

Invasion and persistence of *Salmonella* in human fibroblasts positive or negative for endogenous HLA B27

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Abstract

Objective—Analysis of the interaction of enteropathogenic bacteria with HLA B27 transfected murine fibroblasts showed a specific influence of HLA B27 on microbial invasiveness. This possible novel mechanism for the action of HLA B27 should be verified by using endogenous HLA B27 positive and negative human fibroblasts as a model for the direct interaction of arthritogenic bacteria and host cells.

Methods—Fibroblasts were obtained from healthy donors positive or negative for HLA B27; cultivated as monolayers; and infected with *Salmonella enterica* serovar enteritidis.

Results—Invasion and survival of bacteria in human cells was not influenced by the presence of HLA B27. Enhancement of HLA class I molecule expression by treatment of cultures with interferon gamma decreased invasion and survival of bacteria in both HLA B27 positive and negative cells. After disappearance of live bacteria lipopolysaccharide antigens persisted within cells.

Conclusion—Endogenous HLA B27 does not modulate the direct interaction of *Salmonella* with human cells. Non-professional phagocytes are able to limit bacterial survival in cells, and interferon gamma accelerates killing of bacteria, but arthritogenic antigens persist after disappearance of live bacteria.

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Reactive arthritis triggered by infection with enteropathogenic bacteria is a frequent cause of acute inflammatory joint disease.¹ One to three weeks after infection with or without gastroenteritis patients may suffer from mild arthralgias to severe polyarthritis.² After a few weeks to months most patients recover, but some may have ongoing arthritis or even progress to ankylosing spondylitis.³

Spondyloarthropathies including reactive arthritis are associated with the presence of the human MHC class I antigen HLA B27.⁴ However, it is unknown how HLA B27 and the different bacteria might cooperate to initiate arthritis.⁵ Although constitutive HLA B27 positive and negative human monocytes did not differ in their ability to process and express *Yersinia* antigen,⁶ the monocytic cell line U937

demonstrated impaired elimination of *Salmonella* after three to five days⁷ and the B cell line C1R showed decreased invasion⁸ when these cell lines had been transfected with human HLA B27.

Mouse fibroblast L cells transfected with human HLA B27 were shown to have a decreased invasiveness for *Salmonella*⁹ and a prolonged trypsin elution after infection with *Yersinia*.¹¹ However, another research group found no change in invasion caused by the transfection of HLA B27, but an impaired killing of internalised bacteria¹² possibly because of impaired nitric oxide production.¹³ We could confirm a decreased invasiveness of *Yersinia* in HLA B27 transfected L cells, but found no influence of endogenous HLA B27 on invasion or persistence of *Yersinia* in primary human cell lines expressing constitutive HLA B27.¹⁴ This study was focused upon the following issues: (a) comparison of invasion and persistence of *Salmonella* in HLA B27 positive and negative human fibroblasts; (b) fate of live and dead bacteria in these cells; (c) effect of interferon gamma on the *Salmonella* infection in human fibroblasts.

Methods

CELLS

Primary human fibroblasts derived from skin and maternal abortion material were kindly donated by Dr Höhn (Institute of Human Genetics, University of Würzburg, Würzburg, Germany) and grown in RPMI 1640 supplemented with 10% fetal calf serum and glutamine and propagated with trypsin, as has been described.¹⁵ Cells were used for the experiments during passages 4 to 11. Murine fibroblast L cells, transfected with human HLA B27 or not transfected, were donated by Dr E Herrmann, Mainz, Germany, with the kind permission of Dr J Taurog, Dallas, Texas.⁹

HLA B27

Cells were screened for the presence of HLA B27 by polymerase chain reaction kit using specific primers¹⁶ and following the manufacturer's instructions (Dynal, Oslo, Norway). Expression of HLA B27 was found by indirect immunofluorescence and flow cytometry using a FACScan flow cytometer (Becton-Dickinson, California) and two different antibodies to HLA B27 (HLA-ABC-m3; Chemicon International, Temecula, California; Dianova, Hamburg, Germany). We established 20 HLA B27 negative and six HLA B27 positive cell lines

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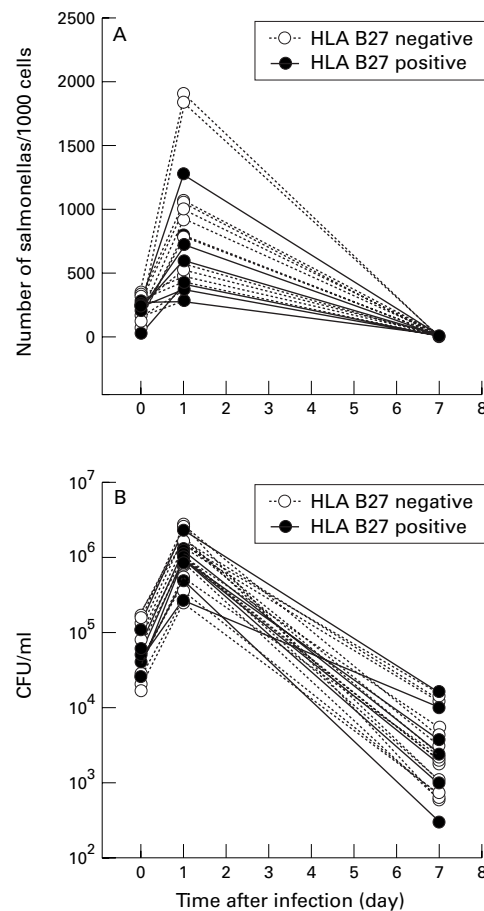


Figure 1 Influence of HLA B27 on invasion and survival of *Salmonella enterica* serotype enteritidis in 26 primary human fibroblast lines, six of which were HLA B27 positive and 20 HLA B27 negative. (A) Number of salmonellas per 100 cells after the infection (invasion) and one and seven days later (survival). Median (range) in HLA B27 positive/negative cells were: day 0: 210 (30–268) / 251 (68–348); day 1: 505 (282–1280) / 794 (282–1910); day 7: 1 (1–6) / 2 (1–4). (B) Colony forming units per monolayer (CFU/ml) after the infection and one and seven days later. Median (range) in HLA B27 positive / negative cells were: day 0: 4.6×10^5 ($2.6–11 \times 10^5$) / 7.4×10^5 ($1.7–17 \times 10^5$); day 1: 9.5×10^5 ($2.7–23 \times 10^5$) / 12.0×10^5 ($2.5–27 \times 10^5$); day 7: 30×10^2 ($3.1–160 \times 10^2$) / 22×10^2 ($6.0–150 \times 10^2$). Comparing the groups of HLA B27 positive and negative cell lines for the number of salmonellas per 100 cells and the CFU/ml at the three time points by U test did not yield any significant differences.

from 26 people. Three of six HLA B27 positive cells have been described before.¹⁴

BACTERIA

Patient isolates of *Salmonella enterica* serotype enteritidis and, for comparison, *Yersinia enterocolitica* O.3 were used. These are the dominant serotypes found in patients with reactive arthritis in Europe. *Salmonella* was grown in brain heart infusion broth (BHIB) overnight. After diluting the culture 1:20 cultivation was continued for three hours at 37°C (mid-log phase). *Yersinia* was grown in BHIB, as has been described.¹⁷ The number of surviving bacteria was determined as colony forming units per monolayer by lysis of cells of infected monolayers with 0.5% tergitol and subsequent plating on Müller-Hinton agar. Giemsa staining of monolayers showed intracellular bacteria

Table 1 Conversion of intact bacterial rods into bacterial 'ghosts', seemingly intact bacterial rods but devoid of DNA. Determination of the percentage of primary human fibroblasts containing bacterial antigen and the percentage of the found bacterial rods that are negative for DNA by propidium iodide. Comparison of cell lines positive or negative for HLA B27

Days after infection	Percentage of cells positive by immunofluorescence for <i>Salmonella</i> antigen		Percentage of bacterial 'ghosts' of all rods	
	HLA B27 neg	HLA B27 pos	HLA B27neg	HLA B27 pos
day 0	50	60	0	0
day 4	70	60	80	70
day 7	75	85	90	90
day 14	85	80	100	100
day 21	90	90	100	100

expressed as the number of bacteria per 100 cells. *Salmonella* antigen was demonstrated by indirect immunofluorescence using a rabbit polyclonal serum against *Salmonella* group D (Behring, Marburg, Germany) or a polyclonal monospecific rabbit serum against *Salmonella enterica* prepared in our laboratory and used at a concentration of 1:10 000¹⁸ and a FITC tagged goat antirabbit immunoglobulin preparation diluted 1:50 (Dianova, Hamburg, Germany). Both serum samples gave similar results. For control uninfected cells were stained or the primary antibody was replaced by normal rabbit serum (Sigma). Propidium iodide (Sigma) was used for staining DNA. Recombinant human interferon gamma was a kind gift from Rentschler (Laupheim, Germany).

INFECTION PROTOCOL

Before infection salmonellas were adjusted to a multiplicity of infection (MOI) of 50 salmonellas per cell in RPMI 1640 cell culture medium. This infectious dose was used as it was not toxic for cells and allowed for a sufficient number of bacteria to enter the cells. MOIs of 0.01 up to 1000 did not increase the number of detached cells in the supernatant nor decrease the number of cells in the culture dishes immediately after the infection and two days later. Subconfluent cells were infected at 37°C with the bacterial suspension for 30 minutes, then washed and incubated for two hours in medium containing 16.2 µg/ml gentamicin

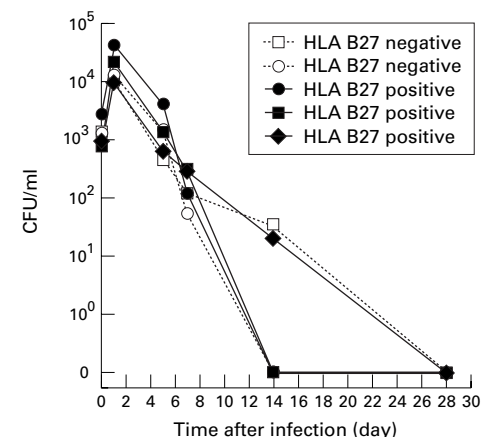


Figure 2 Persistent infection of *Salmonella enterica* serotype enteritidis in primary human fibroblasts. Three cell lines are HLA B27 positive, two lines HLA B27 negative.

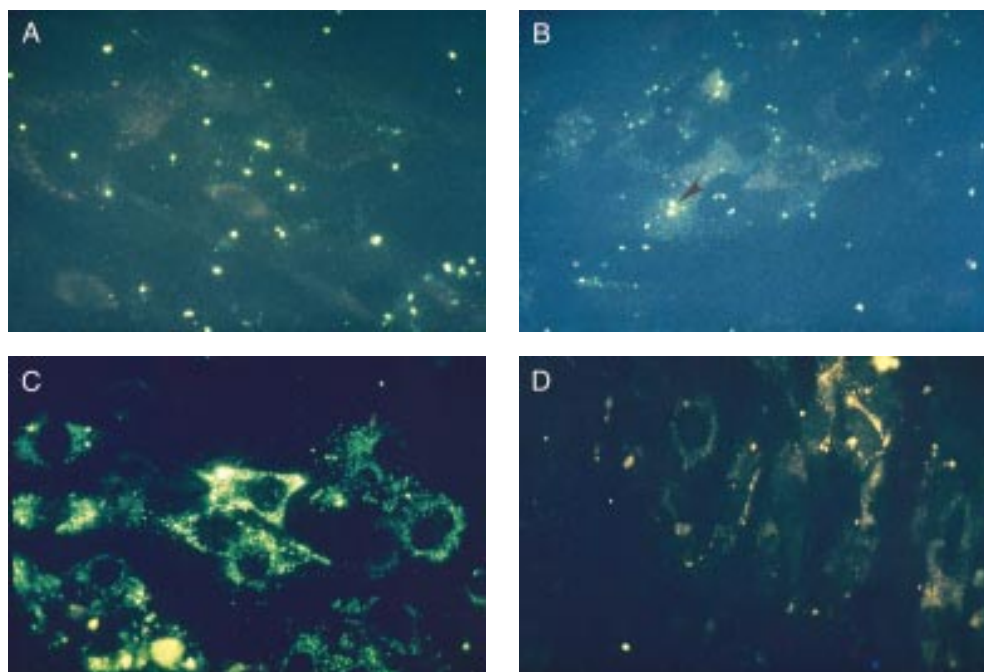


Figure 3 *Salmonella* antigen in primary human fibroblasts by indirect immunofluorescence staining for *Salmonella* lipopolysaccharide antigens directly after infection and two hours of antibiotic treatment (A) and seven (B) and 21 days later (C). Initially intact bacterial rods prevail. After seven days aggregates of cytoplasmic antigen deposits can be seen (arrowhead). After 21 days, at a time when the culture had become sterile, bacterial rods can no longer be identified, but finely dispersed antigen deposit can be seen in the cells. For control a parallel culture was stained with normal rabbit serum after 21 days of infection (D); to show cell morphology exposure time was doubled in comparison with fig 3C. Staining with the normal rabbit serum shows no green colour, but only faint yellow non-specific background staining.

(Merck, Darmstadt, Germany) to kill extracellular bacteria. After a further washing step, cells were grown in medium with 6.5 µg/ml gentamicin. The minimal inhibitory concentration for gentamicin was 1.25 µg/ml. At gentamicin concentrations of 16.2 µg/ml or greater, the number of salmonellas that could be grown from lysed monolayers decreased after five days of culture. Pretreatment of cells for five days with gentamicin at 16.2 µg/ml or greater followed by the standard protocol diminished intracellular survival of salmonellas; pretreatment with 6.5 µg/ml or 3.3 µg/ml or no treatment had no effect. Therefore, the standard protocol permitted protection of internalised salmonellas from gentamicin treatment and killing of extracellular salmonellas.

STATISTICS

Statistical analysis was done with the Mann-Whitney U test or the two way analysis of variance with repeated measures (ANOVA).

Results

When mouse L cells with or without transfected HLA B27 were infected with salmonellas the number of bacteria detected in three different experiments by Giemsa stain or by colony forming units was inferior in transfected cells after incubation of cells with bacteria (invasion) and one and seven days later (survival). To investigate if this was a specific effect of HLA B27 or due to the artificial system of non-human cells transfected with the human gene, we infected 26 primary human fibroblast lines, six of which were HLA B27 positive, with *Salmonella enterica*.

The number of salmonellas per 100 cells and the colony forming units after incubation and one and seven days later were not different between HLA B27 positive and negative cells (fig 1).

To assess if the effect of HLA B27 on the fate of *Salmonella* in human cells might become apparent at a later stage of infection, cultures

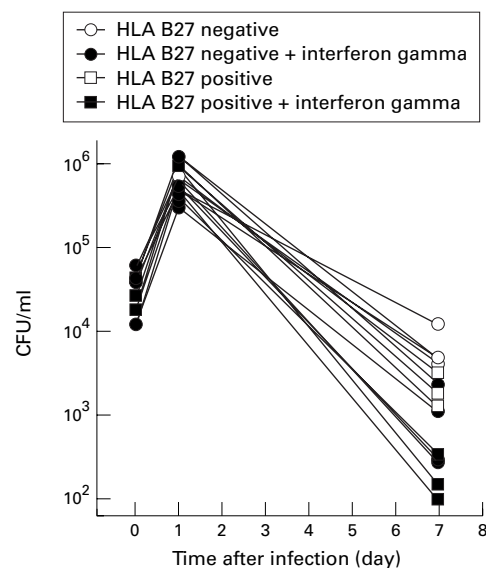


Figure 4 Effect of treatment with interferon gamma on the survival of *Salmonella* in primary human fibroblasts. Seven cell lines were infected with *Salmonella enterica* and then treated with 1000 IU/ml of interferon gamma or left untreated. The three HLA B27 positive cell lines are displayed with square symbols, the four HLA B27 negative cell lines with round symbols. Using ANOVA to compare treated and untreated cells treatment effect ($p < 0.001$) and interaction ($p < 0.0001$) were significantly different.

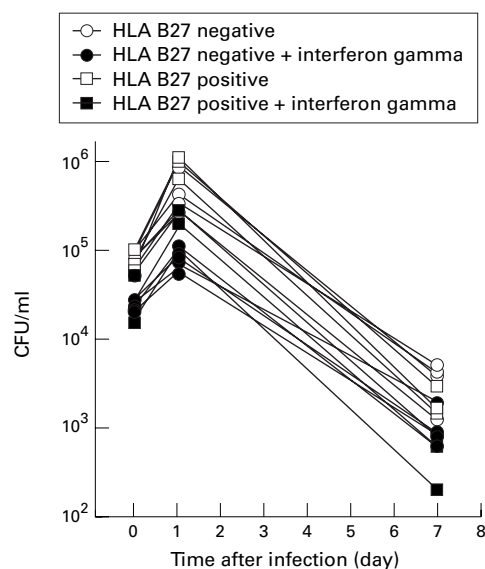


Figure 5 Effect of pre-treatment with interferon gamma on the invasion of *Salmonella* in primary human fibroblasts. Seven cell lines were treated with 1000 IU/ml of interferon gamma for 48 hours or left untreated and then infected with *Salmonella enterica*. The three HLA B27 positive cell lines are displayed with square symbols, the four HLA B27 negative cell lines with round symbols. Using ANOVA to compare treated and untreated cells treatment effect was highly significant ($p < 0.000005$), but interaction was not, confirming the significant influence of interferon gamma on invasion.

were incubated for up to one month after infection. Bacterial titres increased during the first day after invasion, but had decreased at day 5 after infection. Culturable salmonellas could no longer be isolated after two to four weeks (fig 2). All cultures, however, carried intracellular *Salmonella* antigen detected by immunofluorescence at a time when live bacteria had already vanished (fig 3). These results did not differ between HLA B27 positive and negative cells. The results were reproducible in two subsequent experiments. To investigate the transition from cultivable bacterium to antigenic debris cultures were double stained for the presence of DNA and *Salmonella* antigen. After 24 hours a median of 35% (range 10–60%) of salmonellas were converted to bacterial 'ghosts', seemingly intact bacterial rods, but devoid of DNA. Table 1 shows the time course of 'ghost' development. These results did not differ between HLA B27 positive and negative cells.

To assess if increased expression of HLA B27 might influence intracellular survival of *Salmonella*, cells were treated with 1000 IU/ml of interferon gamma after infection. After seven days of infection bacterial titres were significantly lower in interferon gamma treated cultures than in untreated control cultures (fig 4). However, there was no difference between HLA B27 positive and negative cells. The percentage of cells containing *Salmonella* antigen was not changed by treatment with interferon gamma. A repeat experiment yielded similar results. To increase the expression of HLA B27 already at the time of infection cells were treated with interferon gamma for 48 hours before infection with *Salmonella*: invasion of bacteria was significantly decreased to about a

third of the parallel untreated cultures, but there was no difference between HLA B27 positive and negative cells (fig 5). A repeat experiment yielded similar results. The percentage of cells containing *Salmonella* antigen after invasion was reduced by pretreatment with interferon gamma from 60–80% to 20–40%. This difference was still apparent seven days after the infection.

To compare *Salmonella* and *Yersinia* infections, cells were infected in parallel with these pathogens. Initially *Salmonella* titres were significantly higher, but decreased more rapidly than *Yersinia* titres. A repeat experiment yielded similar results.

Discussion

The results from the laboratories of Inman^{8–11} and Granfors,^{7 12 13} showing a modulation of interaction of enteropathogenic bacteria with murine cells that express transfected human HLA B27, prompted us to try to verify their conclusions by using endogenous HLA B27 positive human cells. In this report we have shown that, in contrast with transfected murine cells, endogenous HLA B27 positive primary human fibroblasts do not differ from HLA B27 negative cells in their ability to support invasion and persistence of *Salmonella*.

Our results are in accordance with a recent presentation from Vancouver showing no difference between HLA B27 positive and negative cells in their ability to support invasion by *Salmonella* or *Yersinia*¹⁹ and with results from our laboratory of *Yersinia* infection in primary human fibroblasts.¹⁴ These results cast doubt on the hypothesis that HLA B27 might modulate the interaction of enteropathogenic bacteria and host cells.^{9 12} Although transfected cells allow for the convenient control of experimental conditions, the random insertion of a xenogenetic molecule separated from its normal chromosomal environment and detached from its expression control may lead to artificial conditions unrelated to those found in patients' cells. On the other hand, the experimental design chosen for this study is artificial as it used fibroblast lines that may have changed their phenotype during culture. We believe that primary human cell lines are closer to the situation found in patients than transfected murine cells.

In a previous study we have shown that human cells expressing endogenous HLA B27 have a lower expression of HLA B27 than transfected mouse cells.¹⁴ The overexpression of HLA B27 in transfected murine cells might result in changed mechanisms of infection. Overexpression of HLA B27, however, is probably not the mechanism whereby the transfected HLA B27 genes modulate the infection in murine cells as we have shown in a previous study that treatment with interferon gamma is able to increase and equalise HLA B27 expression in transfected and endogenous cells without changing invasion of *Yersinia*.¹⁴ However, this overexpression is an indicator of a changed HLA B27 metabolism in these transfected murine cells. Results from Vancouver showing no influence of transfected HLA

B27 on bacterial invasion in human cells¹⁹ indicate that the disturbing factor is not the transfection of human HLA B27 itself, but the murine recipient cell that changes invasion of bacteria. In line with this assumption are results from human HLA B27 transgenic rats in which arthritis development depends on the HLA B27 gene copy number, and not expression of HLA B27. Therefore, although transfected cells have been shown to be a valuable experimental tool, we question the relevance of data obtained from transfected murine cells for the infectious pathogenesis of HLA B27 associated diseases.

Interestingly, although fibroblasts are non-professional phagocytes they were able to eliminate intracellular bacteria. *Salmonella* antigens, however, could still be detected at the time when all bacteria had been killed. This antigen load was not diminished when killing of bacteria was accelerated by treatment with interferon gamma. Only when interferon gamma was added before infection could the percentage of cells carrying lipopolysaccharide antigens be reduced. In patients with reactive arthritis interferon gamma found in synovial tissue²⁰ probably is secreted after infection and thus may not be able to diminish the antigen load nor shorten the duration of arthritis. In contrast with fibroblasts, macrophages show apoptosis within a few hours after infection with *Salmonella*.²¹ *Salmonella* enters the cell into an alternative phagosome, which is unable to kill the bacteria and then forms large vacuoles by fusion of several of these phagosomes.²¹ Bactericidal mechanisms of fibroblasts have not yet been elucidated. To determine this important aspect of pathogenesis ultrastructural investigations are under way in our laboratory.

The comparison of infections with *Yersinia* and *Salmonella* showed longer survival of *Yersinia* in human fibroblasts. In contrast with *Yersinia*, *Salmonella* has been shown to induce the formation of filamentous structures in epithelial cells.²² It could be argued that these filamentous structures might render intracellular salmonellas more vulnerable to gentamicin treatment. However, as these structures were not accessible to incoming material from the external medium²² it is unlikely that gentamicin might have gained access to intracellular bacteria. In addition, pretreatments to this study had shown that the concentrations of gentamicin used in these experiments did not affect the number of culturable salmonellas. Finally, other authors had used much higher concentrations of gentamicin than we did.^{21, 23} Although similar in many respects the interactions of *Salmonella* and *Yersinia* with fibroblasts are probably very different at the molecular level²² and the longer survival of *Yersinia* in fibroblasts and the diminished invasion of *Salmonella* after pretreatment with interferon gamma, seen in this study, might be related to fundamental differences in invasion and intracellular survival.^{24, 25} The similarity of the results on the influence of HLA B27 on invasion and survival of *Yersinia* and *Salmonella* despite fundamental differences at the molecular level²⁵ supports the notion that HLA B27 is not important in this interaction.

The results presented in this paper and previous results^{14, 17} offer a comprehensive hypothesis for the pathogenesis of reactive arthritis caused by enteropathogenic bacteria infection. Soon after intestinal infection bacteria might spread through the body of the host and persist for a few days to weeks in resident fibroblast-like cells of synovial tissue. The host defence, including the production of interferon gamma²⁶ and other cytokines,²⁰ results in killing of the bacteria. However, the undegradable bacterial antigens²⁷ remain within the joint and might be liberated from infected cells to induce an inflammatory response. Thus, a few weeks after infection, the host defence might initiate arthritis. At this time traces of degraded bacterial DNA²⁸ and bacterial antigens can be found in synovial fluid²⁹ and synovial tissue of patients with reactive arthritis,³⁰ but culturable bacteria are not present. However, the role of HLA B27 in this scenario remains unclear.

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