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Use and relevance of a bovine mammary gland explant model to study infection responses in bovine mammary tissue

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Our aim was to develop an explant model to define more precisely the early response of bovine mammary epithelial cells to infection. Therefore we investigated the mRNA expression encoding for some soluble immunological factors in lipopolysaccharide (LPS)-treated bovine mammary gland explants. Explants were taken out from the mammary gland of eight lactating cows after slaughter then incubated with LPS (10 μg/ml) for 6 h. The mRNA expression of α-lactalbumin (α-la), various cytokines, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, and two immunoglobulin receptors, the neonatal Fc receptor (FcRn) and polymeric immunoglobulin receptor (pIGR), were assessed with qPCR before and after 3 h and 6 h of LPS challenge. Both immunoglobulin receptors and α-la increased at 3 h then recovered their initial level at 6 h whereas IL-1β, IL-6 and IL-8 increased only after 6 h ($P<0{\cdot}05$). Surprisingly, TNF-α transcripts did not show any regulation in response to the LPS treatment. We nevertheless concluded that our model was valid to examine the short-term response of mammary epithelial cell challenged with LPS.

Keywords: Cytokine, immunoglobulin receptor, LPS, bovine, explant.

Mammary gland immunity, represented as protection and resistance to infectious diseases, is facilitated through a variety of factors. Cytokines are part of the important line of defence represented by both specific and innate soluble factors of defence mechanisms. Numerous reports show immunomodulatory capabilities of cytokines (Sordillo & Streicher, 2002; Alluwaimi, 2004). They are described as cell-free soluble factors that function as communicator molecules between leucocytes and also between leucocytes and tissue. Pro-inflammatory cytokines are thought to initiate the inflammatory reactions in mammary tissues and to induce migration of leucocytes into the udder in response to invading microorganisms or their components such as lipopolysaccharide (LPS) (Riollet et al. 2000a). Indeed, the rapid influx of neutrophils at the site of infection is capital, their ability to phagocytose and kill bacteria being the key to recovery from infection. Interleukin-1 beta (IL-1β), IL-6, IL-8, and tumour necrosis factor-alpha (TNF-α) are some of the cytokines known to play an important role in this process (Okada et al. 1997; Boudjellab et al. 2000; Watanabe et al. 2000).

The inflammatory response is thought to be initiated by leucocytes via the production and release of cytokines but also epithelial cells were recently shown to have the capacity to mount an innate immune response, and they were also demonstrated to express inflammatory mediators (Boudjellab et al. 1998). However, though several studies suggest a non-negligible role of mammary epithelial cell (MEC) in cytokine expression, the extent of this capability remains unclear; precise information about the importance of the part played by MEC would be very useful. Greater understanding of the initial host response to infection may lead to more accurate selection of resistant animals or to novel prophylactic or therapeutic intervention strategies.

To address this concern, studies *in vivo* are not suitable since several types of cells in the mammary gland can produce cytokines, including resident macrophages, endothelial cells, and leucocytes that migrate into the gland in response to the infection. Consequently, several approaches to study bovine MEC are based on cell culture techniques. A homogeneous population of cells is exposed to the same defined infection pressure in the absence of environmental influences. Culture of bovine MEC is considered by many as an established *in-vitro* model to study

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different functions in the mammary gland. However, several aspects of epithelial cell culture show that this model provides only limited information about regulatory mechanisms. Transformed cell lines such as the MAC-T, BME-UV or L1 cells developed to study hormonal influences appeared to be phenotypically unstable and to express milk proteins erratically (Huynh et al. 1991; Zavizion et al. 1996; German & Barash, 2002). To eliminate limits due to a single genotype, cell culture models have been attempted with non-transformed cells, primary cell culture. But still, most investigations with cultured cells were performed on plastic surfaces, which led to drastic alterations of morphology and function from parent tissue. Epithelial cells cultured on plastic dishes lost their mammary specific functions: loss of polarization of organelles, changes in metabolite patterns, alteration of synthesis and secretion of most milk proteins within a couple of days. In most systems the level of milk protein synthesis is either low or non-existent and, if it does occur, it is independent of some or all of the factors (hormones or extra cellular matrix) known to regulate lactogenesis *in vivo* (Rose et al. 2002). Therefore, cell culture is not entirely appropriate for investigations of lactating MEC function.

Incubations of slices of mammary tissue have shown that tissue-specific functions can be maintained for at least 4 d, though this method has never been used to study the inflammation response of MEC (Baumrucker & Stemberger, 1989). Here we report the development of an explant culture model, which allows study of the response of epithelial cells in '*in vivo*-like' configuration while guaranteeing a homogeneous inflammatory pressure. Our particular interest was to determine the role of epithelial cells in the early host responses and their ability to respond rapidly to infection; we therefore focused on the short-term changes.

LPS-induced changes in the transcriptional activity kinetics of pro-inflammatory cytokines such as TNF-α, IL-1B, IL-6, IL-8 were monitored by qRT-PCR until 6 h after challenge. In addition, the transcripts of two immunoglobulin receptors, the neonatal Fc receptor (FcRn) and the polymeric immunoglobulin receptor (pIgR) were also assessed. FcRn and pIgR are expressed on the basolateral surface of mammary alveolar cells to transport immunoglobulins (Ig) from plasma into milk. Transcripts of α-lactalbumin (α-la) were also studied as a control of the lactating properties of the alveolar cells of the explants.

Materials and Methods

Source of tissues

Eight lactating dairy cows were used. Mammary glands were removed from the animals at slaughter and an incision was made aseptically at the proximal part of the gland, through the connective tissue, to expose the secretory tissue. For each of the eight cows, one quarter of the mammary gland was chosen for withdrawal of three samples. All samplings were carried out through the incision, using a Bard magnum Biopsy Instrument (BARD, Covington GA, USA) and a Core Tissue Biopsy Needle (BARD) so that the tissue was removed no later than 20 min after slaughter. Samples were immediately incubated at room temperature in microfuge tubes containing 2 ml of media supplemented or not with LPS until distribution to 12-well dishes under a sterile hood.

Using the biopsy tool allowed a calibration of the samples. Owing to the unchangeable dimensions of the needle (1·6 cm, 2 mm), samples were similar in shape and weight. These very slim and long samples have the advantage of offering a large surface of contact and absorption to the medium and its components and do not require further manipulation.

Explant culture

Explants were incubated in 12-well dishes, one explant per well, in 1 ml of medium, at 37 °C and 5% CO_2. From each cow, explants for all three treatment times of LPS treatment and control were used. The basal medium was DMEM-F12 medium supplemented with insulin (10 μg/ml), hydrocortisone (0·5 μg/ml), penicillin G (100 μg/ml) streptomycin (100 μg/ml), amphotericin B (2·5 μg/ml). Final concentrations of 10 μg/ml of LPS from *Escherichia coli* (0111:B4; cat. No. L4391) were used in the explant cultures for 3 h and 6 h and each stimulation was performed on explants from the eight cows. All chemicals were purchased from Sigma Chemical Co. Explants were processed for RNA extraction either upon arrival at the laboratory for the controls or immediately after being challenged for 3 h and 6 h.

Total RNA extraction and reverse transcription

Tissue homogenization was performed with the homogenizer FastPrep120 centrifuge (Q.Biogene). Each explant was mixed with 1 g Matrix Green and 1 ml TriFast (TriFast, Peqlab, Erlangen, Germany) in Green-Caps and centrifuged twice with the FastPrep120 for 40 s at speed 4·5. Between homogenizations, the samples were stored on ice. Then, total RNA of mammary explants was isolated according to the manufacturer's recommendations (TriFast, Peqlab, Erlangen, Germany).

To quantify the amount of total RNA extracted, the optical density at 260 nm was determined with a photometer (Eppendorf, Hamburg, Germany). RNA integrity was verified by the OD_{260}/OD_{280} nm absorption ratio between 1·7 and 2·0. Total RNA was reverse transcribed to cDNA with reverse transcriptase (MMLV-RT, Promega, Madison WI, USA) and random hexamer primers (Gibco BRL, Grand Island NY, USA) according to the manufacturer's instructions. Final concentration of cDNA was 25 ng/μl.

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Table 1. Sequences of PCR primers of the factors investigated in the experiment

Target†	Sequence (5′-3′)		Product size, bp	EMBL accession number
β-actin	For	AACTCCATCATGAAGTGTGACG	234	U39357
	Rev	GATCCACATCTGCTGGAAGG		
GAPDH	For	GTCTTCACTACCATGGAGAAGG	197	U85042
	Rev	TCATGGATGACCTTGGCCAG		
αla	For	ACCAGTGGTTATGACACACAAGC	233	M18780
	Rev	AGTGCTTTATGGGCCAACCAGT		
IL1-β	For	TTCTCTCCAGCCAACCTTCATT	214	M37211
	Rev	ATCTGCAGCTGGATGTTTCCAT		
TNF-α	For	TAACAAGCCGGTAGCCCACG	277	AF011926
	Rev	GCAAGGGCTCTTGATGGCAGA		
IL-6	For	GCTGAATCTTCCAAAAATGGAGG	188	NM173923
	Rev	GCTTCAGGATCTGGATCAGTG		
IL-8	For	ATGACTTCCAAGCTGGCTGTTG	200	BC103310
	Rev	TTGATAAATTTGGGGTGGAAAG		
FcRn	For	TGAACGGCGAGGAGTTCATG	288	AJ313190
	Rev	GCTCAGGTGGGTAGAAGGAGA		
PigR	For	ATGTGAGCCTGGAGGTCAGCCA	353	AJ313189
	Rev	CTCCAGCACCTGGAGGTCAA		

† See text for abreviations of targets

Quantification by real-time PCR

Quantitative real-time PCR was performed with the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). PCR reactions were carried out using a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) with 1 µl of cDNA (25 ng) in a 10-µl reaction mixture (3 mM-$MgCl_2$, 0·4 µmol/l of each primer, 1× LightCycler DNA Master SYBR Green I). Mixtures underwent the following real time PCR protocol: a denaturation program (95 °C for 30 s) and a three segment amplification and quantification program (95 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s with a single fluorescence acquisition point) repeated for 40 cycles. Primer sequences used for housekeeping gene and target gene amplification are shown in Table 1. The specificity and identity of each PCR product was determined by melting curve analysis (Rotor-Gene 3000 software, version 5.03) and subsequent gel electrophoresis separation. Crossing point (CP) values were acquired by using the comparative quantification method of the Rotor Gene software version 5. CP is defined as the point at which the fluorescence rises appreciably above background.

Relative mRNA expression levels are given by the arithmetic formula $2^{-\Delta\Delta CP}$ where the CP of the target gene is normalized to the mean of housekeeping genes GAPDH and β-actin relative to total RNA (ΔCP) and then compared with the control group collected before LPS stimulation (ΔΔCP): quantity of a transcript in treated explants relative to that in the control groups was expressed as fold change in expression (Livak & Schmittgen, 2001). The mRNA expression of both housekeeping genes resulted in constant expression levels in all samples.

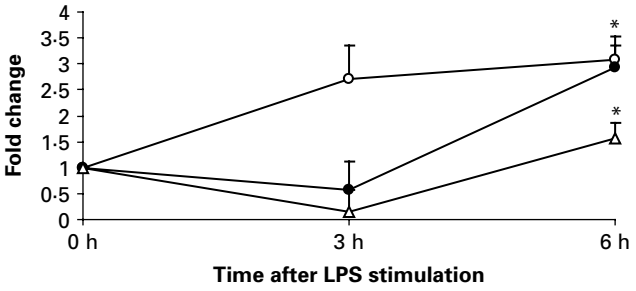

Fig. 1. Relative mRNA expression of Il-1β (○), IL-6 (●) and IL-8 (△) in mammary explants incubated with LPS (10 µg/ml) at 0, 3, and 6 h. Values are the mean ± SEM fold increase of eight cows at each time point. Means with * are significantly different ($P<0·05$).

Statistical analyses

Statistical significance of differences in mRNA expression between control and infection were tested for normal distribution then assessed by a paired *t* test performed with Sigma Stat 3.0 (SPSS Inc., Chicago IL, USA). $P<0·05$ was considered significant.

Results

Cytokines

IL-1β, IL-6, IL-8 mRNA expression increased significantly ($P<0·05$) after 6 h of LPS challenge, about 3-fold higher for Il-1β and Il-6 and 1·5-fold higher for IL8 than in the control (Fig. 1). TNF-α mRNA expression did not change

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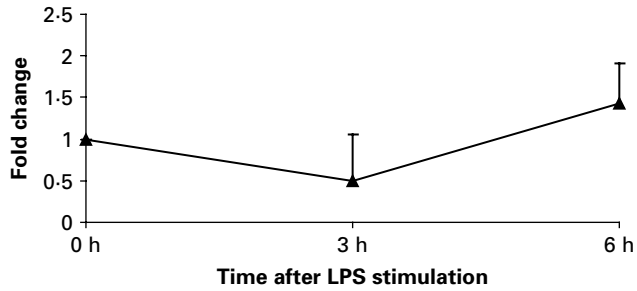

Fig. 2. Relative mRNA expression of TNF-α in mammary explants incubated with LPS (10 μg/ml). Values are means ± SEM of eight cows at each time point. No significant difference was detected.

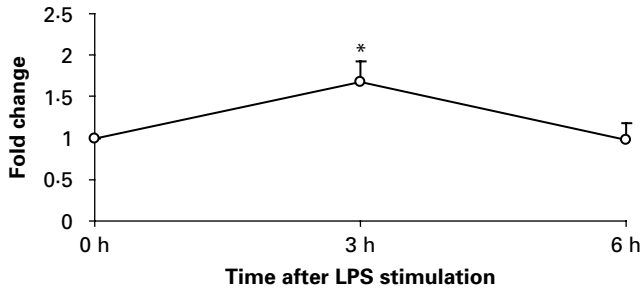

Fig. 3. Relative mRNA expression of α-la in mammary explants incubated with LPS (10 μg/ml). Values are means ± SEM of eight cows at each time point. Means with * are significantly different ($P<0·05$).

significantly although a small rise seemed to take place at 6 h (Fig. 2). IL-6, IL-8 and TNF-α tended to be down-regulated 3 h after the stimulation whereas IL-1β seemed to be already up-regulated.

α-Lactalbumin and immunoglobulin receptors

A transient increase in mRNA expression was observed for α-la (Fig. 3) and both Ig receptors, FcRn and pIgR (Fig. 4), at time 3 h ($P<0·05$). They recovered their basal level of expression after 6 h of stimulation.

Discussion

To our knowledge, mammary gland explants have not been used to measure innate immune response under inflammation conditions. In the current study, we determined whether this model would be relevant to examine the ability of MEC to produce cytokines when stimulated with LPS.

To get information that would be as close as possible to conditions *in vivo*, the first step was to verify that the alveolar cells of the explants were fully functional and kept their lactating phenotype. α-Lactalbumin as one major milk protein was a good candidate. The transient increase of α-la transcripts due to the LPS treatment

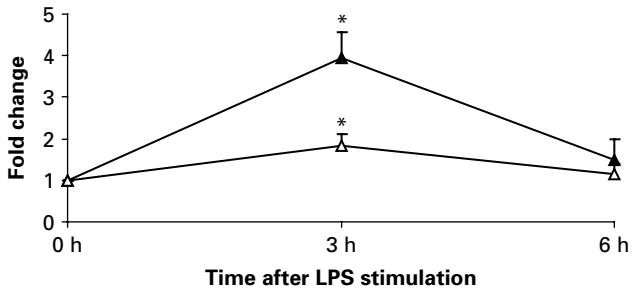

Fig. 4. Relative mRNA expression of immunoglobulin receptors, FcRn (receptor of IgG; ▲) and pIgR (receptor of IgA and M; △), in mammary explants incubated with LPS (10 μg/ml). Values are means ± SEM of eight cows at each time point. Means with * are significantly different ($P<0·05$).

observed in the explants attested to the capability of the epithelial cells to express milk proteins and validated the functionality of the model. However, these results differed from a previous report of α-la mRNA being down-regulated in bovine mammary gland challenged with LPS (Schmitz et al. 2004). One possible explanation is that a reaction to the release of milk from the explants could explain this increase of α-la mRNA whose expression would have been repressed in the mammary glands engorged with milk before slaughter.

Among the cytokines playing key roles in mediating acute inflammatory reactions, IL-1β and TNF-α are extremely potent inflammatory molecules. They recruit and activate neutrophils, induce fever and the production of acute phase reactant proteins by the liver (Feghali & Wright, 1997). They also enhance the expression of vascular endothelial adhesion molecule expression, thereby promoting neutrophil transendothelial migration to the site of infection (Gray et al. 1982; Craven, 1986).

TNF-α seems to be constitutively expressed by cultured bovine MEC but natural coliform mastitis, experimental infection, or LPS exposure increase considerably and rapidly TNF-α protein and mRNA expression (Persson Waller et al. 2003; McClenahan et al. 2005; Strandberg et al. 2005). Surprisingly, LPS stimulation had no significant effect on the production of TNF-α mRNA by explants under our experimental conditions. This could be attributed to marked variability in individual response, as production of TNF-α mRNA was very heterogeneous among the eight mammary glands.

Production of IL-1 by normal MEC *in vitro* has been reported (Okada et al. 1997) but like TNF-α, it experiences a sharp elevation after infection. In *Esch. coli* infection, IL-1β concentration increases in milk (Riollet et al. 2000b) but this increase is detectable only at 40 h post infection (pi) (Bannerman et al. 2004) whereas in cultured and LPS-stimulated ovine MEC, the greatest concentration of IL-1β is reached sooner and peaks at 24 h post challenge (pc) (Okada et al. 1999). As this sharp elevation is also associated with the influx of neutrophils, it is difficult

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to determine whether cytokines in milk originated from immune cells or from mammary cells *in vivo*. However, IL-1β gene expression is markedly up-regulaed by more than 500-fold in bovine MEC. That strongly suggests an active role of MEC in the production of these pro-inflammatory cytokines. The increase in MAC-T was only greater by 4·26-fold after 24 h pc (Strandberg et al. 2005). In our study we were able to detect a significant 3-fold increase of IL-1β mRNA within 6 h.

IL-8 is a powerful chemotactic factor that attracts neutrophils and T lymphocytes and is also involved in neutrophil activation (Harada et al. 1994). If the transcriptional level shows no significant variation at different stages of lactation in the healthy bovine mammary gland (Alluwaimi et al. 2003), it is elevated early in experimental coliform mastitis. The time required for significant elevation of the level of the protein to be detectable *in vivo* varies considerably according to the study. The protein is detected in milk as early as 4 h pi (Persson Waller et al. 2003), 14–16 h pi (Bannerman et al. 2004) or 24 h pi (Shuster et al. 1997). In our study, a significant increase in IL-8 mRNA was detectable after 6 h of exposure to LPS. Many studies indicate that MEC is a major source of IL-8. MEC lines stimulated with LPS or *Esch. coli* copiously produce IL-8. Both bovine MEC and MAC-T cell lines showed a significantly increased production of IL-8 after 24 h of LPS stimulation although the increase was more marked in bovine MEC. Thus, after 24 h IL-8 transcripts are increased by 137-fold in bovine MEC against 22 in MAC-T. IL-8 mRNA peaks within 2 h then remains constant for the following 22 h and within 1 h in the study of (McClenahan et al. 2005). In addition, the protein level assessed with ELISA also showed an up-regulation. To investigate further the main source of production, levels of IL-8 were measured in milk and lymph, revealing a higher amount in milk, indicating that the mammary epithelium rather than the sub-epithelial tissue is the major source of IL-8 (Persson Waller et al. 2003).

IL-6 is one of the proinflammatory cytokines incriminated in the development of signs of acute septic shock in coliform mastitis. It has been postulated that IL-6 facilitates the transition of the inflammatory process from influx of neutrophils to monocytes (Kaplanski et al. 2003). IL-6 mRNA is expressed by normal MEC; a IL6-like activity is shown as well (Okada et al. 1997) and both are up-regulated in the same cell type but stimulated by LPS (Okada et al. 1999). Bovine MEC cell line responds to LPS stimulation in producing IL-6 in a dose-dependent manner. Standberg et al. (2005) showed IL-6 to be up-regulated to a lesser extent than the other cytokines: about 3·32–3·70-fold in bovine MEC and MAC-T. In mammary gland infected with *Esch. coli*, IL-6 expression was detected as early as 14 h pi (Shuster et al. 1997). We demonstrated a significant increase of IL-6 mRNA expression after 6 h of LPS stimulation.

To our knowledge, the present study is the first to examine the regulation of the expression of immunoglobulin receptors in mammary gland under inflammation conditions. FcRn is detected in epithelial cells of mammary gland of many species: mouse (Cianga et al. 1999) ruminant (Kacskovics et al. 2000; Mayer et al. 2002) and human (Cianga et al. 2003). In bovine mammary gland, FcRn is uniformly distributed in epithelial cells before parturition then only on their apical side after calving (Mayer et al. 2005). After LPS challenge, FcRn mRNA was up-regulated at 3 h then recovered its prechallenge level. Transcripts of pIgR showed a similar pattern. Little is known about the distribution of this receptor in mammary gland, the only study reported on ruminant being in sheep. pIgR mRNA is detectable from the third part of pregnancy, accumulates until established lactation when it reaches its highest level (Rincheval-Arnold et al. 2002). These results are important in suggesting a possible role for epithelial cells in the early stage of inflammation. Immunoglobulins are part of the soluble defences that elicit effective protective responses to invading pathogens. IgG and IgM present in serum and milk can opsonize bacteria to enhance phagocytosis (Rainard & Riollet, 2006). IgA contribute to agglutination of bacteria to prevent colonization and neutralize bacterial toxin (Sordillo et al. 1997; Sordillo & Streicher, 2002). Epithelial cells would have the capacity to enhance their production of Ig receptor in order to concentrate Ig at the place of infection to thwart the bacterial invasion. Thus mammary epithelial cells might provide a rapid immunological response to infection.

The response of the explant to LPS stimulation is somehow encompassed between those of the *in-vitro* and *in-vivo* models and corresponded to what would have been expected from a model having the characteristics of its two precedents. However, the degree of change does not exceed a 3-fold increase and is much less than expected on the basis of cell culture studied. Although the medium surrounded the explants and the MEC could be stimulated from their luminal as well as their basal side, we can assume that the number of cells in the explant that could come into contact with LPS and could respond to its stimulation was less than in a cell culture model. Another limit to the model might be trauma caused by the biopsy procedure. The metabolism of the cells injured by the tool could be perturbed and this might explain the unexpected initial trends observed for α-la, IL-6 and IL-8. This trauma might also have been caused by the culture procedure since the explants had not been previously cultivated in the medium before the LPS stimulation.

The underlying assumption made in these analyses is that the enhanced mRNA expression observed has a direct relationship with the quantities of the corresponding proteins. It was not possible to verify whether it was the case in our experimental conditions but several studies report this direct correlation. Several studies report increased IL-8 protein levels secreted into the cell culture medium after IL-8 mRNA up-regulation caused by LPS (Boudjellab et al. 2000; Wellnitz & Kerr, 2004; Strandberg et al. 2005). However, these data concern only one cytokine

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and it must be kept in mind that mRNA expression is not necessarily associated with protein expression.

A tissue explant as an intact and functional piece of the original mammary gland might contain resident macrophages, endothelial cells and cell types other than epithelial cells that could produce cytokines. Thus, the possibility that cytokines may also be produced by other cell types present in the explant cannot be formally ruled out but the cytokine expression by leucocytes that migrate at the place of the infection can be excluded. The clear majority of epithelial cells in the explant leads to the assumption that their contribution is a relevant one. The sheer number of epithelial cells in the explants gives them the potential to be a major contributor to the cytokine secretion.

The current study demonstrates that explant models can be used to study the immune response of MEC. The model developed responded to challenge with LPS by up-regulation of mRNA expression for a range of cytokines and immunoglobulin receptors.

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