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In Vitro Model for *Campylobacter pylori* Adherence Properties

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The adherence of 12 strains of *Campylobacter pylori* was studied on four cell lines. Immunofluorescence and scanning and transmission electron microscopy were used to visualize the bacteria. A heavy adherence to the epithelial cell line HEp-2 and to the intestinal cell line Int-407 was noted. By transmission electron microscopy, a close association between bacteria and cells in the form of cup-like structures was observed, but pedestals were not present.

Campylobacter pylori is a newly discovered bacterium which colonizes the human stomach (11). It has been associated with chronic active antral gastritis, and this association is now considered to be causal. However, little is known about the pathogenic mechanisms of this organism. Cytotoxins different from those usually found have been described for some strains. They induce morphological changes, such as vacuoles, in seven of nine mammalian cell lines tested (9). *C. pylori* has been found in intercellular junctions as well as on the surface of antral cells in vivo but never inside the cells. Therefore, it is not pathogenic due to its invasive properties, but its adherence properties could generate the characteristic histopathological lesions observed (4). Adherence has been identified as a pathogenic mechanism of some strains of *Escherichia coli* (2).

The aim of this study was to develop an in vitro model of adherence for *C. pylori* in order to analyze the bacterium-cell interactions. In addition, electron microscopy of *C. pylori* adherence, which has not been previously reported, is presented.

Strains. The 12 *C. pylori* strains used were initially isolated from patients with antral gastritis referred to the endoscopic unit of Saint André Hospital in Bordeaux, France. Five out of seven patients had chronic active gastritis. This information was lacking for the five others. They were characterized according to previously described procedures (13). Ten strains were of serotype 1 and two strains were of serotype 3 in the Lior schema (10). All these strains were passaged more than 20 times before being tested. One strain of *C. jejuni* isolated from the stools of a patient was also tested in parallel, as were three gastric *Campylobacter*-like organisms no. 2 ($GCLO_2$) (6): reference strain NCTC 11848, and two wild strains, one isolated from an adult human's stomach and one isolated from a child's feces. The organisms were kept frozen at −70°C before being tested.

Cells. The cell lines used were HEp-2, an epithelial cell line, and Int-407, an undifferentiated cell line from the intestine of a human fetus (both obtained from Flow Laboratories, Puteaux, France); AG 2791, an endothelial cell line from the pulmonary artery of a bovine fetus (15) (obtained from the Culture Collection of the Coriell Institute for Medical Research, Camden, N.J.); and HGT1, a gastric cell line from a patient with a gastric carcinoma of the fundus (8) (kindly provided by C. Laboisse, Institut National de la Santé et de la Recherche Médicale U-239, Paris, France). All the cells were maintained in Eagle minimum essential medium (Flow Laboratories) with 10% fetal calf serum (Seromed, Berlin, Federal Republic of Germany) and 10 μg of gentimicin, with the exception of AG 2791 (20% fetal calf serum) and INT-407 cells (5% fetal calf serum). For HEp-2 cells, nonessential amino acids (1%) were also added. They were passaged biweekly.

Adherence tests. To perform the adherence tests, the cells were grown in 24-well microplates (Nunc, Roskilde, Denmark) with cover slips in 1 ml of medium without antibiotics for 24 to 48 h, depending on the cell line, in order to obtain a subconfluent monolayer.

The bacteria were grown for 48 h on chocolate agar, and a loopful was inoculated in a biphasic medium (10 ml of Mueller-Hinton agar plus 5 ml of brucella broth supplemented with vancomycin in 25-cm^2 tissue culture flasks) and incubated overnight without shaking (5). All cultures were incubated at 37°C in a microaerobic atmosphere (GasPak H_2 and CO_2 without a catalyst; BBL Microbiology Systems, Cockeysville, Md.). The broth was tested by phase-contrast microscopy to assure purity and motility of the strains and was adjusted to an optical density of 0.6 at 600 nM. The bacterial suspension (35 μl) was incubated in each well in duplicate. A viable colony count was performed on one strain and was found to be 3×10^7 CFU/ml (mean of three counts). The microplates were then reincubated at 37°C for 3.5 h and subsequently washed three times with strong agitation with phosphate-buffered saline to remove nonadherent bacteria and fixed before being observed microscopically. To ensure that 3 washes were sufficient, the experiment was repeated with 6 and 10 washes.

Light microscopic examination. The fixation was performed by using a mixture of methanol and acetic acid (3:1). The monolayer was stained with bisbenzimidazole (Hoechst stain no. 33258; Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.) for 30 min, mounted in buffered glycerin, pH 5.5, and observed by epifluorescence microscopy (magnification, ×1,000) (12). The percentage of infected cells was determined by counting 200 cells. Each strain was tested twice. Strain CCUG 19110 was also used to determine an adherence index after 1.5, 3.5, and 6 h of contact on HEp-2 cells, by determining the percentage of cells with more than 20 adherent bacteria per cell.

Scanning electron microscopy. For scanning electron microscopy, the monolayer was fixed with 2.5% glutaraldehyde solution, washed in phosphate buffer, and dehydrated in a graded series of ethanol. Then it was dried by a critical point and coated with gold-palladium. Observations were made by using a Philips SEM 515 microscope.

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TABLE 1. Adherence of strains of *C. pylori* and *C. jejuni* to four cell lines

Species and strain[a]	Adherence (% of infected cells) to:			
	HEp-2	Int-407	HGT1	AG 2791
C. pylori				
CCUG 19104	52	41	3	5
CCUG 19106	76	34	2	9
CCUG 19107	51	64	5	12
CCUG 19110	85	37	9	2
CIP 101260	75	53	7	6
Bx 1	62	40	10	4
Bx 13	61	55	2	11
Bx 16	72	54	6	9
Bx 18	83	37	7	6
Bx 22	77	39	15	4
Bx 33	80	59	5	6
Bx 169	51	42	4	10
C. jejuni ($GCLO_2$)				
NCTC 11848	7	26	68	92
87170	4	30	10	72
87255	42	70	78	78
C. jejuni				
Wild strain	1	1	15	9

[a] CCUG, Culture Collection of the University of Goteborg; CIP, Collection de l'Institut Pasteur. These strains were deposited in the collections.

Transmission electron microscopy (TEM). For TEM, cell monolayers were fixed in 2.5% glutaraldehyde in cacodylate buffer, postfixed in osmic acid, dehydrated in graded ethanol solutions, and embedded in Epon. Staining was performed with uranyl acetate. Another stain, composed of tannic acid and potassium ferricyanure, was also used to improve the visualization of the cytoplasmic membranes (3). Examination was performed with a Philips 201 electron microscope.

The results obtained for the different strains tested with the four cell lines are presented in Table 1. *C. pylori* adherence was found to be much greater on Int-407 (46.3 ± 9.7 [mean ± standard deviation]) and HEp-2 cells (68.8 ± 12.2) and significantly different from observations of HGT1 (6.3 ± 3.6) and AG 2791 cells (7.0 ± 3.0). There was no difference between the two *C. pylori* serotypes or between gastritis activities. No change was observed after 6 and 10 washes.

The adherence index obtained with one strain was 19.3% (standard deviation, 3.0%) after 1.5 h, 75.2% (standard deviation, 10.2%) after 3.5 h, and 67.0% (standard deviation, 4.0%) after 6 h. On HEp-2 cells, many organisms were dispersed on the entire surface of the cell and could be observed by light microscopic examination (Fig. 1A) as well as by scanning electron microscopy (Fig. 2A). When the organisms were present on HGT1 cells, they showed a localized adherence (Fig. 1B). With scanning electron microscopy (Fig. 2B), bacteria were observed to be piled up.

C. jejuni exhibited only light adherence properties to the cells in comparison with *C. pylori*. In contrast, $GCLO_2$ strains were adherent, but in a different way than *C. pylori*. They adhered more to HGT1 cells and AG 2791 cells than to the others, with the exception of strain 87255, isolated from the stomach, which adhered heavily to HEp-2 and Int-407 cells. In all cases, a localized adherence was noted (Fig. 3).

On TEM photographs, a close association was noted between the cell cytoplasmic membranes and the cell walls of the bacteria (Fig. 4 and 5). It occurred mainly at the terminal portion of the bacteria.

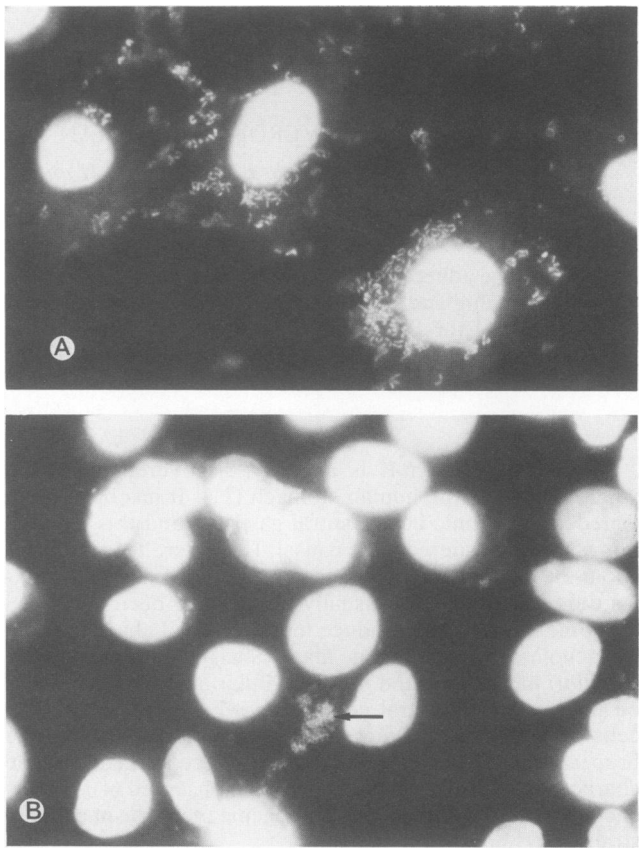

FIG. 1. Adherence of *C. pylori* CCUG 19110 after being stained with bisbenzimidazole. (A) Diffused adherence on HEp-2 cells. (B) Localized adherence on HGT1 cells. Magnification (both panels), ×500.

A salient feature of *C. pylori* strains was their important adherence properties to two of the cell lines tested, HEp-2 and Int-407, which are the ones used in conventional adherence tests. There are presently no epithelial cell lines of antral origin available. The gastric cells used (HGT1) are of fundic origin and are derived from a carcinoma (8). They did not allow an important fixation of *C. pylori*. They probably lack adequate receptors because they are transformed cells. We could not determine the classical adherence index (bacteria per 100 cells), since there were too many bacteria per cell to be counted. We have estimated the percentage of cells with more than 20 bacteria per cell after 1.5, 3.5, and 6 h. This index is at its maximum after 3.5 h. Other adherence techniques, such as colony count, could be used; however, our method has the advantage of visualizing the bacteria on the cells, and culture techniques are subjected to the nonculturable stage of *C. pylori*.

By TEM, it was possible to see an intimate association between bacteria and the cells. A loss of cell surface microvilli was not evident, and we did not observe true projections of the cell surface (e.g., pedestals) where the bacteria were fixed but only saw cup-like projections of the cell surface. Pedestals are observed in large numbers with enteroadherent *E. coli* strains, accompanied by a localized disruption of the cytoskeleton (7). With *C. pylori*, images similar to those we have described were observed in vivo by Goodwin et al. (4). Other authors did not mention this feature (1, 18). Nevertheless, a close association was noted;

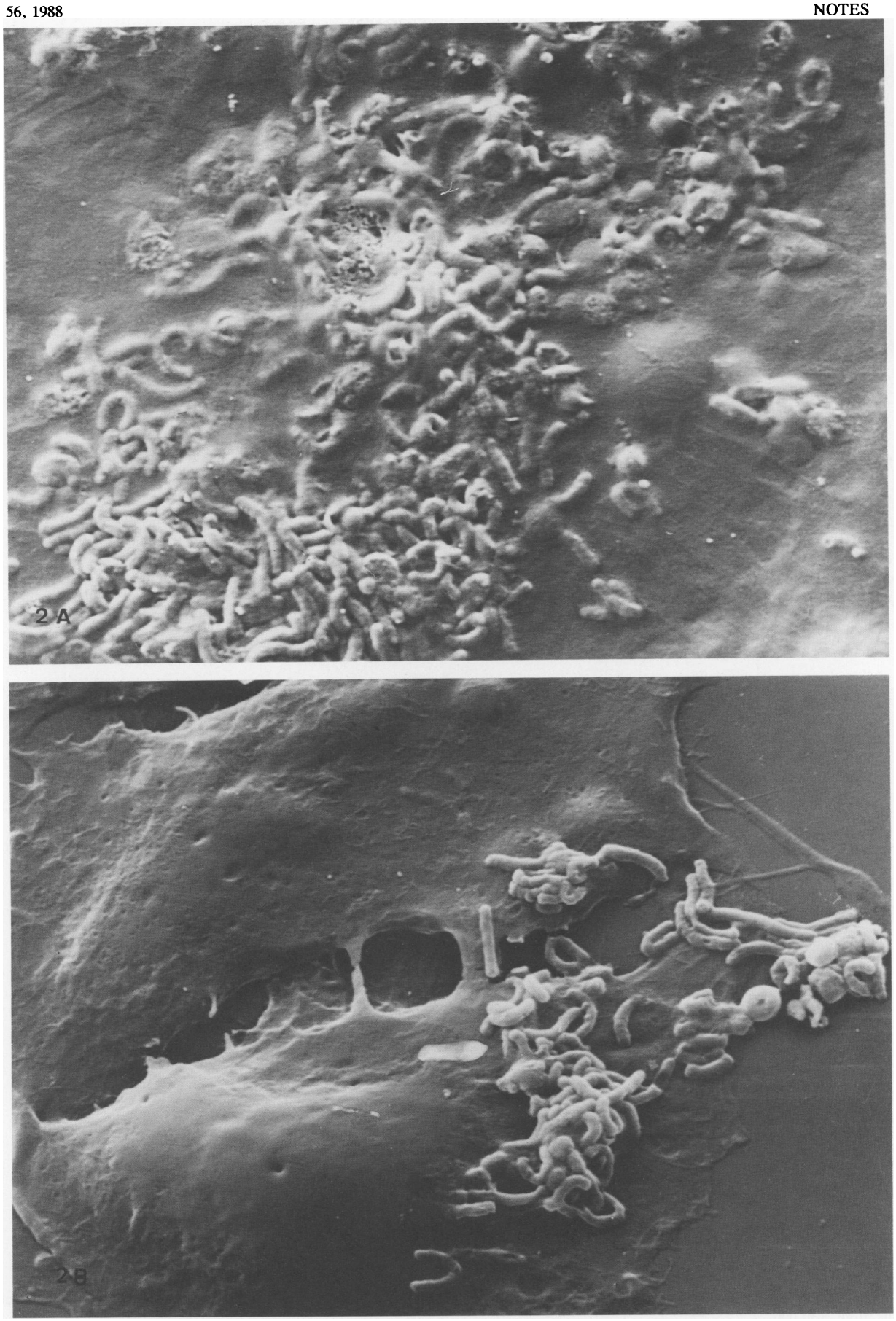

FIG. 2. Adherence of *C. pylori* CCUG 19110 visualized by scanning electron microscopy. (A) Diffused adherence on HEp-2 cells. (B) Localized adherence on HGT1 cells. Magnification (both panels), ×3,441.

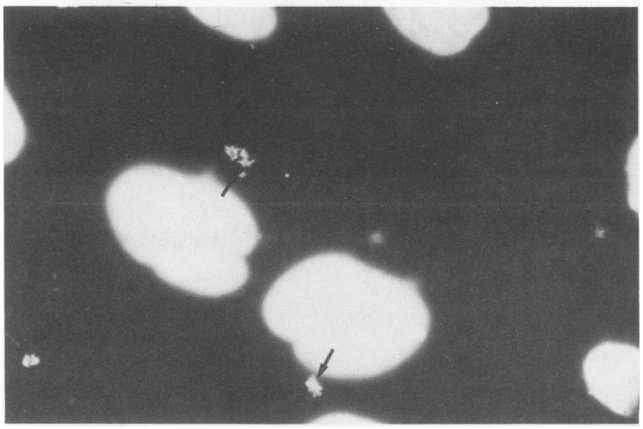

FIG. 3. Localized adherence of *C. jejuni* $GCLO_2$ 87255 on HEp-2 cells after staining with bisbenzimidazole. Magnification, ×500.

Tricottet et al. described a partial fusion (18), and Chen et al. noted the disruption of the cell membrane (1). In all cases, disappearance of the microvilli was observed.

The molecular nature of the adherence is not known, but there is little evidence for its fimbrial nature because of the close contact and apparent damage to the cells, in contrast to that which is seen with enterotoxigenic *E. coli*. The role of the flagella has not been studied but may be important.

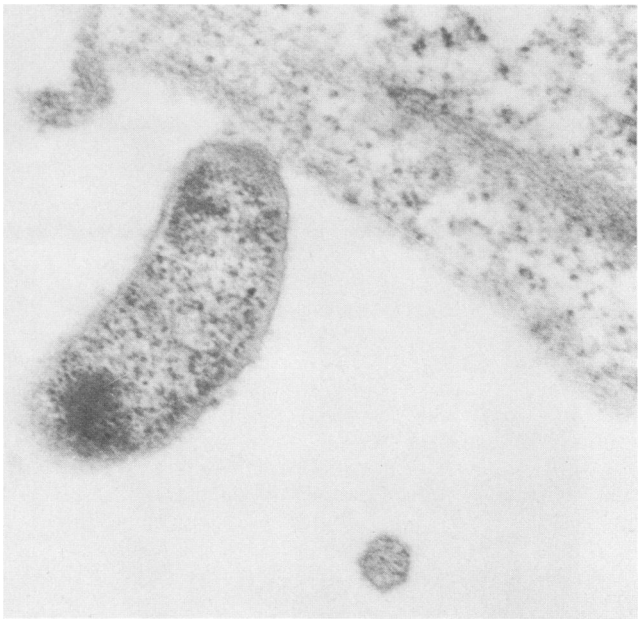

FIG. 5. Adherence of *C. jejuni* $GCLO_2$ 87255 to HEp-2 cells visualized by TEM. Magnification, ×30,000.

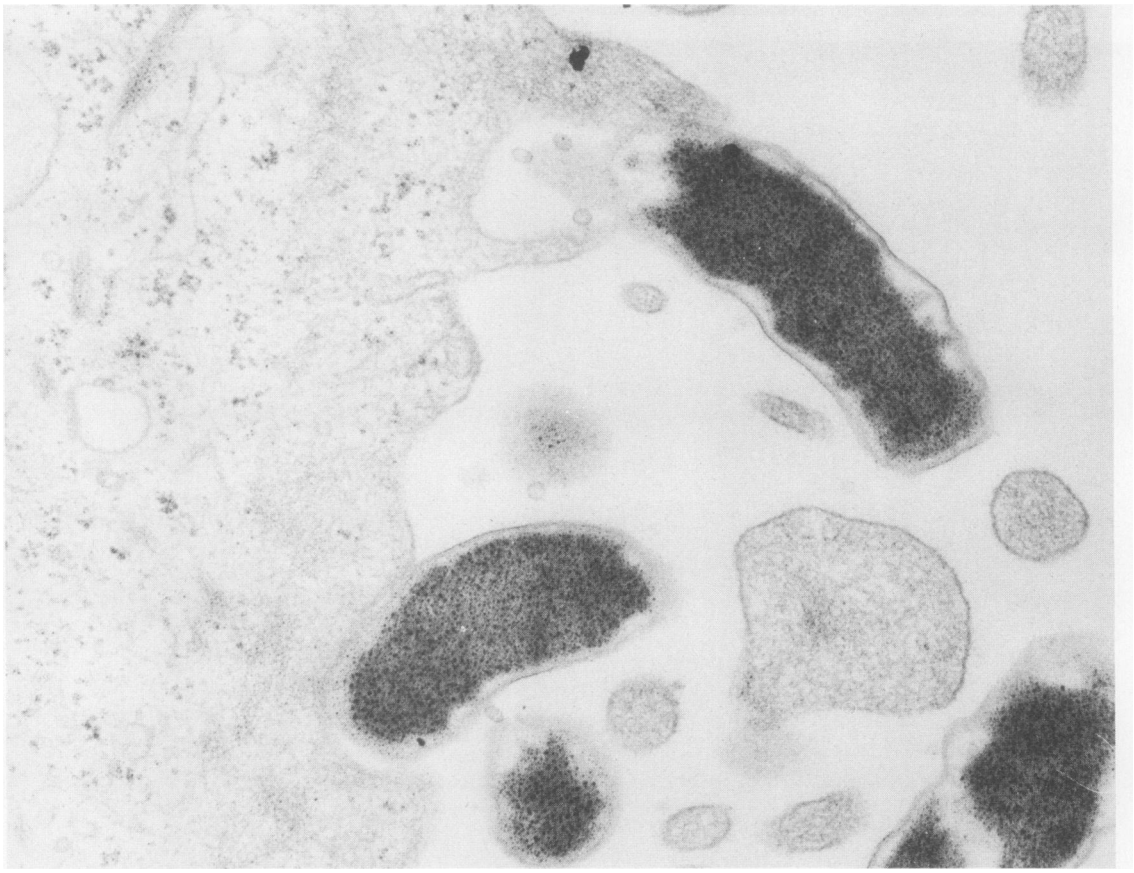

FIG. 4. Adherence of *C. pylori* to HEp-2 cells visualized by TEM. Adherence occurs at the extremity of the bacteria to cup-like structures. Magnification, ×30,000.

An interesting feature was the localized adherence, like that seen with enteropathogenic *E. coli* strains (16), observed for *C. pylori* with some cell lines, such as HGT1, and for $GCLO_2$ with all the cell lines. We hypothesize that receptors to localized adherence are not distributed over the whole surface of HGT1 cells, in contrast to HEp-2 cells, and that receptors for $GCLO_2$ are probably different from those for *C. pylori*. $GCLO_2$ has recently been classified as a *C. jejuni* subspecies on the basis of genotypic characteristics (14). In contrast to *C. pylori*, its ecological niche is not restricted to the stomach and duodenum, and it has been associated with diarrheal disease in Australia (17). Among the three strains tested, two were isolated from gastric biopsies, and one was isolated from feces. Important variations were noted between the strains concerning their adherence properties. The strains isolated from the stomach were markedly more adherent.

This close association could explain part of the pathological findings. The bacteria present on the cell surface, at least, deprive the cell of its nutrients. They may also have an effect on the cytoskeletal structure. Some of the strains have also been shown to produce cytotoxins. These cytotoxins are relatively weak, and they have a better chance to be efficient if they are produced near the cells. This model will allow a more extensive study of *C. pylori*-cell interactions.

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