Invasion of *Salmonella* into human intestinal epithelial cells is modulated by HLA-B27

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Abstract

**Objective.** To investigate the influence of the major histocompatibility complex (MHC) class I molecule HLA-B27 on (i) the invasion of *Salmonella* and *Yersinia* into human intestinal epithelial cells, (ii) the survival of intracellular *Salmonella* in these cells, and (iii) the production of certain inflammatory cytokines by the cells after *Salmonella* infection.

**Methods.** The human intestinal epithelial cell line Henle-407 was transfected with HLA-B27 DNA. These cells and HLA-B27-negative control cells were infected with *Salmonella* or *Yersinia*, and viable intracellular bacteria were determined as colony-forming units. Cytokine production was assayed with ELISA.

**Results.** *Salmonella* invaded HLA-B27-positive Henle cells in higher numbers than HLA-B27-negative control cells. However, HLA-B27 did not affect the invasion of *Yersinia* or the survival of the intracellular bacteria in these intestinal epithelial cells. *Salmonella* infection induced production of interleukin-8 (IL-8), IL-6 and monocyte chemotactic protein 1 (MCP-1) by Henle cells that was not affected by HLA-B27 in a specific way.

**Conclusions.** These findings suggest that HLA-B27 enhances the invasion of *Salmonella* into intestinal epithelial cells. The interaction between bacteria and intestinal epithelial cells plays an important role during the early phases of ReA. HLA-B27-linked modulation of *Salmonella* invasion may lead to an increased load of *Salmonella* in intestinal tissue and thus increased susceptibility to reactive arthritis.

**KEY WORDS:** Intestinal epithelial cells, *Salmonella*, Invasion, HLA-B27, Cytokines.

The intestinal epithelium plays a crucial role in the pathogenesis of infections caused by a variety of enteropathogenic microbes and in host defence against them. Gastroenteritis caused by *Salmonella* or *Yersinia* in susceptible individuals who are positive for the class I major histocompatibility complex (MHC) molecule HLA-B27 may be followed by a joint complication, reactive arthritis (ReA) [1]. The association between HLA-B27 and spondyloarthropathies has been known for decades [2, 3]. However, despite intensive research, the way in which the causative agents of ReA with HLA-B27-positive cells may initiate a cascade of events that alters the host’s defence against the microbes and eventually leads to joint inflammation. In this interaction the HLA-B27 molecule may have a biological function distinct from its conventional role as an antigen-presenting molecule.

Yersinia bacteria have been demonstrated by immunofluorescence in intestinal biopsy specimens of patients with seronegative spondyloarthropathy long after initial infection [4], and bacterial antigens have been detected in the peripheral blood and synovial fluid cells of patients with ReA [5–9]. In addition, antibodies against the triggering microbes have been measured in the sera of these patients for an exceptionally long time after the initial infection [10]. It has been thought that the interaction of the causative agents of ReA with HLA-B27-positive cells may initiate a cascade of events that alters the host’s defence against the microbes and eventually leads to joint inflammation. In this interaction the HLA-B27 molecule may have a biological function distinct from its conventional role as an antigen-presenting molecule.

Intestinal epithelial cells secrete an array of proinflammatory cytokines in various pathophysiological conditions. Many invasive bacteria, including *Salmonella*, can induce the expression and up-regulation of several proinflammatory cytokines in human intestinal epithelial cell lines [11, 12]. Some of these chemokines are needed to attract inflammatory cells to the site of infection, thus giving rise to and maintaining the mucosal inflammatory response. Interleukin (IL)-8 and monocyte chemotactic protein 1 (MCP-1) are examples of the most rapidly expressed chemokines secreted by human intestinal epithelial cells after infection with *Salmonella* [13]. While IL-8 effectively


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attracts neutrophils, MCP-1 is a potent chemoattractant for monocytes and memory T lymphocytes and activates basophils [14]. Thus, they play an important role in initiating the first line defence against pathogenic microbes in the intestinal mucosa. Tumour necrosis factor α (TNF-α) and granulocyte-macrophage colony stimulating factor (GM-CSF) are important proinflammatory cytokines, and IL-6 may also have an anti-inflammatory effect during inflammation [15].

Intestinal epithelial cells form an important barrier against pathogenic microbes in the gut mucosa, and serve thus as an interesting target to study the interaction between arthritogenic bacteria and HLA-B27. Our previous studies have shown that the elimination of Salmonella enteritidis is impaired in HLA-B27-positive human monocytic and murine fibroblast cell lines [16, 17]. Accordingly, we investigated whether the expression of HLA-B27 plays a role in the invasion and elimination of S. enteritidis in human enterocytic cell line Henle-407. As inflammatory cytokines have a central role in effective host defence, the production of particular cytokines by Salmonella-infected, HLA-B27-positive intestinal epithelial cells was also studied. Features such as these may be related in vivo to the accessibility of the intestinal epithelium to arthritogenic bacteria and the modulation of the mucosal immune responses of an HLA-B27-positive person, and thus the development of ReA.

Materials and methods

Cell culture

A 6-kilobase genomic clone of human HLA-B*2705 DNA [18] was obtained from Dr J. Taurog (University of Texas Southwestern Medical Center, Dallas, TX, USA). The human intestinal epithelial cell line Henle-407 (CCL-6; American Type Culture Collection, Rockville, MD, USA) was transfected with HLA-B27 DNA (DOTAP) (Boehringer Mannheim Biochemica). These cells were designated ‘B27-Henle’ and ‘mock-Henle’ respectively. Stable transfectants were obtained by continuous selection with 0.5 mg/ml geneticin (Sigma, St Louis, MO, USA).

Cells were grown in disposable flasks (75 cm²; Greiner, Frickenhausen, Germany) or 24-well dishes (1.9 cm²/well; Greiner). Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (PAA Labor- und Forschungsgesellschaft., Linz, Austria). 1.8 mmol/l l-glutamine and 50 µg/ml gentamicin (both from Biological Industries, Kibbutz Beit Haemek, Israel) in a humidified 5% CO₂ atmosphere at 37°C. All cell lines were routinely tested to be free of mycoplasma contamination.

Cell surface expression of the transfected gene was studied by flow cytometry. For this, cells were detached from the cell culture flask by scraping with a rubber policeman and stained by sequential incubation with primary monoclonal antibodies and fluorescein isothiocyanate (FITC)-conjugated F(ab’)-fragments of anti-mouse IgG (Sigma) diluted in buffers containing serum and sodium azide. The ME1 monoclonal antibody against HLA-B27 and W6/32 against class I MHC were used as culture supernatants of the hybridoma cells [American Type Culture Collection (Rockville, MD, USA) (ATCC)]. A subclass-matched negative control antibody was also used. Flow cytometry was performed using a FACSscan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Bacteria

The S. enteritidis strain used in the infection assays was a stool isolate from a patient with Salmonella-triggered ReA. Before infection, the bacteria were grown in Luria-Bertani (LB) broth overnight at 37°C, diluted 1:20 in fresh prewarmed LB, and grown for an additional 2 h to reach the logarithmic phase of growth. S. typhimurium SL1344 (ATCC) was grown overnight in LB broth at 37°C, diluted 1:100 in fresh prewarmed LB, and grown for an additional 3.5 h before infection. Yersinia enterocolitica O:3 (6471/76) [19] and O:8 (8081) [20] were grown in LB broth on an orbital shaker (150 r.p.m.) for 20 h at room temperature, and were used in the stationary phase of growth. According to earlier work and our present experience, these different growth conditions result in Yersinia and Salmonella bacteria with optimal invasiveness.

Infection protocol

Infection of the cells was performed as described [21]. Briefly, confluent monolayers of Henle-407 cells were washed with Hanks balanced salt solution (HBSS), overlaid with fresh IMDM and co-cultured with bacteria (multiplicity of infection, 40–60 bacteria per cell) for 2 h at 37°C. Cells were washed four times with HBSS and overlaid with fresh IMDM supplemented with 50 µg/ml gentamicin. After the periods of time indicated, live and dead cells were counted using trypan blue exclusion. The cells were lysed in the well, and the number of viable intracellular bacteria was determined as colony-forming units (c.f.u.), as described [21]. Briefly, the cells were lysed with 1% Triton X-100, tenfold dilutions were made in microtitre plates, and 20 µl of these dilutions was cultured on LB plates overnight at 37°C, after which bacterial colonies on the plates were counted.

Enzyme immunoassay for cytokines

IL-8, IL-6, MCP-1, TNF-α and GM-CSF proteins produced by Henle-407 cells were determined by enzyme-linked immunosorbent assay (ELISA). After the periods of time indicated, cell culture supernatants of Salmonella-infected and non-infected cells were
collected and cytokine concentrations were assayed with commercial kits. The IL-8, IL-6, TNF-α and GM-CSF kits were purchased from Amersham (Amersham, UK) and the MCP-1 kit was from R&D Systems (Abingdon, UK). The measurements were performed according to the manufacturer’s specifications. Absorbances at 450 nm were read using a Victor Multilabel Counter (Wallac Oy, Turku, Finland).

**Statistical analysis**

Experiments were done at least three times in duplicate or triplicate wells. Statistical analysis was performed using Student’s t-test.

**Results**

**Expression of transfected gene**

The expression of HLA-B27 molecules on the surface of transfected Henle cells was studied by flow cytometry using the monoclonal antibody ME1. The result was clearly positive in HLA-B27 transfected cells (Fig. 1) and negative in other cells.

**Invasion and elimination of S. enteritidis in transfected Henle cells**

Intestinal epithelial cells, represented here by a Henle-407 cell line, function as a barrier against pathogenic microbes in gut mucosa and thus provide an interesting target to investigate the effect of HLA-B27 on invasion and elimination of *Salmonella*. More *S. enteritidis* invaded HLA-B27-positive intestinal Henle-407 cells than HLA-B27-negative control Henle cells ($P < 0.005$; Fig. 2). To examine whether enhanced invasion into B27-Henle cells was specific only for *S. enteritidis*, invasion of some other enteropathogenic bacteria (*S. typhimurium* and *Y. enterocolitica*) into Henle cells was also studied. Interestingly, as shown in Fig. 2, *S. typhimurium* also invaded B27-Henle cells in higher numbers than control Henle cells ($P < 0.002$). In contrast, *Y. enterocolitica* serotypes O:3 and O:8 invaded HLA-B27-positive and -negative cells to an equal extent.

During the first day after infection, the number of viable intracellular *S. enteritidis* organisms increased in all cell lines (Fig. 3). On the basis of several independent experiments, there was no significant difference in the survival of *S. enteritidis* in B27-Henle cells and control Henle cells. The viability of the cells was determined at each time point using trypan blue exclusion.

**Fig. 1.** Expression of cell-surface molecules in Henle-407 cells. The cells were incubated with specific monoclonal antibodies and FITC-conjugated secondary antibody, and analysed by flow cytometry. The expression of MHC class I molecules is shown as open histograms and that of the negative control as filled histograms. The x-axis shows the fluorescence intensity on a log scale and the y-axis shows the relative number of cells.

**Fig. 2.** Invasion of bacteria into parent (Henle), mock-transfected (mock) and HLA-B27 transfected Henle-407 cells (B27). Cell monolayers were infected with *S. enteritidis*, *S. typhimurium*, *Y. enterocolitica* O:3 or *Y. enterocolitica* O:8 and washed after 2 h, and the intracellular bacteria were determined as c.f.u. after incubation for an additional hour in medium with gentamicin. Values are the mean ± s.d. of six (*S. enteritidis*), three (*S. typhimurium*) or two (*Y. enterocolitica*) experiments with triplicate wells. *$P < 0.005$; **$P < 0.002$ vs control Henle cells.*
Discussion

The primary purpose of the present study was to investigate the effect of HLA-B27 on the invasion of Salmonella into human intestinal epithelial cells and its elimination from the cells. An interesting finding was that the expression of HLA-B27 enhanced the invasion of S. enteritidis and S. typhimurium into Henle cells, while Y. enterocolitica serotypes invaded B27-Henle and control Henle cells in equal numbers. The reason for the difference between these two bacterial genera is unknown, although diverse invasion mechanisms provide one possible explanation.

HLA-B27-positive individuals are predisposed to develop ReA after certain mucosal infections. Despite intensive research, the exact mechanisms behind this susceptibility are not known. The main function of MHC class I molecules has been traditionally thought to be antigen presentation, but there is some evidence that their function might not be so limited. Earlier studies by our group have demonstrated that S. enteritidis can survive better in an HLA-B27-positive human monocytic cell line and a murine fibroblast cell line than in HLA-B27-negative control cells in vitro [16, 17]. Also, MHC molecules may play a role in modulating the replication of intracellular viruses [22]. Furthermore, HLA-B27 has been shown to modulate early signal transduction events in Hela cells after S. typhimurium infection [23].

This is the first report in which intestinal epithelial cells have been used to study the effect of HLA-B27 on the invasion of arthritogenic bacteria into cultured cells. Decreased invasion of the bacteria into HLA-B27-positive fibroblasts has been reported by Kapasi and Inman [24, 25], whereas several others have suggested that HLA-B27 does not have a clear-cut effect on invasion of the bacteria [16, 17, 26–28]. The decreased invasion of Salmonella into HLA-B27-positive mouse fibroblasts reported by Kapasi and Inman was not as clear in our study with mouse fibroblasts [17]. Ortiz-Alvarez et al. [28] studied several types of HLA-B27-positive and -negative cells, and did not detect a significant difference in the invasion of the bacteria, even in a cervical epithelial cell line. The diverse results may reflect the properties of different cell lines and bacterial strains, as it is probable that both of these elements have a crucial effect on the outcome of studies. Intestinal epithelial cells are relevant objects when the invasion of

### Production of cytokines by Henle cells

The finding of increased invasion of Salmonella into HLA-B27-positive Henle cells prompted us to examine whether the higher number of invading bacteria would induce these cells to produce more inflammatory cytokines (IL-8, IL-6, MCP-1, TNF-α, GM-CSF) than control cells. Clear induction of IL-8 secretion was detected in all cell lines after infection with Salmonella (Fig. 4). However, on the basis of five independent experiments, there was no clear trend of one cell line being superior in IL-8 production compared with the others.

IL-6 production by Henle cells was also induced after Salmonella infection, although the concentrations produced were considerably lower than those of IL-8 (Table 1). The kinetics of IL-6 production was very rapid; the highest values were measured 3 h after the start of infection (the time point of 1 h) with lower values after that. Similar amounts of IL-6 were detected in all cell lines. Also, MCP-1 production was induced by Salmonella infection in all cell lines, whereas TNF-α and GM-CSF were not clearly induced after the infection in any of the cells (data not shown).

![Production of IL-8 by parent (Henle), mock-transfected (mock) and HLA-B27 transfected Henle-407 cells (B27) after Salmonella infection. Cells were infected with S. enteritidis and after the periods of time indicated, the cells were lysed and viable bacteria were determined as c.f.u. Values are mean ± S.D. of six (1 h) or two (other time points) experiments.](image1)

![Fig. 4. Production of IL-8 by parent (Henle), mock-transfected (mock) and HLA-B27 transfected Henle-407 cells (B27) after Salmonella infection. Cells were infected with S. enteritidis and after the periods of time indicated, the cell culture supernatants were collected and the IL-8 concentration was measured by ELISA. Values are mean ± S.E.M. of triplicate wells from five (Henle and B27 cells) or three (mock-transfected cells) experiments.](image2)
enteropathogenic bacteria is investigated. In vivo, the majority of Yersinia and Salmonella pass through the intestinal epithelium mainly via specialized M cells [29, 30], but these cells are difficult to cultivate in vitro. The fate of the bacteria internalized by M cells is usually to be phagocytosed by the macrophages residing in the invaginations of these cells. Thus, even though they are not as effective, from the bacterial point of view columnar epithelial cells may be a safer route through the intestinal epithelium than M cells.

In the present study, Salmonella and Y. enterocolitica differed with respect to the effect of HLA-B27; we demonstrated increased invasion of Salmonella into B27-Henle cells whereas the presence of HLA-B27 did not influence the invasion of Yersinia. Although several pathogenic properties of these enteric bacteria resemble each other, there are also many differences, for instance in invasion mechanisms. However, both bacterial genera may trigger ReA. Interestingly, the severity and duration of gastroenteritis preceding arthritis is often different in Salmonella and Yersinia infections. The prolonged diarrhoea during acute illness in Salmonella patients was correlated positively with the development of ReA [31, 32], whereas in patients developing ReA after Yersinia infection the gastrointestinal symptoms have often been reported to be mild or even absent [33]. Furthermore, in our recent study, acute Salmonella infection was shown to be more severe in HLA-B27-positive patients and those developing joint symptoms than in HLA-B27-negative patients and those without joint inflammation [34]. It is possible that the effective invasion of intestinal epithelial cells may be more important for Salmonella as an intracellular parasite than for Yersinia, which replicates mainly extracellularly, for instance in Peyer’s patches [35, 36]. It might be that these two bacteria have different target cells for the interaction with HLA-B27 during the development of ReA.

The finding that HLA-B27 did not influence the survival of S. enteritidis in intestinal epithelial cells seems to be inconsistent with our earlier studies with a human monocytic cell line and a murine fibroblast cell line [16, 17]. However, in vivo the functions of intestinal epithelial cells and professional phagocytes differ from each other. Intestinal epithelial cells are not specialized to eliminate microbes, although they do have some innate antimicrobial defence mechanisms, e.g. lysozyme [37], defensins [38], phospholipase A₂ [39] and histone H1 proteins [40]. Rather, intestinal epithelial cells function as a barrier against microbes and have a signalling role in that they can attract inflammatory cells to the site of infection [41]. Also, intestinal epithelial cells are short-lived cells with a renewal rate of 2–5 days [42], and it is not likely that bacteria can reside in them for a long period. Monocytes and fibroblasts, as long-lived cells, provide a more probable reservoir of persisting bacteria. Thus, in these cell lineages the finding of impaired elimination of Salmonella in HLA-B27-positive cells can also have relevance in vivo. Interestingly, it has been reported that S. typhimurium responds to different cellular environments with specific patterns of protein synthesis unique to each cell type [43]. It may be that the interaction between Salmonella and HLA-B27 leads to different responses in various cell types.

An interesting phenomenon in our present study was that, although S. enteritidis invaded B27-Henle cells in higher numbers than control cells, in 1 day the difference disappeared. One explanation for this finding might be that, in addition to increased invasion into HLA-B27-positive cells, bacteria may also exit these cells more easily than control cells. In this in vitro system, gentamicin would then kill the bacteria which have passed through the cells; this would not happen in vivo.

The production of inflammatory cytokines by Salmonella-infected Henle cells was studied to evaluate whether the increased bacterial load in B27-Henle cells would induce these cells to produce more cytokines than control cells. The results indicate that this was not the case, as we did not find a significant difference in the production of cytokines between HLA-B27-positive and -negative Henle cells. The induction of IL-8 and MCP-1 production by Henle cells after infection with Salmonella was in accordance with a previous report of Salmonella-induced production of cytokines by intestinal epithelial cells [12], while the lack of TNF-α and GM-CSF induction was not. However, the cell lines and Salmonella serovar used by Jung et al. [12] were different from those we used, which may explain the different results. Our finding that S. enteritidis infection of Henle cells induced quite low levels of IL-6 agree with the results of a recent study performed with the same cell line using typhoidal and non-typhoidal Salmonella serovars [44].

Our present findings provide additional information about the role of HLA-B27 in the pathogenesis of ReA.
after enteric infection. We demonstrated that the expression of HLA-B27 in intestinal epithelial cells affects the invasion of these cells by Salmonella but does not influence the survival of intracellular bacteria or the induction of inflammatory mediators produced by the cells after Salmonella infection. Although these in vitro findings should be interpreted cautiously, in vivo they might mean that, even though HLA-B27-positive intestinal epithelial cells permit more Salmonella to invade the host than HLA-B27-negative cells, they do not evoke a stronger response that attracts inflammatory cells to the site of infection.

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