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Morphologic Conversion of Helicobacter Pylori From Spiral to Coccoid Form

Scanning (SEM) and transmission electron microscopy (TEM) suggest viability

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ABSTRACT

Helicobacter pylori is a pathogen associated with type B gastritis, peptic ulcer disease, gastric atrophy, and stomach cancer. H. pylori exists in two morphological forms, spirals and coccoids. The latter has been described as viable but non-cultivable. The role of the coccoid form in the pathogenesis of gastric disease is disputed. Some authors consider the coccoid form to be a degenerative or dead form of H.pylori, while others consider it a resting but still metabolically active form.

This study reports the conversion from spiral to coccoid form ultrastructurally. Dense material is accumulated in the periplasmic space, the spiral bacteria bend and the outer membrane is separated from the inner cell wall layer. Remodeling of inner structures takes place, ending in the coccoid form of the bacteria with preserved light polyphosphate areas. Reduction of surface takes place by production of surface membrane vesicles, which later are squeezed off. The finding of preserved subcellular structures and intact double membranes in combination with degenerative forms suggests that some of the coccoids are viable. Scanning electron microscopy (SEM) demonstrates coccoid form of bacteria with slightly ruffled surfaces but no spiral forms.

INTRODUCTION

Helicobacter pylori is a human pathogen associated with type B gastritis, peptic ulcer disease and gastric atrophy and stomach cancer [32]. An oral challenge with H.pylori caused an infection in human volunteers, monkeys, gnotobiotic pigs, nude and euthymic mice inducing histopathological changes similar to natural Type B gastritis in man [32].

H. pylori exists in two morphological forms, spirals and coccoids. The latter have been described as "viable but non-cultivable"(VBNC). The coccoid form of H. pylori may be viable and can revert to cultivable forms in mice [7, 32] but are no longer cultivable on conventional media [3, 5, 9, 10, 16, 30]. The role of the coccoid form in pathogenesis of H. pylori-associated gastritis has been disputed. Some authors consider the coccoid form as a degenerative or dead form of H. pylori [16, 21, 25], while others consider it as a resting but still metabolic active form [2, 3, 12, 22, 29, 32]. The aim of this study was to demonstrate the conversion of spiral form of H. pylori to coccoid forms, and to show both degenerative and intact subcellar morphology suggesting that some of the coccoid bacteria may be viable.

MATERIAL AND METHODS

Bacterial strains and culture

H. pylori strain 553/93 was a freshly isolate from human gastric biopsies. The strain was grown on GAB-CAMP agar supplemented with horse serum, 10% and incubated for 48 h at 37° C under microaerophilic conditions to obtain a high yield of spiral shaped H.pylori [32]. To obtain viable but non-cultivable H.pylori bacteria, 3-5 day-old agar cultures were harvested and resuspended in 20 ml of Ham´s F12 medium supplemented with 10% calf serum (Flow laboratories, Irvine), and incubated in a micro-aerobic environment for 3 days at 37° C and then stored at 4°C. If no growth was observed after incubation for 5 days on GAB-CAMP agar at 37°C, harvested cells were defined as viable but non-cultivable [32].All bacteria were analyzed for the presence of spiral forms in the coccoid suspension. No spiral forms were found.

Transmission electron microscopy(TEM)

During the spiral to coccoid conversion period samples were taken and immediately fixed in 2%glutaraldehyde, in 0.1 M sodium cacodylate buffer (pH 7.2), postfixed in 2% osmium tetroxid, in S-colloidine buffer (pH 7.2), dehydrated in graded ethanol and embedded in agar resin 100. Semithin section was cut and examined by light-microscopy. A representative area was chosen and ultra-thin sections, 50 nm were cut on a Reichert-Jung Super Nova Ultramicrotome and contrasted with uranyl acetate and lead citrate. The grids were examined in a Philips CM 10 transmission electron microscope at 60 kV.

Scanning electron microscopy (SEM)

Coccoid Helicobacter cells were placed in a fixative solution containing 2.5%(wt/vol.) glutaraldehyde for 24 h at 22° C. The preparations were rinsed 3 times in Soerensen´s phosphate buffer, and then dehydrated in a graded alcohol series ending with 99% ethanol containing a molecular sieve, and then critical point-dried in liquid carbon dioxide. The specimens were mounted on metal stubs and sputtered in a Polaron E 5400 sputter coated with gold for SEM. Each specimen was studied in a Philips SEM 515 unit at a magnification of x 312 to x 5700.Photographs of representative findings were obtained.

RESULTS

SEM showed rounded coccoid H. pylori after 5 days of cultivation on GAB-CAMP agar. No spiral shaped H. pylori were detected. The coccoids were seen in clusters and some debris was noted (Fig 1).

Fig 1. Clusters of Coccoid bacteria after 5 days cultivation on GAB-CAMP agar with rounded contours and some debris. No spiral forms are detected. SEM x 5,700 (original magnification).

In TEM transformation of the spiral form of H.pylori to coccoid form occurred via an intermediate U-shaped form. It is initiated by ingrowth of the periplasmic space on one side of the bacteria separating the double membrane into two membranes (Fig 2).Accumulation of cell dense material could be seen. Later the U-shaped form became predominant with enhancement of electron dense material (Fig 3). Transection of such U-bent bacteria yielded two forks with a light space in-between. The membranes were separated into an inner and outer part, where the forks were either covered by a single layer at the center and double layer at the periphery (Fig 4). Sometimes at this development stage a part of the spiral form was clearly visible while the other part became more diffuse (Fig 5).

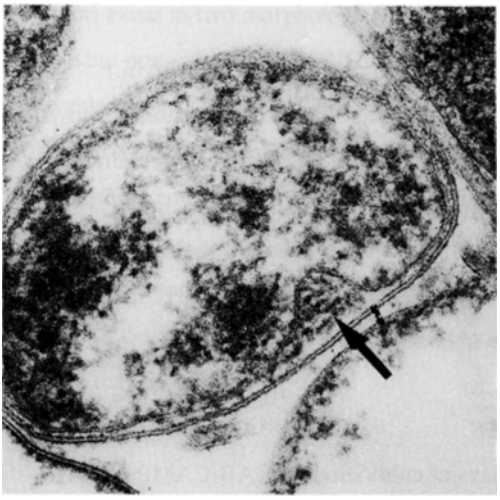

Fig 2

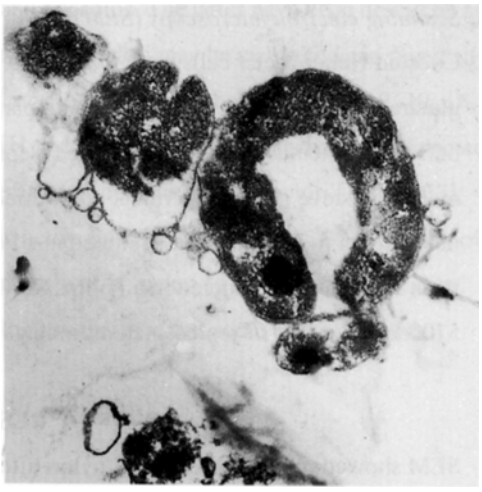

Fig 3

Fig 2.H.pylori spiral form with separation of inner and outer membrane by the periplasmic space with accumulation of dense granular material (Arrow). TEM 105,000(original magnification).

Fig 3.U-formed spiral bacteria. Note cytoplasmic vesicles, polar density and light areas, supposedly polyphosphate-rich areas. Diameter 1.34 μm. TEM x 21,000 (original magnification).

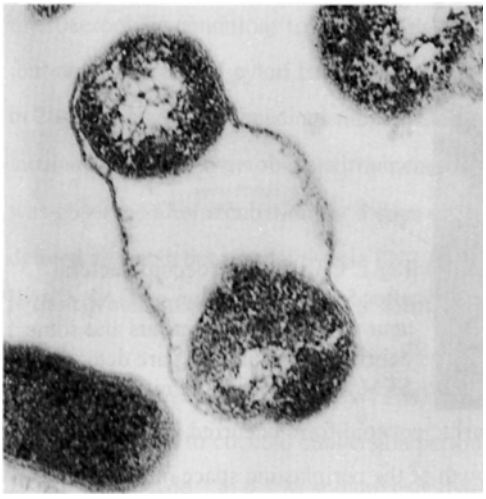

Fig 4

Fig 5

Fig 4.Transection of U-bent H.pylori spiral form with a clear space between forks. Note remains of inner membranes around light and dense structures. TEM x 52000 (original magnification).

Fig 5.Partly formed coccoid H.pylori. Condensation of dense material in one part of the bacteria, double membranes and shedding of cytoplasmic vesicles. TEM x 52000 (original magnification).

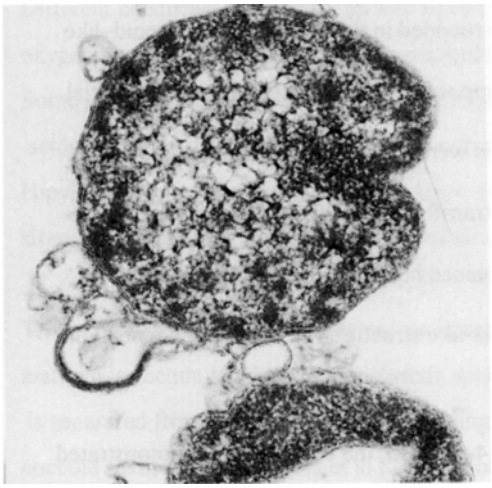

Fig 6

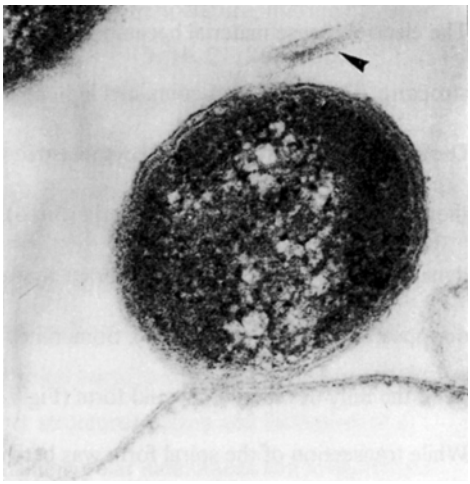

Fig 7

Fig 6.Nearly finished H.pylori coccoid. Note light areas and cytoplasmic vesicles sometimes containing dense material. TEM x 52,000 (original magnification).

Fig 7.Fully developed H. pylori coccoid. Note double membranes, dense and light areas and part of a flagella (arrow). TEM x 105,000 (original magnification).

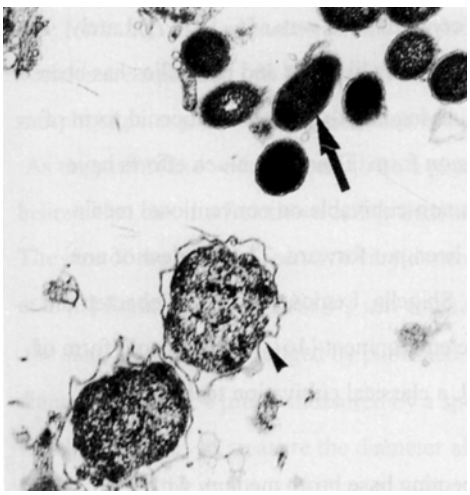

Fig 8

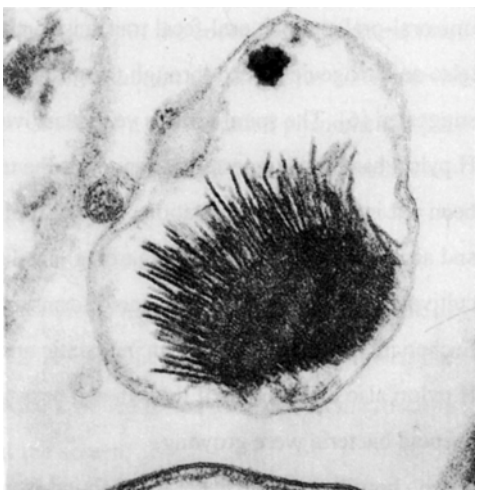

Fig 9

Fig 8.Mixture of transected spiral H. pylori (arrow) (Diameter 0.41-0.45 μm) and coccoid forms (arrow head) (1.04-1.16 μm). TEM x 15,500 (original magnification). Fig 9.Coccoid H. pylori with morphologically clear degeneration and fragmentation of membranes and central dense area. TEM x73,000 (original magnification).

The electron dense material became gradually more rounded in the center of the coccoid–like structure. The center also contained light spaces, supposedly holding phosphate-rich material. During the transformation many cytoplasmic vesicles were produced, sometimes squeezed in the space outside the coccoid bacteria (Fig 6). The transformation strongly suggests an dynamic process. The double membrane system remained but periplasmic dense material disappeared subsequently (Fig 7). Sometimes flagella-like structures were seen in connection with the fully developed coccoid form (Fig 7).

While transection of the spiral form was between 0.4-0. 8 μm, the coccoid form demonstrated a diameter of 0.9-1.4 μm (Fig 8). A progressive loosening and detachment of the outer membrane vesicles accompanied this transformation. Degenerated coccoid bacteria were also found with fragmentation of membranes and disruption of centrally dense material (Fig 9).

DISCUSSION

The mode of transmission of H.pylori from man to man is still unclear. Two theories exist: one oral-oral and one oral-fecal transmission through contaminated water [6, 11, 17]. Lately also an iatrogenic spread through the gastroscope and vectors like cats and houseflies has been suggested [6]. The spiral form is very sensitive for air drying and therefore the coccoid form of H.pylori has been suggested to represent the transmission form. Therefore much efforts have been put into its characterization. The coccoid form is non-cultivable on conventional media and accordingly pro and cons regarding viability have been put forward. The problem of non-cultivable but in water viable coccoid forms as Vibrio, Shigella, Legionella, Campylobacter and Escherichia coli are well known in aquatic and marine environment [16]. Is the coccoid form of H.pylori also an example of this? In egg passages [17], a classical cultivation technique, no coccoid bacteria were growing.

Lately, however, Andersen et al [1] found by supplementing base broth medium with 2 % newborn calf serum, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+} and lysed human erythrocytes that cocci reverted to spiral forms at Ph 8.0-8. 5 with recovery of urease activity.

In order to find out whether and when certain coccoid H.pylori are viable a vast amount of work with many techniques has been used.

In order to induce coccoid forms of H. pylori in vitro several methods were scrutinized.

Different chemicals as bismuth, as well as bile acids, antibiotics, temperature, nutritients, oxygen tension, starvation and aging are some examples [3, 10, 15, 16, 21, 25].

Some antibiotics seem to create greater changes to the cell wall than bismuth salts and bile acids [25]. In vivo changes and transformation mechanisms are less well known. Coccoid H.pylori bacteria seem to be relatively more common in the duodenal mucosa than in the stomach in humans, [26].Several TEM studies have analyzed the transformation of spiral H. pylori bacteria into a coccoid form [2-5, 8, 10, 12]. SEM-investigations are few [15].Our TEM findings of coccoid formations are in accordance with others [2-5, 8, 10, 12]. Dense material is accumulated in the periplasmic space, the spiral bacteria bend and the outer membrane is separated from the inner layer. Remodeling of inner structures occurs and formation of a coccoid takes place. Benaissa et al [2] described remaining polar membranes and invagination of surface membranes. We also found flagella structures, found by several other authors [3, 10], but denied by others [19, 25, 29]. The coccoid center seems to contain light areas. Bode et al [3] demonstrated that they contain polyphosphate-rich material by electron spectroscopic imaging, which is used for basic energy requirements. These light areas were found by us and others [2, 4, 12]. Membranes were found to be intact in most coccoids but also degenerative forms were found [10].

The formation of membrane vesicles is a striking finding. Later in the coccoid development these vesicles are squeezed off. This might be a remodeling with reduction of surface for better survival, morphologic expression of detoxification mechanism or simply a degenerative phenomenon [21]. As the membrane vesicles seem to be a phenomenon characteristic of all coccoid formation we believe that this is a functional rather than a degenerative event.

The size of the coccoid seems to vary depending on the method used to produce them. Bode et al [3] found a mini-form of 0.3 μm when using antibiotics. Other methods yielded bacteria of the size 0.8-1.5 μm as judged by published photos [2, 3, 10]. Our coccoid bacteria showed the diameter of 0.9-1.4 μm as measured by a special device installed inside the electron microscope which enabled us to measure the diameter already at the screen.

SEM showed clusters of coccoid bacteria with slight uneven surfaces. No spiral forms were detected after 5 days cultivation. Clustering seems to be a feature in the human stomach [12] as well as in vitro [15].

Marked differences between the spiral and coccoid form were discovered [8, 16] by chemical and physiological measurements as well as immuno-, DNA, RNA and ATP-analyses.

A substantial amount of similarities were, however, discovered with intact or similar properties in both forms of H. pylori. Thus, DNA and RNA were sometimes preserved , especially in younger coccoid cultures [16, 18, 22, 30] with incorporation of BrdU [31] as a sign of protein synthesis [31], a finding however denied by others [21]. Some authors have reported decreased RNA /DNA content with non-random fragmentation but rarely nil-values [16, 21, 23]. Levels of increased ATP have also been found after addition of fresh medium [30]. Specific cellular antigens sometimes remained [28] but a substantial modification in the cell wall of peptido-glycans occurred [14]. Immunoblot and RAPD-fingerprinting report loss of 160 kD, 120 KD (vac A) and a 35 k D cell surface protein while most other protein bands were weakened or preserved at normal levels [25, 30, 31].

Adherence of H. pylori coccoid to gastric epithelia and glycocalyx was the same for cocccoid and spiral forms as was also staining for 8 types of lectins [27]. Cole at al. [13] reported poor adherence to gastric epithelial cells with little or no interleukin-8 secretion. Adhesion with pedestal formation was seen between H. pylori coccoid and epithelial cells [19, 31] as well as in a Balb/cA mice H.pylori infection model [32]. Plasminogen and lactoferrin bind to the coccoid form of Helicobacter, and also to extracellular matrix protein considered to be an important mechanism for tissue adhesion [20].

Moran [24] suggests that we can not exclude that two different types of H. pylori coccoid forms exist; one that is degenerative and one viable but not (at that time) cultivable and that the viable form later on can develop to a degenerative form. The possibility that a minority of bacteria, not detected by methods commonly used, can stay in coccoid or resting state can not be ruled out [16]. This view might explain the contradictory findings present today regarding viability of the coccoid form of H.pylori. The advantage of TEM is that single cells are analyzed compared to most other methods and that fully intact as well as degenerated bacteria can be recorded.

We strongly believe that some of the coccoids are viable due to the fact that subcellar structures and intact membranes were found in our TEM and SEM studies. We were also able to see coccoid bacteria attached to gastric epithelia in Balb/cA mice with subsequent development of gastritis after 16 weeks [32] .Cellini et al. [7] observed the same in Balb/c mice. This further strengthens our view that the coccoid form of H. pylori is viable.

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