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

Junko Minoura-Etoh,[1] Kazuyo Gotoh,[1] Ryugo Sato,[2] Masao Ogata,[2] Naomi Kaku,[3] Toshio Fujioka[4] and Akira Nishizono[1]

Microbiology[1], Second Internal Medicine[2], Oral-Maxillary Surgery[3] and General Medicine[4], Department of Infectious Diseases, Faculty of Medicine, Oita University, Oita, Japan

Correspondence
Akira Nishizono
a24zono@med.oita-u.ac.jp

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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 (NH_2Cl), 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 NH_2Cl treatment. Lytic infection, determined by morphological change and EA-D antigen expression, occurred immediately after treatment with *in vitro*-synthesized NH_2Cl. 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 NH_2Cl 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.

EBV preferentially infects B lymphocytes via the EBV receptor (CD21), and the major envelope protein of EBV gp350/220 is a ligand for CD21 (Kieff & Rickinson, 2001). Although EBV primarily infects the mucosal epithelium of the oropharynx, such epithelial cells do not possess CD21 molecules on their surface, unlike B cells (Janz *et al.*, 2000). The mechanisms of the primary viral infection of epithelium, especially gastric cells, are not understood. According to a recent report, EBV infections are detected in non-carcinoma gastric epithelium characterized by chronic atrophic gastritis accompanied by intestinal metaplasia (Yanai *et al.*, 1997). Whether such gastric epithelium latently infected with EBV functions as the progenitor of gastric carcinoma or as a producer of progeny virus is not understood. Although the EBV genome is latent in B cells and oropharyngeal/gastric epithelial cells in the carrier state, reactivation of the EBV genome occurs after various stimuli, whereby it enters into the viral lytic cycle (Kieff & Rickinson, 2001). A number of different stimuli or agents *in vitro* have been shown to trigger the switch from the latent infection state into the lytic infection. These include halogenated pyrimidines, nutrient starvation, phorbol esters, calcium ionophores, transforming growth factor beta 1 (TGF-β1), *n*-butyrate, histone deacetylase inhibitors, azacytidine, and cross-linking of surface immunoglobulin (Gradoville *et al.*, 2002). Of these lytic-infection-inducing stimuli, several are capable of leading to cell cycle arrest or cell death (Kudoh *et al.*, 2003).

H. pylori-colonized gastric mucosa is characterized by dominant accumulation of neutrophils and lymphocytes in the infected gastric mucosa; activated neutrophils generate O_2^- and H_2O_2 (Morris & Nicholson,1987). Myeloperoxidase in neutrophils catalyses the oxidation of chloride by H_2O_2 to yield hypochlorous acid (HOCl). The interaction between *H. pylori*-derived NH_3 and HOCl produces monochloramine (NH_2Cl), which is reactive and toxic, and leads to gastric mucosal cell injury via destruction of DNA double strands and chromatin condensation because of its lipophilic properties and low molecular mass (Murakami *et al.*, 1995; Sato *et al.*, 1999; Suzuki *et al.*,1992). In addition, NH_2Cl is known to induce detachment of various types of cells (fibroblasts, epithelial cells and endothelial cells) from culture dishes, with concomitant cell shrinkage (Nakamura *et al.*, 1995).

Thus, both *H. pylori* and EBV infect the human stomach, and therefore may have a synergistic relationship or some other form of impact on each other. The aim of the present study was to examine whether *H. pylori* induces reactivation of the EBV lytic cycle in the human gastric epithelium which these two pathogens commonly colonize, via a cell damage process induced by NH_2Cl production.

METHODS

Cell culture. AGS-neo cells were transfected with recombinant EBV containing the neomycin resistance (Neo^r) 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 (5×10^4 cells ml^{-1}) in F-12 (HAM) Nutrient Mixture medium (Gibco) supplemented with 10 % (v/v) non-heat-inactivated 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 NH_2Cl *in vitro*. *In vitro* synthesis of NH_2Cl was carried out according to the method of Grisham *et al.* (1990). Briefly, NH_2Cl was synthesized by adding NaOCl to a solution of ammonium chloride in 0·05 M phosphate buffer (pH 8·0). The concentration of NH_2Cl 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 NH_2Cl. NH_2Cl 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, NH_2Cl 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 NH_2Cl or 100 μM NH_2Cl combined with 20 mM methionine (Sigma), which is an NH_2Cl 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 immunofluorescent flow cytometric analysis using a FACSCalibur (BD Immunocytometry 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 1×10^5 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 NH_2Cl treatment *in vitro*

Firstly, we determined whether or not *in vitro* synthesized NH_2Cl could induce lytic infection in B-cell lines, namely B 95-8 and Raji cells, latently infected with EBV. As shown in Fig. 2(A, B), apparent deviations of EA-D expression in B 95-8 and Raji cells were observed in the presence of 100 μM NH_2Cl. Although minimal increases were observed after 1 h of NH_2Cl treatment, the degree of increase was clearer after 48 h of treatment.

In order to investigate the induction of EBV lytic infection by NH_2Cl produced by *H. pylori* infection in gastric mucosa, we studied the effect of *in vitro* NH_2Cl on AGS-neo cells. Increased expression of EA-D was observed in a dose-dependent manner after the addition of *in vitro*-synthesized NH_2Cl (Fig. 2C). Such expression was detected as early as 2·5 min after the addition of NH_2Cl, and was sustained for almost 60 min (Fig. 2D). More than 2 h treatment of AGS-neo cells with 100 μM NH_2Cl 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 NH_2Cl at 100 μM. The addition of methionine (20 mM), a well-known specific scavenger of NH_2Cl, prior to the treatment with NH_2Cl completely inhibited the detachment of cells from the bottom of the culture dish (Fig. 3D). Furthermore, the addition of methionine to NH_2Cl-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

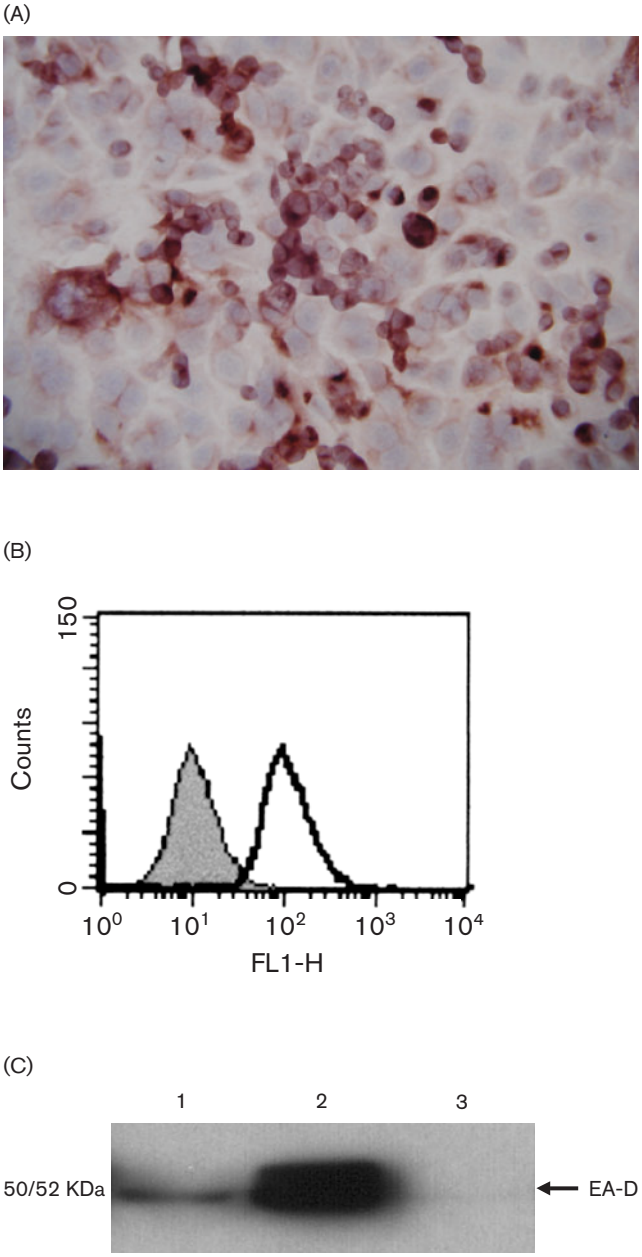

Fig. 1. Characteristics of AGS-neo cells with respect to EBV latent infection and lytic conversion induced by TPA: *in situ* detection of EBER-1 expression in AGS-neo cells. Strongly positive reactions of EBER-1 were seen in AGS-neo cells by ISH (A) and the expression of EBNA1 antigen (white peak) was examined by FACS analysis (B). The grey peak shows the expression of EBNA1 signal without primary antibody. Monolayer-cultured AGS-neo cells were treated with 1 μg TPA ml^{-1} in six-well plates, and cells were then collected separately after 2 days incubation. (C) Western blotting was carried out for the detection of EBV EA-D. Lane 1, cell lysate obtained from adherent AGS-neo cells treated with 1 mg TPA ml^{-1}; lane 2, lysate from detached AGS-neo cells treated with the same concentration of TPA; lane 3, lysate from AGS-neo cells without treatment with TPA.

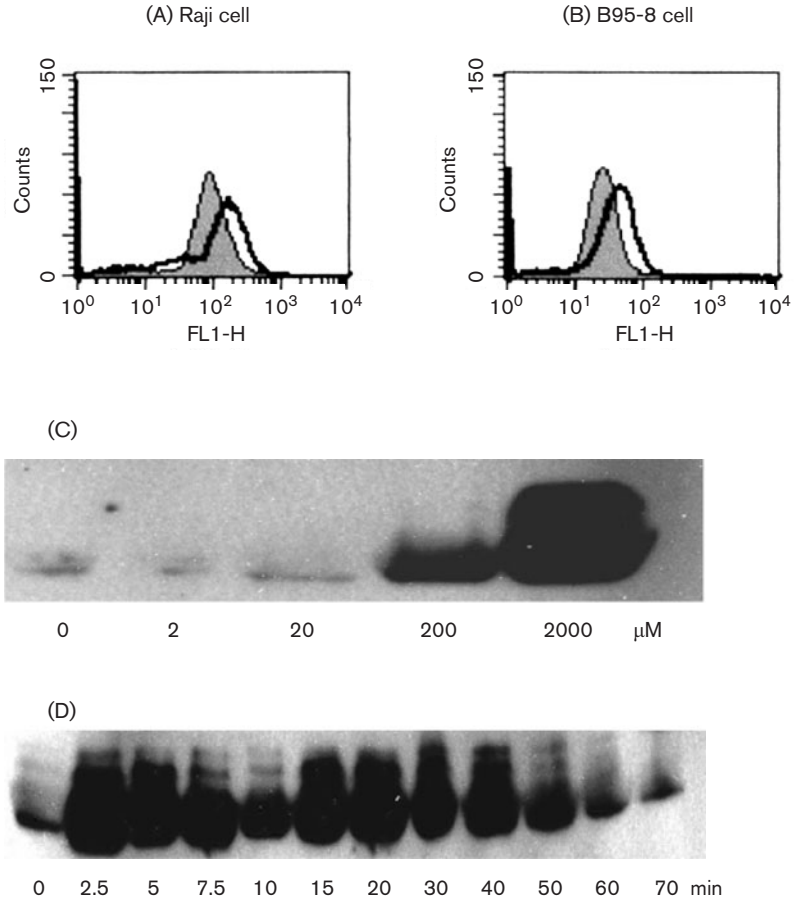

Fig. 2. Effect of NH_2Cl on EBV cells and AGS-neo cells. (A, B) Expression of EA-D antigen on the surface of Raji (A) and B95-8 (B) cells treated with NH_2Cl: after 48 h, the bold line (white peak) shows the level of EA-D antigen in cells treated at a concentration of 100 μM NH_2Cl and the thin line shows the level without NH_2Cl treatment (grey peak). (C) NH_2Cl was added at concentrations of 0, 2, 20, 200 and 2000 μM to AGS-neo monolayers placed in six-well plates. After incubation for 5 min, the cells were collected, and Western blotting was performed with anti-EBV EA-D p52/50 antibody (×5000) as the primary antibody. (D) NH_2Cl was added at a concentration of 100 μM to AGS-neo monolayers in six-well plates. After incubation for 0, 2·5, 5, 7·5, 10, 15, 20, 30, 40, 50, 60 and 70 min, the cells were collected, and Western blotting was performed. EBV EA-D was detected within 2·5 min of stimulation with NH_2Cl.

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 aetiologic 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

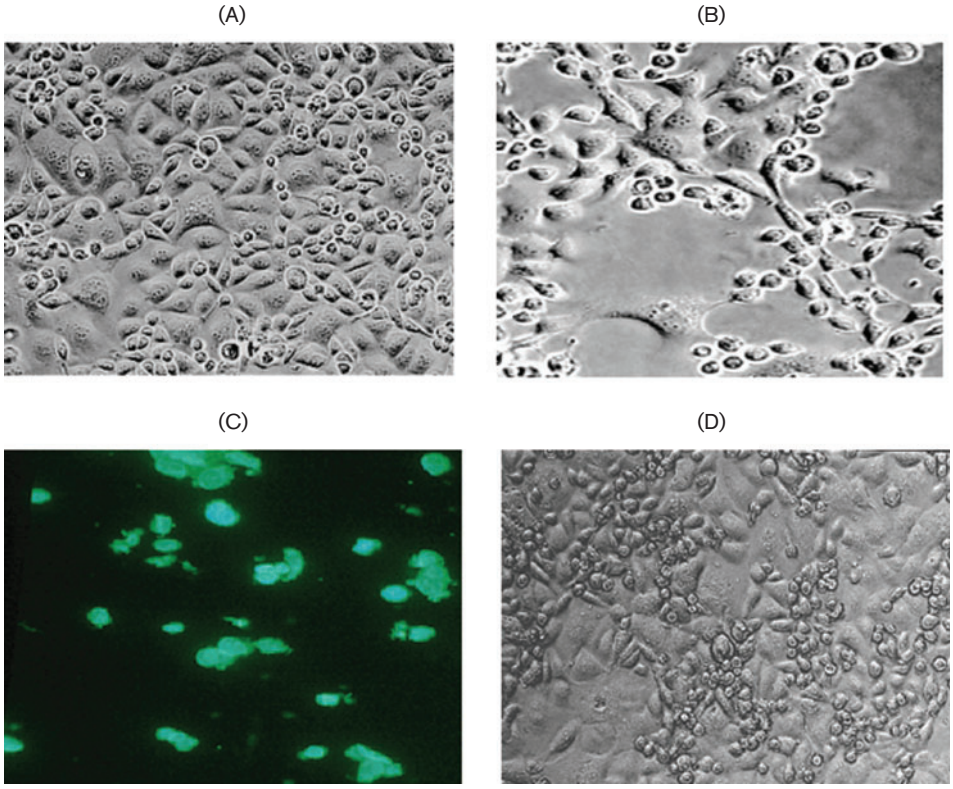

Fig. 3. NH_2Cl induces morphological changes and EA-D expression in AGS-neo cells. Microscopic findings of the effect of NH_2Cl on AGS-neo cells are shown. The cells were cultured for 48 h at 37 °C in F-12 (HAM) Nutrient Mixture medium supplemented with 10 % (v/v) non-heat-inactivated FBS, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 500 μg geneticin ml^{-1} (A). (B) NH_2Cl at 100 μM was added to the confluent AGS-neo cells and the cells were incubated for 60 min. Most cells were round, shrunken and detached from the bottom of the flask. Such damaged cells showed expression of EA-D antigen (C). When 20 mM methionine was added prior to treatment with 100 μM NH_2Cl, the cells completely retained adhesion to the culture dishes (D).

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_2Cl 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.

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

+, *H. pylori* infected; −, uninfected.

Patient group	Incidence		
	EBER-1	EBNA1	LMP-1
H. pylori (+)*	24/48	18/48	13/48
H. pylori (−)	2/30	0/30	0/30

*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 $P < 0{\cdot}01$ by the chi square test.

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

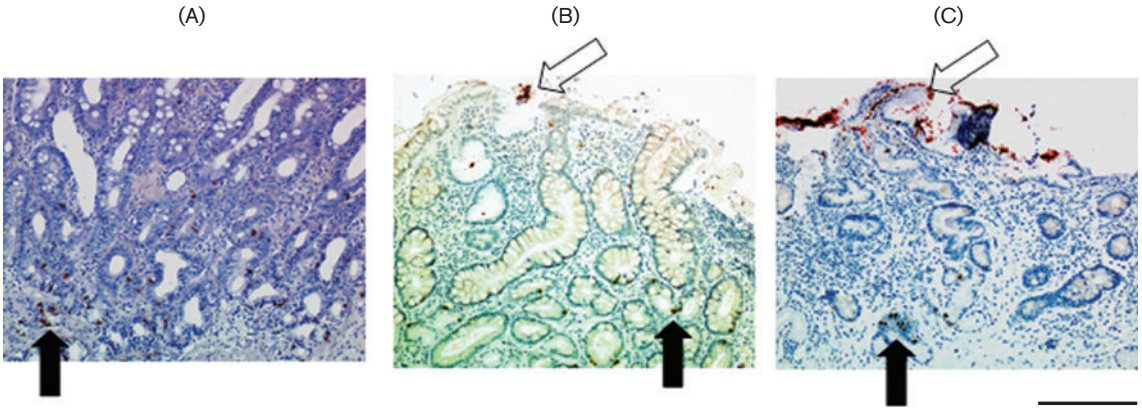

Fig. 4. Detection of EBV latent infection-associated antigens and *H. pylori* in gastric specimens colonized with *H. pylori*. (A) EBER-1 signals (closed arrow) were noted in a deep region of gastric glands with heavy goblet cell metaplasia. (B, C) *H. pylori* bacteria (open arrow) were observed on the surface of gastric epithelium. EBV latent antigen EBNA1 (B, closed arrow) and LMP-1 (C, closed arrow) showed positive reactions in the middle or lower layer of the gastric glandular region with inflammation. Bar, 0·4 mm.

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_2Cl 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 μM) of NH_2Cl 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 μM NH_2Cl, and whereas the proportion of viable cells in the presence of 100 μM NH_2Cl 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_2Cl (data not shown). Following the reactivation of the expression of EBV early gene products by *H. pylori* infection or NH_2Cl 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_2Cl, 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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