 94 (78%) patients with newly diagnosed type 1 diabetes had IA2Ab >3 U. In the International IA-2A Proficiency Test #1, the assay scored 100% for sensitivity, specificity, validity, and consistency. Detectable antibodies were defined as IAA >5.5%, GADAb >5 U, or IA2Ab >3.0 U.

Antibody data were classified in three categories: 1) one antibody (IAA or GADAb or IA2Ab) detected on one occasion only, *n* = 52 of 317 (16.4%), 2) one antibody raised repeatedly, *n* = 18 of 317 (5.7%), or 3) two or more antibodies raised on more than one occasion, *n* = 22 of 317 (6.9%).

Antibodies detected during the first 9 months after birth were not classified as islet autoimmunity when the proband was the child's mother because of the likelihood of transplacental transfer.

**HLA typing.** HLA class I (A,B) typing was performed by a standard serological microlymphocytotoxicity assay using T-cells separated from whole blood using magnetic beads coated with a T-cell monoclonal antibody (Dynal, Oslo, Norway). Class II (DR) typing was performed on polymerase chain reaction-amplified DNA using sequence-specific oligonucleotides according to the 11th International Histocompatibility protocol with minor modifications (14).

**Statistical analysis.** For each of the infants, the duration of exclusive breast-feeding and of total breast-feeding were determined. In addition, the time intervals from birth until the introduction of infant formula, the introduction of cow's milk, and the introduction of other dairy products were also determined. Kaplan-Meier survival analysis was used to describe the distribution of times to each of these events. For infants in whom any of these events had not yet occurred during the study, the date of last follow-up was used to determine the interval, and the observation was treated as censored.

Cox proportional hazards regression was used to determine the relationship between islet autoimmunity and duration of exclusive breast-feeding, total duration of breast-feeding, and age of introduction of cow's milk. Antibody data were classified for each infant as one antibody raised transiently, one antibody raised repeatedly, or two or more antibodies raised. The dates when antibodies were detected were used to enter this information into the Cox model as time-dependent covariates.

## RESULTS

Characteristics of the infant feeding of subjects with and without islet antibodies are shown in Table 1. Because only four mothers avoided dietary dairy products while breast-feeding, this group of infants was too small to analyze separately.

Cox proportional hazards modeling survival analysis showed no relationship between breast-feeding (duration of exclusive breast-feeding, total duration of breast-feeding), or age of introduction of cow's milk protein (either cow's milk protein infant formulas, full strength cow's milk, or other dairy products) and the development of islet autoimmunity. Infants with one antibody detected transiently, one antibody detected repeatedly, or two or more antibodies detected were analyzed separately in comparison with the rest of the study population, who did not have raised islet antibodies during follow-up (225 of 317 [71%]; Table 2 and Fig. 1). Four of

TABLE 2

Cox proportional hazards modeling survival analysis of relationship between duration of breast-feeding and age of introduction of cow's milk protein and islet autoimmunity

	<i>n</i>	Duration of breast-feeding		Age of introduction of cow's milk protein		
		Exclusive	Total	Infant formula	Cow's milk	Dairy products
One antibody detected once	52	1.01	1.00	1.00	1.09	1.01
One antibody detected repeatedly	18	0.97	0.99	1.00	0.91	0.99
2 antibodies detected	22	0.99	1.00	0.99	0.95	0.92

Data are relative risks.

the 22 subjects with two or more antibodies detected have developed type 1 diabetes, at 18, 24, 30, and 42 months of age.

The incidence of HLA DR3, DR4, and DR3/4 did not differ between the groups with two or more antibodies raised (16 of 19 [84%]), with one antibody raised repeatedly (10 of 16 [62.5%]), with one antibody raised transiently (20 or 30 [66.7%]), or without raised antibodies (110 of 157 [70.1%]), using  $\chi^2$  analysis.

## DISCUSSION

There was no statistical relationship between the development of islet autoimmunity and either the duration of breast-feeding or the introduction of cow's milk protein in the infant diet. This applied irrespective of the number of antibodies detected, and to exclusive and total duration of breast-feeding and introduction of cow's milk protein as cow's milk protein infant formulas, cow's milk, or other dairy products.

The strength and originality of the present study lies in its prospective design. Previous studies have been cross-sectional with retrospective analyses, including 23 retrospective association studies and one retrospective study of children with islet antibodies (6). These have the potential for biased recall of infant feeding, with some data collected from parents who already know the child has diabetes, which may influence the questionnaire response. Interestingly, two of the retrospective studies reported higher relative risks in children with HLA susceptibility genes for type 1 diabetes (15,16). One would anticipate, therefore, that a harmful effect of cow's milk or a protective effect of breast milk would be more readily detected in the children in the present study who have an increased prevalence of high-risk haplotypes than in the normal population. Our study involved separate analysis of introduction of cow's milk protein through a variety of sources in the infant diet and consideration of both exclusive and total duration of breast-feeding; this detail in analysis has, to our knowledge, not been reported previously.

Recently (6), the Diabetes Autoimmunity Study in the Young (DAISY) reported a retrospective analysis that showed no difference in cow's milk protein introduction at 3 and 6 months of age and total duration of breast-feeding in 18 children with islet autoimmunity, five of whom had two or more antibodies (6). The median recall time for the history of infant feeding was 4 years (6). Our prospective study confirms these findings in more detail, and with greater numbers of subjects, we were able to use more powerful continuous analyses. A preliminary report from the German Baby DIAB Study also showed no association between cow's milk introduction and the development of islet antibodies in early life (17).

Our end point was islet autoimmunity, not type 1 diabetes; four children within the group with two or more antibodies have developed type 1 diabetes. While it is established that the risk of progression to type 1 diabetes is increased when one antibody is persistently present and is further increased when more than one antibody is present, the significance of transient autoimmunity in early life is uncertain. Lifetime risk of type 1 diabetes for these infants with a first-degree relative with type 1 diabetes approximates 5%. Therefore, we would anticipate that ~15 of 317 children in the present study would ultimately develop type 1 diabetes. The relatively large percentage of children who had transient islet autoimmunity (52 of 317 [16.4%]) argues that they are not a high-risk group. The prevalence of transient islet autoimmunity in our population was higher than that previously reported (18). The prevalence of infants with one or more antibodies raised repeatedly was similar to that previously reported (6,18).

Breast-feeding practices of the mothers in this study did not differ significantly from those of the general population in Australia in terms of total numbers of infants breast-fed and duration of breast-feeding (both exclusive and total) (19). We had anticipated that the study population may have been influenced by suggestions in the popular press of a possible protective effect of breast-feeding. However, no feeding advice was given to these families by the investigators.

Our study does not exclude the possibility of a complex interaction between infant feeding and another environmental factor(s) in genetically at-risk children. There is strong evidence that breast-feeding decreases the incidence and severity of diarrheal illnesses in infancy (20,21). Some of the Coxsackie viruses and rotaviruses have been implicated as triggers of  $\beta$ -cell immunity (22–24). Therefore, breast-feeding could confer a protective effect indirectly by reducing the risk of exposure to potential diabetogenic viral infections. To unravel a complex interaction between infant feeding and early gut infection would require a more detailed understanding of which infectious agents are associated with the development of islet autoimmunity and the relationship between infection and infant feeding practices.

The American Academy of Pediatrics has recommended that children with high genetic risk of type 1 diabetes are breast-fed and not exposed to cow's milk protein during the first year of life (25). In a more recent policy statement on breast-feeding, the Academy again referred to the possible protective effect of human milk feeding on the development of type 1 diabetes (26). In addition, primary nutrition intervention trials during the first year of life to prevent type 1 diabetes through avoidance of cow's milk protein during the first 6 months of life have now begun (27,28). Our prospec-

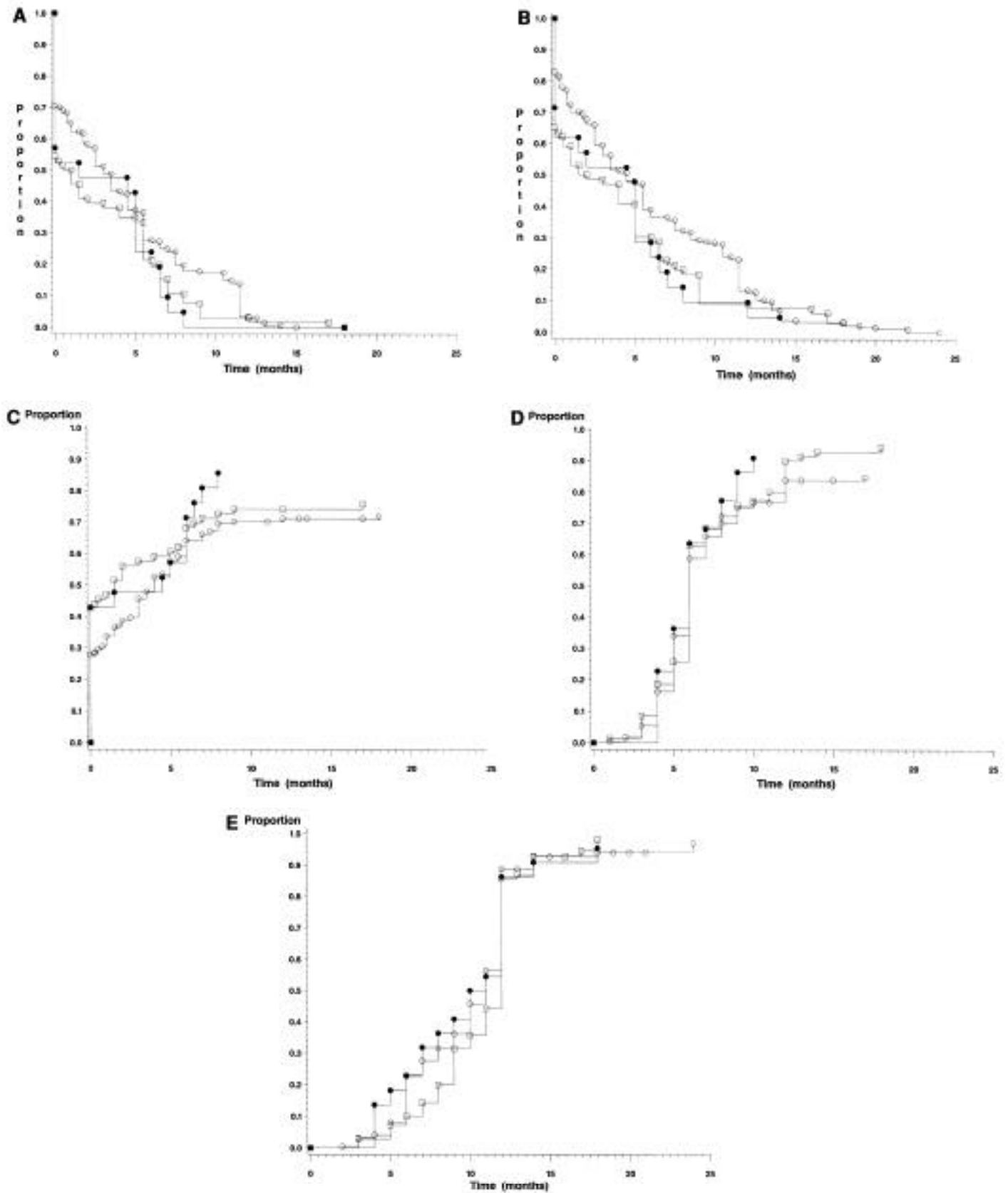


FIG. 1. Kaplan-Meier survival analyses in infants with no islet antibodies (○), one islet antibody raised (□), and two or more islet antibodies raised (●) for duration of exclusive breast-feeding (A), total duration of breast-feeding (B), age of introduction of cow's milk protein infant formulas (C), age of introduction of dairy products (D), and age of introduction of full strength cow's milk (E).

tive findings are, therefore, timely and question the basis of these recommendations.

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