JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1993, 44, 1, 17—22

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HELICOBACTER PYLORI TOXIN INHIBITS GROWTH AND PROLIFERATION OF CULTURED GASTRIC CELLS-KATO III

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The presence of Hp is strongly associated with chronic type B gastritis and peptic ulcer disease. The pathogenic mechanisms of *Helicobacter pylori* (Hp) infection are still unclear. Hp produces toxins which are capable of exerting cytotoxic effects. Whether these changes result in decreased cell proliferation has not been previously demonstrated. Our results show that a 2 hour incubation of gastric Kato III cells with Hp cytotoxin produces a 60% decrease in cell proliferation. In conjunction with a decreased cell proliferation, cell growth is also decreased on days 5 and 7. The ability of Hp to retard cell proliferation may play a role in the pathogenesis of Hp-associated diseases by inhibiting the normal mechanisms of gastric mucosal protection and repair.

Key words: *Helicobacter pylori; cytotoxin; gastric cells; cell proliferation;*

INTRODUCTION

Helicobacter pylori (Hp) is a spiral bacterium that colonizes the gastric mucosa. It is usually found in the mucus layer, in close proximity to the gastric surface epithelial cell (1). The presence of Hp is strongly associated with chronic type B gastritis and peptic ulcer disease (2—5). Most recently, an association has been identified between Hp and gastric carcinoma (6—9). The pathogenic mechanisms of Hp-associated diseases are not clear. Hp is known to produce extracellular toxins that cause vacuolization of mammalian cells in culture (10). Hp infected patients possess antibodies that neutralize this toxin indicating an *in vivo* production (11). Approximately 55% of Hp strains exhibit this cytotoxic effect and these toxic strains are found more frequently in patients with peptic ulcer disease as opposed to those with chronic gastritis only (12). Various investigators have correlated the toxin production with both a 128-kDa protein band and a 87,000 kDa protein band identified by immunoblotting (13,14). Although the cytotoxic effect of vacuolization is

evident both in vitro and in vivo (15), the toxin does not appear to be lethal to cultured cells (including HeLa, Intestine 407, HEp-2, Y-1, and Kato III cell lines) (10).

Some of the cytotoxic effect of Hp culture supernatant has also been attributed to its urease activity. Although urease alone does not cause cell injury, its degradation of urea liberates ammonia which is known to be toxic to epithelial cells (16,17). The combination of the extracellular toxin and the production of ammonia may have a synergistic cytopathic effect (18).

The aim of this work was to determine if Hp supernatant containing cytotoxin affects gastric cell growth and proliferation in vitro.

MATERIAL AND METHOD

Culture Supernatant

Hp strain Cag, an isolate with known cytotoxic activity was used for this study. The endoscopic biopsy specimen was minced and cultured on TSA agar with 5% sheep RBC at 37°C under microaerophilic conditions with 5% CO2(19). Hp was identified by standard procedures (20) including urease, catalase, and oxidase activity, as well as Gram stain and colony morphology. Colonies from day 3 culture were transferred to Brucella broth with 10% fetal calf serum and grown for 72 hours at 37°C in a 5% CO_2 atmosphere. Culture supernatant was separated from bacteria by centrifugation. The supernatant was then dialyzed against phosphate buffered saline (PBS) for 42 hours to remove small molecular weight molecules, including ammonia. Then it was concentrated 10-fold with a speed-vac concentrator (Savant, Farmingdale, NY, USA). Brucella broth was dialyzed and concentrated identically to serve as a control. Supernatant was diluted at 1/20, 1/100, 1/1000 with PBS.

Cell Culture

Kato III, which is a cell line derived from a gastric carcinoma (ATCC HTB 103) was grown in 25cm² flasks of RPMI 1640 tissue culture medium with 20% fetal calf serum added. Cells (3.5×10^5 cells/ml) were grown at 37°C in a 5% CO_2, 95% O_2 atmosphere. On day 0, 2.3×10^4 cells/ml were placed in each microtiter well. Culture supernatant at various concentrations was then added to the culture media. All tests were run in quadruplicate.

Cell Growth

Cell growth was assessed by counting the number of cells per ml in a cytometer on days 0, 1, 3 and 7.

Cell Proliferation

Cell proliferation was assessed by a immunohistochemical method using 5'-bromo-2'-deoxyuridine (BrdU), a thymidine analogue which binds to single stranded DNA during the S phase of the cell cycle (21). The Kato III cells were incubated with either Hp supernatant or Brucella broth (control) for 2 hours. BrdU (Sigma Chemical Co., St. Louis, MO) at a concentration of 0.1 M was then added for 1 hour. Five slides were made by cytospin for each

group. Slides were dried for 1 hour at room temperature and fixed with acetone for 10 minutes in a 4°C refrigerator. After rinsing three times in PBS, the slides were incubated with 1 : 100 dilution of anti-BrdU monoclonal antibody (Becton-Dickson, Mt. View, CA) for 1-2 hours at room temperature. The sections were rinsed three times for 5 minutes each in PBS, and biotinylated second antibody (50 ul/10ml PBS) was applied for 30 minutes using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), followed by three 10-minute rinses in PBS. Sections were then incubated in avidin-biotin-peroxidase conjugate (Vector) for 60 minutes at room temperature and rinsed for 15 minutes in PBS. Diaminobenzidine tetrahydrochloride (DAB; Sigma) solution was applied for 15 minutes. Sections were counterstained with hematoxylin. Cells with labeled nuclei (being in DNA synthetic phase) were counted under 400 magnification. The labeling index was calculated as the ratio of stained cells to total cells.

Statistical Method

The ANOVA followed by the Fisher PLSD test was used to determine statistically significant differences from 95 to 99.99% confidence.

RESULTS

Cell Growth

Hp supernatant at a titer of 1 : 20 significantly inhibited cell growth on days 5 and 7 compared to control (Brucella broth) while Hp supernatant at a titer of 1 : 100 inhibited cell growth significantly only on day 7 (*Fig. 1*). This cytotoxic effect was not inhibited by boiling the supernatant prior to incubation, ie it is heat-stable.

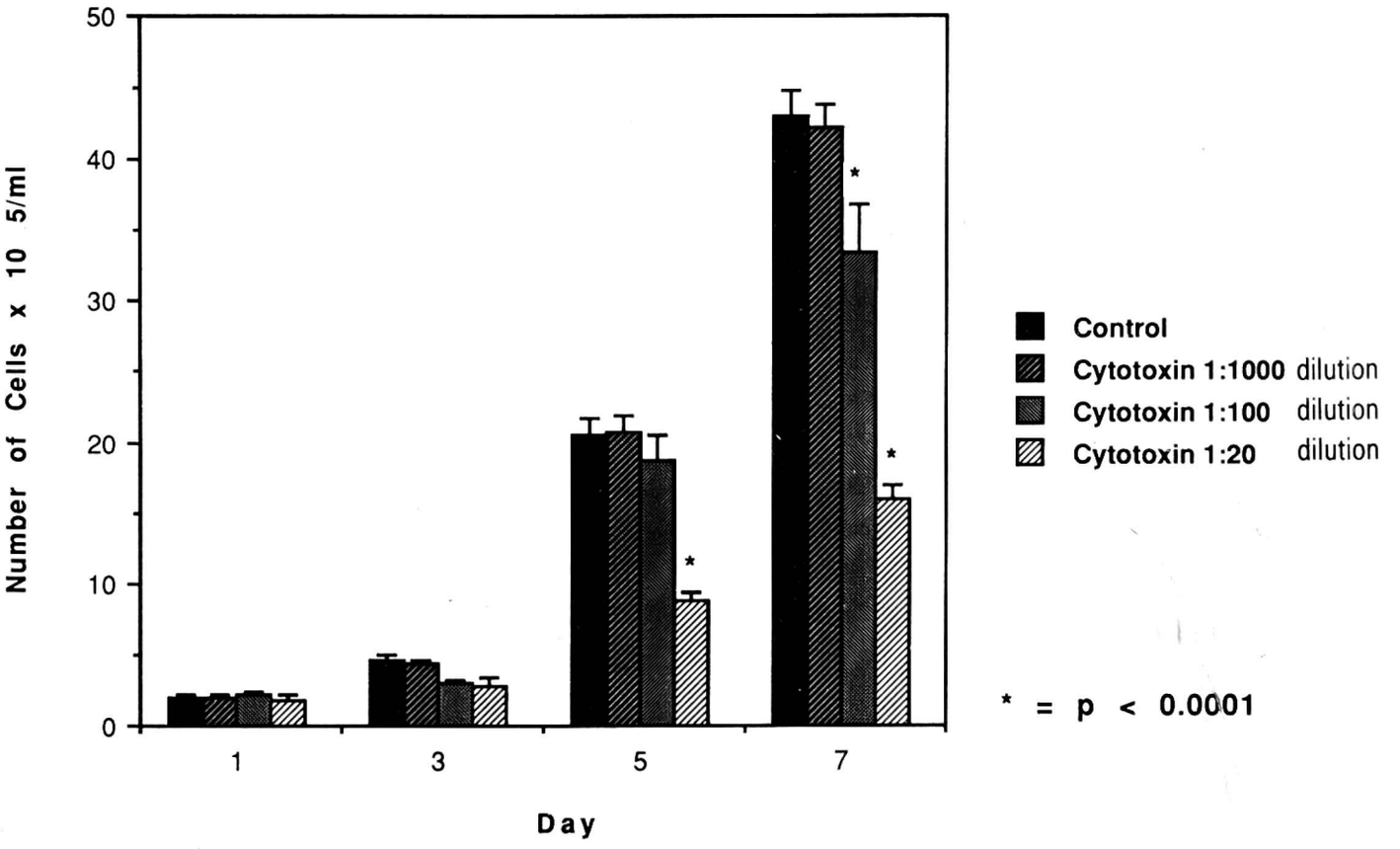
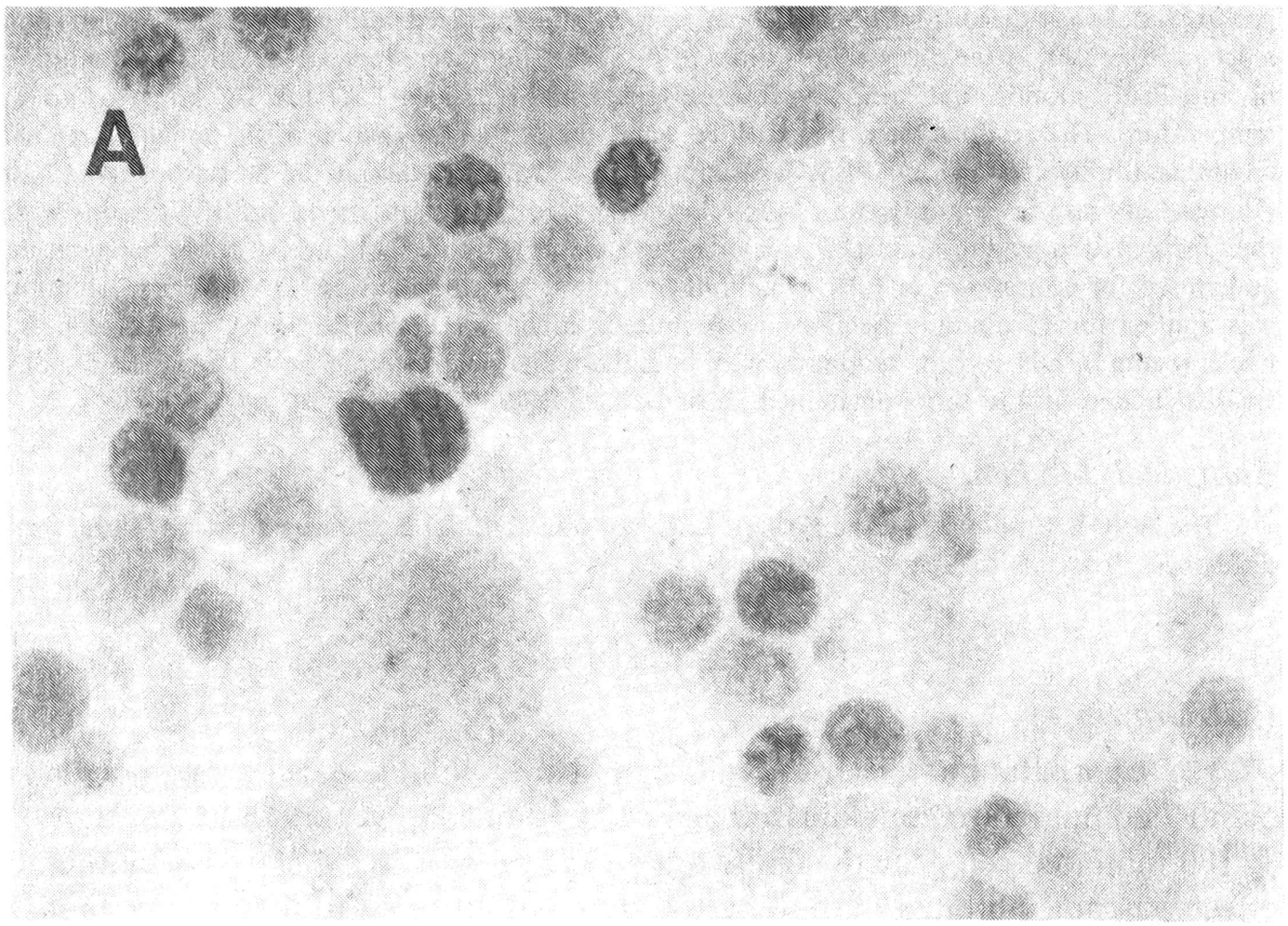


Fig. 1. Comparison of cell counts between Kato III cells exposed to Hp cytotoxin at different titers vs. control on days 1, 3, 5 and 7.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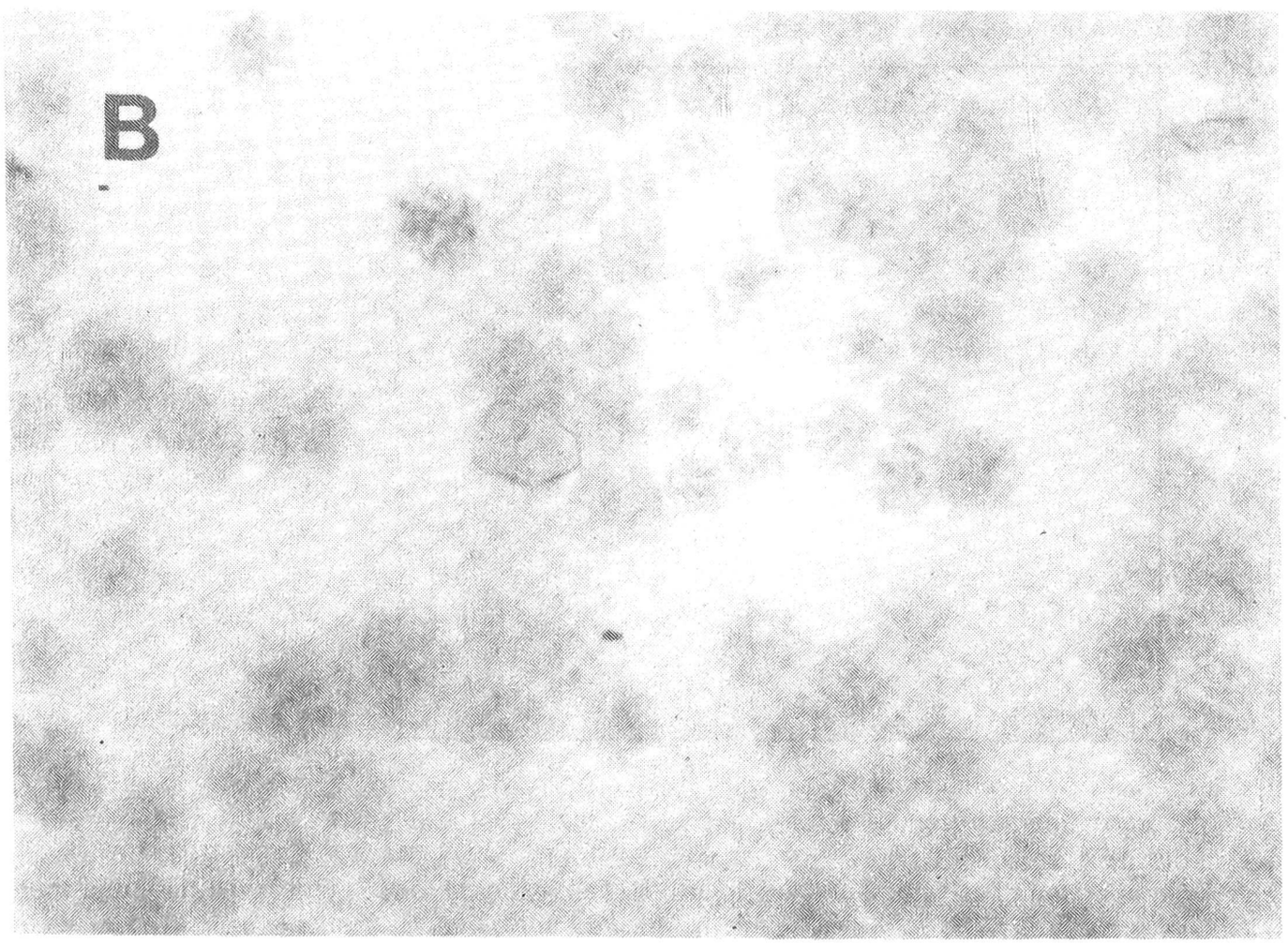
B

Cell Proliferation

After 2 hours' incubation of Kato III cells, the labeling index for BrdU was 8.9% in the cells treated with supernatant at a 1 : 20 titer vs 19.8% in the control ($p < 0.01$) (*Fig. 2*).

DISCUSSION

Cytotoxin from isolates of Helicobacter pylori can cause cytotoxic changes in gastric cells (10,12). These changes are manifested as vacuolization of cells without cell death. Vacuolizing cytotoxin activity is usually observed at 24 to 48 hours (13,18). Whether this cytotoxic activity results in decreased cell proliferation has not been previously demonstrated. We have shown in this report that after a 2 hour incubation, Hp cytotoxin at a titer of 1 : 20 can decrease BrdU uptake by the cell nucleus. This indicates that the cytotoxin may work by inhibiting DNA synthesis, a crucial step in the normal cell cycle. The effect of cytotoxin on cell proliferation is thus very rapid, appearing after a 2 hour exposure.

The decrease in cell proliferation is manifested by the decrease in cell growth, with a significant fall in the growth curve seen by day 5. Whether this cytotoxic effect of Hp occurs in vivo is unknown. If Hp elicits a cytotoxin which causes decreased cell proliferation in vivo, it would impair the gastric mucosa's normal ability to repair itself from various noxious agents. Nakajima et al showed that Hp filtrate inhibits gastric epithelial proliferation stimulated by epidermal growth factor in rabbit fetal gastric cells after a 18 hour incubation (22). Interference with epidermal growth factor or its receptor is a possible mechanism for Hp's effect on cell proliferation and growth. The ability of Hp to retard cell proliferation may attenuate the normal mechanisms of gastric mucosal protection and repair. This may play a significant role in the pathogenesis of Hp-associated diseases.

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Fig. 2. Photomicrograph of Kato III cells stained with BrdU. A) Two-hour culture with medium alone. B) After a two-hour exposure to Hp cytotoxin, BrdU uptake is reducted the reflecting inhibition of DNA synthetic phase.

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Received: September 8, 1992
Accepted: December 21, 1992
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