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Invasion of Burkitt's Lymphoma Cell Lines by *Yersinia enterocolitica* (41553)

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Abstract. We attempted to determine whether or not *Yersinia enterocolitica* could invade two cell lines derived from Burkitt's lymphoma (BL), P3HR-1, and Raji cells, and if the expression of Epstein–Barr virus (EBV) genome could be activated by the invasion of the organisms into the cells. The invasiveness of *Y. enterocolitica* into BL cells was examined morphologically, using transmission and scanning electron microscopy, and the induction of EBV antigens in the BL cells was examined by indirect immunofluorescence. *Y. enterocolitica* was clearly observed to invade P3HR-1 and Raji cells within 2 hr incubation at 37°, after bacterial challenge. However, the invaded BL cells did not show significant induction of EBV early antigen after cultivation for 72 hr at 37°. Although the present experiment failed to yield positive findings pertaining to the activation of EBV genome, our experimental system per se may still be a useful model when attempting to assess the effects of invading bacteria on the viral genome persistently carried in the host cells.

It has been reported that a repressed Epstein–Barr virus (EBV) genome in BL cells can be activated by halogenated pyrimidines (1, 2), sodium butyrate (3), or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4). The malarial infections in Africa may be possible cofactors in the induction of Burkitt's lymphoma (5, 6). We consider that other microorganisms may also activate a repressed EBV genome in BL cells.

Since the organisms invading BL cells may activate a repressed EBV genome, we decided to use microbes with the ability to invade human cells. There are reports on the intracellular invasiveness of enteropathogenic *Escherichia coil* (7), *Salmonella* (8), *Shigella* (9), *Y. pseudotuberculosis* (10), and *Y. enterocolitica* (11) in human cell cultures. Of these, *Y. enterocolitica* was considered to be a suitable organism as it will invade HeLa cells, thus indicating its pathogenicity (11). We studied the invasiveness of *Y. enterocolitica* into BL cells, morphologically using electron microscopy and the effect of the invasion of the organisms into BL cells on the expression of EBV antigen was examined using an indirect immunofluorescence method.

Materials and Methods. *Cell lines.* Three cell lines, P3HR-1, Raji, and HeLa, were used. P3HR-1 cells (12) were producers and were positive for EBV antigens, viral capsid antigen (VCA), and early antigen (EA) by immunofluorescence tests. Raji cells (13) were nonproducers and negative for their EBV antigens. Both P3HR-1 and Raji cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (250 μg/ml).

HeLa cells were used as controls. The monolayer cultures were grown in Eagle's MEM medium supplemented with 60 μg/ml of kanamycin and 10% calf serum at 37° for 4 days. After trypsinization, the cells were harvested by centrifugation and resuspended in the same medium, at a concentration of 1×10^6 cells/ml.

Bacterial strain. The *Y. enterocolitica* MYO(X)O-3 strain used in the present study was a gift from Mr. T. Morigaki, Kyoto Prefectural Institute of Hygienic and Environmental Sciences. The organisms were initially grown on an agar plate of brain heart infusion (BHI, Difco) at 27° for 24 hr. One loopful of organisms from the agar plate was then suspended in 10 ml of antibiotic-free Eagle's MEM medium (0.2 mg wet weight of bacteria/ml). The resulting suspension was used for bacterial challenge. In addition, one portion killed by heating at 100° for 10 min served as the control.

Invasion of cell lines by organisms. The

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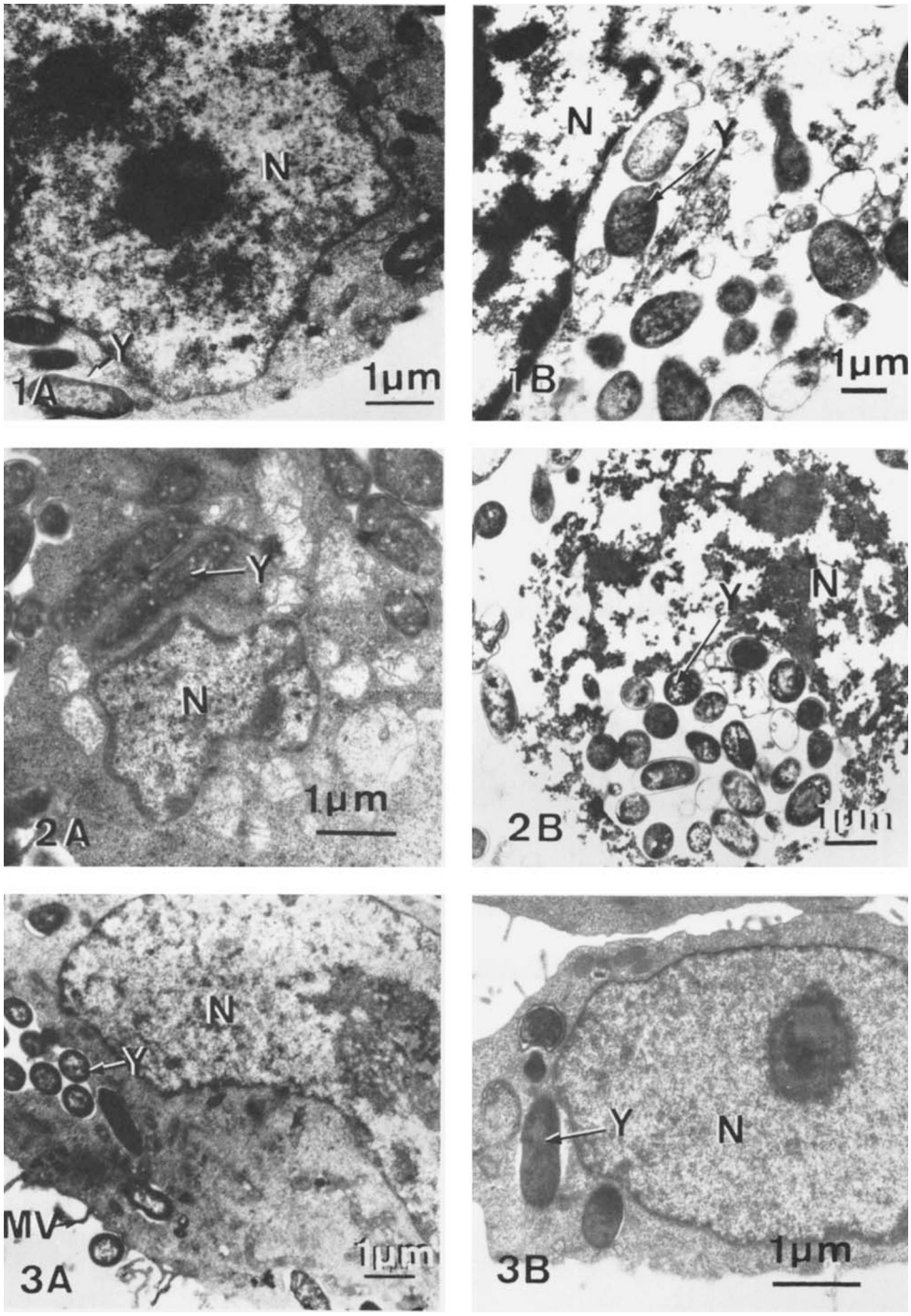

FIG. 1. Transmission electron micrographs showing the presence of *Y. enterocolitica* in the cytoplasm of P3HR-1 cell after bacterial challenge. (A) Incubation period: 2 hr, intracellular *Y. enterocolitica* was not surrounded by a clear peribacillary space. (B) Incubation period: 18 hr, the intracellular *Y. enterocolitica* is present in vacuoles of P3HR-1 and the cell is losing integrity. Y, *Y. enterocolitica*; N, nucleus.

three cell lines, P3HR-1, Raji, and HeLa, were used as target cells. A bacterial challenge experiment was performed using the same procedure as reported by Une (11). Two milliliters of the BL cell suspension containing 1 $\times 10^6$ cells/ml was mixed with an equal volume of the bacterial suspension adjusted to 0.2 mg wet weight of bacteria/ml in antibiotic-free RPMI 1640 medium and the preparation was incubated at 37° for 2 hr. After bacterial challenge, the BL cells were harvested by centrifugation and thoroughly

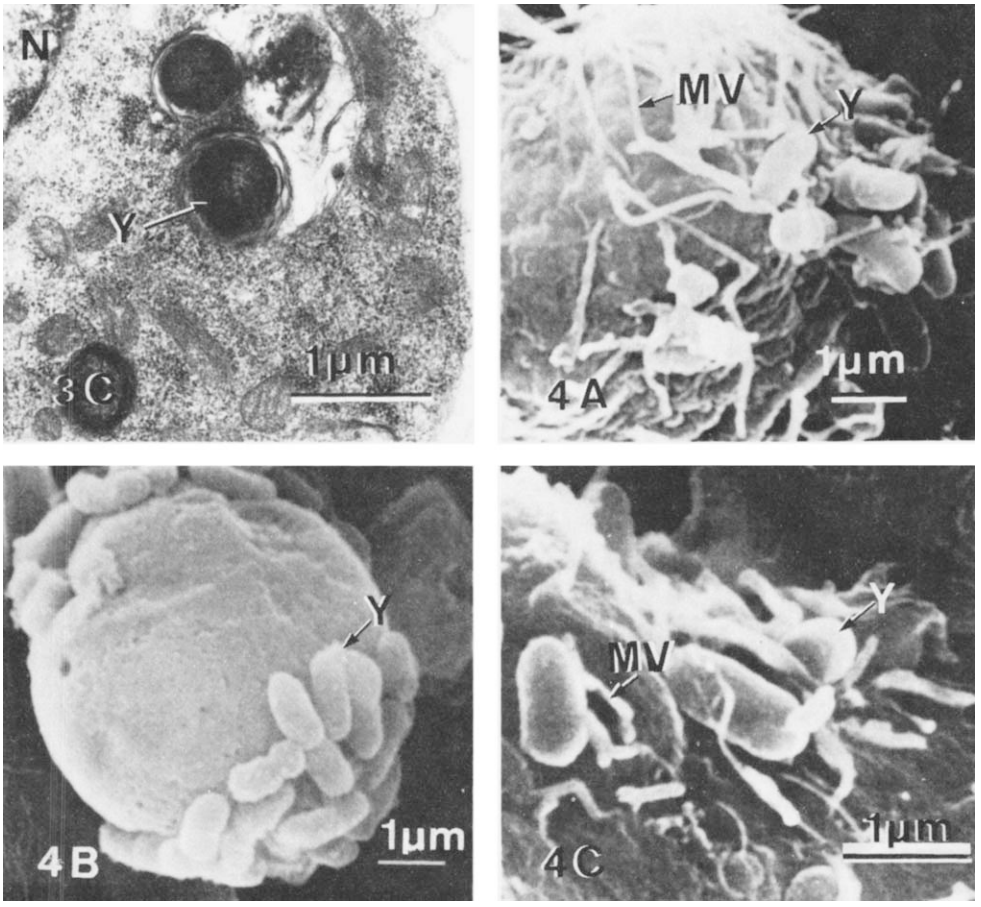

FIG. 2. Transmission electron micrographs showing the presence of *Y. enterocolitica* in the cytoplasm of Raji cell after bacterial challenge. (A) Incubation period: 2 hr, the intracellular *Y. enterocolitica* is not surrounded by a clear peribacillary space. (B) Incubation period: 48 hr, the intracellular *Y. enterocolitica* is present in vacuoles of Raji cell, and the cell is losing integrity. Y, *Y. enterocolitica*; N, nucleus.

FIG. 3. Transmission electron micrographs showing the presence of *Y. enterocolitica* in the cytoplasm of HeLa cell after bacterial challenge. (A) Incubation period: 2 hr, the intracellular *Y. enterocolitica* is enclosed in a phagocytic vesicle. (B) Incubation period: 18 hr, the intracellular *Y. enterocolitica* is digested in part by a phagocytic-like procedure. (C) Incubation period: 72 hr, the disruption of the intracellular *Y. enterocolitica* is proceeding. Y, *Y. enterocolitica*; N, nucleus; MV, microvilli.

FIG. 4. Scanning electron micrographs of *Y. enterocolitica* attached to the three cell lines for 2 hr incubation after bacterial challenge. (A) Attachment of *Y. enterocolitica* to a P3HR-1 cell with microvilli. (B) Attachment of *Y. enterocolitica* to Raji cell without microvilli. (C) Attachment of *Y. enterocolitica* to HeLa cell with microvilli. Y, *Y. enterocolitica*; MV, microvilli.

washed with the same medium to minimize extracellular organisms. The cells were then further incubated for 2, 18, 24, and 48 hr with fresh RPMI 1640 medium containing 5% fetal calf serum and 60 μg/ml of kanamycin to protect the cells from reinfection with the live organisms. The cells were then resuspended in the medium at a concentration of 1×10^6 cells/ml for subsequent electron microscopic observations. With the bacterial challenge experiment using HeLa cells as control, the medium used was Eagle's MEM in place of the RPMI 1640. The challenge procedure and subsequent treatment was carried out in the same order described above, plus an additional 72 hr of incubation.

To determine whether killed *Y. enterocolitica* could invade the three cell lines, the bacterial suspensions were heated at 100° for 10 min and tested for the challenge experiment.

Transmission electron microscopy. The BL cells challenged with the organisms were harvested by centrifugation, fixed by adding 2% glutaraldehyde for 1 hr at 4°, washed twice with 0.1 *M* cacodylate buffer (pH 7.2), and treated with 1% osmiun tetroxide for 1 hr. After postfixation, HeLa cell monolayers were removed from the glass bottle using a rubber policeman and the cells were harvested by centrifugation. The three cell specimens were then dehydrated in a graded series of 30 up to 100% ethanol, cleared in propylene oxide, and embedded in Epon 812. These preparations were sectioned on an LKB 8800 ultrotome III, followed by staining with 10% alcoholic uranyl acetate, 0.1% citrate, and were then examined using JEM 100B transmission electron microscope (JEOL) at 80 kV.

Scanning electron microscopy. The specimens were prepared by the same procedure as described above prior to the dehydration in ethanol. After critical-point drying, they were coated with gold in a JFC-1100 ion sputter (JEOL), and were then examined with a JSM-35C scanning electron microscope (JEOL) at 25 kV.

Immunofluorescence tests. The immunofluorescence was carried out using the method of Henle and Henle (14). P3HR-1 or Raji cells challenged with *Y. enterocolitica* were incubated for 24, 48, and 72 hr at 37°. In the control culture, P3HR-1 cells were adjusted to a concentration of 1×10^6 cells/ml and incubated for the same periods at 37°, with or without 5 m*M* *n*-butyrate (15), and Raji cells were treated with or without 4 m*M* *n*-butyrate plus 25 ng/ml of TPA (16). After incubation, the cells were resuspended in 0.15 *M* phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1×10^6 cells/ml.

A small drop of such suspension was smeared on a slide glass and allowed to dry at room temperature. The smears were then fixed with acetone for 10 min and then dried at room temperature. The smears were incubated for 1 hr at 37° with a 1:40 dilution of standard serum from a patient with nasopharyngeal carcinoma (NPC) (anti-EA titer; 1:1280; anti-VCA, 1:2560). The cell specimens were washed with PBS three times and stained with a 1:20 dilution of FITC conjugated anti-human IgG for 1 hr at 37°. After washing three times with PBS, a cover slip was mounted on the cell smears using 50% glycerol in PBS. Each specimen was examined under a Nikon FT type fluorescence microscope. The induction of EBV antigen-positive cells was examined by counting EA positive in comparison with controls.

Results. *Invasion of cells by Y. enterocolitica.* Observations using the transmission electron microscope revealed that *Y. enterocolitica* invaded the cytoplasm of the P3HR-1 cells within 2 hr after bacterial challenge. Both bacterial and cellular structures were completely maintained for the incubation period (Fig. 1A). On the 18 hr incubation after bacterial challenge, the structures of a few intracellular organisms remained intact, but the cellular structures of P3HR-1 were all but disrupted by that time (Fig. 1B).

In Raji cells challenged with the organisms, the invasion by the organisms occurred within 2 hr of incubation (Fig. 2A). The structures of a few intracellular organisms were stable for 48 hr incubation (Fig. 2B), while the structures of Raji cells began to disrupt. No EBV particles were observed in any of the nuclei or cytoplasms in the P3HR-1 and Raji cells during the first 48 hr of incubation. Slight morphological alteration of the structures of the HeLa cells was observed during the 72-hr incubation after bacterial challenge (Fig. 3C). The electron micrographs showed numerous organisms invading the cytoplasm of the HeLa cells within the first 2-hr incubation (Fig. 3A)

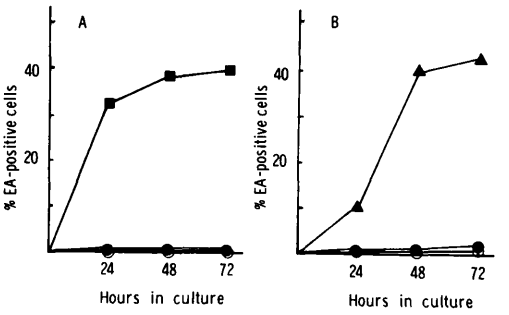

FIG. 5. Effect of invasion of BL cells by *Y. enterocolitica* on the expression of EA. (A) Cultures of Raji cells were incubated (○) without treatment, (●) with *Y. enterocolitica*, and (■) with 25 ng/ml of TPA + 4 m*M* *n*-butyrate. (B) Cultures of P3HR-1 were incubated (○) without treatment, (●) with *Y. enterocolitica*, and (▲) with 5 m*M* *n*-butyrate.

and partial structure disruption of the intracellular organisms by the 18-hr incubation (Fig. 3B). The structures of the organisms were partially disrupted after 72 hr incubation (Fig. 3C).

No organisms were seen in any nuclei of the three cell lines after bacterial challenge. The organisms presented in the BL cells were rarely surrounded by a clear peribacillary space within 2 hr incubation (Figs. 1A and 2A). In contrast, nearly all the organisms observed in the cytoplasms of HeLa cells were surrounded by a clear peribacillary space within the same incubation period. In the case of control studies of the killed organisms, electron microscopy results revealed that the organisms were unable to invade any of the three cell lines during the 48-hr incubation. No morphological alteration of their three cell lines without bacterial challenge was observed during 72 hr incubation.

Scanning electron microscopic findings. The scanning electron microscope was used to visualize the surface features of the three cells during challenging with *Y. enterocolitica*. Organisms were attached to the three cell surfaces without altering the morphological integrity of the cells during the 2-hr incubation after bacterial challenge (Figs. 4A–C). We also observed that the organisms were randomly distributed on the cell surfaces and could be entwined by the microvilli of the P3HR-1 or HeLa cells (Figs. 4A and C). In addition, the micrographs showed that the organisms could attach to the cell surface, even when Raji cell surfaces were smooth and lacked the microvilli (Fig. 4B).

Effect of invasion of BL cells by Y. enterocolitica on the expression of EA. The effect of bacterial challenge on the expression of EA was examined by an indirect immunofluorescence technique. As shown in Fig. 5, the results revealed no significant increases in the percentage of EA-positive cells in both the cultures tested and which were challenged with the organisms.

Figure 5A shows that the EA-positive cells increased little in Raji cultures after bacterial challenge, compared to the untreated control, and the maximum rate of response obtained was 0.1% (72 hr). In the control culture, the positive cells in the Raji cultures treated in combination with 4 m*M* *n*-butyrate and 25 ng/ml of TPA increased 32.2% in the first 24 hr and reached a maximum of 40.1% after 72 hr of continuous treatment. The frequency of EA-positive cells in the untreated cultures was below 0.01%.

Figure 5B shows a slight increase of EA-positive cells in the P3HR-1 cultures after bacterial challenge compared to the untreated control and the maximum rate of response obtained was 1.5% even for up to 72 hr. In the control culture, the treatment of the P3HR-1 cells with 5 m*M* *n*-butyrate markedly increased the number of EA-positive cells and the maximum rate was 43.0% (72 hr), while the frequency of EA-positive cells in the untreated cultures reached a maximum of 0.8% for 72 hr. Attachment of the organisms to the BL cells may slightly increase the number of nonspecific fluorescence-positive cells. Therefore, there is probably little significant difference in the number of EA-positive cells in the BL cells challenged with the organisms, as compared to the untreated controls. A minimum of 500 cells were counted in each preparation.

Discussion. The infectivity of *Y. enterocolitica* to monolayer cell cultures may not be limited to the HeLa cells since its potential invasiveness into porcine kidney cells has been demonstrated by Pedersen *et al.* (17). Thus, the primary purpose of the present experiment was to determine whether or not the organisms were capable of invading the BL cells.

An assessment was made of the fine structure of the BL cells invaded by the organisms and the findings were compared with HeLa cell structures. The transmission electron micrographs showed that the organisms invaded the cytoplasm of the P3HR-1 and the Raji cells within the first 2 hr of incubation, after bacterial challenge. The structures of a few intracellular organisms in the BL cells appeared to be intact during 18 hr incubation after bacterial challenge, while the primary BL cell structures were all but disrupted during the same period. The structures of the HeLa control cells were maintained for 72 hr incubation after bacterial challenge, whereas the structures of the intracellular organisms were partially disrupted during the same incubation.

Heating of the organisms to 100° for 10 min completely inhibited their capability to invade the three cell lines. The HeLa cells are considered to be essentially somewhat phagocytic as it has been reported that there are enclosed small particles such as carbon in the phagocytic vesicles (18, 19), whereas the lymphoblastoid cells such as BL cells are thought to have little phagocytic ability (20). For these reasons, it was considered that the structures of a few intracellular organisms in the BL cells might have been morphologically maintained in a normal state, even when the BL cell structures were disrupted. On the other hand, the structures of the intracellular organisms in HeLa cells seemed to have been disrupted during the phagocytic digestion process. Furthermore, the characteristic feature of the organisms within the BL cells was the predominant absence of an enveloping clear peribacillary space. This may be because the BL cells do indeed have little phagocytic ability. Directly after infection, a few organisms could be detected in the three cell lines and after 18 hr incubation several organisms were present in the cells. These findings suggest that the bacteria multiplied within the cells.

Among the specimens so far examined electron microscopically, no induction of EBV particles was observed in either P3HR-1 or Raji cells, within 48 hr after the bacterial challenge.

In the immunofluorescence tests, the challenge with *Y. enterocolitica* did not significantly increase the number of EA-positive cells either in P3HR-1 or Raji cells, during the 72-hr incubation period.

Although our present experiment failed to yield positive results pertaining to the activation of the EBV genome in BL cells, the experimental system may still be considered to provide a useful model in studying the effects of invasive bacteria on the repressed viral genome.

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