The natural history and incidence of *Yersinia pestis* and prospects for vaccination

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Plague is an ancient, serious, infectious disease which is still endemic in regions of the modern world and is a potential biothreat agent. This paper discusses the natural history of the bacterium and its evolution into a flea-vectored bacterium able to transmit bubonic plague. It reviews the incidence of plague in the modern world and charts the history of vaccines which have been used to protect against the flea-vectored disease, which erupts as bubonic plague. Current approaches to vaccine development to protect against pneumonic, as well as bubonic, plague are also reviewed. The considerable challenges in achieving a vaccine which is licensed for human use and which will comprehensively protect against this serious human pathogen are assessed.

**Plague**

Plague is an ancient disease, which has caused many millions of deaths in the course of three pandemics during the last 15 centuries (Perry & Fetherston, 1997). Recent research, using a phylogenetic comparison of 17 *Yersinia* isolates from global sources, indicates that the causative bacterium, *Yersinia pestis*, originated in or near China and subsequently was transmitted by various routes, for example, via the Silk Road to West Asia and to Africa, to establish pandemics (Morelli *et al.*, 2010). The first of these, known as the Justinian Plague, reached East Africa around 540 AD and spread north to the entire Mediterranean region. This pandemic is thought to have lasted up to 60 years, killing an estimated 40 million people. The second pandemic, during the 14th century, spread along the silk routes through China and India, eventually reaching the Middle East, North Africa and Europe, and decimating up to one-third of Europe’s population (approx. 25 million people). This pandemic became known as the Black Death due to the blackening of the hands and feet of its victims. To become a pandemic, it is thought that as well as being flea-vectored, aerosolized plague must also have been directly transmitted from person-to-person by coughing. The third pandemic, which began in south-west China in the latter part of the 19th century, had spread to Hong Kong by 1894, from where the disease was carried by rats on ships to Calcutta and Bombay by 1896, and to San Francisco in 1899. Since 1970, most human cases of plague have occurred in New Mexico with the remaining cases in Arizona, Colorado and California (Eisen *et al.*, 2007; CDC, 2006). The causative organism of plague is *Y. pestis*, named after Alexandre Yersin, a member of the French Colonial Health Service, who was sent to Hong Kong in 1894 where he identified bacilli in victims of the bubonic plague outbreak (Hawgood, 2008). This Gram-negative bacterium was originally an enteric pathogen, and is thought to have evolved from *Yersinia pseudotuberculosis* (Achtman *et al.*, 1999) over a possible 20 000 years to become a flea-vectored pathogen. Fleas feed on infected rodents and then transmit bacteria to a susceptible mammal by flea bite (Perry & Fetherston, 1997). Man is an accidental host in (WHO, 2005). During the 1950s and 1960s, the greatest incidence of cases occurred in Asia; however, during the 1990s, the incidence of plague increased in Africa and maintained at a steady level in Asia (Alvarez & Cardineau, 2010).

The manner in which plague can be established as a zoonotic infection is illustrated by the natural history of the disease in the US. The south-western US remains an endemic region for the disease (Ben Ari *et al.*, 2008). Following the introduction of plague into urban rat populations in San Francisco from 1899, as well as to several other port cities including Seattle, New Orleans, Galveston and Pensacola, hundreds of human plague cases resulted. Rat and flea control measures contained the disease, but domestic rats became reservoirs for the bacteria. Subsequently, domestic rats in the San Francisco Bay area transmitted the disease to Californian ground squirrels in the early 1900s and the spread of plague continued through wild rodents, rabbits and carnivores throughout the Western states. By 1938, plague had spread to prairie dogs in New Mexico, with first reports of human plague cases here in 1949. Since 1970, most human cases of plague have occurred in New Mexico with the remaining cases in Arizona, Colorado and California (Eisen *et al.*, 2007; CDC, 2006).

Some would argue that the third pandemic is still ongoing since plague is still present in endemic regions of the modern world and results in approximately 3000 cases reported to the World Health Organization each year.
Evolution of the organism, and biovars and virulence factors in \( Y. \) \( pestis \)

\( Y. \) \( pestis \) exists in three biovars, Antigua, Orientalis and Medievalis (Devignat, 1951; Perry & Fetherston, 1997; Pollitzer, 1960). Whilst Antigua strains are isolated in Africa and Medievalis strains in Central Asia, Orientalis strains are widespread and appear to be the cause of the current pandemic. In the course of the transition of \( Y. \) \( pestis \) from an environmental enteropathogen to flea-vectored systemic pathogen, genes have been acquired, inactivated and rearranged (Wren, 2003; Parkhill et al., 2001). The 4.56 Mb genome from the clinical isolate CO92, a biovar Orientalis strain, has been sequenced (Parkhill et al., 2001), as has the much smaller 4.6 kb genome from a strain, KIM, of the Medievalis biovar (Deng et al., 2002). In addition to the three biovars, there is a proposed fourth biovar: microtus (glycerol-positive, arabinose-negative and nitrate-negative) (Zhou et al., 2004). Members of the microtus biovar are characterized biochemically by their ability to ferment rhamnose and melibiose: this is probably the most ancient branch of \( Y. \) \( pestis \) phylogenetically (Motin et al., 2002; Achtmann et al., 2004) and comprises the Pestoides strains, originating from the Former Soviet Union (Garcia et al., 2007). Subgroup F of the Pestoides lacks pPst and is thus attenuated by dermal routes but virulent by aerosol challenge (Worsham & Roy, 2003).

In common with \( Y. \) pseudotuberculosis and \( Yersinia \) enterocolitica, \( Y. \) \( pestis \) possesses a 75 kb \( Yersinia \) virulence plasmid, pYV/pCD1 (Fig. 1). Unlike the other \( Yersinia \) species, however, \( Y. \) \( pestis \) has two further plasmids, namely pFra/pMT1 (100–110 kb) and pPst/pPCP/pPla (9.5 kb) (Hinnebusch et al., 1998; Easterbrook et al., 1995; Williamson et al., 1995; Hu et al., 1998; Lindler et al., 1998; Perry et al., 1998), which carry many of the exclusive virulence factors of \( Y. \) \( pestis \). Sequencing of the \( Y. \) \( pestis \) CO92 genome revealed evidence of frequent intragenomic recombinations, a process which may still be ongoing (Parkhill et al., 2001). A recent publication which reported the reconstruction of the genome of the 14th century \( Y. \) \( pestis \) bacterium from DNA fragments isolated from the teeth of Black Death victims concluded that there were no significant differences between the ancient bacterium and the CO92 strain; however, since the latter was used as a reference strain for the reconstruction, it would be difficult to identify sequence in the medieval strain not found in CO92 (Bos et al., 2011). The genome sequences from 17 \( Y. \) \( pestis \) strains are known (Morelli et al., 2010) and indicate that during evolution, the organism has acquired genes encoded on the two smaller plasmids relating to transmission from the flea which control the expression of pesticin, coagulase and plasminogen activator. It has also lost or has inactivated genes relating to adherence, invasion and dissemination (yadA, invasion), biofilm formation and inducing a defensive response (O-antigen expression) in the host (Zhou & Yang, 2009). The bacterium also has many pseudogenes in pathways that are no longer essential to its survival. The identification of many pseudogenes in the genomes of fully virulent \( Y. \) \( pestis \) strains indicates that genome reduction processes are under way, suggesting that \( Y. \) \( pestis \) may not be able to evolve much further in the environmental niche it now occupies.

The plasmid common to the \( Yersinia \), pYV/pCD1, encodes a type three secretion system which involves the elucidation of a needle-like structure when the bacterium makes contact with a host cell and through which the \( Yersinia \) outer proteins (Yops), with cytotoxic and anti-phagocytic activity, are delivered into the host cell (Cornelis, 1998). The needle structure comprises \( Yersinia \) secretory factor \( F \) (YscF) (Matson et al., 2005) tipped with \( V \) antigen (Mueller et al., 2005). Whilst the \( V \) antigen is secreted by the bacterium during this process, there are also reserves within the bacterial cell, where it exerts regulatory activity. The two plasmids unique to \( Y. \) \( pestis \) carry the caf operon encoding the capsular Fraction 1 protein (\( F1 \) antigen) from pFra/pMT1; and pesticin and plasminogen activator (Pla) from pPst/pPCP/pPla (Hu et al., 1998; Lindler et al., 1998; Perry et al., 1998). During infection, Pla was originally thought to contribute to transmission by blocking the flea. This hypothesis was disproven, and it is now thought that Pla facilitates the dissemination of plague bacilli from the site of the flea bite (Hinnebusch et al., 1998). Antibody to Pla was detected in convalescent human sera (Easterbrook et al., 1995), and Pla has more recently been tested as a candidate DNA vaccine but was poorly immunogenic and provided only partial protection against lethal plague in a mouse model (Latham et al., 2007). The \( F1 \) antigen is the major protein in the bacterial capsule and, during infection, has anti-phagocytic properties (Cavanaugh & Randall, 1959; Williams et al., 1972; Du et al., 2002).

All of these bacterially secreted proteins have been isolated and evaluated as potential vaccine antigens, and although some such as Pla, YscF (Matson et al., 2005; Swietnicki et al., 2005) and some Yops (Leary et al., 1999; Andrews et al., 1999; Benner et al., 1999; Li et al., 2009) show some partial protective activity, it is only the \( F1 \) (Andrews et al., 1996) and \( V \) (Leary et al., 1995) antigens which individually provide substantial protective immunity, which is additive when they are used in combination (Williamson et al., 1995; Heath et al., 1998). The protective efficacy of the combined recombinant \( F1 \) and \( V \) (r\( F1 \)+r\( V \)) subunits against \( Y. \) \( pestis \) has now been reported by a number of
laboratories and in a range of laboratory animal models (reviewed by Williamson, 2009). Immunization with rF1/V has been shown to protect animal models against flea-vectored plague (Jarrett et al., 2004) as well as against experimental exposure to Y. pestis (Powell et al., 2005; Williamson et al., 1997). A number of studies have now demonstrated that rF1 and rV in combination, or genetically fused (rF1–V), constitute an efficacious vaccine which has been shown to protect macaques against inhalational challenge with Y. pestis, providing a real prospect for a vaccine effective against pneumonic plague (Table 1). However, complacency does not make for progress, particularly since F1-negative strains exist in nature which can infect humans and are virulent in animals (Ivanov et al., 2008) and since polymorphisms of the V antigen exist in Y. pestis, Y. pseudotuberculosis and Y. enterocolitica (Anisimov et al., 2010; Motin et al., 1992; Roggenkamp et al., 1997). Furthermore, during infection, V antigen has been shown to exert a local immunomodulatory effect by suppression of the pro-inflammatory cytokines TNF-α and IFN-γ through the induced secretion of IL-10 (Nakajima et al., 1995). Neutrophil activation has been shown to be important in the early control of lung infection with Y. pestis (Laws et al., 2010) and evidence has been gained that the pYV plasmid in Y. pestis has the effect of suppressing the IL-17-mediated recruitment of neutrophils to the lymph nodes, thus delaying the host inflammatory response (Comer et al., 2010). A truncated form of the V antigen (rV10) has been produced which lacks residues 271–300 and the immunomodulatory effect of the intact protein (DeBord et al., 2006). The rV10 protein is also being considered as a candidate vaccine for plague (Quenee et al., 2011). Much effort has also been placed into the development of live, rationally attenuated strains of Y. pestis which protect against both bubonic and pneumonic plague and which could provide broader protection should the protection provided by one or two subunit antigens be somehow subverted (Sun et al., 2011).

F1/V vaccination protects against plague

The F1 antigen is a 15 kDa monomer which can polymerize to a large mass (approx. 3 MDa) in a stacked ring structure (Tito et al., 2001a). The V antigen, on the other hand, exists as a 37 kDa protein, but with a tendency to form dimers and tetramers (Tito et al., 2001b). In combination, or as a genetic fusion, they are potently immunogenic and protective, most critically against pneumonic plague. This sets the experimental rF1/V vaccine apart from the previous killed whole-cell vaccines, which have been demonstrated to protect animal models against injected challenge with Y. pestis, but which cannot protect against inhaled challenge (Williamson et al., 1997). The protective efficacy achieved with rF1/V in animal models is equivalent to that afforded by live attenuated vaccine strains, such as EV76, but without the risks inherent to a live vaccine (Oyston & Williamson, 2011).

The differential between live attenuated and killed vaccines in efficacy against pneumonic plague has been attributed to
related to the presence of a neutralizing antibody response, can be demonstrated in the selected animal models and (Williamson et al.) correlates with protection as observed in mice. Whilst the development of an IgG titre to these plague and a titre of specific antibody correlated with protection. Immune correlates of protection in plague (Heath et al., 1995) and inferred from passive transfer studies with clinical trial serum (Williamson et al., 2005), neutralizing antibody alone does not describe the entire mechanism of protection against this virulent pathogen (Williamson, 2009; Smiley, 2007; Parent et al., 2005). The assay of functionality of the induced antibody may be more instructive. If protection can be demonstrated in the selected animal models and related to the presence of a neutralizing antibody response, then the identification of the same neutralizing antibody within serum samples from human clinical trial volunteers indicates an immune correlate of protection and potential surrogate marker of efficacy. Thus immune macaque (Williamson et al., 2007) and human Phase I trial volunteers’ (Williamson et al., 2005) sera have been demonstrated to compete with the plague-protective mAb (mAb 7.3) for binding to the V antigen on solid phase in vitro; protected J774 cells in vitro from the cytotoxic effect of V antigen secreted by Y. pseudotuberculosis, and passively protected naive mice from challenge with Y. pestis. The passive transfer of protective immunity in human serum into mice also correlated significantly with the total IgG titre in the human donors to rF1 + rV at days 21 (P<0.001) and 28 (P<0.03) (Williamson et al., 2005).

Subsequently, however, competitive ELISA has not been shown to be consistent between laboratories as a correlate of protection assay (Anderson et al., 2009), likely due to the existence of more than one protective B-cell epitope on the V antigen (Hill et al., 2007). Thus a pragmatic approach towards assays, showing a correlation between immunological readouts in relevant animal models and man, needs to be taken to thoroughly test such assays for consistency and utility.

Researchers from several groups have reported a strong cell-mediated immune (CMI) response to be operating (Lin et al., 2010, 2011; Parent et al., 2005; Smiley, 2007) and in response to the alhydrogel-adsorbed formulation of the rF1 + rV vaccine, this generally has been observed to be a CD4+ Th2-biased CMI response (Williamson et al., 1999). However, alternative formulations of the rF1 + rV vaccine in which different adjuvants have been substituted for alhydrogel have also been demonstrated to induce protective immunity in a CD4+ Th1-biased setting (Elvin & Williamson, 2000, 2004). Additionally, strains of mice with targeted gene deletions affecting antibody production by B cells (µMT B cell knockouts or SCID/beige) or the nature of the Th cell response including Stat 4/Stat 6 knockouts and IL4/IL10 knockouts were studied (Williamson, 2009; Elvin et al., 2007; Parent et al., 2005). The assay of functionality of the induced antibody may be more instructive. If protection can be demonstrated in the selected animal models and related to the presence of a neutralizing antibody response, then the identification of the same neutralizing antibody within serum samples from human clinical trial volunteers indicates an immune correlate of protection and potential surrogate marker of efficacy. Thus immune macaque (Williamson et al., 2007) and human Phase I trial volunteers’ (Williamson et al., 2005) sera have been demonstrated to compete with the plague-protective mAb (mAb 7.3) for binding to the V antigen on solid phase in vitro; protected J774 cells in vitro from the cytotoxic effect of V antigen secreted by Y. pseudotuberculosis, and passively protected naive mice from challenge with Y. pestis. The passive transfer of protective immunity in human serum into mice also correlated significantly with the total IgG titre in the human donors to rF1 + rV at days 21 (P<0.001) and 28 (P<0.03) (Williamson et al., 2005).

Table 1. Published studies in non-human primates to evaluate rF1/V vaccines

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Non-human primate</th>
<th>Immunogenic</th>
<th>Protective</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1/V</td>
<td>African Green</td>
<td>+</td>
<td>0–75 % aerosol Y. pestis CO92</td>
<td>Pitt (2004)</td>
</tr>
<tr>
<td></td>
<td>Cynomolgus macaque</td>
<td>+</td>
<td>80–100 % aerosol Y. pestis CO92</td>
<td></td>
</tr>
<tr>
<td>Nicotiana F1 + V</td>
<td>Cynomolgus macaques</td>
<td>+</td>
<td>100 LD50 aerosol Y. pestis CO92</td>
<td>Mett et al. (2007)</td>
</tr>
<tr>
<td>V</td>
<td>Baboons</td>
<td>+</td>
<td>Serum passively protected mice</td>
<td>Stacy et al. (2008)</td>
</tr>
<tr>
<td>F1 + V</td>
<td>Cynomolgus macaque</td>
<td>+</td>
<td>104 c.f.u. Y. pestis CO92</td>
<td>Cornelius et al. (2008)</td>
</tr>
<tr>
<td>Flagellin-conjugated F1–V</td>
<td>African Green and Cynomolgus macaque</td>
<td>+</td>
<td>ND</td>
<td>Mizel (2009)</td>
</tr>
<tr>
<td>Nicotiana LcrV-F1</td>
<td>Cynomolgus macaque</td>
<td>+</td>
<td>105 c.f.u. aerosol Y. pestis CO92</td>
<td>Chichester et al. (2009)</td>
</tr>
<tr>
<td>F1–V</td>
<td>Cynomolgus macaque</td>
<td>+</td>
<td>Serum passively protected mice</td>
<td>Fellows et al. (2010)</td>
</tr>
<tr>
<td>F1 + V</td>
<td>Cynomolgus macaque</td>
<td>+</td>
<td>104 c.f.u. aerosol Y. pestis CO92</td>
<td>Williamson et al. (2011)</td>
</tr>
<tr>
<td>F1 + rV270</td>
<td>Rhesus macaques</td>
<td>+</td>
<td>106 c.f.u. s.c. Y. pestis strain 141</td>
<td>Qiu et al. (2010)</td>
</tr>
</tbody>
</table>

ND, Not determined.

Mechanisms of protection

Abrogation of mAb protection with anti-TNF-α and anti-IFN-γ

In animal models, the administration of mAbs with specificity for F1 and V has been shown to protect mice infected with Y. pestis, even when the mAbs were administered at 48 h post-exposure (Hill et al., 2003). However, the protective effect of the anti-V mAb 7.3 was abrogated by the co-administration of anti-TNF-α and anti-IFN-γ, indicating that a cellular pro-inflammatory response is also contributing to protection (Lin et al., 2010). There is scope for combining immuno- and antibiotic therapy post-exposure to Y. pestis to shorten the duration of antibiotic therapy required.

Immune correlates of protection in plague

Immunization of mice with either (Andrews et al., 1996; Anderson et al., 1996) or both (Williamson et al., 1995; Heath et al., 1998) F1 and V proteins was protective against plague and a titre of specific antibody correlated with protection. Whilst the development of an IgG titre to these proteins correlates with protection as observed in mice (Williamson et al., 1999), guinea pig (Jones et al., 2003) and non-human primate (Williamson et al., 2007, 2011) and inferred from passive transfer studies with clinical trial serum (Williamson et al., 2005), neutralizing antibody alone does not describe the entire mechanism of protection against this virulent pathogen (Williamson, 2009; Smiley, 2007; Parent et al., 2005). The assay of functionality of the induced antibody may be more instructive. If protection can be demonstrated in the selected animal models and related to the presence of a neutralizing antibody response,
& Williamson, 2000, 2004; Reithmeier-Rost et al., 2007); rF1 + rV-immunized Stat 4-deficient mice, which have low levels of IFN-γ production, were found to be poorly protected from Y. pestis challenge, despite producing similar antibody titres to rF1 + rV as the intact controls (Elvin & Williamson, 2004). Moreover, the rF1 + rV vaccine was able to induce protective immunity in IL-4 knockout mice despite a Th1-biased environment operating in these animals (Elvin & Williamson, 2000). Indeed, Stat 4-mediated immune mechanisms leading to a Th1 response were found to be essential for protection, whereas Stat 6/Th2-mediated responses were not (Elvin & Williamson, 2004). Thus for the rF1 + rV vaccine, the induction of specific antibody neutralizing the F1 and V antigens is a significant immune correlate of protection; however, the supporting CM1 response is not necessarily Th2-polarized and indeed the operation of Th1 mechanisms during infection appears to be essential for full protection and recovery (Williamson, 2009).

Potential surrogate markers of efficacy for countermeasures to plague

From the published data to date, several serological surrogate markers of efficacy have emerged for human volunteers immunized with the rF1/V subunit vaccine (Williamson et al., 2005). The ability of human serum taken from volunteers immunized with the rF1/V antigens to inhibit in vitro the cytotoxicity caused by V antigen secreted from a Y. pseudotuberculosis construct is a potential surrogate marker of efficacy. Qualitative data from this assay have been published (Williamson et al., 2005); however, the assay has subsequently been improved and made quantitative (Welkos et al., 2008). It has been demonstrated that a decrease in Caspase-3 activity in macrophages exposed to immune non-human primate (nhp) serum correlated with an increase in survival of those nhp to Y. pestis infection.

Passive transfer of immune serum, including human, has been demonstrated to protect naive mice against plague infection, in a dose-related manner (Fellows et al., 2010). The passive transfer of human immune serum into the mouse conferred protection against subsequent challenge with Y. pestis, and this correlated significantly with total IgG titre to rF1 + rV at days 21 ($r^2=98.6\%$; $P<0.001$) and 28 ($r^2=76.8\%$; $P<0.03$) (Williamson et al., 2005).

Assays for cellular surrogate markers of efficacy are practically more challenging, particularly in a clinical setting, since they require fresh whole blood samples and relatively prompt analysis. Attempts to analyse changes in cell surface markers on peripheral blood mononuclear cells (PBMCs) collected from vaccinees and restimulated ex vivo with both recombinant antigens, during the course of a small Phase 1 clinical trial for rF1/V, did not reveal any. Flow cytometry was used to detect the specific activation of the PBMCs ex vivo; however, due to the large variation in response between individuals, no significant trends were detected (Williamson et al., 2005). A more practical alternative may be to use an ELISpot assay, where for example, interferon γ secretion from PBMCs restimulated ex vivo with vaccine antigens is detected (Smiley, 2007).

The mapping of CD4+ T-cell epitopes for F1 and V would provide more specific probes for the ex vivo stimulation of PBMCs. Progress is being made in this area since CD4+ T-cell epitopes in F1 and V have been identified in mice (Musson et al., 2006; Shim et al., 2006) and an N-terminal H-2d-restricted murine T-cell epitope in F1 has been shown to be essential for protection in BALB/c mice (Chalton et al., 2006). The significance of certain CD4+ T-cell epitopes in the F1 antigen for protection has been demonstrated in a study in which a monomeric stable circularly permuted F1 (cpF1) protein was created by relocating the N-terminal donor strand, via a flexible linker, to the C-terminus (Chalton et al., 2006). The cpF1 protein retained immunogenicity in both H-2d and H-2k mouse strains, but the circularization had the effect of disrupting the H-2d restricted T-cell epitope (at primary sequence position 7–20) in the N-terminal donor strand, with loss of protective efficacy in BALB/c mice, but with no effect on protective efficacy in H-2k mice. Recently, protective CD8+ T-cell epitopes have been identified in the Yersinia outer protein YopE (Lin et al., 2011) and in the V antigen (Wang et al., 2011).

Similarly, human leukocyte antigen (HLA)-restricted T-cell epitopes have been mapped in F1 (Musson et al., 2010) and are being sought in V antigen using HLA transgenic mice. These data may in the future provide functional targets for human T-cell memory responses, recognition of which by immune PBMCs could provide a cellular surrogate marker of efficacy.

Conclusions

Much work is ongoing to identify statistically valid immune correlates of protection for plague, particularly since a clinical demonstration of efficacy would be difficult to achieve, given the spasmodic and unpredictable nature of the outbreaks that occur in regions of the world endemic for plague. This has required the development of non-clinical models which authentically represent the human infection. As far as possible, the immune correlate should be demonstrated in more than one non-clinical model. Whilst the immune correlate(s) may not describe all the immune mechanisms operating in protection against a pathogen, they should be reproducibly consistent between the non-clinical models and the clinic and should be quantitative, to assess the likely benefit to be conferred on the vaccinee. With an increasing understanding of the molecular basis of pathogenicity and of the innate and adaptive immune response mechanisms required to counter Y. pestis, immune correlates of protection are being identified and reported and this in turn will expedite the development of next generation vaccines and immunotherapies for both biodefence and protection against endemic disease purposes.
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