Interaction of Insulin with *Pseudomonas pseudomallei*

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*Pseudomonas pseudomallei* is the causative agent of melioidosis, a disease being increasingly recognized as an important cause of morbidity and mortality in many regions of the world. An intriguing observation regarding melioidosis is that a significant percentage of patients who develop the disease have preexisting diabetes mellitus. In this regard, we have tested the hypothesis that insulin may modulate the growth of *P. pseudomallei*. We have demonstrated that insulin markedly inhibits the growth of *P. pseudomallei* in vitro and in vivo. The growth rate of *P. pseudomallei* in minimal medium containing human recombinant insulin was significantly lower than that of control cultures containing no insulin. *P. pseudomallei* grew at an increased rate in serum samples obtained from diabetic rats compared with that in serum samples obtained from control animals. When the insulin level was restored by the addition of human recombinant insulin, the growth rate was reduced to a level similar to that seen in control serum. *P. pseudomallei* also grew significantly better in insulin-depleted human serum than control human serum. 125I-insulin binding studies demonstrated that *P. pseudomallei* possesses a specific, high-affinity binding site for human insulin. In in vivo studies, rats made diabetic by streptozotocin injection (80 mg/kg of body weight, intraperitoneally) were significantly more susceptible to *P. pseudomallei* septicemia than control rats. Thus, it appears that serum insulin levels may play a significant role in modulating the pathogenesis of *P. pseudomallei* septicemic infections.

*Pseudomonas pseudomallei* is the causative agent of melioidosis, most cases of which occur in the latitudes between 20°N and 20°S. The disease is rare in the Western Hemisphere but has been identified in Panama, Ecuador, Haiti, Brazil, Peru, and Guyana (1, 4, 11). Melioidosis can be seen as an inapparent infection, as an asymptomatic pulmonary infiltration, as an acute localized suppurative infection, as an acute pulmonary infection (most commonly), as an acute septicemic infection, or as a chronic suppurative infection (8, 28). The incubation can be as short as 2 days, or the organism can remain latent in the body for as long as 26 years (20).

The pathogenic determinants of *P. pseudomallei* have not been well studied. A thermolabile toxin and a protease have been described, though not well characterized. Endotoxin has also been considered in the pathogenesis of the disease; however, the toxicity of *P. pseudomallei* endotoxin is reportedly low (5, 9). Even with vigorous use of antibiotics and supportive therapy, the mortality in patients with septicemic melioidosis is 70% or greater (4). A consistent and intriguing observation regarding septicemic melioidosis patients is that a significant percentage of these patients are diabetic (4, 22).

Diabetes mellitus occurs in all parts of the world, but with striking variations in prevalence in different races and geographical locations. The two main types are insulin-dependent (type 1) diabetes and non-insulin-dependent (type 2) diabetes. In the tropics, another type of diabetes known as tropical pancreatic disease has to be considered (2). Type 1 diabetes is associated with pancreatic beta cell destruction and insulin insufficiency, whereas type 2 diabetes involves resistance of target organs to the effects of insulin. Each of these types results in an upward shift in blood glucose, and treatment of diabetes involves normalization of blood glucose levels by insulin injections (type 1) or the administration of oral hypoglycemic drugs (type 2) (2). It is well established that patients with diabetes are prone to infection, including fungal infections, necrotizing infections, and osteomyelitis. This has been attributed to the poor circulation and the lack of local sensation in these patients and to the ketoacidosis associated with diabetes (26).

In a 1986 prospective study of all patients with melioidosis admitted to a large provincial hospital in northeast Thailand, those with septicemic melioidosis presented mainly in the rainy season; the disease occurred predominantly in rice farmers and their families and was significantly associated with preexisting diabetes mellitus (4). Of the 63 patients with melioidosis, 20 (32%) had underlying diabetes mellitus (4). In a recent review of 50 cases of septicemic melioidosis from Malaysia, it was reported that 38% of these patients had underlying diabetes mellitus (22). The patients in each of these two studies were shown to suffer from type 1 diabetes. On the basis of these observations that patients with insulin-dependent (type 1) diabetes are significantly at risk for acquiring septicemic melioidosis, the present studies were designed to test the hypothesis that insulin may modulate the growth of *P. pseudomallei* and in this manner may influence the pathogenesis of disease due to this organism.

**MATERIALS AND METHODS**

**Bacterial strains.** *P. pseudomallei* clinical isolates used in these studies were generously provided by D. A. Dance, Oxford Tropical Medicine Research Program, Wellcome-Mahidol University, Bangkok, Thailand. Clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Pseudomonas cepacia* from blood cultures were obtained from the Clinical Microbiology Laboratory of the Foothills Hospital, Calgary, Alberta, Canada.

**In vitro experiments.** (i) Effects of insulin on bacterial growth. The effects of insulin on bacterial growth were examined in a series of clinical isolates. All strains tested were cultured from frozen stock onto M9 agar containing...
0.5% glucose (24). This culture was used to inoculate a starter culture of M9 broth with 0.5% glucose which was grown overnight at 37°C. One hundred microliters of this starter culture containing approximately \(10^8\) organisms (giving a final concentration of \(10^7\) organisms per ml in each flask) was transferred to separate flasks containing M9 broth plus insulin (Humulin R [Eli Lilly, Indianapolis, Ind.]), a human biosynthetic insulin, at a concentration of 10 U/ml. In control cultures, human serum albumin was added to a final protein concentration equivalent to 10 U of insulin per ml. These growth experiments were performed in triplicate, and the growth rate was determined by measuring the \(A_{690}\) of the cultures at 0, 4, 8, and 24 h following inoculation and incubation in a shaking water bath (200 rpm) at 37°C.

(ii) \textit{P. pseudomallei} growth in rat serum. Rats were made diabetic by intraperitoneal streptozotocin (STZ) injection (80 mg/kg for each of 2 consecutive days according to the method of Tancredi et al. [27]). Induction of diabetes was monitored by measurement of urine and blood glucose levels (29). All animals with a blood glucose concentration greater than 248 mg/dl were considered diabetic. Whole blood obtained from diabetic rats and nondiabetic control rats was centrifuged (7,000 \(\times\) g for 15 min), and the serum was removed. The complement was inactivated by placing the serum into a 56°C water bath for 30 min.

To each of nine 125-ml flasks were added 5 ml of complement-inactivated serum, 5 ml of phosphate-buffered saline (PBS), and 100 \(\mu\)l of a dilution of an overnight culture of \textit{P. pseudomallei} containing approximately \(10^9\) organisms (giving a final concentration of \(10^7\) organisms per ml for each flask). The first triplicate set of flasks contained 5 ml of control serum each, the second triplicate set contained 5 ml of diabetic rat serum each, and the third triplicate set contained 5 ml of diabetic rat serum each with 0.1 U of human recombinant insulin per ml. At 0, 4, 8, and 24 h following inoculation of the flasks with bacterial organisms, 100-\(\mu\)l samples were removed from the flasks and the numbers of bacteria were determined.

(iii) \textit{P. pseudomallei} growth in human serum. The growth of \textit{P. pseudomallei} in complement-inactivated human serum depleted of insulin was examined. Insulin was removed from normal human serum by affinity chromatography using anti-insulin monoclonal antibody coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). CC9C10 cells (ATCC HB 123, graciously donated by J. W. Yoon, University of California), which produce an antibody reactive with insulin, were grown in Dulbecco’s modified Eagle’s medium with 4.5 g of glucose per liter and 10% fetal calf serum (GIBCO Canada, Inc., Mississauga, Ontario, Canada). Culture supernatants were collected and precipitated in 70% ammonium sulfate. The precipitates were redissolved in 0.1 M NaHCO\(_3\) (pH 8.3) containing 0.5 M NaCl (coupling buffer) and dialyzed overnight against this buffer. The antibody was coupled to CNBr-activated Sepharose 4B according to the manufacturer’s directions (Pharmacia). Insulin depletion was monitored by immunoassay, and the insulin-depleted serum was complement inactivated and filter sterilized.

The growth of \textit{P. pseudomallei} in human serum was examined as described above for growth in rat serum, except that the first triplicate set of flasks each contained 5 ml of control human serum obtained from a healthy volunteer with no history of diabetes; the second triplicate set of flasks each contained 5 ml of insulin-depleted serum; and the third triplicate set of flasks each contained 5 ml of insulin-depleted serum plus 0.1 U of human recombinant insulin per ml.

(iv) Insulin binding to \textit{P. pseudomallei}. The binding of \(^{125}\text{I}\)-insulin to \textit{P. pseudomallei} was examined. Humulin R (Eli Lilly), a human biosynthetic insulin, was iodinated with \(^{125}\text{I}\) by using Iodo-Beads (Pierce Chemicals, Rockford, Ill.) according to the established procedures (12, 19). Insulin-bound label was separated from unbound \(^{125}\text{I}\)-insulin by membrane ultrafiltration (Ultrafree-MC; Millipore Corp., Bedford, Mass.) according to the method of Lipford et al. (17). Protein concentrations of the radiolabelled ligand were determined with the Bio-Rad Protein Assay System (Bio-Rad, Richmond, Calif.). \textit{P. pseudomallei} cells were grown for 18 h at 37°C in M9 broth containing 0.5% glucose. The cells were centrifuged (10,000 \(\times\) g for 15 min), washed twice in PBS, and resuspended to a final \(A_{690}\) of 0.3 (approximately \(5 \times 10^9\) cells per ml). Into 1.5-ml microfuge tubes were placed 100 \(\mu\)l of cells, \(^{125}\text{I}\)-insulin ranging in final concentrations from 1.0 to 10 \(\mu\)g/ml in 10-\(\mu\)l aliquots, and 150 \(\mu\)l of PBS containing 0.1% (wt/vol) albumin (7). Following incubation at 24°C for 20 min (i.e., under standard assay conditions for insulin binding proposed by Cuatrecasas [6]), the cells were washed three times in cold PBS. The amounts of free \(^{125}\text{I}\)-insulin in the supernatant and of cell-bound \(^{125}\text{I}\)-insulin were determined. These experiments were performed in triplicate.

The specificity of insulin binding was determined by examining the competition of unlabelled insulin with \(^{125}\text{I}\)-insulin for binding to \textit{P. pseudomallei}. \(^{125}\text{I}\)-insulin (50 ng) was incubated (24°C for 20 min) with \textit{P. pseudomallei} (5 \(\times 10^9\) cells) and increasing amounts of unlabelled insulin. These experiments were performed in triplicate.

In vivo experiments. Two types of animal experiments were performed with rats which had been insulin depleted by STZ injection. The first set of experiments was performed with infant rats intraperitoneally injected with \textit{P. pseudomallei}. These experiments represented our initial attempts to differentiate diabetic from nondiabetic animals in terms of susceptibility to \textit{P. pseudomallei}. The end point of these experiments was death, and results were reported as relative 50% lethal dose (LD\(_{50}\)) values noted with diabetic versus nondiabetic rats. The second set of animal experiments employed a rat lung infection model, which is representative of a more natural exposure route than intraperitoneal injection. Adult animals were used in these studies because of the technical difficulties associated with intratracheal inoculation of infant rats. In these experiments, infected diabetic animals were compared with their nondiabetic counterparts with regard to quantitative lung bacterial counts and the numbers of animals with positive blood cultures. All animals were sacrificed at 2 days postinoculation, and the infections were not allowed to proceed to death, so that quantitative lung bacteriology and septicemia could be assessed.

(i) Infant rat model. Thirty male Sprague-Dawley rats with an average weight of 50 g were used. Animals were given intraperitoneal injections either of PBS or of STZ (80 mg/kg of body weight for each of 2 consecutive days) to induce diabetes (25). Urine glucose levels were monitored to assess the onset of diabetes. Diabetic animals were given 10 mU of Neutral Protamine Hagedorn (NPH) insulin per g of body weight intraperitoneally when urine glucose levels reached 2 g/dl. Three days postinjection, groups of animals (five diabetic and five nondiabetic) were inoculated intraperitoneally with \(10^8\), \(10^7\), or \(10^6\) \textit{P. pseudomallei}. The experimental end point was death, and LD\(_{50}\) values were determined by the method of Reed and Muench (23).

(ii) Adult rat model. Twenty-four 200-g male Sprague-Dawley rats were used. Diabetes was induced in 12 animals by using intraperitoneal injections of 80 mg of STZ per kg on each of two consecutive days. The remaining animals re-
receiving intraperitoneal injections of PBS. Urine glucose levels were monitored to assess the onset of diabetes. Diabetic animals were given 10 mU of NPH insulin per g intraperitoneally when urine glucose levels reached 2 g/dl. Anesthetized and tracheotomized rats were inoculated by deposition of 0.1 ml of a suspension of *P. pseudomallei* encased in agar beads into the lower lobe of the left lung with a bead-tipped curved needle by the methods of Cash et al. (3). Two days postinoculation, the animals were sacrificed and the lungs were removed for bacteriological examination. For quantitative bacteriological studies, the entire lung was removed, and lobes were excised from the hilus and placed in 3 ml of sterile PBS. The tissue was homogenized with a Polytron Homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). Serial dilutions of the homogenate in PBS were plated on tryptic soy agar plates.

**RESULTS**

*In vitro experiments.* For the studies of the growth of *P. pseudomallei* in medium, rat serum, and human serum and for the studies of the binding of insulin to *P. pseudomallei*, similar results were obtained with a number of different *P. pseudomallei* strains tested, and representative results obtained from the use of one strain, strain 316c, are shown in Fig. 1. *P. pseudomallei* 316c is a blood culture isolate from a septicemic melioidosis patient and is a ribotype A1 organism (25).

At a concentration of 10 U of insulin per ml, the growth rate of *P. pseudomallei* was significantly inhibited compared with that of cultures containing no insulin (Fig. 1). Insulin had no effect on the growth of *P. aeruginosa*, *P. cepacia*, or *E. coli* at any concentration up to 10 U/ml (data not shown). The effects of insulin on the growth of *P. pseudomallei* in rat serum were examined by using complement-inactivated normal rat serum, complement-inactivated serum from STZ-treated rats, and complement-inactivated serum from STZ-treated rats with insulin levels restored by the addition of 0.1 U of human recombinant insulin per ml of rat serum (Fig. 2). After 24 h, growth in serum from STZ-treated rats (insulin depleted) was significantly greater than growth in control serum (insulin sufficient), while growth in serum from STZ-treated rats with insulin added back was significantly reduced compared with that in serum from STZ-treated rats and control serum. The reduction in growth below that seen in control serum was attributed to the large excess of insulin added.

The effects of insulin on the growth of *P. pseudomallei* in human serum were examined, and over a 24-h period, the growth rate of *P. pseudomallei* was significantly greater in insulin-depleted serum than in control human serum. The addition of 0.1 U of human recombinant insulin to insulin-depleted serum reduced the growth to a rate below those seen in insulin-depleted human serum and in control human serum (Fig. 3). Again, the reduction in growth rate below that seen in control serum was attributed to the excess insulin added.

Binding of $^{125}$I-insulin to *P. pseudomallei* was observed to be a saturable phenomenon at 24°C, indicating a finite number of specific binding sites for insulin on the *P. pseudomallei* cell surface. Saturation occurred at approximately 50 ng of $^{125}$I-insulin (Fig. 4). Approximately $5 \times 10^{8}$ cells per ml were present in the incubation mixture. Therefore, approximately $10^{-4}$ pg of $^{125}$I-insulin bound to an individual organism. Based upon an estimated molecular weight of 6,000 for insulin, the calculations predict that there are approximately $5.2 \times 10^{3}$ insulin binding sites per *P. pseudomallei* cell. Calculations performed from the data obtained from the Scatchard plot (Fig. 4, inset) and specific affinity measurements ($1.9 \times 10^{3}$ cm²/mg of protein) predict a value of approximately $7.5 \times 10^{7}$ insulin binding sites per
allowed to Scatchard plot serum in 4048 WOODS ET AL.
organisms was of P. serum. human inoculation and following ml of
by restored which had been serum anti-insulin monoclonal antibody; A,
standard deviation.

FIG. 3. Effects of insulin on the growth of P. pseudomallei in human serum. A 100-µl portion of a dilution of an overnight culture of P. pseudomallei 316c corresponding to approximately 10⁶ organisms was added to culture flasks (giving a final concentration of 10⁷ organisms per ml in each flask) containing 10 ml of 50% human serum in PBS (pH 7.2). Growth was examined at 4, 8, and 24 h following inoculation and reported as mean CFU/ml of serum (10³ ± standard deviation). ●, Control (normal human serum); ■, human serum which had been depleted of insulin by anti-insulin monoclonal antibody; ▲, human serum which had been depleted of insulin by anti-insulin monoclonal antibody and whose insulin levels had been restored by the addition of 0.1 U of human recombinant insulin per ml of serum.

P. pseudomallei cell and a Kᵦ of 1.5 × 10⁻¹⁰. The predicted number of binding sites agrees well with the previously predicted value of 5.2 × 10⁶ insulin binding sites per cell based upon saturation binding experiments. The specificity of insulin binding was demonstrated by the ability of unlabelled insulin to compete with ¹²⁵I-insulin for binding to P. pseudomallei in a concentration-dependent manner (data not shown). The saturability and specificity of insulin binding provide evidence for the presence of a specific, high-affinity binding site for human insulin on the surface of P. pseudomallei.

In vivo experiments. The LD₅₀ values for infant rats infected with P. pseudomallei 316c are demonstrated in Table 1. The LD₅₀ values for STZ-treated diabetic rats were markedly lower than those for nondiabetic PBS-treated animals. In three separate experiments, the LD₅₀ values for P. pseudomallei in diabetic animals were 7.3 × 10³, 4.3 ×

**TABLE 1.** LD₅₀ values for diabetic versus nondiabetic infant rats with P. pseudomallei

<table>
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<th>Treatment⁴</th>
<th>LD₅₀ for rats in exp⁵:</th>
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<tr>
<td>PBS</td>
<td>&gt;1 × 10⁶</td>
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<tr>
<td>STZ</td>
<td>7.3 × 10³</td>
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<td>1.7 × 10³</td>
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⁴ PBS-treated animals were nondiabetic; animals were made diabetic by STZ injection (80 mg/kg).
⁵ Determined by the method of Reed and Muench (23); n = 30 (15 diabetic and 15 nondiabetic) rats for each experiment.

FIG. 4. Binding of ¹²⁵I-insulin to P. pseudomallei whole cells. ¹²⁵I-insulin was added to 10⁷ cells in the amounts indicated, and binding was allowed to proceed for 20 min at 24°C, at which time bound ¹²⁵I-insulin was separated from unbound ¹²⁵I-insulin by centrifugation. Inset, Scatchard plot of the binding data. All data are means of triplicate values and are representative of at least three separate experiments.
10^3, and 1.7 x 10^4. The LD_{50} values seen for P. pseudomallei in nondiabetic animals were greater than 10^6 in each of the three experiments.

The data obtained from quantitative bacteriological lung cultures of diabetic and nondiabetic adult rats infected with P. pseudomallei 304b are as follows (mean ± standard deviation, in CFU per lung): the lungs of nondiabetic (PBS-treated) animals yielded counts of 3.4 x 10^7 ± 6.8 x 10^6 (experiment 1) and 1.6 x 10^6 ± 2.9 x 10^5 (experiment 2), whereas those of diabetic (STZ-treated) animals yielded counts of 2.5 x 10^6 ± 3.9 x 10^5 (experiment 1) and 2.4 x 10^6 ± 6.4 x 10^5 (experiment 2). Similar results were obtained for a number of P. pseudomallei strains tested in this model. Strain 304b is also a ribotype A1 organism (25). There was a 100-fold increase in the numbers of organisms recovered from STZ-treated diabetic rats compared with those from PBS-treated nondiabetic animals, which represented a significant difference in the mean bacterial counts in the lungs of diabetic animals compared with those in nondiabetic animals (P < 0.05, Student's t test). Additionally, P. pseudomallei was isolated from the blood of 8 of the 12 diabetic animals, whereas none of the nondiabetic animals were septic with P. pseudomallei.

**DISCUSSION**

Hormones and hormone-binding proteins resembling those of vertebrates are widespread in fungi, yeasts and bacteria (13, 14). E. coli grown in synthetic medium has been found to produce materials that resemble vertebrate insulins (15, 16); however, the only evidence for the existence of an insulin-responsive signal transduction system in a microorganism has been obtained from studies with Neurospora crassa (15). In the present studies, insulin was not detected in the growth medium of P. pseudomallei cultures (data not shown); however, our studies do provide the first evidence for the ability of a human hormone, insulin, to suppress the growth of a bacterial organism, P. pseudomallei. We have demonstrated specific and saturable insulin binding to P. pseudomallei and the presence of receptors for approximately 5,000 molecules of insulin on the surface of P. pseudomallei. Cuatrecasas (6) has demonstrated that approximately 11,000 molecules of insulin can bind to adipose-tissue cells. Thus, while P. pseudomallei possesses less than half of the number of insulin receptors present on adipose-tissue cells, binding of insulin to these receptors results in a demonstrable physiological response, i.e., depression of the growth rate of P. pseudomallei.

Physiological concentrations of insulin suppressed the growth of P. pseudomallei in vitro in broth cultures. This effect was shown to be specific for P. pseudomallei, as the growth rates for P. aeruginosa, P. cepacia, and E. coli were not affected by insulin. Additionally, P. pseudomallei growth in serum, both rat and human, was suppressed by the presence of insulin. The normal range for serum insulin levels reported by the Foothills Hospital is 10 to 24 µU/ml, and approximately 10^7 bacteria per ml were inoculated with P. pseudomallei cultures at the beginning of the incubation period. Thus, the ratio of micromolars of insulin to bacterial cells in the cultures containing 10-U/ml insulin was approximately 1:1. Though quantitative studies have not been done with P. pseudomallei, in studies with a related organism, P. aeruginosa, it has been reported that in the vast majority of cases the number of organisms recovered per milliliter of blood in septicemic infections due to P. aeruginosa is less than one (10). If one conservatively estimates the normal insulin level in 1 ml of human serum to be 10 µU and the number of P. pseudomallei organisms present in 1 ml of blood in septicemic melioidosis to be one, the normal insulin/bacterial-cell ratio approximates 10:1. Thus, it is clear that the levels of insulin reported in our present studies which demonstrated an effect on growth are well within the physiological range. We recognize that the relatively large inocula used in our studies represent a limitation in our study design. We have, however, performed other studies using smaller inocula, and when the starting inoculum of P. pseudomallei in the growth medium was reduced to 10^4 organisms per ml, growth was completely inhibited by as little as 0.01 U of insulin per ml.

Rats made diabetic by STZ treatment, which attacks pancreatic beta cells and thus induces type 1 diabetes, were significantly more susceptible to P. pseudomallei infection than nondiabetic rats, an effect which was constant for both infant and adult rats.

Porot et al. (21) reported that binding of the cytokine interleukin 1 (IL-1b) to the surface of E. coli enhanced the growth of virulent clinical isolates of E. coli grown in culture medium and that this enhancement was blocked by an IL-1 receptor antagonist (IL-1ra). In addition, radio-labelled IL-1 bound to virulent E. coli strains in a specific and saturable fashion and IL-1ra inhibited this binding. More recently, it has been demonstrated that tumor necrosis factor alpha binds to Shigella flexneri by utilizing a high-affinity receptor and that this results in an enhancement of virulence as demonstrated by enhanced cell-invasive properties (18). In the present studies, we have provided the first evidence for the presence of a specific insulin receptor on P. pseudomallei. Further, our studies have demonstrated that insulin binding appears to have an effect opposite to that of cytokines in that the growth and the potential virulence of a microorganism are depressed by the binding of insulin to the P. pseudomallei surface.

Clinical studies have conclusively demonstrated that insulin-dependent diabetes predisposes an individual to septicemic melioidosis (4, 8, 22). The results from the present studies provide a plausible rationalization for this phenomenon. We have clearly demonstrated that insulin suppresses the growth of P. pseudomallei both in vitro and in vivo, and studies to define the mechanism(s) whereby insulin interacts with P. pseudomallei to modulate the growth of this micro-organism are under way.

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