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# A RECOMBINANT FRAGMENT OF *HELICOBACTER PYLORI* CagA AFFECTS PROLIFERATION OF HUMAN CELLS

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The outcome of *H. pylori* infectins depends on proliferation of various host cells, including lymphocytes, monocytes and epithelial cells. In this study we showed that a recombinant fusion protein carrying an immunodominant region of *H. pylori* CagA antigen affected the proliferation of human cells. The rCagA inhibited PHA-driven T cell proliferation but enhanced the growth of epithelial HeLa cells, especially in the presence of granulocyte macrophage colony stimulating factor (GM-CSF). When THP-1 monocytes and Kato-3 epithelial cells from metastasis of gastric carcinoma were stimulated with GM-CSF, they were also susceptible to the inhibitory effect of rCagA. These results confirmed our earlier suggestion on the inhibition of T cell function by *H. pylori* CagA protein. However, antiproliferative activity of CagA antigen appears to be not restricted to T lymphocytes but modulatory effect of this protein seems to depend on the cell type.

Key words: *Helicobacter pylori, rCagA, cell proliferation*

## INTRODUCTION

*Helicobacter pylori* bacteria colonize host gastrointestinal epithelium with various consequences dependent on the expression of virulence factors by an infective agent (1—2) and on a host immunological status (3, 4). It could explain why prevalence of *H. pylori* infections estimated on the basis of serological tests is much higher than the frequency of *H. pylori*-induced diseases. Moreover, it could explain a great variation in the outcome of *H. pylori*-induced diseases, from a mild non-ulcer chronic gastritis and duodenal ulcer to gastric carinoma (5). Clinical *H. pylori* infections result from multiple gastrointestinal response to bacterial products, including the reactions of epithelial cells as well as the cells of immune system. At least to a certain degree, the outcome of *H. pylori* infections depends on cell proliferation: the

proliferation of monocytes/macrophages which constitute a common cellular element of *H. pylori*-induced inflammation (6), the expansion of T lymphocytes which are effector cells in the development of specific cellular response and helper cells in the differentiation of antibody producing B cells, the proliferation of epithelial cells during ulcer healing and unrestricted multiplication of tumor cells during development of gastric cancer. Recently, the inhibition of cell proliferation by *H. pylori* products was demonstrated in experimental models. The supression of blastogenic response of mitogen- or antigen-stimulated human blood mononuclear cells as well as the inhibition of growth of six different cell lines (937, Jurkat, Ags, Kato-3, Hep-2, P 388D) were observed by Knipp *et al.* (7, 8). The bacterial product (Proliferation-Inhibiting Protein-PIP) affecting the proliferation of human mammalian cell lines has been partially characterized as a protein with an apparent native molecular mass of 100+/− 10 kDa (8). By using isogenic knockout mutant strains the authors excluded the urease, CagA protein and vacuolizing cytotoxin as a cause of PIP activity. The anti-proliferative effect of a soluble extract of *H. pylori* was reversible. In the cultures of U 937 cells it was associated with a $G_0/G_1$ cell cycle arrest (9). Indirect evidence for antiproliferative activity of *H. pylori* in infected patients was also presented (10—12). Peripheral blood lymphocyte responses to *H. pylori* were reduced in *H. pylori*-colonized as compared with uninfected dyspeptic patients. Similarly, antigen specific proliferative responses and interferon $\gamma$ production by gastric lamina propria lymphocytes were depressed in *H. pylori* positive as compared with *H. pylori* negative patients (13). An increase in percentage of lamina propria $CD8^+$ T cells in *H. pylori* positive patients suggested activation of antigen specific suppresion.

Our previous study revealed that *H. pylori* but not *H. felis* bacteria used in a high number completely inhibited blastogenic response of human blood T cells to bacterial antigens and PHA (14, 15). Antiproliferative activity was localized in bacterial somatic fraction. Neither *H. pylori* acid glycine extract containing surface bacterial structures nor sialic acid-specific haemagglutinin expressed suppresive activity. We also excluded bacterial LPS as a possible cause of paralysing effect of somatic fraction. SDS-PAGE analysis revealed the presence of high molecular weight proteins (110 kDa-200 kDa) in *H. pylori* somatic fraction which were absent in bacterial acid glycine extract (14). This suggested that CagA protein (128 kDa) could have been responsible for antiproliferative activity of *H. pylori* somatic fraction. In this study we investigated the influence of a recombinant fusion protein (rCagA) on the PHA-driven response of freshly isolated T lymphocytes of seronegative healthy blood donors and on the growth of three human cell lines (HeLa, Kato-3, THP-1). We found that rCagA inhibited by 50% the proliferative response of T lymphocytes to PHA.

It also affected the growth of immortal cells lines; rCagA stimulated the growth of ephitelial-like HeLa cells but inhibited the proliferation of THP-1 monocytes and Kato-3 cells from gastric carcinoma.

## MATERIAL AND METHODS

### *rCagA*

A recombinant CagA fusion protein carrying an immunodominant region of *H. pylori* CagA antigen, expressed in *E. coli*, was used (2, 16).

### *Mononuclear cells (MNC's)*

Buffy coat samples from healthy seronegative blood volunteers served as a source of peripheral blood leukocytes. Mononuclear cells (MNC) were separated by Lymphoprep gradient centrifugation as recomended by the manufacturer (Nycomed Pharma AJ, Norway). The RPMI-1640 medium containing 10% heat-inactivated FCS, 200 mM L-glutamine and 50 μg/ml gentamicin, all from ICN Pharmaceuticals, Costa Mesa, US (complete culture medium), was used for cell cultures. MNC suspensions ($5 \times 10^6$/ml) were depleted of adherent cells by incubation on plastic tissue culture dishes, for 90 min at 37°C in an atmosphere of 5% $CO_2$. Non-adherent dendritic cells were removed by Metrizamide-gradient centrifugation (Nycomed) (17). Lymphocytes were passed over a nylon wool column to obtain a cell suspension enriched in T cells. To obtain fraction enriched in B cells, lymphocytes were incubated overinght with neuraminidase treated sheep erythrocytes. Rosetted cell suspensions were separated by a Lymphoprep-gradient centrifugaton and non-T cells (B cells) were collected at the interface (14, 15). The viability of cell suspensions was > 90% as determined by Trypan blue exclusion. The purity of T cell (> 90%) and B cell (> 70%) suspensions was examined in cytotoxic assay with DAKO mAb to human T cells or B cells respectively. The cells were incubated with mAb in the presence of fresh rabbit serum as a source of complement and the cell viability was estimated by Trypan blue exclusion.

### *Cell lines*

Three human cell lines from European Cell Culture Collection (ECACC, Salisbury, Wiltshire, UK) were used for the study: HeLa cells-epithelial like cells, Kato-3 cells-derived from metastasis of gastric carcinoma, THP-1 monocytes derived from monocytic leukemia. The cells were grown in complete culture medium for 3—4 days at 37°C in humidified atmosphere of 5% $CO_2$. For estimation of cell proliferation, the cells were harvested from culture flasks, washed once by centrifugation and resuspended in culture medium at $10^6$ cells/ml.

### *Cell cultures for DNA synthesis measurement*

The fractions of MNC (non-separated cells, non-adherent cells, the suspensions enriched in T or B cells) and the cells of human cell lines ($10^5$ in 100 μl) were cultured for 72 h with 100 μl of stimulant in 96 well microplates (Falcon, Becton Dickinson California, US). The mononuclear cells were stimulated with rCagA (5 μg/ml), rCagA and PHA (Sigma, St. Louis, US; 2.5 μg/ml), PHA and urea (0.1 M, a solvent of rCagA fusion protein), PHA alone or urea alone. The HeLa, Kato-3 and THP-1 cells were cultured in medium containing rCagA (5 μg/ml), 1 ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF, Sigma), rCagA and GM-CSF, rCagA or

GM-CSF and urea (0.1 M) or urea alone. The MNC, HeLa, Kato-3 and THP-1 cells in some wells were cultured in medium alone to estimate a spontaneous cell proliferation.

The cell viability in 72-h cultures was determined by Trypan blue exclusion. All cultures contained a similar percentage (90—95%) of viable cells.

To assess the DNA-synthesis, 18 h before the end of cultivation 5 μCi ($^3$H) thymidine was added to each well. Incorporation of the thymidine was measured in LKB-liquid scintillation counter (Wallac Oy Turku, Finland after harvesting the cells onto fibre filters. All cultures were settled in triplicate. The results are expressed as mean cpm/culture ±standard deviation (SD).

## *H. pylori antibody test*

The lack of anti- *H. pylori* antibodies in the sera from blood donors was proved in ELISA test with an acid-glycine extract of *H. pylori* strain 17874 (18).

## *Statistics*

Statistical analysis was performed by Student's t test. The results are expressed as mean values of at least three separate experiments ±standard deviation (SD).

## RESULTS

Although PHA is a T-cell specific mitogen, the proliferation of T lymphocytes stimulated with this mitogen depends on the presence of accessory cells (*Fig. 1*). The response of T cells to PHA was more intensive in

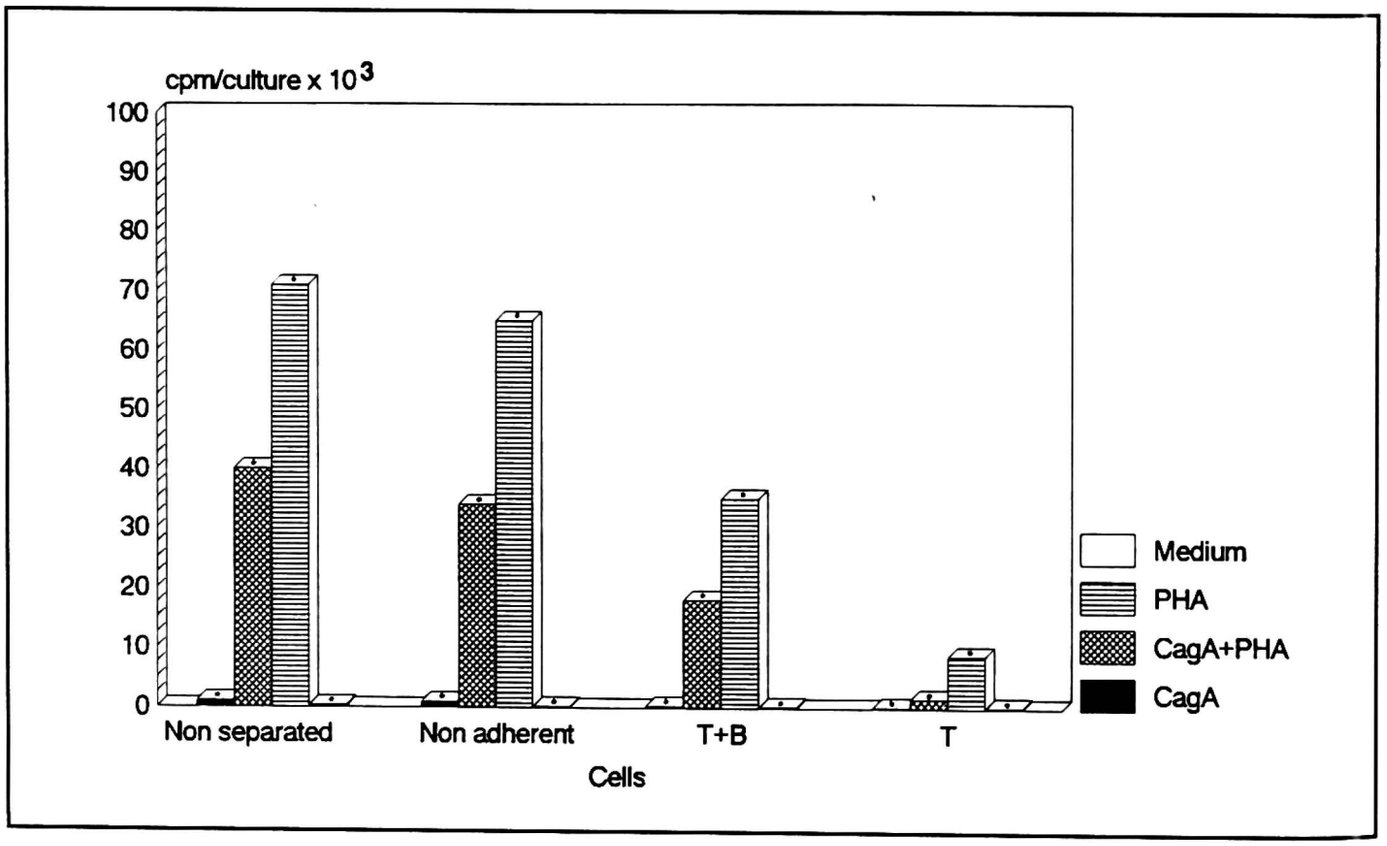


*Fig. 1.* Inhibition of PHA-induced T cell proliferation by rCagA. Blood mononuclear cells MNC: non separated (macrophages, dendritic cells and lymphocytes), non adherent cells (dendritic cells and lymphocytes), T and B lymphocytes, were cultured for 72 h in medium alone (Medium, empty bars) or in medium containing 5 μg/ml rCagA (CagA, black bars), rCagA and 2.5 μg/ml PHA (CagA + PHA, crossed bars) or PHA alone (PHA, lined bars), the last 18 h in presence of 5 μCi ($^3$H) thymidine. Incorporation of isotope was measured in scintillation β counter.

the cultures containing lymphocytes, macrophages and dendritic cells (non separated mononuclear cells) than in the cultures of purified T lymphocytes. However, rCagA (5 μg/ml) inhibited PHA-driven T cell response in the cultures with (inhibition 52%) or without (inhibition 87%) accessory cells (*Fig. 1*). The urea (0.1 M) used as a solvent of rCagA fusion protein slightly increased (6—14%) PHA-induced T cell proliferation (data not shown).

Data in *Fig. 2* demonstrate the effect of rCagA on the growth of human monocyte (THP-1) and epithelial cell (HeLa and Kato-3) lines. Neither rCagA alone nor GM-CSF alone influenced the growth of THP-1 monocytes or Kato-3 epithelial cells. However rCagA affected the proliferation of those cells when they were stimulated with GM-CSF. In the presence of GM-CSF the proliferation of THP-1 and Kato-3 cells was inhibited by 35% and 22%Ø respectively. The proliferation of HeLa epithelial cells was increased by rCagA alone (30% increase) and more intensively by rCagA in the presence of GM-CSF (62% increase) (*Fig. 2*). The urea used as the solvent of rCagA did not influence the proliferation of HeLa cells.

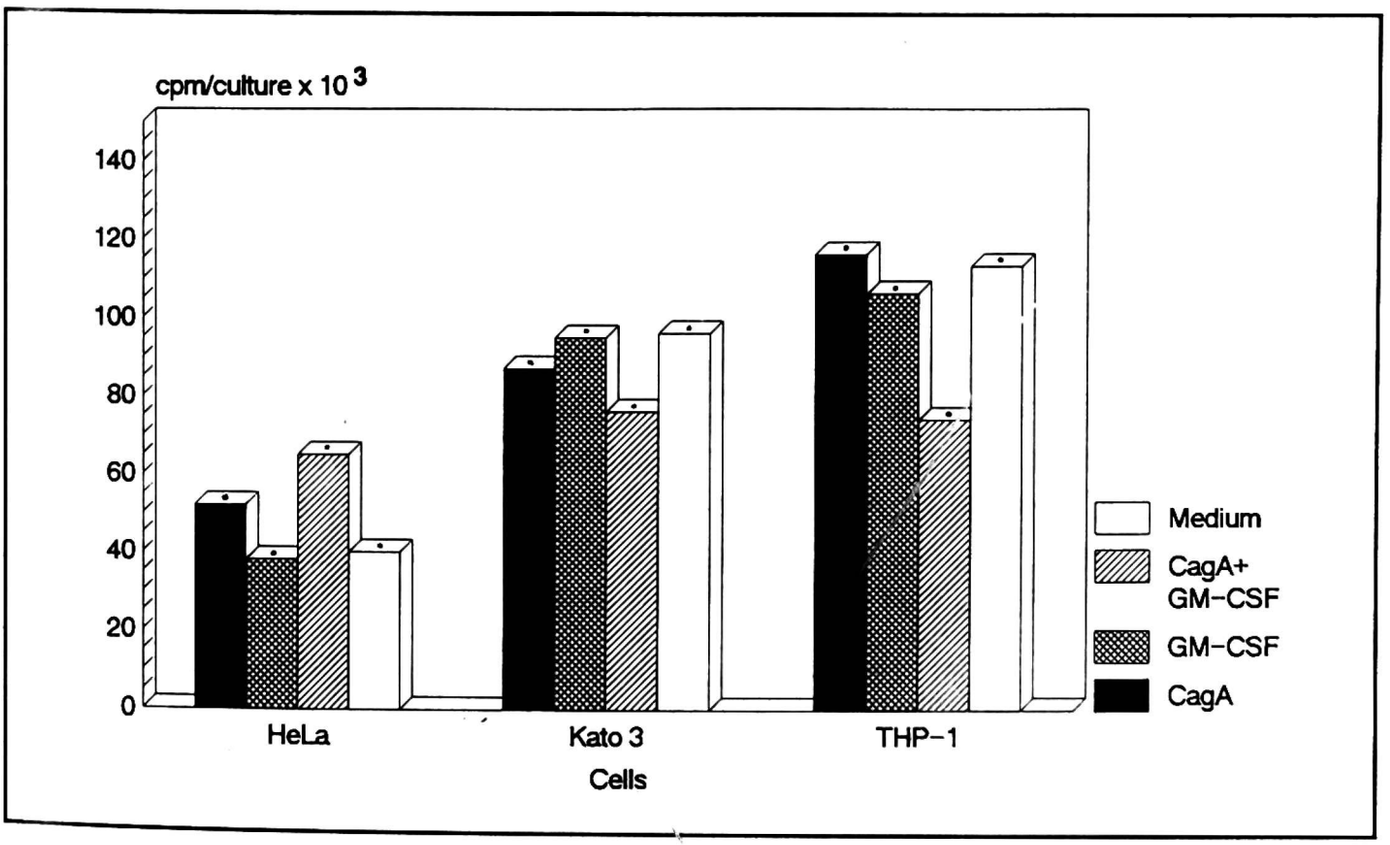


*Fig. 2.* The influence of rCagA on proliferation of human epithelial cells in a presence of GM-CSF. The human cell lines: HeLa, Kato-3, THP-1 cells were cultured for 72 h in medium alone (Medium, empty bars) or in medium containing 5 μg/ml rCagA (CagA, black bars), 1 ng/ml GM-CSF (GM-CSF, crossed bars), or rCagA and GM-CSF (CagA + GM − CSF, lined bars), the last 18 h in a presence of 5 μCi ($^3$H) thymidine. Incorporation of isotope was measured in scintillation β counter.

## DISCUSSION

In this study, T cells from healthy blood donors who did not produce anti-*H. pylori* antibodies were used. Such cells did not proliferate when stimulated with rCagA even in the presence of accessory cells, dendritic cells or dendritic cells and macrophages. In contrast, rCagA (5 μg/ml) inhibited the proliferative response of T lymphocytes to PHA, a known T-cell specific mitogen. This inhibition was stronger in the cultures of purified T lymphocytes (87%) than in the cultures of T cells and accessory cells 52%) suggesting a direct interaction of rCagA with T lymphocytes. On the other hand, the stronger suppresive effect of rCagA in the cultures of purified T cells could be a result of a weak proliferation of T lymphocytes in the absence of accessory cells, especially dendritic cells.

The inhibition of PHA-driven T cell response by rCagA confirmed our previous suggestion that native CagA protein was partly responsible for anti-proliferative activity of somatic fractions of *H. pylori* strains (14, 15). However, these fractions completely inhibited PHA driven T cell response while rCagA reduced proliferation by about 50%. These results suggested that CagA might be one but not the only one bacterial inhibitor of T-cell function. It is very likely that inhibition of proliferative activity of T cells in *H. pylori* infections, may prevent development of specific cellular immunity and elimination of bacteria but favour long-standing infections as well as reinfections in patients who were eradicated in the past by effective treatment. Such an explanation of our results corresponds to the reports on the depression of blastogenic response to *H. pylori* antigens, of peripheral blood and lamina propria lymphocytes from *H. pylori*-infected as compared with uninfected dyspeptic patients (10—12).

It has been demonstrated that rCagA fusion protein used by us presents antigenic determinants recognized by anti-CagA antibodies produced by humans infected with CagA-positive *H. pylori* strains (16, 18). Its is also probable that the interaction of rCagA with T lymphocytes observed by us reflects the interaction of *H. pylori* CagA with these cells. If so, our data on suppressive activity of rCagA towards T cells could explain why duodenal ulceration is more common in subjects infected with CagA-positive versus CagA-negative strains of *H. pylori* (16, 19, 20). Moreover, infection with CagA-positive strains appears to be associated with increased risk of gastric atrophy and gastric caner of intestinal type (6). Although some authors could see no correlation between the CagA expression and the severity of disease (21, 22). An indirect evidence for pathogenic activity of CagA was achieved in mice infected with *H. pylori* isolates. In this model, a persistent colonization of stomach with CagA-positive strains caused cell infiltration and epithelial erosions, while the infection with CagA-negative strains caused a mild gastritis.

Immunization with CagA and *E. coli* LT enterotoxin used as an adjuvant, protected mice against infection with CagA-positive but not CagA-negative *H. pylori* strains (4, 23). So, it appears that neutralization of CagA activity may reduce pathological effects of *H. pylori* bacteria.

Our results revealed that anti-proliferative activity of rCagA was not restricted toT lymphocytes stimulated with PHA. When immortal cell lines were stimulated with GM-CSF, they were also susceptible to modulatory activity of rCagA. The growth of THP-1 monocytes was inhibited by 35%. Obviously, in a CagA-positive *H. pylori* infection characterized by enhanced chemokine production (24), monocytes and macrophages of gastric mucosa cannot avoid activation by the mediators of inflammation. So, it is quite possible that they are susceptible to inhibitory activity of *H. pylori* CagA.

The modulatory effect of rCagA on the growth of epithelial cell lines depends on the cell type. Although the proliferation of Kato 3 epithelial cells was slightly inhibited by rCagA, the proliferation of HeLa epithelial cells was increased by 30% by rCagA alone and by 62% by rCagA in the presence of GM-CSF. Our results do not allow to know if CagA produced *in situ* can stimulate proliferation of epithelial cells as it has been found for rCagA fusion protein. The proliferation of epithelial cells can be beneficial for the host if it concerns restoration of ulcering epithelium (25). However, it can be harmful if it refers to the tumor cells. The association of CagA-positive *H. pylori* infections with gastric cancer has already been demonstrated (6).

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