The modulation of chlamydial replication by HLA-B27 depends on the cytoplasmic domain of HLA-B27

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Abstract

Objective

The intracellular persistence of viable Chlamydia trachomatis (CT) within the joint is thought to initiate and maintain the inflammatory process in CT-induced arthritis. CT-induced arthritis is associated with HLA-B27. Recently it was shown that HLA-B27, besides being a T-cell restriction element, can directly influence the invasion and/or replication of enterobacteriae and alters salmonella-induced signal transduction. It was the aim of this study to analyze the effect of HLA-B27 on CT-invasion and replication in human host cells.

Methods

Human Hela cells and Hela cells transfected with either HLA-B27 cDNA or controls (HLA-A1 cDNA; HLA-B27 mutant = HLA-B27 without cytoplasmic tail; B27Q10 = HLA-B27 Exon 1-4 linked to Exon 5 of murine Q10) were infected with CT. By direct immunofluorescence chlamydial invasion was determined 4 hours post infection (p.i.), chlamydial replication 2 days and 4 days p.i. The number of infective CT in the different cell lines was determined by titration of the cell lysates on Hep-2 cells with subsequent immunoperoxidase staining.

Results

Invasion was not affected by HLA-B27. However, formation of chlamydial inclusion bodies and replication was suppressed by HLA-B27. Genetically engineered mutants of HLA-B27 (HLA-B27 mutant, B27Q10) lacking the cytoplasmic tail of HLA-B27 did not affect replication.

Conclusion

The reduction of chlamydial replication by HLA-B27 depends on the cytoplasmic domain of HLA-B27, thus providing a new hypothesis for chlamydial persistence in HLA-B27 positive reactive arthritis.

Key words
Chlamydia trachomatis, HLA-B27, reactive arthritis.

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Introduction

The association of *Chlamydia trachomatis*- or Salmonella-induced reactive arthritis and HLA-B27 was initially thought to be mediated by altered antigen presentation by the MHC class I molecule HLA-B27, leading to insufficient elimination of the persisting bacterial organisms from the joint or giving rise to T-lymphocyte mediated autoimmune reactions due to arthritogenic peptides presented by HLA-B27 (1, 2). However, recently it was shown that HLA-B27, besides being a T-cell restriction element, can directly influence enterobacterial invasion and replication and alter enterobacteria-induced signal transduction (3-6).

Yu and co-workers showed that in Hela cells, the expression of c-fos was induced by Salmonella invasion only when the cells expressed the transfected HLA-B27 gene, but not the HLA-A1 gene or a truncated HLA-B27 gene lacking the exons encoding the cytoplasmic domain (3). This study elucidated for the first time the mechanisms by which HLA-B27 can modulate bacterial infections.

No data are available on the effect of HLA-B27 on chlamydial infection. In this regard it is important to stress that Chlamydia-induced arthritis is the only HLA-B27 associated arthritis in which intra-articularly persisting organisms have been unequivocally demonstrated by different groups (reviewed in 7). Additionally, in a rat model of chlamydial infection it has been demonstrated that HLA-B27 transgenic rats develop more severe urogential tract infection compared to control rats (8). Furthermore Chlamydiae are - in contrast to enterobacteriae - obligate intracellular pathogens. Therefore, the analysis of the effect of HLA-B27 on chlamydial infection of the host cell may elucidate the hitherto unsolved mystery of the pathophysiology of persisting chlamydial infections (7).

The aim of this study was to analyze the effect of HLA-B27 on *Chlamydia trachomatis* invasion and replication in human host cells from the site of the original infection and to pinpoint the domain of HLA-B27 reponsible for the modulation of bacterial infection.

Materials and methods

Preparation of Chlamydia trachomatis elementary bodies (EB)

C. trachomatis elementary bodies (EB) were prepared as described previously (9). In brief, infectious EB of *C. trachomatis* serovar K (UW/31/Cx; Washington Research Foundation, Seattle, WA, USA) were grown in HEp-2 cells (human larynx carcinoma epithelial cell line). Serovar K was chosen for analysis because it causes urogenital tract infections and has been shown to induce reactive arthritis. The organisms were purified in a discontinuous gradient of Urografin (Schering, Berlin, Germany) by ultracentrifugation as described (9). Purified EB were resuspended in 1 ml of sucrose phosphate buffer (0.01 M sodium phosphate, 0.25 M sucrose, 5 mM L-glutamic acid, pH 7.2, all chemicals from Sigma, St. Louis, USA) and stored at -80°C. By titration on Hep-2 cells and subsequent indirect immunoperoxidase assay 4.8 x 10^6 inclusion forming units (IFU)/ml were determined.

Flowcytometry analysis

Immunofluorescence detection of surface HLA-B27 was carried out as described previously (3). In brief, cells were harvested and washed twice with HBSS containing 1% bovine serum albumin and 0.2% sodium azide and incubated at 4°C for 60 min with saturating amounts of monoclonal antibody ME1 in the form of culture supernatant (ATCC, Rockville, MD) or isotype control. Besides HLA-B27, ME1 also recognizes HLA-B7, B22, B42, B67, and B73. After being washed the cells were incubated with Fluorescein-isothiocyanat (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin G (Jackson Immunresearch Labs, West Grove,PA) at 4°C for another 60 min. The cells were then washed and fixed with 2% paraformaldehyde in phosphate-buffered saline, and 10^4 cell per sample were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Transfection of Hela cells

The cDNA of HLA-B*2705 and of HLA-A1 inserted into the RSV5.neo

vector (a kind gift of Dr. B. Carreno, Seattle, WA) were transfected into Hela cells (human cervix carcinoma cell line, HLA-typing: HLA-A68,-B72,-Bw6,-Cw12) using Lipofectin following the protocol provided by the manufacturer (GIBCO BRL, Gaithersburg, MD). Mutant HLA-B27 lacking the sixth and seventh exons, i.e. the cytoplasmic tail, was generated as described (3). B27Q10 is a soluble non-membrane expressed molecule containing Exons 1 through 4 of HLA-B*2705 linked to Exon 5 of the secretory murine HLA-molecule Q10. Using PCR, the cDNA of exons 1-4 of HLA-B*2705 was linked to the cDNA of exon 5 of the murine Q10 molecule and cloned into the mammalian expression vector RSV.5neo (10).

The correctness of both mutant HLA-B27 and B27Q10 was proven by DNA sequencing. These constructs were transfected into Hela using Lipofectin. Successful transfection was demonstrated for G418-resistant cell colonies by flowcytometry for the expression of HLA-B27, mutant HLA-B27 and HLA-A1 as described (3). Successful transfection and production of B27Q10 by the transfected Hela cells was demonstrated by intracellular flowcytometry with monoclonal antibody ME1 and by the demonstration of soluble B27Q10-molecule in the supernatant of the transfected cells by isoelectric focusing (10). B27Q10 is a secretory molecule and is therefore not expressed in the cell membrane, as demonstrated by flowcytometry using monoclonal antibody ME1 (10).

These stable transfectants were designated as the "B27-Hela", "B27mutant-Hela", "B27Q10-Hela" and the "A1-Hela". Cells were cultured in RPMI with 10% fetal calf sera at 37°C in 5% CO_2. Media for the transfectants were supplemented with 0.5 mg/ml G418.

Subcloning

B27-Hela is a polyclonal transfected cell line. To control for artefacts due to transfection, subclones of this cell line were generated. For this purpose cells were diluted in RPMI1640 containing 0.5 mg/ml G418 supplemented with 10% FCS and seeded into 96-round bottom plates (Fa. Greiner, Wiesbaden, Germany) with 0.5 cells/well. Growing clones were analyzed for HLA-B27 expression by staining with monoclonal antibody ME1 and flowcytometry. 5 clones with only slightly differing HLA-B27 expression (data not shown) were chosen for further analysis.

Infection of Hela, B27-Hela, A1-Hela, B27mutant-Hela and B27Q10-Hela with Chlamydia trachomatis elementary bodies (EB)

Infectious EB were added to the cells at a multiplicity of infection (MOI) of 0.005 or 0.05 IFU/cell. For identical MOI, one aliquot of purified chlamydial EB was used for infection of the different cell lines. Unabsorbed chlamydiae were removed 2 hours post-infection (p.i.) by washig 5 times in Hanks' balanced salt solution (HBSS). Fresh medium was added to the cells. A control microscopic examination was performed daily. At day 2 and day 4 p.i. the cells were harvested by trypsin digestion and washed.

In a blinded fashion the number of inclusion bodies per cell were determined by immunofluorescence, and the number of infective EB per cell were determined by titration of the cell lysates on Hep-2 cells and a subsequent indirect immunoperoxidase assay. The viability of the infected cells analyzed was > 85% 2 days p.i. and > 80% 4 days p.i. No differences in viability were observed for the different cell lines.

Each experiment was performed at least in quadruplicate using one *C. trachomatis* aliquot for infection for all the cell lines tested to avoid differences due to any variation in the number of Chlamydiae present in different aliquots. Experiments were repeated on different days using different *C. trachomatis* aliquots.

For the analysis of chlamydial invasion, infectious EB were added to the cells at a multiplicity of infection (MOI) of 0.05 IFU/cell. Cells were incubated in infection medium for 4 h at 37°C and washed in HBSS 3 times. Surface bound bacteria were removed as described (11). Briefly, cells were incubated with 4 mg pronase per ml (Pronase, typ XIV, from *Streptomyces griseus*, Sigma). Then cells were sedimented through a fetal calf serum gradient, washed 3 times at 75 g, cytocentrifuged and analysed by direct immunofluorescence in a blinded fashion. The viability of the cells was > 95%; no differences between the different cell lines were observed.

Immunofluorescence microscopy of chlamydial particles

Chlamydial major outer membrane protein (MOMP) was detected by direct immunofluorescence as described (9), with a fluorescein-conjugated murine monoclonal antibody (MAb) (MicroTrak, Syva, Palo Alto, CA, USA). All samples were screened with an epifluorescence microscope (Leitz, Wetzlar, Germany). Only brightly shining green particles or inclusion bodies were counted. Analysis was performed in a blinded fashion.

Indirect immunoperoxidase assay

Cells were incubated for 1 h with the serum of a *C. trachomatis* antibody-positive patient and further incubated with a peroxidase-conjugated goat-anti-human IgG antibody (Sigma) diluted 1 to 40 in PBS. After addition of the substrate 4-chloro-1-naphthol, 1 to 2 in PBS, inclusion bodies were defined as black dots which were counted by light microscopy. Analysis was performed in a blinded fashion.

Statistical analysis

Kruskal-Wallis non-parametric ANOVA, followed by Dunn's multiple comparisons test or one-way ANOVA, followed by Tukey-Kramer multiple comparisons tests, were used. A p-value less than 0.05 was defined as significant.

Results

Invasion was defined as the number of intracellular chlamydial organisms determined by immunofluorescence 4 hours post infection (p.i.). No differences in the invasion of *Chlamydia trachomatis* (CT) into Hela cells, B27-Hela, B27mutant-Hela, B27Q10-Hela and A1-Hela were found (Table I).

Formation of inclusion bodies, i.e. the

Table I. Invasion of *Chlamydia trachomatis* (MOI 0.05 IFU/cell) into Hela, A1-Hela, B27-Hela, B27mutant-Hela and B27Q10-Hela, defined by the number of intracellular chlamydial elementary bodies 4 hours p.i. Results of two different experiments,each performed in quadruplicate on different days, are shown. No significant differences in invasion were found (Kruskal-Wallis non-parametric ANOVA).

Cell line	Mean	SD	No.
Experiment 1			
HELA	29	4	4
A1-HELA	29	3	4
B27-HELA	28	12	4
B27 mutant-HELA	44	1	4
B27Q10-HELA	25	11	4
Experiment 2			
HELA	30	8	4
A1-HELA	42	4	4
B27-HELA	61	2	4
B27 mutant-HELA	53	26	4
B27Q10-HELA	71	14	4

Mean = mean number of intracellular chlamydial elementary bodies per 105 cells; SD = standard deviation; no. = number of different culture wells analyzed.

Table II. Early replication of *Chlamydia trachomatis* in Hela, B27-Hela and A1-Hela defined by the number of inclusion bodies per 10^5 cells 2 days p.i. The cell lines are listed in the order of early replication, i.e. the cell line with the least replication, B27-Hela, is listed first. Results of three different experiments, each performed at least in quintuplicate on different days, are shown.

Cell line	Mean	SD	No.
Experiment 1 (MOI 0.005)			
B27-HELA**	17	5	6
A1-HELA	24	6	5
HELA**	36	13	8
Experiment 2 (MOI 0.05)			
B27-HELA#	414	146	6
HELA*	476	209	6
A1-HELA*#	840	325	6
Experiment 3 (MOI 0.05)			
B27-HELA*	414	118	6
HELA	715	204	6
A1-HELA*	1155	627	5

Mean = mean number of inclusion bodies per 10^5 cells; SD = standard deviation; no. = number of different culture wells analyzed; MOI = multiplicity of infection (IFU/cell) used.
Significant differences between two cell lines (* or # $p < 0.05$; ** $p < 0.01$) on Kruskal-Wallis non-parametric ANOVA are shown.

early replication of CT in Hela, B27-Hela and A1-Hela, was defined 2 days p.i. as the number of chlamydial inclusion bodies determined by immunofluorescence. Early chlamydial replication was significantly reduced only in B27-Hela compared to controls (Table II).

To analyze the possibility that, due to artefacts of the transfection, reduced replication in the HLA-B27 positive cells was observed, five subclones of B27-Hela with only slightly differing HLA-B27 expression as determined by staining with ME1 and flowcytometry (data not shown) were infected with *Chlamydiae*. Early replication was analyzed by immunofluorescence 2 days p.i. and compared to untransfected Hela cells and the original polyclonal B27-Hela cell line. Both the original B27-Hela cell line and all subclones showed significantly reduced chlamydial replication compared to untransfected Hela cells. No significant differences between the different subclones and/or the original B27-Hela cell line were observed (Table III).

Late chlamydial replication was determined 4 days p.i. by immunofluorescence. The number of infective chlamydiae generated (infection forming units) were determined 4 days p.i. by titration of the cell lysates on Hep2-cells and immunoperoxidase staining. Significantly less chlamydial inclusion bodies and infective Chlamydiae were generated in the B27-Hela (Table IV).

To analyze which domain of HLA-B27 is responsible for the modulation of chlamydial infection, early replication in B27 mutant-Hela and B27Q10-Hela were compared with Hela,A1-Hela and B27-Hela. Reduced chlamydial replication was only observed in the B27-Hela, expressing full length HLA-B27. In B27 mutant-Hela and B27Q10-Hela, both producing genetically modified HLA-B27 without cytoplasmic tail, no reduction of chlamydial replication was observed (Table V).

Table III. Early replication of *Chlamydia trachomatis* in Hela, B27-Hela, and 5 different B27-Hela subclones (clones #1,#2, #8, #9,#13) defined by the number of inclusion bodies per 10^5 cells 2 days p.i. Chlamydial replication in Hela was significantly (Kruskal-Wallis non-parametric ANOVA) higher compared with B27-Hela ($p < 0.01$) and subclones #1 ($p < 0.001$),#2 ($p < 0.05$) and #13 ($p < 0.05$). No significant differences between B27-Hela and the subclones were observed. The results of two separate experiments were analyzed.

Cell line	Mean	SD	No.
B27-Hela	113	40	8
clone #1	87	31	8
clone #2	129	39	8
clone #8	146	61	8
clone #9	146	62	8
clone #13	126	34	8
HELA	420*	206	8

MOI = multiplicity of infection (IFU/cell) used; mean = mean number of inclusion bodies per 10^5 cells; SD = standard deviation; no. = number of different culture wells analyzed.

Discussion

The hallmarks of *Chlamydia trachomatis* (CT)-induced arthritis are the persistence of viable, metabolically active *Chlamydia* and the high association of HLA-B27 especially with chronic forms of the disease (1, 7). To date several hypotheses have been examined to link these two characteristic findings (1). Being a MHC class I molecule, HLA-B27 presents antigenic peptides derived from intracellular pathogens to cytotoxic T-cells, leading ultimately (by destruction of the infected cell) to the curing of the intracellular bacterial infection. Therefore, a search was started to find T-cells in synovial fluid specific for CT-derived peptides presented by HLA-B27. So far *Chlamydia*-specific cytotoxic T-cell clones have been described in the mouse only (12). In contrast to *Yersinia*-induced reactive arthritis, where HLA-B27 restricted autoreactive T-cell clones were described (2), such T-cells have not been found in *Chlamydia*-induced arthritis. Other models of altered antigen-presentation, such as HLA-B27 as the potential autoantigen presented by MHC class II molecules (1), therefore have been proposed. However, none of these hypotheses has yet been validated by experimental data.

For this reason an alternative approach to examine the association of HLA-B27 with reactive arthritis by analyzing a direct effect of HLA-B27 on bacterial invasion and replication was initiated

Table IV. Replication of *Chlamydia trachomatis* in B27-Hela and A1-Hela defined by the number of inclusion bodies per 10^5 cells and the number of infection forming units (IFU) per cell 4 days p.i. The B27-Hela and A1-Hela are listed in their order of replication,i.e. the cell line with the least replication, B27-Hela,is listed first. The p-value for the comparison of B27-Hela versus A1-Hela,as determined by Kruskal-Wallis non-parametric ANOVA, is given. The results of 4 different experiments, each performed at least in quadruplicate on different days, are shown.

Cell line	p-value	Mean no. of inclusion bodies	SD	N
Experiment 1 (MOI 0.005)				
B27-HELA	< 0.01	21	6	4
A1-HELA		65	18	4
Experiment 2 (MOI 0.005)				
B27-HELA	< 0.05	13	3	5
A1-HELA		35	13	6
Cell line	p-value	Mean no. of IFU per cell	SD	N
Experiment 3 (MOI 0.05)				
B27-HELA	< 0.001	2.50	0.58	8
A1-HELA		4.45	0.46	8
Experiment 4 (MOI 0.05)				
B27-HELA	< 0.001	0.12	0.02	7
A1-HELA		0.44	0.03	8

MOI = multiplicity of infection (IFU/cell) used; inclusion bodies:mean number of inclusion bodies per 10^5 cells; IFU = mean number of IFU per cell; SD = standard deviation; n = number of different culture wells analyzed.

Table V. Early replication of *Chlamydia trachomatis* in Hela,A1-Hela, B27-Hela,B27 mutant-Hela and B27Q10-Hela, as defined by the number of inclusion bodies per 10^5 cells 2 days p.i. The cell lines are listed in the order of early replication,i.e. the cell line with the least replication,B27-Hela,is listed first. The p-value determined by one-way ANOVA for the comparison of each cell line with B27-Hela is given; no differences were observed between the other cell lines.

Cell line	p-value	Mean	SD	No.
B27-HELA		145	33	8
A1-HELA	< 0.05	256	55	8
HELA	< 0.01	279	76	8
B27 mutant-HELA	< 0.01	297	70	8
B27Q10-HELA	< 0.001	308	75	8

MOI = multiplicity of infection (IFU/cell) used was 0.05; mean = mean number of inclusion bodies per 10^5 cells (the results of 2 separate experiments were analyzed); SD = standard deviation; no. = number of different culture wells analyzed.

recently for enterobacteriae. Kapasi and Inman described the reduced invasion of different enterobacteriae (4, 5) and Granfors *et al.* desribed unaltered invasion but increased replication in HLA-B27 positive host cells (6), whereas others found no effect of HLA-B27 on enterobacterial invasion or replication (3, 13, 14).

Two main explanations for the observed differences are conceivable. Firstly, enterobacteriae are facultative intracellular pathogens. Therefore, the effect of HLA-B27 on enterobacterial invasion or replication in the host cell may vary with each bacterial strain and may not reflect the situation *in vivo*. Secondly, technical reasons – such as the use of different cell lines (epithelial, fibroblast, myelomonocytic of either human or murine origin) or of different controls (untransfected cell lines or human cells from healthy controls) – may account for these differences. For example, in one study cell lines from different individuals were analyzed, leading to very high inter-individual variability and obscuring the potential effects of HLA-B27 on enterobacterial infection (13); in another, controls transfected with other MHC class I genes were missing (14); and in a third study reproduction of the data in multiple HLA-B27 transfected clones was not performed, thus allowing for artefacts due to transfection (6).

In the study presented here, a potential direct effect of HLA-B27 on chlamydial invasion and replication in human host cells from the original site of infection was analyzed for the first time. To understand the biological relevance of an effect of HLA-B27 on bacterial invasion and replication, it is important to stress two differences between *Chlamydiae* and enterobacteriae. Firstly *Chlamydia trachomatis* is an obligate intracellular pathogen in contrast to the facultative intracellular enterobacteriae. Secondly, CT-induced reactive arthritis represents the only HLA-B27 associated arthritis, for which bacterial persistence has been unequiviocally demonstrated (7).

To control for the above mentioned artefacts, untransfected controls, HLA-A1-transfected controls, as well as multiple different HLA-B27 transfected clones and controls transfected with two different forms of mutated HLA-B27 were used in this study. Infection experiments were performed at a low multiplicity of infection, firstly to mimic the situation *in vivo*, where only a very little number of *Chlamydiae* is persisting, and secondly to avoid unspecific cytopathic effects by *Chlamydiae*.

CT primarily infects epithelial cells of the urogenital tract where it also can cause persistent bacterial infection (15). It is for the original site of infection that published evidence suggests a direct effect of HLA-B27 on chlamydial infection. Whittum-Hudson *et al.* reported that HLA-B27 transgenic rats develop more severe urogenital tract infection compared to control rats vaginally infected with *C. trachomatis* (8).

Therefore, Hela cells, a human epithelial cell line from the urogenital tract, was used in this study.

Using a set of different transfectants, we were able to show that HLA-B27 does not modulate invasion, but does suppress chlamydial replication to a certain extent. This suppression is not the result of an artefact due to transfection, because multiple clones transfected with HLA-B27 showed reduced chlamydial replication compared to untransfected or control transfected Hela cells.

Our data allow the hypothesis that HLA-B27 may contribute to chlamydial persistence by suppressing bacterial replication. Suppressed chlamydial replication with reduced presentation of chlamydia-derived peptides to CD8+ T-cells may lead to evasion from efficient immune recognition. Further studies addressing the effect of reduced chlamydial replication on the peptide presentation by HLA-B27 are needed and are currently underway in our laboratory.

We were able to demonstrate that the modulation of chlamydial infection depends on the cytoplasmic domain of HLA-B27. In transfectants with genetically engineered mutants of HLA-B27, with both mutants having in common the lack of the cytoplasmic domain of HLA-B27, the modulation of chlamydial replication was absent. This observation also argues against the modulation being an artefact due to transfection, since the B27 mutant-Hela and the B27Q10-Hela are transfected with the same vector as B27-Hela and differ from this cell line only in terms of the described modifications of the HLA-B27cDNA-insert. Furthermore, it is in line with a recent report that bacteria-induced signal transduction depends on the cytoplasmic domain of HLA-B27 (3). The precise mechanism by which this domain alters chlamydial infection is unknown and needs further investigation. However, with c-fos being selectively induced upon Salmonella invasion only in HLA-B27 positive cells, processes downstream of c-fos induction such as the induction of MCP-1 may be involved (3). The fact that the signal transducing domain of HLA-B27 mediates the modulation of chlamydial infection warrants the analysis of host cell genes induced by Chlamydiae *in vitro* and *in vivo* in order to better understand the pathophysiology of the slow-bacterial infection in reactive arthritis.

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