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Na^+,K^+-ATPase of gastric cells

A target of *Helicobacter pylori* cytotoxic activity

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The present study shows a direct impairing action of a cytotoxin-producing *Helicobacter pylori* strain on the Na^+,K^+-ATPase (evaluated as K^+-dependent phosphatase activity) of human gastric epithelial cells in culture. The toxin itself is likely involved in this action which may also account for the cell edema found in vivo in *Helicobacter pylori*-colonized stomach.

Na^+,K^+-ATPase; *Helicobacter pylori*; Cytotoxicity; Cell vacuolation; K^+-dependent phosphatase activity; Cultured gastric cells

1. INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium which plays a central role in the etiology of chronic gastritis and peptic ulcer disease [1–3]. Recently, *H. pylori* infection has been also related to gastric cancer [4,5]. However, the mechanisms whereby *H. pylori* exerts its pathogenetic action remain poorly understood. An important virulence factor of *H. pylori* is a heat-labile cytotoxin produced in vitro by approximately 50% of *H. pylori* strains and inducing cell vacuolation [6]. Recently, Cover and Blaser [7] have shown that the toxin migrates as an 87 kDa protein under denaturing conditions and in nature forms aggregates with a molecular mass > 972 kDa. The amino-terminal sequence of this toxin shows no homology with the sequences of other known bacterial toxins, while being partially homologous with internal sequences of various ion-transporting ATPases [7]. Therefore, Cover and Blaser [7] suggested that ion-motive ATPases may be targets of *H. pylori* cytotoxin and proposed that the toxin could act by altering ATPases function.

The aims of the present study were to verify whether the broth culture filtrate (BCF) of a cytotoxin-producing *H. pylori* strain exerts any action on the Na^+,K^+-ATPase of human gastric epithelial cells in culture and whether this action could be accounted for by Cover and Blaser's toxin.

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2. MATERIALS AND METHODS

Two *H. pylori* strains were used: (1) CCUG 17874 (from Culture Collection University of Göteborg, Göteborg, Sweden), a cytotoxin-producing strain [8], and (2) G21 (kindly given by N. Figura, Siena, Italy), which does not produce the toxin [8]. Bacteria were grown in Brucella broth, supplemented with 5% fetal calf serum (FCS) (Gibco, Grand Island, NY), for 24–36 h at 37°C in a thermostatic shaker under microaerophilic conditions. Bacteria were then removed by centrifugation and the supernatants were sterilized by passage through a 0.22 μm cellulose acetate filter (Nalge Co., Rochester, NY) to obtain the BCFs [9]. Aliquots of BCF from the CCUG 17874 strain were incubated for 15 min in boiling water in order to inactivate the heat-labile cytotoxin [10]. BCF from the cytotoxin-producing CCUG 17874 strain was also fractionated by using ultrafiltration membranes with different molecular mass cutoff (Amicon Inc., Beverly, MA). Each fraction was brought back to BCF starting volume with Hanks's balanced salt solution (HBSS) to obtain the same concentration of BCF for all bacterial substances in the fractions. Moreover, since ammonia plays an important role in cytotoxin-induced cell vacuolation [7,11], the ammonia content of each fraction was equalized to that of the BCF (4 mM). The presence of the toxin in the BCF fractions was assayed with SDS-PAGE under denaturing conditions followed by silver staining according to Cover and Blaser [7]. Aliquots of the toxin-containing fraction were incubated for 5 h at 37°C with insoluble proteinase K attached to 4% cross-linked beaded agarose (Sigma, St. Louis, MO) [11]. The insoluble protease was then removed by centrifugation.

A gastric epithelial cell line, namely MKN 28, derived from a human gastric tubular adenocarcinoma and showing gastric-type differentiation [12,13], was used. MKN 28 cell monolayers were grown in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (1:1) supplemented with 10% FCS and 1% antibiotic-antimycotic solution (both from Gibco) in 75 cm^2 tissue culture flasks (Greiner GmbH, Frickenhausen, Germany) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Subconfluent cell cultures were washed twice with HBSS and then incubated for 16 h with *H. pylori* BCFs or fractions of the CCUG 17874 strain BCF, diluted 1:3 in HBSS without FCS, at 37°C in a humidified atmosphere of 5% CO_2 in air. Uninoculated broth filtrate

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and its fractions served as controls. At the end of incubation, cell monolayers were washed twice with ice-cold 154 mM NaCl solution. The cells were then harvested using a cell scraper in ice-cold buffer containing 5 mM EDTA and 10 mM Tris-HCl (pH 7.8). The K^+-dependent phosphatase activity of the cells was measured according to Murer et al. [14]. This ouabain-sensitive [15] enzymatic activity represents the terminal hydrolytic step of the overall enzymatic cycle of the Na^+,K^+-ATPase [16,17]. The degree of cell vacuolation was assayed by means of Neutral red dye uptake according to Cover et al. [11]. Cell K^+-dependent phosphatase activity and cell vacuolation were both expressed per mg protein. Protein content of cell monolayers was measured according to Lowry et al. [18].

All the results were expressed as the mean ± S.E.M. of 5 experiments. The statistical significance of the differences was evaluated by the analysis of variance followed by Newman–Keuls's Q-test [19].

3. RESULTS AND DISCUSSION

The BCF from the cytotoxin-producing CCUG 17874 strain induced a statistically significant decrease in cell K^+-dependent phosphatase activity as compared with control (Fig. 1). This decrease was completely prevented by heat inactivation of the BCF. No effect was observed with the BCF from the cytotoxin-negative G21 strain (Fig. 1). Moreover, BCF from the cytotoxin-producing CCUG 17874 strain induced a higher degree (about 2.5-fold) of cell vacuolation as compared to that induced by BCF from the cytotoxin-negative G21 strain (Fig. 2). Heat inactivation of CCUG 17874 BCF reduced its vacuolating activity to a level similar to that induced by G21 BCF (Fig. 2). The vacuolating activity of both G21 BCF and CCUG 17874 heat-inactivated BCF may be accounted for by their ammonia contents [9]. Cover and Blaser's vacuolating toxin was detectable by means of SDS-PAGE only in the CCUG 17874 BCF fraction containing proteins with native molecular mass >100 kDa (data not shown). A bacterial substance sensitive to digestion with protease accounted for the higher degree of cell vacuolation induced by the BCF fraction with molecular mass >100 kDa (Fig. 3) as compared to that of other fractions vacuolating mainly due to their ammonia content [9]. Moreover, the decrease in the cell K^+-dependent phosphatase activity induced by BCF from the CCUG 17874 strain was also due to a

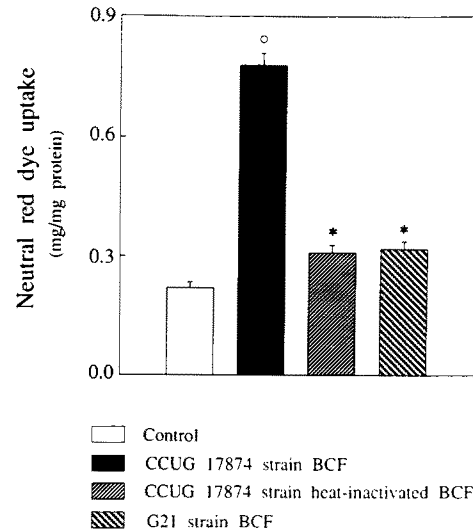

Fig. 2. Cell vacuolation induced by *H. pylori* broth culture filtrates (BCFs). MKN 28 cells were incubated as in Fig. 1. Cell vacuolation was evaluated as neutral red dye uptake. Means ± S.E.M. (n = 5). $*P < 0.05$ vs. control; $°P < 0.001$ vs. control and all other conditions.

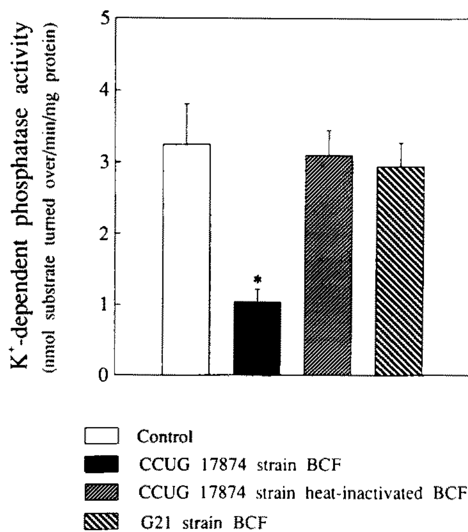

Fig. 1. Effects of *H. pylori* broth culture filtrates (BCFs) on the K^+-dependent phosphatase activity of MKN 28 cells. Cells were incubated with: (1) uninoculated broth filtrate (control), (2) BCF from the cytotoxin-producing CCUG 17874 strain, (3) heat-inactivated BCF from the cytotoxin-producing CCUG 17874 strain, (4) BCF from the cytotoxin-negative G21 strain. Means ± S.E.M. (n = 5).$*P < 0.001$ vs. control and all other conditions.

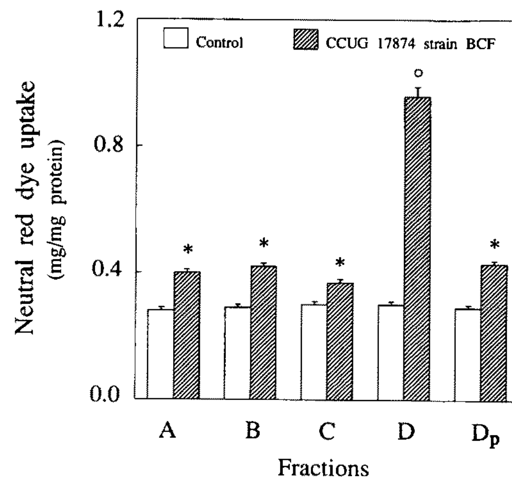

Fig. 3. Cell vacuolation induced in MKN 28 cells by fractions of different molecular mass from the CCUG 17874 strain broth culture filtrate. Respective fractions from uninoculated broth filtrate served as controls. Fractions: A, <10 kDa; B, >10 kDa and <30 kDa; C, >30 kDa and <100 kDa; D, >100 kDa; Dp, protease-treated fraction D. Cell vacuolation was evaluated as neutral red dye uptake. Means ± S.E.M. (n = 5). $*P < 0.05$ vs. all controls; $°P < 0.001$ vs. all other conditions.

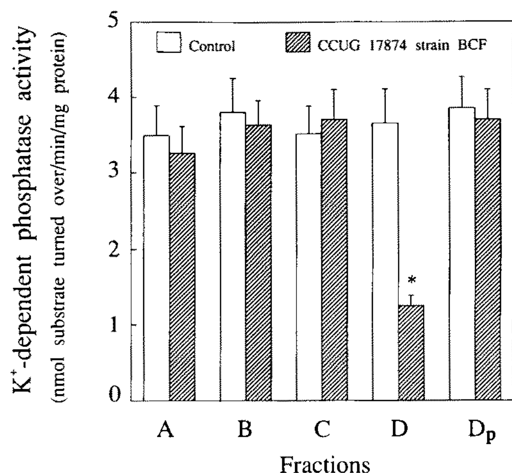

Fig. 4. Effects of fractions of different molecular mass from the CCUG 17874 strain broth culture filtrate on the K^+-dependent phosphatase activity of MKN 28 cells. Respective fractions from uninoculated broth filtrate served as controls. Fractions as in Fig. 3. Means ± S.E.M. ($n = 5$). $*P < 0.001$ vs. all other conditions.

protease-sensitive bacterial substance present in the fraction with molecular mass > 100 kDa (Fig. 4).

Since only the BCF from the cytotoxin-producing CCUG 17874 strain (and not the BCF from the cytotoxin-negative G21 strain) impairs the Na^+,K^+-ATPase, and since the impairing agent is a protein of native molecular mass > 100 kDa, just like the vacuolating cytotoxin [6,7], the toxin itself may be involved in the Na^+,K^+-ATPase impairment. This conclusion would fit with the observed sequence homology [7] between the toxin and the Na^+,K^+-ATPase. In addition, the higher degree of cell vacuolation obtained with the Na^+,K^+-ATPase impairing BCF compared to the non-impairing BCFs is in agreement with Cover et al.'s observation [20] that ouabain, a specific inhibitor of Na^+,K^+-ATPase, potentiates the vacuolating activity of purified *H. pylori* cytotoxin. Unfortunately, the toxin purified from the CCUG 17874 strain was not used in our experiments. Attempts to purify the vacuolating toxin in a functionally active state from the CCUG 17874 strain by means of column chromatography have been disappointing since the toxin lost most of its activity during the purification steps and the yield was extremely low. Moreover, the available anti-toxin antibodies are characterized by low specificity and affinity (R. Rappuoli, personal communications).

In conclusion, the present study provides the first demonstrations that cytotoxin-containing *H. pylori* BCF directly impairs the Na^+,K^+-ATPase of gastric cells and that the toxin itself is likely involved in this action. Since the inhibition of Na^+,K^+-ATPase causes cell edema [21], our results may explain the swelling and endoluminal bulging of gastric cells found in vivo in *H. pylori*-colonized stomach [22].

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