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Association with HeLa Cells of *Campylobacter jejuni* and *Campylobacter coli* Isolated from Human Feces

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We developed a rapid in vitro test for determining the association of *Campylobacter jejuni* and *C. coli* with HeLa cells. Association was expressed as a weighted mean of the number of bacteria associated with one cell in an association index (AI). The reproducibility of the AI was checked by repeating the test six times, using four strains chosen at random. Means and standard deviations of the means were 7.3 ± 1.2, 6.8 ± 0.9, 1.8 ± 1.2, and 0.1 ± 0.2. The experimental conditions for which the results are reliable have been standardized. Among 42 strains from human feces, two groups appeared: for 22 nonassociative strains (52%), AI values ranged from 0.0 to 2.1 (mean ± SD, 0.5 ± 0.6); for 20 associative strains (48%), AI values ranged from 3.5 to 8.3 (mean ± SD, 6.2 ± 1.4). Of these 42 strains, 17 were clinically documented. Diarrhea occurred more frequently in patients infected with associative strains than in those infected with noninvasive strains (7/7 versus 3/10, $P = 0.01$). Fever also occurred more frequently in patients infected with associative strains (6/7 versus 2/10, $P = 0.03$). Transmission electron microscopy and viable counts made after killing of extracellular bacteria by gentamicin support the fact that associated *Campylobacter* spp. are adherent to the cell membrane and are internalized into cytoplasmic vacuoles. The described test seems to be a convenient and rapid method for estimating the pathogenicity of a given strain.

Campylobacter jejuni and *C. coli* are microaerophilic gram-negative bacteria which can now be routinely isolated and identified. They are today the most frequently isolated enteropathogens, found as often in industrialized countries as in the third world. The pathophysiology of *Campylobacter* infections is still partially unknown (30). Some strains produce a heat-labile enterotoxin (7, 13, 26), but *C. jejuni* is also undoubtedly an enteroinvasive organism as demonstrated by observation in humans (18) and in experimentally infected animals (1, 4, 6, 11, 20).

At the present time, one of the least-known aspects of *C. jejuni* virulence is the nature of the interaction between this organism and intestinal cells. On the basis of in vitro (19, 21) or in vivo (4, 6, 25) studies, some researchers assume that *C. jejuni* can adhere to enterocytes and even invade these cells. Lee et al. explain the pathogenicity of *C. jejuni* by its particular adaptation to the mucus of intestinal crypts. Klipstein et al. (13) demonstrated that invasive strains (which induce bloody diarrhea) share common antigens and produce cytotoxin. Thus, the nature of the interaction between *Campylobacter* spp. and enterocytes still remains unclear.

The relationship between *C. jejuni* and enterocytes can be studied by in vivo models, but these methods are cumbersome and do not allow control of each experimental parameter. *C. jejuni* has not been found to be invasive by the Sereny-Anton test, a standard reference for bacterial invasiveness (19). Thus, in vitro models, which are simpler and more easily monitored, were required in this field (13, 30). Such models using mammalian cultivated cells exist for other enteropathogenic organisms (2, 3, 8, 9, 12, 14, 15, 17, 19, 22–24, 27); these models are simple and reproducible, they closely simulate studied phenomena in allowing direct observation, and their results generally correlate with the virulence of bacteria. Only a few studies for determining

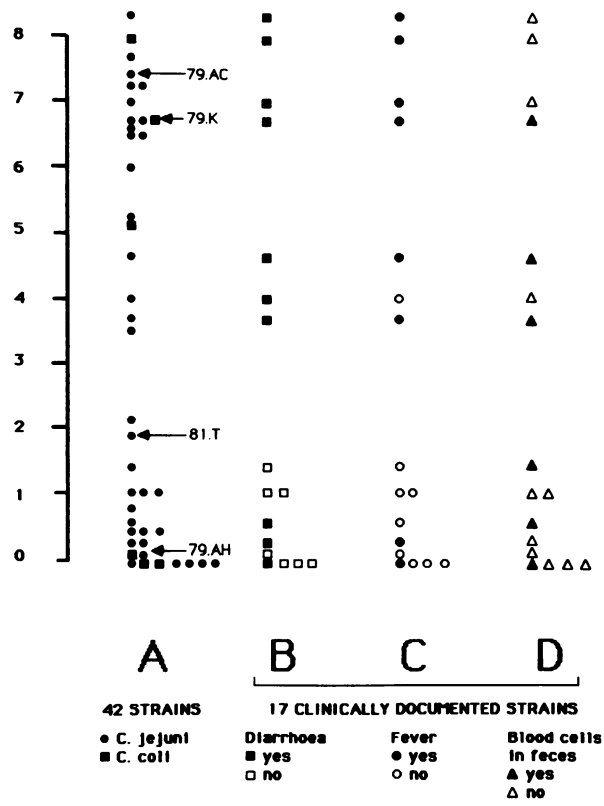

FIG. 1. Association of 42 strains of *C. jejuni* and *C. coli* with HeLa cells. (A) AI distribution of 42 strains from fecal isolates (AI, *y* axis). (B, C, and D) AI distributions of the 17 strains clinically documented in terms of the absence or presence of diarrhea, fever, and blood cells in feces.

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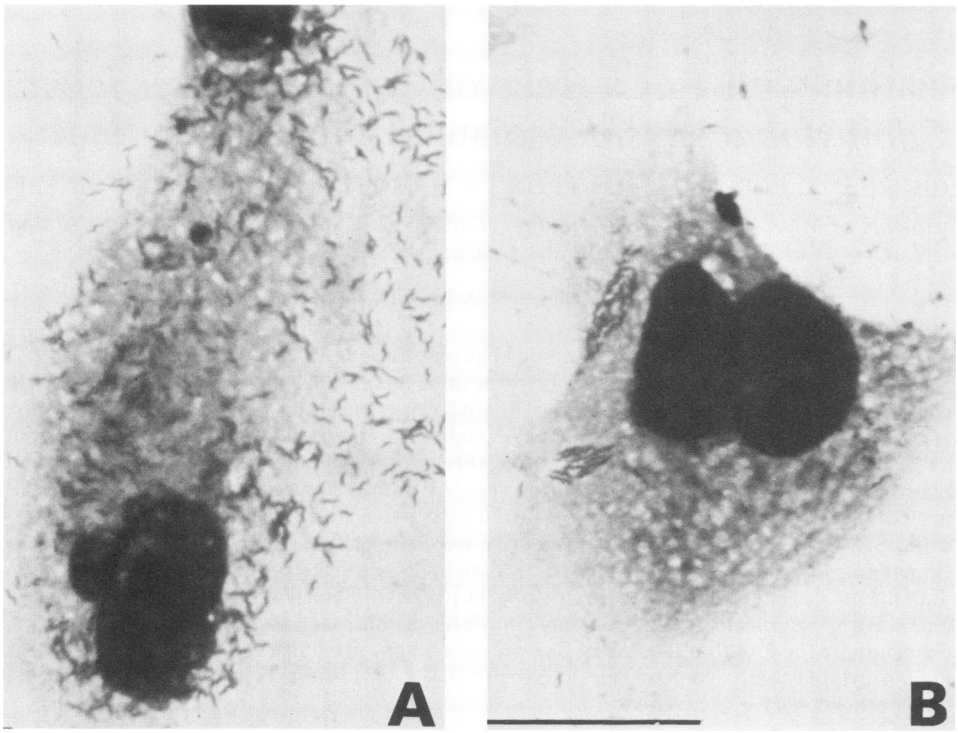

FIG. 2. Microscopic aspects of association between HeLa cells and *C. jejuni* and *C. coli*. (A) Bacteria dispersed over the whole area of the cell. (B) Bacteria gathered in particular cytoplasmic sites. These two patterns of association were seen with each of the 20 associative strains. Giemsa staining; bar, 10 μm.

Campylobacter association with cultivated cells have so far been reported. Manninen et al. (19) studied 15 *Campylobacter* strains, all of which associated with cultivated HeLa cells; Newell and Pearson (21) studied 2 *Campylobacter* strains, both of which associated with cultivated INT 407 cells. However, in these studies, microscopic evaluation of this association was difficult because only a few bacteria were seen associated with cells even after centrifugation. Moreover, the association was not clearly correlated with the expression of pathogenicity in vivo.

In this study, we developed an in vitro test to evaluate the association of *C. jejuni* and *C. coli* with HeLa cells and to generate quantitative data. Using this test, we studied 42 strains from human isolates and compared the results with clinical symptoms. Evidence is brought for the ability of *C. jejuni* and *C. coli* to be internalized into eucaryotic cells.

MATERIALS AND METHODS

Bacterial strains. Forty-two *Campylobacter* strains were isolated from human feces onto Skirrow medium (BioMérieux, Marcy l'Etaile, France). These strains were assigned to the *C. jejuni* or the *C. coli* group on the basis of morphology, Gram staining, motility, microaerophilicity, oxidase and catalase tests, cultivation temperature, and susceptibility to cefalotin and nalidixic acid (29). The 42 strains were distinguished on the basis of the hippurate hydrolysis test (10): 36 strains were positive and 6 were negative, and they were conventionally designated *C. jejuni* and *C. coli*, respectively. All of the strains were stored at −80°C in brucella broth (BB; Difco Laboratories, Detroit, Mich.) supplemented with 10% dimethyl sulfoxide.

Of the 42 strains, 17 were from patients who were clinically documented. The strains were isolated from children under 7 years of age. Three clinical symptoms were noted in all 17 patients: diarrhea (defined by watery or unformed stools), the presence of blood cells in feces (determined by phase-contrast microscopic observation of a fresh stool specimen), and fever (temperature over 38°C on the day of specimen collection).

HeLa cells. HeLa 229D cells (Flow Laboratories, Inc., McLean, Va.) were maintained in minimum essential medium (MEM) (BioMérieux) supplemented with 10% newborn calf serum, 200 IU of penicillin G, 50 μg of streptomycin, and 2.5 μg of amphotericin B per ml. Cells were grown routinely in plastic tissue culture flasks in an atmosphere containing 5% CO_2. Confluent stock cultures were trypsinized and adjusted to 10^5 cells per ml. For the culture used in the association test, 1 ml of the cell suspension was introduced into a tissue culture chamber slide (Lab-Tek Products, Div. Miles Laboratories, Naperville, Ill.) and was incubated for 24 to 48 h at 37°C under a 5% CO_2 atmosphere to obtain a nonconfluent cell monolayer. Two hours before the test, the cell monolayers were washed three times with MEM without antibiotic or serum.

Association test in standard conditions. For the association test, each bacterial strain was cultivated in BB at 37°C for 48 h under microaerophilic conditions. A 5-ml sample of culture was centrifuged (400 × *g* for 20 min), and the bottom layer was resuspended in MEM and adjusted to 10^7 to 10^8 bacteria per ml. The bacterial suspension was retrospectively checked by viable count on Columbia agar (Difco). A portion (1 ml) of bacterial suspension was added to the cell monolayer, and the mixture was incubated for 1 h at 37°C under a 5% CO_2 atmosphere to allow bacterial adhesion to the cells. The cell monolayer was then washed five times with MEM and reincubatd for 3 h in the same conditions to allow bacterial invasion of the cells. After incubation, the

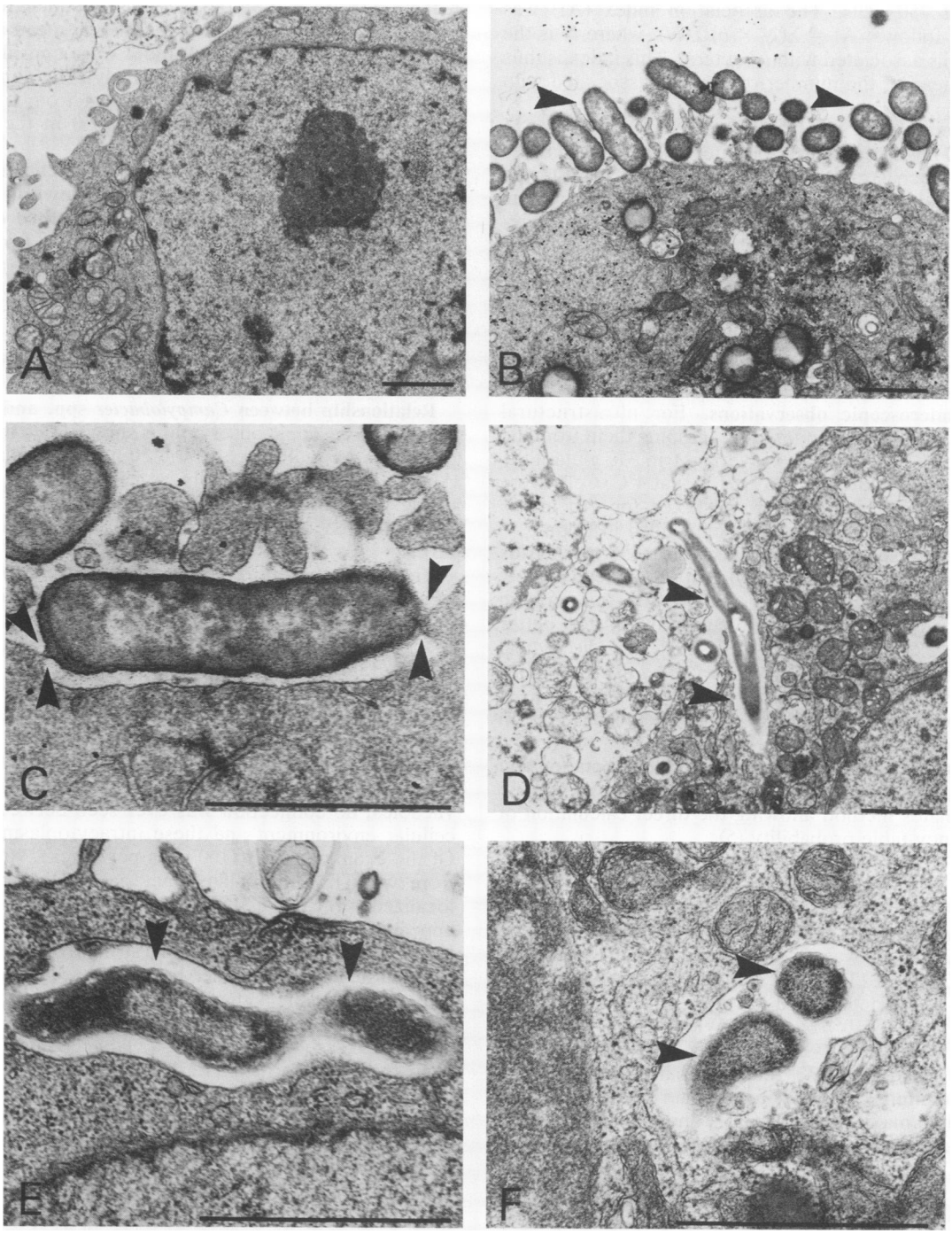

FIG. 3. Electron micrographs of HeLa cells inoculated with *C. jejuni*. (A) Noninvasive strains show an absence of bacteria. (B to F) Invasive strains show multiple bacteria (arrows) in various stages of penetration in cells. (B) Adhesion of bacteria to cell membrane. (C) Close associations between *C. jejuni* outer membrane and HeLa cell membrane. (D) Internalization of *Campylobacter* spp. into the cell. (E) Characteristic S shape of *C. jejuni* in cytoplasmic vacuole. (F) Enclosed bacteria in a phagosome. Staining: A, D, E, and F, ultrathin sections contrasted with uranyl acetate-lead citrate; B and C, sections stained by the periodic acid-thiocarbohydrazide-silver proteinate method. Bars, 1 μm.

cell monolayer was washed thoroughly 10 times, and the last washing liquid was checked for viable bacteria; each time, it contained less than 0.01% of the initial inoculum. Cell viability was checked after the test by microscopic observation after trypan blue staining; in all cases, cell monolayers included more than 99% viable cells. Cells were then fixed in methanol and Giemsa stained for microscopic examination and enumeration.

The degree of *Campylobacter* association with HeLa cells was quantified by counting the number of bacteria associated

with each of 250 cells. The association index (AI) was calculated as follows: $AI = \Sigma(N \times b)/\Sigma(N)$, where N is the number of cells associated with b bacteria. This index is thus a weighted mean of the number of bacteria associated with one cell.

For enumeration of viable intracellular bacteria, MEM containing 50 mg of gentamicin per liter (more than 50 times the MIC for each strain) was added onto the monolayers after the second incubation. After a 2-h contact at 37°C, the monolayers were washed thoroughly 10 times with antibiotic-free MEM and were treated with 0.5% sodium deoxycholate to lyse the HeLa cells. The lysates were then appropriately diluted in BB and plated onto Columbia agar to determine the number of intracellular CFU. For enumeration of total viable associated bacteria, the same procedure was followed, replacing gentamicin with antibiotic-free MEM.

Electron microscopic observations. For ultrastructural studies, cells were first recovered by scraping them from the glass cover slip and then fixed overnight in 3% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) at 4°C. Cells were then rinsed in phosphate buffer, postfixed for 1 h in 1% osmium tetroxide, dehydrated, and embedded in Epon.

Serial ultrathin sections were collected on 150-mesh grids and then counterstained with uranyl acetate and lead citrate. In addition, some sections were treated with the periodic acid-thiocarbohydrazide-silver proteinate method to better visualize the external structures with high glucidic radical content. Ultrathin sections were observed by using a Philips EM 300 transmission electron microscope at 80 kV. At least 100 HeLa cells from each tissue block were analyzed on successive sections.

Statistics. Correlations between HeLa cell association and clinical manifestations were tested by the χ^2 test by using the Fisher and Yates method allowing the direct calculation of the contingency table probability (5).

RESULTS

Standardization of the association test. The effect of several parameters on the AI was assessed using four strains chosen at random: 79AH, 81T, 79AC, and 79K. The AIs of these four strains were determined by increasing the bacterial inocula from 10^3 to 10^{11} bacteria. With strains 79AH and 81T, the AI remained lower than 1 for all inoculum sizes. With strains 79AC and 79K, the AI remained lower than 1 when the inoculum contained fewer than 10^7 bacteria and rapidly reached maximum values (7.3 and 6.8, respectively) when the inoculum exceeded the threshold value of 10^7 to 10^8 bacteria.

The influence of time (from 10 min to 4 h) during the first contact between cells and bacteria was tested. With the four studied strains, the AIs regularly increased until 60 min after the inoculation of cells, and then the AIs remained steady at their maximum values.

Six successive association tests were carried out with each strain in standard conditions to estimate the accuracy of the AI (inoculum, 10^7 to 10^8 bacteria; first contact, 1 h). The observed means, standard deviations (SD), and ranges of AI, respectively, were as follows: strain 79AC, 7.3 ± 1.2 (6.6 to 9.1); strain 79K, 6.8 ± 0.9 (5.6 to 7.4); strain 81T, 1.8 ± 1.2 (0.0 to 3.7); and strain 79AH, 0.1 ± 0.2 (0.0 to 0.4).

Distribution of the AI. The distribution of AI is shown in Fig. 1A for the 42 strains studied. In this distribution the strains appeared clustered in two groups. The first group included 22 strains (52%) showing a low AI (range, 0 to 2.1; mean ± SD, 0.5 ± 0.6); the second group included 20 strains (48%) showing high AI (range, 3.5 to 8.3; mean ± SD, 6.2 ± 1.4). The strains in groups 1 and 2 were considered nonassociative and associative, respectively. The number of bacteria associated with each cell ranged from 0 to 5 in the nonassociative group and from 0 to 50 in the associative group.

Of the 42 strains, 17 were clinically documented. Distributions of AI for these 17 strains are shown in Fig. 1B, C, and D according to the presence or absence of the three recorded clinical symptoms. Diarrhea and fever were reported more frequently for patients infected by a strain with a high AI (7 children) than for those infected with a strain with a low AI (10 children); the differences were significant for diarrhea (7/7 versus 3/10, $P = 0.01$) and fever (6/7 versus 2/10, $P = 0.03$). The frequencies of blood cells in feces were the same in the two groups.

Relationship between *Campylobacter* spp. and HeLa cells. Optical microscopic observations showed two main patterns of association with each of the 20 strains displaying a high AI. (i) The bacteria usually appeared dispersed over the whole cell surface (Fig. 2A). (ii) Sometimes the bacteria were gathered in packets in particular cytoplasmic sites (Fig. 2B).

Transmission electron microscopic observations were performed with two nonassociative strains (79AH and 81T) and two associative strains (79K and 79AC). In the nonassociative strains, no bacteria were found associated with cells and no endocytic vacuoles appeared (Fig. 3A). In the associative strains, bacteria were seen either in close association with the cell membrane (Fig. 3B, C, and D) or included inside cytoplasmic vacuoles (Fig. 3E and F). Examination of successive sections in a given tissue block demonstrated the endocytic nature of bacterium-containing vacuoles; no connection was ever seen between the extracellular environment and these intracytoplasmic vacuoles. Of the examined cells (100 cells per block), 90% contained from two to five internalized bacteria, which were frequently localized in the Golgi area. Some of the internalized bacteria appeared to be partially lysed, and sometimes they appeared to be associated with myelin structures of intracellular degradation, whereas other bacteria that had retained their size, characteristic S shape, and outer membrane seemed to be still alive.

In the 20 associative strains, a viable count of intracellular bacteria was performed after killing extracellular bacteria by treatment with gentamicin (more than 50 times the MIC for 2 h). The percentages of intracellular bacteria ranged from 18 to 100% (mean ± SD, 46 ± 26) with regard to total viable associated bacteria. Each of the 20 associative strains showed both extracellular and internalized viable bacteria. In contrast, the same test performed with the nonassociative strains showed percentages of intracellular bacteria ranging from 0 to 5% (mean ± SD, 0.97 ± 2).

DISCUSSION

We evaluated in this study the degree of *C. jejuni* and *C. coli* association with HeLa cells. We standardized the experimental conditions giving reliable results and conclude that the procedure appears to be easier to perform than those previously described (19, 21).

These results demonstrate that less than half of the *C. jejuni* and *C. coli* strains isolated from human feces can associate with eucaryotic cells in vitro. Some researchers who have used similar tests have concluded that all such

strains can associate (19, 21), but they used somewhat different procedures and only a few strands.

As with other enteropathogenic organisms (8, 9, 17), in vitro association of *C. jejuni* with eucaryotic cells is related to various clinical symptoms: the associative strains were more frequently isolated from patients with febrile diarrhea than from patients without diarrhea or fever. In contrast, we did not find any correlation between HeLa cell association and the presence of blood cells in feces. Fever and blood cells in feces are two classical clinical manifestations of enteroinvasion, but in fact these symptoms depend as much on the host as on the bacteria.

Transmission electron microscopic examination and viable counts made after killing of extracellular bacteria support the fact that *Campylobacter* spp. associated with cells are either adherent to the cell membrane or internalized in endocytic vacuoles. Although the adherent bacteria were usually more numerous than the internalized bacteria, our results support the invasion capacity for associative strains of *C. jejuni*. The two patterns of association (diffused or localized bacteria) observed by optical microscopy were seen for each of the 20 associative strains, in contrast to the results of Scaletsky et al. (28), who distinguished two types of *Escherichia coli* strains which adhere locally or diffusely to HeLa cells. The images of gathered *Campylobacter* spp. were always rare and could be a consequence of cell reaction against bacterial aggression.

We conclude that HeLa cell association with *C. jejuni* is a convenient and rapid method for predicting the pathogenicity of a given strain. This method may also be useful for evaluating the biological modality of *Campylobacter* invasion of mammalian cells.

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