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Breast Milk Lymphocyte Response to K1 Antigen of *Escherichia coli*

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Comparison milk and blood lymphocyte blastogenic responses to the K1 antigen of *Escherichia coli* and lipopolysaccharide (LPS) from *E. coli* O127,B8 were examined in 16 postpartum women by [^{3}H]thymidine uptake. Rabbit hemolysin-coated sheep erythrocyte monolayers were used to deplete macrophages from milk lymphocyte preparations and to enrich for T lymphocytes in order to make milk preparations more comparable to blood preparations. Response was defined as a stimulation index of ≥2.0. There was no evidence of selective response to K1 antigen by milk lymphocytes, since both blood and milk lymphocytes responded in four women and neither blood nor milk lymphocytes responded in nine. Milk lymphocytes alone responded to K1 in one woman, whereas blood lymphocytes alone responded in two women. Additional nonpaired milk or blood cultures were available from three women. None of these responded to K1 antigen. Corresponding lymphocyte cultures were stimulated with LPS. A positive K1 response was always accompanied by an LPS response, and the LPS response correlated with the K1 response in 17 of 19 women. Stool cultures examined with an antiserum agar showed no correlation between the presence of K1 *E. coli* in the stool and milk or blood lymphocyte response to K1 antigen. In the system used here, no selectivity of response of breast milk lymphocytes to K1 antigen was noted.

Previous investigators (19–21) have proposed that a selective homing or tracking of lymphocyte populations to the human mammary gland results in a milk lymphocyte population different from the peripheral blood lymphocyte population. In vitro lymphocyte stimulation with several antigens has shown that breast milk lymphocyte responses do not always correspond with peripheral blood lymphocyte responses. The K1 antigen of *Escherichia coli* (a gut-associated antigen) is the only antigen studied that has been reported to stimulate breast milk lymphocytes but not peripheral blood lymphocytes. These data of Parmely et al. (20) support the concept of selective tracking of lymphocytes from the human gut to the human mammary gland.

Homing of B lymphocytes from the gut to the mammary gland had been suggested before the studies of Parmely et al. by other investigators who used both animal and human subjects (1, 5, 13). In their studies of the gut-associated lymphoid systems in mice, Guy-Grand et al. (15) demonstrated tracking of T and B lymphoblasts to the lamina propria and intestinal epithelium from thoracic duct lymph and mesenteric lymph nodes. More recently, Roux et al. (25) have performed experiments in mice with radiolabeled mesenteric lymph node lymphoblasts which suggest possible homing of immunoglobulin A (IgA)-secreting plasma cells to the mammary gland late in pregnancy and during lactation, in addition to a homing back to the intestinal epithelium. The K1 data of Parmely et al. (20) and their subsequent hypothesis of selected T-cell populations in human milk are consistent with existing information from animal and human studies.

A problem with comparison blood and milk lymphocyte stimulation studies is that previous work may not have adequately controlled for the high macrophage content of human milk and the variable T-cell content of human milk. Since the cell population in human breast milk contains approximately 80% macrophages, 15% lymphocytes, and 5% polymorphonuclear cells (23), depletion of macrophages was attempted before lymphocyte stimulation (20). In the studies of Parmely et al., passage over glass bead columns was used to deplete macrophages. However, since milk macrophages did not adhere optimally, resulting lymphocyte preparations varied considerably, ranging from 35% to 80% lymphocytes. In addition, the milk lymphocyte percentage of T cells has been shown by Ogra and Ogra (18) to decrease rapidly in the days

postpartum. Thus, the percentage of T cells in milk and blood lymphocyte preparations is also important in comparing milk and blood lymphocyte cultures.

In the experiments described here, rabbit hemolysin-coated sheep erythrocyte (SRBC) monolayers were used to deplete macrophages and enrich for T lymphocytes in milk lymphocyte preparations. By such processing, milk lymphocyte preparations were more comparable in percentage of T lymphocytes and in monocyte/macrophage content to blood lymphocyte preparations.

MATERIALS AND METHODS

Milk specimens were obtained by using the Lopuco hand pump (Lopuco, West Laurel, Md.) from women 2 to 4 days postpartum. Heparinized blood (10 to 15 U/ml of blood) was obtained by venipuncture.

Cell preparation. Peripheral blood lymphocytes were prepared by Ficoll-Hypaque differential centrifugation and extraction of the mononuclear layer (22).

Breast milk cells were centrifuged at 400 × *g* for 15 min. The top fat layer and supernatant were removed. Cells were then suspended in calcium- and magnesium-free Hanks balanced salt solution (HBSS). Cells were washed three times in HBSS by centrifugation at 400 × *g* for 10 min. An erythrocyte antibody (EA) monolayer was prepared by using a modification of the method of Kedar et al. for preparing peripheral blood cells (17). Plastic petri dishes (Falcon, Cockeysville, Md.) were treated with poly-L-lysine (50 μg/ml) for 45 min followed by rinsing with HBSS and addition of a 1.5% washed SRBC suspension for 45 min at room temperature. Three washes with HBSS were followed by addition of heat-inactivated 1:100 rabbit hemolysin (Colorado Serum, Denver, Colo.). Incubation at 37°C for 25 min was followed by rinsing and priming of the preparation with RPM1-1640 (5% heat-inactivated pooled human AB sera) for 10 min. RPMI-1640 and AB sera were obtained from GIBCO Laboratories, Grand Island, NY.

Breast milk cells suspended in RPM1-1640–5% AB sera were suspended on monolayers and incubated for 45 min at 37°C. Nonadherent cells were removed from the monolayer, and the monolayer was washed once with HBSS. Cells from wash were added to nonadherent cells. These nonadherent, T-cell-enriched, macrophage-depleted cells were centrifuged at 400 × *g* for 10 min and resuspended in RPM1-1640–5% AB sera. If the percentage of macrophages was not less than 25%, milk cells were placed on a second EA monolayer.

Cell were counted by toluidine blue staining in a wet preparation. In addition, for breast cell preparations, a supervital stain (29) was used to differentiate phagocytic and nonphagocytic cells. Verification of monocyte content was done by using alpha-napthyl butyrate (2) nonspecific esterase stain on smears made by 1:1 suspension of cells in patients' plasma. The percentage of lymphocytes that formed E rosettes was determined in both milk and blood lymphocyte preparations by the following procedure: 0.5×10^6 lymphocytes in 0.25 ml of HBSS were incubated with 0.25 ml of 1.5% SRBC at 37°C for 15 min. Cells were spun at 200 × *g* for 5 min and incubated on ice overnight (3). The pellet was resuspended, mixed with toluidine blue stain (9), and counted for percentage of rosetting lymphocytes (T lymphocytes).

Lymphocyte cultures. Lymphocyte preparations were suspended in RPM1-1640 with 5% pooled AB sera, additional L-glutamine, an antibiotic/antimycotic preparation (GIBCO), and gentamicin (10 μg/ml). Cultures were standardized for the total number of lymphocytes. Then 5×10^4 lymphocytes (milk or blood) suspended in 0.1 ml of RPMI-1640 (5% AB sera) were added to each well of microtiter plates. This concentration was used by previous workers (20), and the yield of lymphocytes in human breast milk necessitates use of lower cell numbers per well. Triplicate cultures were done. Labeling with [^{3}H]thymidine was done with 0.4 μCi in 0.1 ml of RPMI-1640 (20% fetal calf serum). All cell (milk and blood) cultures were labeled on day 6. In addition, duplicate blood cultures were labeled at day 3. Duplicate milk cultures were also labeled on day 3 when the yield of cells permitted. Cultures were incubated for 24 h with [^{3}H]thymidine, and these cells were harvested with a Mash II harvester.

K1 antigen was kindly supplied by Ann Sutton and John Robbins at the Bureau of Biologics. Lipopolysaccharide (LPS) was obtained from Sigma Chemical Co., St. Louis, Mo., and was prepared from *E. coli* O127,B8 by phenolic extraction (30). The preparation had 3.9% nitrogen and 13.9% lipid A. Phytohemagglutinin P was obtained from Burroughs Welcome Co., Research Triangle Park, N.C.

Rectal cultures. Stool cultures were performed by streaking rectal swabs onto antiserum agar prepared with Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), 1.2% agarose, and 5.5% equine antiserum to group B meningococcus, which has a capsular polysaccharide nearly identical to the K1 antigen of *E. coli* (14). Antiserum was supplied by A. Sutton and J. Robbins at the Bureau of Biologics. Stool cultures were screened for halo-forming colonies, and subsequently these halo-forming colonies were confirmed as *E. coli* by an enteric differential system.

LPS measurement. *Limulus* lysate assay (24) was used for quantitating endotoxin in K1 antigen preparations.

RESULTS

Macrophage/monocyte contamination of milk and blood lymphocyte preparations. Before passage over the EA monolayer, the mean percentage of macrophages in milk preparations was 48%. After monolayer passage, macrophage contamination was 13%, with a range of 3.0 to 21.0% (toluidine blue and supervital stain wet preparations). By α-naphthyl butyrate nonspecific esterase staining, macrophage content after monolayer passage ranged from 4 to 17%.

Blood mononuclear cell preparations had a monocyte content ranging from 1 to 10% by wet preparation. By esterase stains, monocyte content varied from 1% in one patient to 12%.

Milk lymphocyte preparations contained 79% lymphocytes, 8% polymorphonuclear cells, and 13% macrophages, whereas blood cells contained 92% lymphocytes, 3% polymorphonuclear cells, and 5% monocytes. Thus, in terms of monocyte/macrophage contamination, milk lymphocyte preparations that had been passed over the EA monolayers were comparable to the blood lymphocyte preparations.

T- and B-cell content of milk and blood lymphocyte preparations. Yield of T lymphocytes from monolayer passage varied from 10 to 75.0%, with a mean of 44%. The mean T-cell content of milk lymphocytes was 21% before and 41% after monolayer passage. Blood lymphocytes were 50% T cells (Table I). Milk and blood lymphocyte preparations for lymphocyte culture were generally comparable in terms of proportion of T lymphocytes and total number of T lymphocytes.

Mitogen stimulation of lymphocytes. Control milk and blood lymphocytes were stimulated with phytohemagglutinin P (Burroughs Welcome) in the dose range of 0.16 to 3.3 μg/ml. Cultures were labeled with [^{3}H]thymidine after 3, 5, or 7 days of culture. Maximal response occurred in day 3 or day 5 cultures (Table 2). Maximal responses only are presented, with maximal response occurring at a PHA dose ranging from 0.83 to 3.3 μg/ml. Of the five control patients studied, milk lymphocytes from one patient showed no response to PHA, whereas blood lymphocytes always responded. Hyporesponsiveness of milk lymphocytes to PHA has been previously reported (10, 18, 20).

Dose response. Response to K1 antigen was seen in the range of 10 to 50 μg/well (final concentration in well, 67 to 335 μg/ml), with the most consistent response at 50 μg/well. A stimulation index of ≥2.0 was defined as a positive response.

Responses to LPS were observed in the range of 0.01 to 10 μg/well (0.067 to 67 μg/ml). Maximal response was usually observed in the range of 0.1 to 1 μg/well (0.67 to 6.7 μg/ml).

Examination of data with both 3- and 6-day labeling revealed (with only one exception) responses to K1 antigen on day 6 with no response on day 3. In addition, seven blood lymphocyte cultures were labeled with K1 and LPS on day 5 in addition to days 3 and 6. Results on day 5 agreed with those on days 3 and 6 with one exception, a response (stimulation index of >2.0, but $P > 0.05$) to LPS on day 5 with none on day 6 or day 3.

Antigen stimulation of lymphocytes. Comparison blood and milk lymphocytes were available from 16 women, and nonpaired milk or blood specimens were available from 3. Stimulation indices for milk and blood lymphocytes to K1 antigen and LPS are plotted for each patient in Fig. 1. Since multiple doses of K1 antigen and LPS were used, the maximal response for any day at any dose is plotted. The most important observation from these data is that there was no evidence for a selective response of either milk or blood lymphocytes to K1 antigen of *E. coli*. In four women both milk and blood lymphocytes responded to K1 antigen.

TABLE 1. *Cell content of lymphocyte preparations*

Cell type	% in:[a]		
	Milk		Blood
	Pre-monolayer passage	Post-monolayer passage	
T lymphocytes	21 ± 10	41 ± 14	50 ± 17
Macrophages/monocytes[b]	50 ± 13	13 ± 5	5 ± 2

[a] Standard deviation.

[b] 5×10^4 lymphocytes were dispensed into each well of a microtiter plate at a concentration of 5×10^5 lymphocytes/ml for both milk and blood. For milk, total cell concentration varied from 5.2×10^5 to 6.0×10^5 cells/ml, with a mean T-cell concentration of $2.1 \pm 0.7 \times 10^5$ T cells/ml. For blood, total cell concentration varied from 5.2×10^5 to 5.9×10^5 cells/ml, with a mean T-cell concentration of $2.5 \pm 0.9 \times 10^5$ T cells/ml.

TABLE 2. *Phytohemagglutinin responses of milk and blood lymphocytes*

Control no.[a]	Milk lymphocytes		Blood lymphocytes	
	cpm[b]	% Macrophages/monocytes[c]	cpm	% monocytes
1				
NS	160 ± 49	6	86 ± 26	2
PHA	163 ± 33		80,464 ± 4,006[d]	
2				
NS	213 ± 107	23	219 ± 155	7
PHA	23,103 ± 9,622[d]		18,896 ± 1,822[d]	
3				
NS	689 ± 328	18	137 ± 67	8
PHA	99,254 ± 4,029[d]		6,497 ± 1,094[d]	
4				
NS	74 ± 37	19	70 ± 13	14
PHA	346 ± 284		96,255 ± 9,785[d]	
5				
NS	44 ± 8	17	136 ± 52	9
PHA	834 ± 496[d]		32,838 ± 1,968[d]	

[a] NS, Control, nonstimulated wells; PHA, phytohemagglutinin.

[b] ± Standard error.

[c] As estimated by toluidine blue wet preparations. Esterase stain results were similar.

[d] $P \leq 0.05$ by the Student t test.

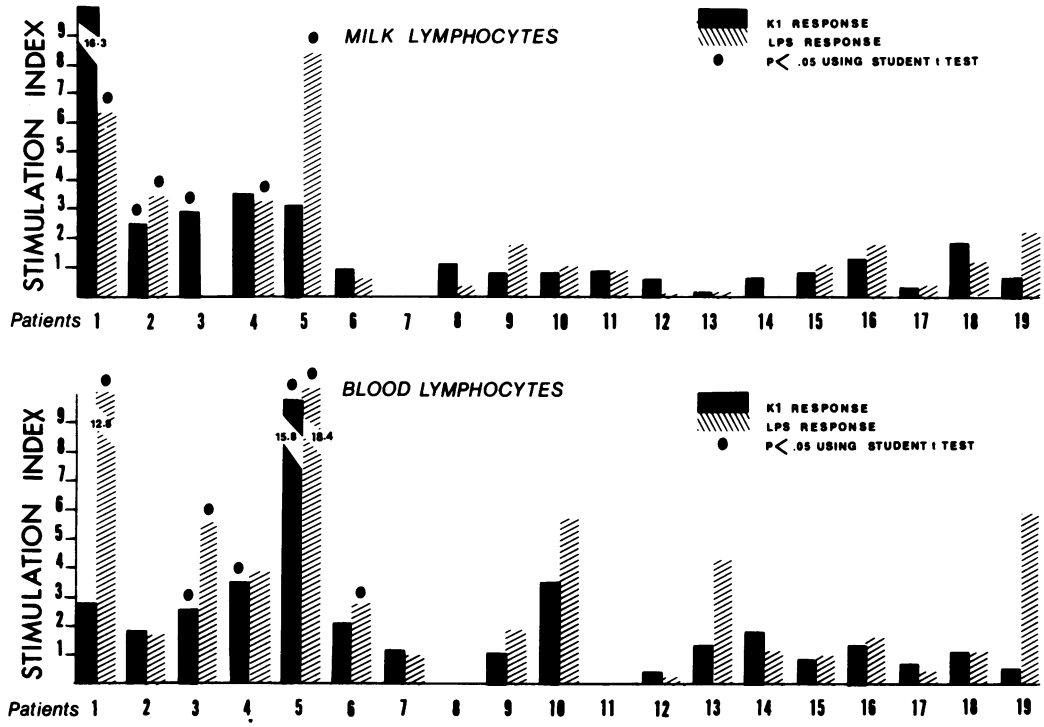

FIG. 1. *Milk and blood lymphocyte responses to K1 antigen of E. coli and to LPS. Stimulation indices for K1 antigen and LPS are recorded for each patient. (●) Significance by the Student t test.*

Corresponding lymphocyte cultures were also stimulated with LPS. A positive K1 antigen response was always accompanied by a positive LPS response. In 17 of 19 women, LPS and K1 antigen response correlated. A positive response to K1 antigen was never observed without a positive response to LPS, although lymphocytes from two women had a positive LPS response without a K1 response.

Since much variation was found in counts per minute in each of triplicate cultures, the counts per minute in stimulated versus nonstimulated cultures were not always significant ($P \leq 0.05$) despite a stimulation index of ≥2.0. The Student *t* test (11) was used to determine significance. Data from women with positive responses are presented in Table 3.

Presence of K1 *E. coli* in rectal swab. Rectal swabs streaked on to antiserum agar were examined for the presence of K1 *E. coli*. There was no correlation between blood or milk lymphocyte responses to K1 and presence of K1 *E. coli* on rectal swab.

LPS contamination of K1 antigen. The K1 antigen used was contaminated with LPS (0.01 to 0.02%) as measured by the *Limulus* lysate assay (24). Correlation of K1 antigen lymphocyte stimulation response with LPS suggests that this amount of contamination may be significant.

Before the experiments summarized above, preliminary studies were performed with a less purified K1 antigen (0.1 to 0.2% LPS) and with more macrophage contamination of milk cultures. Results verified the previous results of Parmely et al. (20). Only two of five women studied had milk responses to K1 antigens, and none of the five had blood lymphocyte responses. Additional preliminary studies with a more purified K1 antigen (LPS, 0.01 to 0.02%) but still with macrophage contamination (35 to 85% of cells in culture) showed no selectivity of response to K1 antigen by milk lymphocytes. These additional preliminary data support the conclusions of the present experiments concerning the significance of LPS contamination of K1 antigen in stimulating lymphocyte responses.

Although maximal lymphocyte blastogenesis to LPS was generally observed at 0.67 to 6.7 μg/ml, responses were seen at 0.067 μg of LPS per ml. This LPS concentration is comparable to the LPS contamination of the K1 antigen (0.034 to

TABLE 3. *Milk lymphocyte and peripheral blood lymphocyte responses to K1 antigen and LPS*

Patient no.	Milk lymphocyte response (cpm ± SE)[a]			Blood lymphocyte response (cpm ± SE)		
	NS[b]	K1 antigen	LPS	NS[b]	K1 antigen	LPS
1 (12/5)[c]	78 ± 9	1,270 ± 858 (16.3)	496 ± 113 (6.3)	73 ± 16	206 ± 79 (2.8)	940 ± 87 (12.8)[d]
2 (12/10)	406 ± 97	1,029 ± 155 (2.5)[d]	1,392 ± 166 (3.4)[d]	374 ± 43	691 ± 33 (1.8)	585 ± 239 (1.6)
3 (18/3)	80 ± 42	229 ± 27 (2.9)[d]		105 ± 24	267 ± 59 (2.6)[d]	580 ± 96 (4.8)[d]
4 (10/5)	169 ± 37	605 ± 236 (3.5)	541 ± 221 (3.2)	146 ± 15	510 ± 85 (3.5)[d]	
				59 ± 9		223 ± 86 (3.8)
5 (7/3)	75 ± 31	231 ± 84 (3.1)	496 ± 138 (8.4)[d]	54 ± 10	932 ± 287 (15.8)[d]	1,087 ± 528 (18.4)[d]
6 (/8)				82 ± 7	171 ± 85 (2.1)	224 ± 74 (2.7)
10 (14/1)	59 ± 12	45 ± 10 (<1.0)	71 ± 19 (1.2)	130 ± 51	437 ± 334 (3.4)	578 ± 611 (5.7)
13 (12/33	333 ± 167	41 ± 15 (<1.0)	32 ± 2 (<1.0)	72 ± 5.0	96 ± 14 (1.3)	302 ± 199 (4.2)
19 (16/8)	81 ± 36	65 ± 21 (<1.0)	195 ± 118 (2.2)	151 ± 11	114 ± 22 (<1.0)	889 ± 570 (5.9)

[a] SE, Standard error. Stimulations index is given in parentheses.

[b] NS, Control, nonstimulated wells.

[c] Numbers in parentheses indicate percentage of monocytes and macrophages in milk/percentage of monocytes in blood as measured by toluidine blue wet preparation. Esterase stain results were similar.

[d] $P \leq 0.05$ by the Student t test comparison of mean counts per minute in stimulated and nonstimulated wells.

0.067 μg of LPS per ml in 335 μg of K1 per ml) and further supports the influence of this contaminant on lymphocyte stimulation results.

DISCUSSION

Previous investigators (20) studying antigen-stimulated lymphocyte blastogenesis have suggested the presence of selected lymphocyte populations in human milk, particularly lymphocytes responsive to the K1 antigen of *E. coli*. Alternative explanations of previous data exist, however, including macrophage enhancement of milk lymphocyte responses or relatively increased B-lymphocyte numbers in milk preparations. In this study, we attempted to minimize these differences between milk and blood lymphocyte cultures by preparing milk and blood lymphocyte cultures that were comparable in terms of macrophage/monocyte and T-cell/B-cell content.

In our system, we did not find a selective response to K1 antigen in breast milk lymphocytes, and we did not find a correlation between the presence of *E. coli* K1 in stool and breast milk lymphocyte response to K1 antigen. The K1 antigen used was contaminated with LPS (0.01 to 0.02%) as measured by the *Limulus* lysate assay (27). Correlation of K1 antigen stimulation response with LPS response suggests that this amount of contamination may be a significant factor in lymphocyte stimulation. A previously published study (20) was performed with a K1 antigen prepared as a broth supernatant of *E. coli* K1 dialyzed against saline. This antigen would probably contain more LPS and other cell wall constituents, and differences in antigens used could explain differences in results. LPS can stimulate macrophages to secrete B-cell-activating factor (32), and macrophages contaminating milk lymphocyte cultures could be so stimulated by an antigen contaminated with LPS. In addition, the presence of contaminating macrophages in milk preparations could change dose-response relationships, resulting in a response of milk lymphocytes at an antigen dose for which blood lymphocytes remain unresponsive.

In a recent publication (14), Goodman and Sultzer have demonstrated that endotoxin protein, an outer membrane protein of gram-negative bacteria associated with LPS, is an effective activator of human peripheral blood lymphocytes. This endotoxin protein can be extracted from LPS by using trichloroacetic acid, and LPS purified in this manner did not stimulate human peripheral blood lymphocytes. Since the commercial LPS preparations used in our study were contaminated with protein (≈3.9% nitrogen), the lymphocyte stimulation observed could be due to the endotoxin-associated outer membrane protein and not LPS itself.

Alternatively, the B-cell/T-cell content of the lymphocyte cultures is an important determinant of lymphocyte blastogenesis since mitogens and antigens can selectively stimulate T or B cells. LPS is a known B-cell mitogen in mice (8, 12, 28), although blastogenic response to LPS by human peripheral blood lymphocytes has been much lower or absent (24). Schroder et al. have shown responses of human cord blood B cells to LPS (26). When comparing milk and blood lymphocyte responses, it may therefore be important to determine whether the T- and B-cell contents of the specimens are comparable. In one patient in our study, a milk lymphocyte response to K1 antigen and LPS was noted in the absence of blood lymphocyte response. In this particular case, the T-cell content of milk preparations was only 21% of the lymphocyte content, whereas 70% of the blood lymphocytes

were T cells. These data support a possible B-cell response rather than a selective response of milk lymphocytes in this case.

A recognized problem of adapting the method of Kedar et al. (17) for preparation of milk lymphocytes is that lymphocyte subpopulations bearing IgG Fc receptors (7, 33) may be removed with the macrophages and B lymphocytes. In human peripheral blood samples, IgG Fc receptors have been reported to be present on 4.5 to 10% of T lymphocytes.

These data do not disprove the concept of homing as applied to humans. However, they do suggest caution in interpreting comparison milk and blood lymphocyte responses to gut-associated antigens in the presence of LPS or endotoxin-associated protein contamination. In addition, comparison milk and blood lymphocyte preparations should be similar in terms of macrophage/monocyte and T-cell/B-cell content before conclusions about selected T-cell populations in milk can be made.

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