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外源性诱导原发性胆汁性胆管炎动物模型研究进展

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【摘要】 原发性胆汁性胆管炎(primary biliary cholangitis, PBC),又称原发性胆汁性肝硬化,是一种少见的自身免疫性肝病,发病率低,发病机制不明确,无法治愈。研究表明外界环境因素导致发病率逐年升高,所以构建发病因素相似的动物模型研究 PBC 的发病和治疗都具有重要意义。现通过阅读 20 年来国内外相关文献,总结各种外源性物质(2-辛炔酸偶联牛血清白蛋白、聚肌苷酸胞嘧啶核苷酸、新鞘氨醇杆菌、抗线粒体抗体抗原、同源胆管蛋白)诱导 PBC 动物模型的方法,分析各种造模方式的特点。为研究者选择更合适的动物模型,探索 PBC 发病机制和研发新药提供参考。

【关键词】 原发性胆汁性胆管炎;动物模型;抗线粒体抗体;外源性诱导
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Research progress on animal models of exogenously induced primary biliary cholangitis

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【Abstract】 Primary biliary cholangitis (PBC) is an incurable rare autoimmune liver disease with low incidence and an unclear pathogenesis. Studies have shown that external environmental factors have led to a yearly increase in incidence. Therefore, it is of great significance to establish an animal model with similar pathogenesis to study the pathogenesis and treatment of PBC. After reading relevant domestic and foreign literature over the past 20 years, we summarized the method to establish PBC animal models induced by various exogenous substances. Various exogenous (2-OA-BSA, polyI:C, *N. aromaticivorans*, AMA antigen and BDP) and analyze the characteristics of various modeling method. Induction method of PBC animal models are summarized and the characteristics of various modeling method were analyzed. We provide a reference for researchers to choose more suitable animal models, explore the pathogenesis of PBC and develop new drugs.

【Keywords】 primary biliary cholangitis; animal model; antimitochondrial antibody; exogenous induction

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原发性胆汁性胆管炎(primary biliary cholangitis, PBC),又称原发性胆汁性肝硬化,是一种慢性肝内胆汁淤积性肝病,其特征是肝内小胆管破坏,门脉周围炎症、纤维化,最终导致肝硬化。PBC 的血清学特征是抗线粒体抗体(anti-mitochondrial antibody, AMA)阳性,这是一种在约 95%的 PBC 患者中发现的高度疾病特异性抗体[1]。多个国家流行病学研究表明 PBC 的总体发病率为 8.55/100 万/年,患病率为 118.75/100 万/年,我国目前无论是发病率还是患病率都要高出平均水平[2]。国外流行病学显示尿路感染、香烟、有毒废弃物和工业地区等环境污染以及贫困等因素容易诱发本病[3-5]。Chen 等[6]在对上海部分地区 19012 名居民抗线粒体抗体 M2 亚型(anti-mitochondrial antibody subtype M2, AMA-M2)的筛选调查中发现住在垃圾填埋场和高架附近,吸烟以及使用染发剂等被认为是危险因素。最新流行病学也显示城市工业区,环境污染和社会贫困地区人群的 PBC 患病率较高[5]。Probert 等[7]在垃圾填埋场发现了可以抑制线粒体氧化磷酸化和诱导肝祖细胞凋亡的化学物质,其结构是一种类似硫辛酸的人肝细胞代谢产物,并且可以通过外源性脂酰化途径在酶催化下与丙酮酸脱氢酶复合物的 E2 成分相结合。多项研究显示外源性化学药物或异物可能通过修饰丙酮酸脱氢酶复合物 E2 亚单位(pyruvate dehydrogenase complex E2 subunit, PDC-E2)或其他自身抗原,导致自身免疫易感个体耐受性丧失从而诱发 PBC 的发病[8-9]。外源性诱导动物模型更加类似环境因素对 PBC 发病的影响,所以有必要对外源性诱导 PBC 动物模型的方法和特点进行系统总结(见表 1),以便根据研究目的选择恰当的模型。

1 药物诱导模型

1.1 2-辛炔酸偶联牛血清白蛋白(2-octynoic acid BSA conjugate, 2-OA-BSA)诱导模型

绝大多数 PBC 患者都伴有不同程度的抗线粒体抗体反应,这些抗体识别的自身抗原是 2-氧酸脱氢酶复合体(2-oxo-acid dehydrogenase complex, 2-OADC)的成员,特别是 PDC-E2。实际上,这些抗体也会与许多化学修饰过的类似物发生交叉反应[9,24]。2-辛炔酸(2-octynoic acid, 2-OA)不仅具有在体内修饰 PDC-E2 的潜力,而且重要的是,它被广泛应用于环境中,包括香水、口红等化妆品和许多常见的食品调味料中[9]。

Wakabayashi 等[10]首先使用外源性 2-OA 与牛血清白蛋白(bovine serum albumin, BSA)偶合(100 μg/25 μL),对雌性 C57BL/6 小鼠进行免疫,成功诱导出类 PBC 动物模型。造模方法:2-OA-BSA 与完全弗氏佐剂(Freund's adjuvant complete, CFA)偶合,进行首次腹腔注射,其后将 2-OA-BSA 与不完全弗氏佐剂(Freund's adjuvant incomplete, IFA)进行偶合,每隔 2 周对模型小鼠腹腔注射 1 次,持续 12~24 周。模型特征:最早在免疫 4 周后血清中即可检测到 PDC-E2 抗体阳性并显著升高,8 周即达 100%。肿瘤坏死因子 α(tumor necrosis factor, TNF-α)和干扰素 γ(interferon-gamma, IFN-γ)在免疫 4 周后也显著增高。IL-4、IL-5 和 IL-10 基本没有变化。组织学上免疫 14 周后肝切片显示门管区淋巴细胞浸润,小胆管损伤或消失,肝内或见肉芽肿形成。肝内 CD4/CD8T 细胞的比值降低,而 $CD8^+$ T 细胞的数量显著增加。Lleo 等[11]发现在 PBC 患者中,受损胆管周围的门管区也存在典型的 $CD4^+$T 和 $CD8^+$ T 淋巴细胞浸润。虽然该模型有 PBC 典型胆管周围淋巴细胞浸润,但是纤维化改变和反映胆汁淤积程度的相关酶测定却仍然存在缺陷。然而,2-OA 可用于多种小鼠品系和转基因小鼠[25-26],达到模型的理想病理改变,也表明 2-OA 诱导模型的广泛应用前景,值得进一步探索。

Wu 等[12]、Chang 等[13]发现半乳糖苷基神经酰胺(α-GalCer)激活 iNKT 细胞会加重汇管区炎症反应、胆管损伤、肉芽肿形成和肝纤维化发生的趋势。所以 Chang 等[14]修改造模的实验方案,除以上干预措施外,另加 α-GalCer 用 0.5%吐温 20 溶解成 0.2 mg/mL,取 10 μL 分别于第 1、2、3 次免疫接种的前 1 d 静脉注射至小鼠体内。方案修改后造模小鼠在免疫 4 周后即出现更加明显的门脉炎症、胆管损伤。更为欣喜的是修正后 12 周的模型可提高 AMA 产生,肉芽肿形成和肝纤维化改变征象。Zeng 等[27]、Miyake 等[28]根据这种免疫小鼠模型还发现 iNKT 细胞和自身抗体的关系也具有一致性。这个发现说明不仅适应性免疫参与 PBC 的发展,而且先天性免疫在本病的发生发展中也起着重要的作用。

1.2 聚肌苷酸胞嘧啶核苷酸(polyinosinic-polycytidylic acid, polyI:C)诱导模型

PolyI:C 是一种干扰素诱导剂,触发 I 型干扰素(IFN-1)的产生和下游 IFN-α/β 受体依赖信号通

表 1　不同外源性物质诱导 PBC 动物模型总结

Table 1　Summary of animal models of PBC induced by different exogenous substances

造模物质 Inducer	处理方法 Processing method	模型优点 Advantages of model	模型缺点 Model shortcomings	应用 Application	参考文献 Reference
2-辛炔酸偶联牛血清白蛋白 2-OA-BSA	2-OA-BSA 分别与 CFA 和 IFA 耦合，腹腔注射 C57BL/6 小鼠 2 周/次，造模 12~24 周。修正方案在前基础上辅用 α-GalCer。 2-OA-BSA was coupled with CFA and IFA, respectively. C57BL/6 mice were intraperitoneally injected for 2 weeks/time, and the model was established for 12 ~ 24 weeks. The modified scheme was supplemented with α-GalCer.	4 周可见 M2 抗体阳性，TNF-α 和 IFN-γ 升高；14 周汇管区淋巴细胞浸润，胆管损伤或者消失。修正后肝病理表现更符合。 At 4 weeks, M2 antibody was positive, TNF-α and IFN-γ levels increased. At week 14, lymphocytes were infiltrated in portal area and bile ducts were damaged or disappeared. The pathological manifestations of the liver were more consistent after modification.	药物昂贵，造模周期长，实验成本高；肝纤维化改变不明显。 Drugs are expensive, the modeling cycle is long, and the experiment cost is high. There was no obvious change of liver fibrosis.	环境无染与 PBC 发病相关性研究。修正后先天性免疫对 PBC 的发病研究。 Study on the correlation between environmental non-staining and PBC incidence. Study on the pathogenesis of PBC by modified innate immunity.	[10-14]
聚肌苷酸胞嘧啶核苷酸 PolyI:C	PolyI:C(5 mg/mL)腹腔注射 C57BL/6 小鼠 2 次/周，持续 28 周。 PolyI: C (5 mg/mL) was intraperitoneally injected into C57BL/6 mice twice a week for 28 weeks.	实验成本低，8 周 M2 抗体阳性，IFN-α 高表达，胆管损伤，胆管周围淋巴细胞浸润明显，16 周达到最高。 Experimental cost was low. At 8 weeks, M2 antibody was positive, IFN-α expression was high, the bile duct was damaged, and the infiltration of lymphocytes around the bile duct was obvious, and the peak was reached at 16 weeks.	造模周期长，唾液腺、胰腺和肾也出现类似 PBC 的肝外炎性病变，肝特异性弱。 Extrahepatic inflammatory lesions similar to PBC also appeared in salivary glands, pancreas and kidney, and liver specificity was weak.	PBC 治疗药物研究。 Drug research for the treatment of PBC.	[15-16]
2-OA-BSA 联合聚肌苷酸胞嘧啶核苷酸 2-OA-BSA combination polyI:C	依据上述方法联合使用，造模 8 周。 According to the combination of the above methods, the model was built for 8 weeks.	造模时间短，淋巴细胞和促炎细胞因子增多和肝病理特征。 Model time was shortened, lymphocytes and proinflammatory cytokines were increased and liver pathological features were observed.	实验成本高，动物死亡率增高。 Cost of the experiment is high, and the mortality rate of animals increases.	PBC 的“二次打击”假说研究。 Study on the “Second Strike” hypothesis of PBC.	[17]
新鞘氨醇杆菌 *N. aromaticivorans*	*N. aromaticivorans* 悬液第 1、14 天静脉注射到 NOD 小鼠，至少 4~8 周。 *N. aromaticivorans* suspension was intravenously injected into NOD mice on days 1 and 14 for at least 4~8 weeks.	感染后持续高水平的 IgG，汇管区炎症细胞浸润，胆管损伤和肉芽肿改变。 After infection, high levels of IgG persist, inflammatory cell infiltration in the portal area, bile duct damage and granuloma changes.	实验繁琐，实验条件要求高；肝纤维化改变不明显。 Experiment is cumbersome and requires high experimental conditions; the changes in liver fibrosis are not obvious.	环境微生物导致 PBC 发病的机制研究。 Study on the pathogenesis of PBC caused by environmental microorganism.	[18-19]
抗线粒体抗原 Anti-mitochondrial antigen	C57BL/6 小鼠腹腔注射重组抗原蛋白三联体 100 μg/200 μL，造模 66 周。 C57BL/6 mice were injected intraperitoneally with 100 μg/200 μL of recombinant antigen protein triplet for 66 weeks.	M2 抗体阳性率高，碱性磷酸酶升高；汇管区炎症细胞浸润明显，肝特异性强。 Positive rate of M2 antibody is high, alkaline phosphatase is elevated. Inflammatory cells in the portal area are infiltrated obviously, and the liver specificity is strong.	实验条件高，造模周期长，肝纤维化改变不明显。 Experimental conditions are high, the modeling cycle is long, and the changes in liver fibrosis are not obvious.	PBC 早期诊断研究。 Study on early diagnosis of PBC	[20-22]
胆管蛋白 Bile duct protein	BDP 与 CFA 和 IFA 乳化皮下注射 C57BL/6 小鼠 1 次/周，造模 4 周。 BDP, CFA and IFA were emulsified subcutaneously into C57BL/6 mice once a week, and the mice were modeled for 4 weeks.	造模周期短；M2 抗体阳性率高，淋巴细胞活性增强；汇管区炎症细胞浸润。 Short mold making cycle. The positive rate of M2 antibody was high and the lymphocyte activity was enhanced. Inflammatory cells infiltrate the portal area.	病理上未见胆管损伤和肉芽肿改变。 Pathologically, there were no changes in bile duct damage and granulomas.	PBC 发病的分子拟态研究。 Molecular mimicry for the pathogenesis of PBC.	[23]

路,通过打破人体耐受性促进后续自身免疫反应[29-31]。持续产生 IFN-1 会使免疫系统几乎所有成分转向病理功能,导致组织损伤和疾病[32]。许多系统性自身免疫性疾病患者有持续产生 IFN-1 的迹象,并显示 IFN-α 调控基因表达增加[33-34]。此外,多个病例报道在没有自身免疫性疾病的情况下使用干扰素治疗期间患者出现了自身免疫性肝炎的临床和病理特征[35-37]。提示 IFN 系统在自身免疫性疾病的发病机制中起关键作用。

Okada 等[15]使用 IFN-1 的诱导剂 polyI:C 建立自身免疫性胆管炎动物模型。造模方式:6~8 周的雌性 C57BL/6 小鼠,每周腹腔注射 2 次 polyI:C,以 5 mg/kg 为造模最佳浓度,连续造模 28 周,成功建立 PBC 小鼠模型。模型特点:造模 8 周后组织学可见胆管损伤,胆管周围淋巴细胞浸润明显,门管区淋巴细胞的浸润数量在造模 16 周达到最高,此后基本稳定维持这一水平。模型小鼠血清在 8 周后可以检测到自身抗体,到 24 周后 87%的模型小鼠可检测 AMA。此外,IFN-α 在造模 4 周已经高表达,但到 16 周后开始降低,24 周处于较低的状态。Hanada 等[38]认为 IFN-β 和 IFN-α 可以直接破坏胆管细胞紧密连接的屏障功能,通过改变胆管细胞旁路途径来增加胆管细胞通透性。然而 Stewart[16]认为降低干扰素会减少自身抗体和组织免疫损伤,IFN-α 低表达的情况下,IFN-β 也会出现低表达。奇怪的是这种现象与病理进展相互矛盾,可能是因为其他细胞因子对干扰素的调节所致,至于具体内在联系仍需进一步研究。

1.3 2OA-BSA 联合 polyI:C 诱导模型

Ambrosini 等[17]认为 2OA-BSA 诱导的 PBC 模型肝纤维化的病理特征不符合后期肝纤维化的病理征象,所以在此基础之上联合 polyI:C,达到了较好的效果。造模方法:在首次 2OA-BSA 联合 CFA 腹腔注射诱导后每 3 d 加用 5 mg/kg 剂量的 polyI:C,干预 8 周后处死小鼠。和 2OA-BSA 诱导模型相比有以下异同。相同:两者肝组织有相同程度的淋巴细胞浸润、肉芽肿形成和胆管损伤。两者产生的抗体和 $CD4^+$T 细胞水平相当。不同点:联合 polyI:C 诱导的模型 Th1 细胞因子、IL-12、IFN-γ 和 TNF-α 增多,$CD8^+$T 细胞和嗜酸粒细胞数量增多,门脉汇管区炎症和纤维化形成明显,此外造模时间只需要 8 周。但是 polyI:C 可有效激活免疫系统,联合使用后促使造成严重的腹腔炎症,提高了动物死亡率。polyI:C 在 2OA 免疫诱导下进行二次免疫,证实 PBC 如同其他自身免疫性疾病一样,属于多系统协调免疫反应。

2 抗原诱导模型

2.1 微生物诱导模型

流行病学研究表明 PBC 与复发性尿路感染有关[39-40]。Selmi 等[41]发现 PBC 患者血清对(*Novosphingobium aromaticivorans*, *N. aromaticivorans*)的反应活性比对大肠杆菌(*Escherichia coli*,*E. coli*)要高出 100~1000 倍。其后又进一步将 *N. aromaticivorans*、*E. coli* 等多种细菌的 PDC-E2 内结构域氨基酸序列对比发现前者与人类的 PDC-E2 具有最高的同源性,并且在对比几种细菌诱导 PBC 模型特点后,认为 *N. aromaticivorans* 比之前提出的 *E. coli*、螺杆菌(*Helicobacter species*, Hp)要更可能诱导 PBC 的发病[42]。

Mattner 等[18]使用 *N. aromaticivorans* 打破免疫耐受造出具有显著自身免疫特点的 PBC 小鼠模型。造模方法如下:制备 *N. aromaticivorans* 细菌悬液(每 100 μL 含有 5×10^7 个)。分别于第 1 天和第 14 天将细菌悬液静脉注射到 4~20 周非肥胖型糖尿病(non-obesity diabetes, NOD)小鼠体内进行活体感染。模型特点:活体感染 1 个月后小鼠肝脾质量较前增加;主要组织相容性复合体(major histocompatibility complex, MHC)II 类分子在受损小胆管的表达;可见严重的门脉浸润和胆管损伤,并伴有部分纤维化改变。这种表现在感染 *E. coli* 的小鼠肝组织中未发现。造模 6 个月后门脉炎症、胆管损伤和肉芽肿形成相比 *E. coli* 感染要具有明显差异。血清检测表明感染 2 周后小鼠即出现 PDC-E2 和 AMA 的聚集,伴有 T 淋巴细胞浸润小胆管的现象,并持续长时间高水平的 IgG。尽管如此,这种模型却未出现与人类 PBC 发展进程中相似的肝纤维化表现。但有研究表明这种肝纤维化改变不明显可能是由于 NOD 遗传背景之下 IFN 的高表达[43]。这也提示 PBC 疾病的发生可能与某种遗传易感性基因有关。Wang 等[19]分别用 *E. coli* 和 *N. aromaticivorans* 感染 NOD 小鼠建立 PBC 模型,发现 *E. coli* 感染小鼠会导致更加明显的胆管炎症损伤并且产生更高浓度的 AMA,然而与 PBC 患者血清相比,*E. coli* 感染小鼠对 PDC-E2 的血清学抗体反应却相对较弱[44],尽管如此 Wang 认为在感染 *E. coli*

的早期足以打破免疫耐受并导致类似于 PBC 的肝病理表现。

2.2　抗线粒体抗体抗原(AMA 抗原)诱导模型

英国的一项临床研究发现检出 M2 抗体阳性的无症状患者在出现 PBC 症状和体征之前,时间间隔长达 10 余年之久[45]。目前依靠常规使用的血清 AMA 检测,其特异性和敏感性还不够,仍有一部分病人出现漏诊[46]。所以提高对 PBC 诊断的敏感性显得尤为重要。Kikuchi[47] 和 Miyakawa 等[48] 将 AMA 反应的主要自身抗原克隆并鉴定为 2-氧代酸脱氢酶复合体家族成员:丙酮酸脱氢酶复合体 E2(PDC-E2),支链 2-氧代酸脱氢酶复合体-E2(BCOADC-E2)和 2-氧戊二酸脱氢酶复合体-E2(OGDC-E2)。并且也发现有些患者的血清与 BCOADC-E2 和 OGDC-E2 是可以反应的。多年前 Moteki 等[49] 将牛、大鼠和人类线粒体抗原的混合物,即 3 个不同支链结构域杂交克隆,命名为 pML-MIT3,用于 PBC 病人检测发现可以明显提高检出率。

Jiang 等[20] 根据基因工程的方法将人源靶抗原连接加工后获得重组抗原蛋白三联体(重组 AMA 抗原),据此成功模拟 PBC 模型[21]。造模方法:每只小鼠(C57BL/6)一次腹腔注射重组抗原蛋白三联体 100 μg/200 μL。模型特点:在免疫 66 周后病理显示肝小叶无明显淋巴细胞浸润和肝细胞坏死表现,并且可见汇管区淋巴细胞浸润和小胆管损伤。血清中 AMA-M2 抗体阳性率达 100%,并且谷丙转氨酶(ALT)、总胆红素(TBil)无明显变化,以上特征与早期 PBC 相似。此外具有肝特异性,灵敏度高,已尝试投入临床用于辅助 PBC 疾病的筛查[22]。Miyakawa 等[50] 通过克隆人源 cDNA 利用新开发的酶联免疫吸附法来表达重组蛋白,用于 PBC 阴性病人的检测,有 90%与至少 1 个重组蛋白发生免疫印迹反应。

2.3　胆管蛋白(bile duct protein, BDP)诱导模型

根据 PBC 发病的典型特征,Ma 等[23] 通过同源 BDP 免疫诱导方式建立 PBC 小鼠模型。造模方法:8~12 周龄小鼠(C57BL/6)通过门静脉灌注原位胶原酶 IV,10 min 后剪裁肝,用软牙刷刷去肝细胞,直到可见整个胆管树。将胆管树置于原位胶原酶 IV 中震荡 10 min,分离粘附的肝细胞。在磷酸盐缓冲盐水(PBS)中洗涤 3 次后,将胆管树置于 PBS,制备胆管细胞蛋白匀浆,并进行蛋白定量。将 4 mg/mL 的 BDP 与 CFA 等体积乳化,完全乳化后,取 BDP-CFA 乳剂在小鼠背部多点皮下注射,每周 1 次,免疫 3 次。随后将 BDP 与 IFA 乳化,BDP-IFA 乳剂仅在最后一次免疫中使用,BDP-IFA 处理 1 周后处死小鼠,进行分析。模型特点:在病理中发现门静脉汇管区胆管周围严重的肝特异性炎症,肝和脾中 $CD4^+$ T 和 $CD8^+$ T 细胞的数量和激活状态增加。以及由 T 细胞抗原呈递细胞组成的树突状细胞在肝和脾的数量也有所增加。此外,该模型中脾的生发中心反应更强。血清中 AMAs 100%阳性,并增加了血清中的 AMAs,包括抗 PDC-E2、BCOADC-E2 和 OGDC-E2。该模型的建立主要依赖于胆管抗原,而在人类中,胆管抗原再次表明分子拟态参与触发或促进 PBC 发病。

3　讨论与展望

除了临床样本、环境研究、体外实验和流行病学数据外,动物模型是理解和寻找 PBC 临床方案不可或缺的工具。到目前为止,PBC 的发病机制仍然不够清晰,只是对该疾病发展过程和预后有一定了解。熊去氧胆酸(ursodeoxycholic acid, UDCA)是唯一经 FDA 认证的一线药物[51],但也只能改善患者胆汁淤积的状态,无法根本治愈。在实际临床中有高达 30%的 PBC 患者对 UDCA 应答欠佳,应答欠佳不仅会提高并发症的发生率,更容易进展为肝硬化,甚至需要肝移植,这类患者是快速发展至晚期肝病的高风险人群[52-54]。临床二线贝特类药物联合 UDCA 也仅仅可以改善血清相关指标,与单独使用 UDCA 相比,对症状和死亡率的影响并没有显著差异[55]。因此,当务之急在于尽早探明本病的发病机制以研发更适合 PBC 的新药。

目前建立的动物模型只能模拟 PBC 某一病理阶段,还不能完整模拟其病理过程。人体生活在复杂环境中,自身免疫性疾病是环境多因素刺激和遗传因素导致免疫耐受破坏所致。以上构建的小鼠模型各有特点,启示我们单因素造模方法只能拟合出接近 PBC 某一阶段病理特点的动物模型,联合使用比如 2OA-BSA 联合 polyI:C 可以较好提高动物模型拟合度。对于这种病理过程复杂的疾病模型应该打破传统单因素造模思路,从多因素(不局限外源性诱导)考虑,或者结合肝纤维化、肝硬化疾病动物模型造模方法,采取复合多因素造模方法,探索出更加符合人类 PBC 发病特点的动物模型。

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