Helicobacter pylori-associated oxidant monochloramine induces reactivation of Epstein–Barr virus (EBV) in gastric epithelial cells latently infected with EBV

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To investigate the possibility of an interaction between two ubiquitous human pathogens, Helicobacter pylori and Epstein–Barr virus (EBV), the effect of monochloramine (NH2Cl), locally produced by H. pylori infection, on gastric epithelium latently infected with EBV was examined, by assessing the induction of EBV lytic infection. AGS cells harbouring latently infected EBV were used as the indicator of lytic change caused by NH2Cl treatment. Lytic infection, determined by morphological change and EA-D antigen expression, occurred immediately after treatment with in vitro-synthesized NH2Cl. Analysis of EBV infection in human gastric tissue revealed that out of 48 H. pylori-positive patients, 24 were positive for EBER-1, and 18 and 13 were positive for EBNA1 and LMP-1 antigen, respectively. The results suggest that H. pylori-associated NH2Cl induces EBV lytic conversion in gastric epithelium latently infected with EBV.

INTRODUCTION

Chronic microbial infections induce malignant tumours in humans and animals. It is well known that H. pylori infection is closely associated with gastric mucosal atrophy, intestinal metaplasia and subsequent well-differentiated adenocarcinoma of the stomach via a cause–effect relationship between microbial infection and gastric carcinogenesis (Nomura et al., 1991; Parsonnet et al., 1991). Epstein–Barr virus (EBV) is a ubiquitous herpes virus that is well documented to be causally associated with various malignant tumours, including Burkitt’s lymphoma, nasopharyngeal carcinoma, and B-cell lymphoma in immunodeficient individuals, and is also known to be an important aetiologic agent of gastric carcinoma, as is H. pylori (Rickinson & Kieff, 2001). Approximately 7% of gastric carcinomas are reported to be monoclonal proliferations of gastric epithelial cells infected by EBV (Imai et al., 1994), and lymphoepithelioma-like carcinoma of the stomach is the most prevalent phenotype in EBV-infected gastric carcinoma (Wu et al., 2000). All EBV-carrying gastric carcinomas have been reported to have individual single clonotypes of EBV DNA, as determined by terminal repeat (TR) analysis and the presence of EBV-encoded small RNA1 (EBER-1) (Fukayama et al., 1994).

Abbreviations: EBV, Epstein–Barr virus; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; ISH, in situ hybridization; TPA, 12-O-tetradecanoylphorbol-13-acetate.
**METHODS**

**Cell culture.** AGS-neo cells were transfected with recombinant EBV containing the neomycin resistance (Neo') gene at the BZLF1 site of EBV DNA, which is non-essential for infection and replication in AGS cells. The original AGS cell line was a gastric carcinoma cell line derived from a signet ring cell carcinoma of the stomach. This cell line was kindly provided by Professor K. Takada and Dr H. Yoshiyama, Hokkaido University School of Medicine (Yoshiyama et al., 1997). Cells were subcultured twice a week (5 x 10^6 cells ml^-1) in F-12 (HAM) Nutrient Mixture medium (Gibco) supplemented with 10% (v/v) non-hepatinactivated fetal bovine serum (FBS) (Equitech-bio, ICN Pharmaceuticals), 100 U penicillin ml^-1, 100 μg streptomycin ml^-1 and 500 μg geneticin ml^-1. B95-8 and Raji cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated FBS, 100 U penicillin ml^-1 and 100 μg streptomycin ml^-1.

**Preparation of NH2Cl in vitro.** In vitro synthesis of NH2Cl was carried out according to the method of Grisham et al. (1990). Briefly, NH2Cl was synthesized by adding NaOCl to a solution of ammonium chloride in 0.05 M phosphate buffer (pH 8.0). The concentration of NH2Cl was determined assuming a molar extinction coefficient of 429 at 242 nm.

**Detection of EBV EA-D by Western blotting and immunofluorescence microscopy.** Western blotting was performed as described previously (Sumie et al., 2001). Briefly, the cell lysate was subjected to SDS-12.5% PAGE and the resolved proteins were electroblotted onto a PVDF membrane. The blotted membrane was reacted with 1: 5000-diluted mouse anti-EBV EA-D-p52/50 monoclonal antibody (R3, Chemicon International) and subsequently reacted with 1: 5000-diluted peroxidase-conjugated goat anti-mouse IgG. The membrane was treated with the ECL kit-WB detection system (Amersham) and exposed to X-ray film. Monolayer-cultured AGS-neo cells were treated with 1 μg ml^-1 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma) in six-well plates and cultured. After incubation for 2 days, cells were collected and Western blotting was carried out to detect EBV EA-D. Similarly, EBV EA-D was detected after stimulation of AGS-neo cells by in vitro-synthesized NH2Cl. NH2Cl was added at concentrations of 2, 20, 200 and 2000 μM to AGS-neo monolayers in six-well plates. After incubation for 5 min, the cells were collected, and Western blotting was carried out. Alternatively, NH2Cl was added to AGS-neo monolayers in six-well plates at a concentration of 100 μM. After incubation for 2-5, 5-7.5, 10, 15, 20, 30, 40, 50, 60 or 70 min, the cells were collected and Western blotting was performed. The cells were cultured for 48 h at 37°C under the conditions described above. Then, 100 μM NH2Cl or 100 μM NH2Cl combined with 20 mM methionine (Sigma), which is an NH2Cl scavenger, was added prior to immunofluorescent staining for EA-D.

Immunofluorescent staining for EA-D was carried out by indirect immunofluorescence with 1: 40-diluted mouse anti-EBV EA-D-p52/50 monoclonal antibody, as described above, followed by FITC-conjugated anti-mouse IgG (Cappel) as the secondary antibody.

**Flow cytometry.** The expression of EBV-associated antigen, EBNA1, by AGS-neo cells, and of EA-D by Raji and B95-8 cells, was also measured by indirect immunofluorescence flow cytometric analysis using a FACSCalibur (BD Immunocytochemistry Systems) flow cytometer and CellQuest software. Prior to reaction with the primary antibody, cells were treated with permeabilization reagent using IntraPrep Reagent (Immunotech) according to the manufacturer’s procedures, and then 1 x 10^5 cells were incubated on ice for 1 h with 1: 40-diluted anti-EBNA1 rat monoclonal antibody (2B4-1; Dako) and anti-EBV EA-D-p52/50 mouse monoclonal antibody. FITC-conjugated anti-mouse (1: 50) monoclonal antibody was used as the secondary antibody.

**Gastric biopsy specimens.** Seventy-eight gastric biopsy specimens were obtained from 78 patients who presented with gastrointestinal symptoms and were encouraged to undergo stomach biopsy at Oita University Hospital. All patients were examined regarding H. pylori infection status, which was determined by bacterial culture, histology, and urea breath test. Forty-eight patients were positive for H. pylori infection and the remaining 30 patients were negative. The specimens were fixed in formalin, embedded in paraffin wax, and used for in situ hybridization (ISH) and immunostaining. Informed consent was obtained from all patients before study participation. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Oita University.

**Immunohistochemistry for EBNA1, LMP-1 and H. pylori, and ISH for EBER-1.** Anti-EBNA1 rat monoclonal, anti-LMP-1 mouse monoclonal (S-12, Dako) and anti-H. pylori rabbit polyclonal (B0471, Dako) antibodies were used for the identification of the latent infection state of EBV and the localization of H. pylori. A standard immunoperoxidase staining procedure (Vectastain ABC kit, Vector Laboratories) was used for the antigen detection. EBER-1 was detected with a digoxigenin-labelled 30-base oligomer probe (Research Genetics), using procedures described elsewhere (Yanai et al., 1997). Paraffin-embedded sections (5 μm thick) of biopsy specimens were deparaffinized, rehydrated, pre-digested with Pronase and pre-hybridized, and then hybridized overnight at 37°C. After washing with 0.5 × SSC, hybridization was detected using an anti-digoxigenin antibody alkaline phosphatase conjugate (Boehringer Mannheim) according to the manufacturer’s instructions. Confluent cultured AGS-neo cells in a chamber slide (Lab-Tek, Nalge Nunc) were fixed with cold acetone and subjected to a similar ISH technique for the detection of EBER-1.
RESULTS

Characterization of EBV infection status of AGS-neo cells in the resting state and after TPA treatment

The ISH assay demonstrated that EBER-1 was abundantly transcribed in all AGS-neo cells without any lytic stimuli (Fig. 1A). Flow cytometry analysis revealed that AGS-neo cells showed apparent expression of EBNA1 in the resting state (Fig. 1B). The phorbol ester TPA is the most reproducible and widely used inducer of lytic EBV infection. The expression of EA-D antigen in AGS-neo cells was detected in the presence of TPA at a final concentration of 1 μg ml⁻¹. After the treatment with TPA, AGS-neo cells that appeared to be detached from the culture flask (lane 2, Fig. 1C) showed stronger EA-D expression than adherent cells (lane 1, Fig. 1C).

Expression of EBV EA-D after NH₂Cl treatment in vitro

Firstly, we determined whether or not in vitro synthesized NH₂Cl could induce lytic infection in B-cell lines, namely B95-8 and Raji cells, latently infected with EBV. As shown in Fig. 2(A, B), apparent deviations of EA-D expression in B95-8 and Raji cells were observed in the presence of 100 μM NH₂Cl. Although minimal increases were observed after 1 h of NH₂Cl treatment, the degree of increase was clearer after 48 h of treatment.

In order to investigate the induction of EBV lytic infection by NH₂Cl produced by H. pylori infection in gastric mucosa, we studied the effect of in vitro NH₂Cl on AGS-neo cells. Increased expression of EA-D was observed in a dose-dependent manner after the addition of in vitro-synthesized NH₂Cl (Fig. 2C). Such expression was detected as early as 2.5 min after the addition of NH₂Cl, and was sustained for almost 60 min (Fig. 2D). More than 2 h treatment of AGS-neo cells with 100 μM NH₂Cl caused a higher proportion of damaged cells, cell detachment and cell death.

Although confluent AGS-neo cells show an adherent and polygonal appearance (Fig. 3A), cell detachment (Fig. 3B) and EBV EA-D-positive cells (Fig. 3C) were observed following the addition of NH₂Cl at 100 μM. The addition of methionine (20 mM), a well-known specific scavenger of NH₂Cl, prior to the treatment with NH₂Cl completely inhibited the detachment of cells from the bottom of the culture dish (Fig. 3D). Furthermore, the addition of methionine to NH₂Cl-treated AGS-neo cells resulted in inhibition of not only the cell damage but also EA-D antigen expression (data not shown).

Immunohistochemistry of EBNA1, LMP-1 and H. pylori antigen expression and ISH for EBER-1 in gastric biopsy specimens

Next we explored whether EBV-infected gastric epithelium could interact with H. pylori, and also examined the

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associated inflammatory changes in human specimens. Of 48 H. pylori-positive patients, 24 were positive for EBER-1, as determined by ISH (Table 1). In comparison, only two of 30 H. pylori-negative patients were positive for EBER-1 (Table 1). As shown in Fig. 4(A), the EBER-1 signal was localized in a relatively deep area of gastric mucosal layer, and goblet cell metaplasia was occasionally observed close to EBER-1-positive cells. Immunohistochemical analyses using anti-EBNA1 and anti-LMP-1 monoclonal antibodies revealed that among 48 H. pylori-positive patients, 18 and 13 cases were positive for EBNA1 and LMP-1 antigen, respectively (Table 1 and Fig. 4B, C). Positive expression of latent antigen was also localized in relatively deeper areas of the gastric glands. Live H. pylori cells densely colonized the mucosal surface of the H. pylori-infected gastric tissue, but not the deeper area. Inflammatory reactions characterized by dense infiltration of neutrophils and lymphocytes were recognized in the interstitial area of the gastric mucosa (Fig. 4C).

**DISCUSSION**

Infection with EBV occurs in most individuals. EBV is associated with several forms of human neoplasm (Rickinson & Kieff, 2001) and is also known to be an important aetiological agent of gastric carcinoma (Shibata & Weiss, 1992). EBV infection may cause gastric carcinoma in areas of damaged gastric tissue, which is also a well-known pathological consequence of H. pylori infection. Previous studies of mutual interactions between H. pylori and EBV in the human stomach show that the clinicopathological relationship in gastric carcinogenesis remains unclear (Wu et al., 2000; Shinohara et al., 1998). In the present study, we speculated that these two ubiquitous pathogens, H. pylori and EBV, have direct or indirect relevance to environmental changes in the infected host. We assumed that H. pylori or its products could affect the EBV life cycle, especially the viral reactivation process.

To date, the pathogenicity of H. pylori has been demonstrated in terms of epithelial damage, and in particular in terms of the association with gastric carcinogenesis. We demonstrated that half of the gastric biopsy specimens from H. pylori-infected individuals showed positive signals for EBER-1 (Table 1), and such cryptic EBV infection might be affected by H. pylori infection. Firstly, we hypothesized that intracellular signal modification by cagA injection into the AGS-neo cells via type IV secretion machinery might be responsible for the EBV reactivation process (Asahi et al., 2000; Higashi et al., 2002; Mimuro et al., 2002). However, the induction of EA-D antigen was observed at equal levels for H. pylori strains that possessed cagPAI and those that did not.
not (data not shown). Therefore, other mechanisms may be involved in EBV reactivation in the AGS-neo cells. The neutrophil-derived membrane-permeating oxidant NH$_2$Cl is known to induce detachment of cultured cells, concomitant with DNA damage (Suzuki et al., 1997, 1998), as well as morphological changes characteristic of those caused by dephosphorylation of p-Tyr and an increase in intracellular Ca$^{2+}$ concentration (Nakamura et al., 1995). Mobilization of intracellular Ca$^{2+}$ is closely correlated with the induction of ZEBRA protein, which is known to be the immediate early gene product encoded by BZLF1.

Reactivation of the EBV lytic programme in the epithelium, unlike in B lymphocytes, is not clearly understood (Faulkner et al., 2000). EBV BZLF1 protein is known to inhibit host-cell proliferation by causing cell-cycle arrest in G0/G1 in several epithelial tumour cell lines (Cayrol & Flemington, 1996; Rodriguez et al., 2001). After activation of the lytic programme by BZLF1 induction, B95-8 cells are arrested mainly around the G1/S boundary, and the progression from G1 to S phase, in which cellular DNA synthesis is inhibited, appears to favour viral lytic replication (Kudoh et al., 2003). Therefore, the virus replication/reactivation from the latent stage and the host intracellular environment are closely related. It may be considered that EBV makes rapid and strong attempts to survive or to preserve its genome information when the infected host cells

**Table 1.** Incidence of EBV gene (EBER-1) and associated antigens (EBNA1 and LMP-1) by ISH and immunostaining in *H. pylori*-infected and -uninfected individuals

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Incidence</th>
<th>EBER-1</th>
<th>EBNA1</th>
<th>LMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> (+)*</td>
<td>24/48</td>
<td>18/48</td>
<td>13/48</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> (−)</td>
<td>2/30</td>
<td>0/30</td>
<td>0/30</td>
<td></td>
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</tbody>
</table>

*Significantly higher frequency of EBER-1, EBNA1 and LMP-1 in the *H. pylori* (+) group in comparison with the *H. pylori* (−) group at a significance level of $P < 0.01$ by the chi square test.
are confronted with a crisis or death situation (Hoshikawa et al., 2002).

Our study indicates for the first time that the *H. pylori*-associated oxidant NH$_2$Cl is able to induce the lytic conversion of latent EBV infection. Moreover, the switching of EBV from the latent to the lytic phase occurred very rapidly, which should enable the virus to survive or maintain the viral genome, because exposure to higher concentrations (>100 mM) of NH$_2$Cl for over 2 h resulted in cell death (Nakamura et al., 1995), mediated by a ‘peeling off’ phenomenon characterized by marked morphological changes with cell detachment, ballooning and hypercontraction. We speculate that the expression of EA-D antigen in AGS-neo cells is closely associated with the morphological changes that occur during the EBV reactivation process. In a preliminary study, we found by fluorescence-activated cell sorting (FACS) analysis that the expression of Annexin V of AGS-neo cells increased immediately after treatment (within 2–5 min) with 100 mM NH$_2$Cl, and whereas the proportion of viable cells in the presence of 100 mM NH$_2$Cl was more than 90% up to 2–5 min, the proportion decreased by more than 50% after 60 min in the presence of the same concentration of NH$_2$Cl (data not shown). Following the reactivation of the expression of EBV early gene products by *H. pylori* infection or NH$_2$Cl treatment, progeny virus will be released from the AGS-neo cells. Supernatants from *H. pylori*-infected AGS-neo cells successfully transformed peripheral B lymphocytes and showed EBNA1 expression in their cytoplasm (data not shown). These observations are consistent with the hypothesis that EBV reactivation and newly synthesized viral production occur during *H. pylori* infection in the human stomach.

In conclusion, *H. pylori*-associated gastric inflammation induces the production of the oxidant NH$_2$Cl, derived from infiltrating neutrophils, and this converts the latent EBV infection to infection with activation of the early gene, resulting in the induction of lytic EBV infection.

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**REFERENCES**


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