·脑脊液细胞学研究·

T-SPOT.TB法检测脑脊液单个核细胞对结核性脑膜炎早期诊断价值的研究

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【摘要】 研究背景 结核性脑膜炎为严重危害人类健康的中枢神经系统感染性疾病,传统的脑脊液分析和放射学检查方法敏感性和特异性较低,延误治疗时机,因此临床急需敏感性更高的快速检查方法用于结核性脑膜炎的诊断。本文采用T细胞酶联免疫斑点试验(ELISPOT)检测结核性脑膜炎患者外周血和脑脊液单个核细胞,比较两种检查方法诊断结核性脑膜炎的敏感性和特异性,以为结核性脑膜炎的早期诊断提供有效检查方法。方法 分别收集结核性脑膜炎(30例)和非结核性脑膜炎(30例)患者外周血和脑脊液,分离提取单个核细胞,经冻存和复苏后采用结核杆菌感染T细胞斑点试验(T-SPOT. TB)检测外周血和脑脊液中干扰素-γ分泌的T细胞数目,分别计算其诊断结核性脑膜炎的敏感性和特异性。结果 脑脊液ELISPOT试验显示,结核性脑膜炎组阳性检出率为93.33%(28/30)、非结核性颅内感染组为3.33%(1/30);检测灵敏度93.33%、特异度96.67%,假阳性率3.33%、假阴性率6.67%,阳性似然比28.33、阴性似然比0.07。外周血ELISPOT试验显示,结核性脑膜炎组阳性检出率为76.67%(23/30)、非结核性颅内感染组为13.33%(4/30),检测灵敏度76.67%、特异度86.67%,假阳性率13.33%、假阴性率23.33%,阳性似然比5.79、阴性似然比0.15。外周血ELISPOT试验阳性检出率与脑脊液阳性检出率比较,差异无统计学意义(Fisher确切概率法:$P = 0.254$)。结论 采用ELISPOT试验检测外周血和脑脊液单个核细胞结核杆菌感染率有助于提高结核性脑膜炎的诊断率,而且脑脊液单个核细胞ELISPOT试验检测效率呈高于外周血检测效率之趋势。

【关键词】 结核,脑膜; 脑脊髓液; 敏感性与特异性; 酶联免疫吸附测定; 单个核细胞

Study on the early diagnostic value of T-SPOT.TB assay detecting mononuclear cells in cerebrospinal fluid of patients with tuberculous meningitis

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【Abstract】 Background Tuberculous meningitis (TBM) is a worldwide central nervous system infectious disease and is seriously harmful to human beings. The traditional normal assay of cerebrospinal fluid (CSF) and radiological examination always delay the clinical treatment because of low sensitivity and specificity. Therefore, a more sensitive method for the clinical diagnosis of TBM is badly in need. This article uses T lymphocytes enzyme-linked immunospot assay (ELISPOT) to detect mononuclear cells in peripheral blood (PB) and CSF of TBM patients, and compares the sensitivity and specificity in the diagnosis of TBM between the two methods, for the purpose of providing effective examination method for the early diagnosis of TBM. Methods PB and CSF samples were collected from 30 cases of TBM (case group) and 30 cases of non-TBM (control group) respectively, and mononuclear cells were separated and extracted through cryopreservation and rapid thawing. A spot test using T lymphocytes infected with tubercle bacillus (T-SPOT.TB) was applied to check T lymphocytes secreted by interferon-γ in PB and CSF,

so that the sensitivity and specificity for diagnosing TBM by this method were evaluated. **Results** CSF mononuclear cells ELISPOT assay showed that 28 cases in the case group were diagnosed as TBM and other 2 cases were diagnosed as non-TBM, with the positive rate being 93.33% (28/30); as for control group, 1 case was diagnosed as TBM, and 29 cases were diagnosed as non-TBM, with the positive rate being 3.33% (1/30). In CSF mononuclear cells ELISPOT assay, the sensitivity and specificity were 93.33% and 96.67%; false positive rate was 3.33%; false negative rate was 6.67%; positive likelihood ratio was 28.33; negative likelihood ratio was 0.07. PB mononuclear cells ELISPOT assay showed that 23 cases in the case group were diagnosed as TBM and 7 cases were diagnosed as non-TBM, with the positive rate being 76.67% (23/30); as for control group, 4 cases were diagnosed as TBM, and 26 cases were diagnosed as non-TBM, with the positive rate being 13.33% (4/30). In PB mononuclear cells ELISPOT assay, the sensitivity and specificity were 76.67% and 86.67%; false positive rate was 13.33%; false negative rate was 23.33%; positive likelihood ratio was 5.79; negative likelihood ratio was 0.15. Fisher exact probability test showed the difference between the TBM positive rate of PB and CSF ELISPOT assay was not statistically significant ($P = 0.254$). **Conclusion** PB and CSF mononuclear cells ELISPOT assay can be effectively favorable for the current diagnosis of TBM as an auxiliary diagnostic method. Besides, CSF mononuclear cells ELISPOT assay is more efficient than PB assay.

【Key words】 Tuberculosis, meningeal; Cerebrospinal fluid; Sensitivity and specificity; Enzyme-linked immunosorbent assay; Mononuclear cells

结核性脑膜炎(TBM)是由结核分枝杆菌(结核杆菌)引起的脑膜非化脓性炎症,其发病率约占全身结核病的6%,表现形式严重,早期诊断困难,病残率和病死率高,是结核病死亡的主要原因[1-3]。但在临床实际工作中,早期结核性脑膜炎患者临床表现和脑脊液改变大多不典型,易与病毒性脑膜炎、化脓性脑膜炎、新型隐球菌性脑膜炎(CM)等中枢神经系统感染相混淆,误诊率极高[4]。在传统的结核性脑膜炎的实验室诊断方法中,脑脊液涂片镜检阳性检出率约为10%,细菌培养阳性检出率为10%～30%[5],因而不能及时指导临床诊断与治疗。临床常用细菌感染免疫学检测方法,其阳性检出率随着病程的延长而逐渐增加,但对早期结核性脑膜炎的诊断无益。而且对于机体免疫力低下或应用免疫抑制药的患者,可能出现5%～45%的假阴性结果;另外因抗原特异性、试剂质量及操作方法的不同,可产生2%～15%的假阳性结果[5]。因此,临床工作迫切需要新的高敏感性和特异性的实验室诊断方法以降低早期结核性脑膜炎的误诊率。结核杆菌感染T细胞斑点试验(T-SPOT.TB)是利用酶联免疫斑点试验(ELISPOT)检测结核杆菌感染特异性T细胞诊断结核性脑膜炎的新方法,业已成为目前敏感性较高的细胞学检测技术[6]。在本研究中,我们应用T-SPOT.TB法检测外周血单个核细胞(PBMC)和脑脊液单个核细胞中分泌干扰素-γ(IFN-γ)的T细胞,探讨此项脑脊液细胞学检查方法对早期结核性脑膜炎的诊断价值。

资料与方法

一、研究对象

1. 结核性脑膜炎组(TBM组) 病例来源为2011年1月–2012年6月在宁夏医科大学总医院心脑血管病医院神经内科住院治疗且诊断明确的结核性脑膜炎患者,入选标准参考Skinner等[7]、Ahuja等[8]和Pehlivanoglu等[9]的临床诊断标准:(1)临床表现有发热、头痛、呕吐,脑膜刺激征阳性或伴有局灶性神经损害体征,结核杆菌培养阳性或聚合酶链反应(PCR)阳性。(2)脑脊液检查淋巴细胞比例增加、蛋白定量逐渐升高,结核杆菌培养阴性。(3)CT或MRI检查表现为脑水肿、肉芽肿或渗出。(4)发现中枢神经系统以外结核杆菌感染的证据。(5)抗结核药物治疗有效[10-14]。共30例患者,男性15例,女性15例;年龄17～74岁,平均(42.80±10.76)岁;发病时间5～120 d,其中≤14 d者10例、15～30 d者17例、>30 d者3例。其中单纯结核性脑膜炎26例,合并颅外结核杆菌感染4例。头部CT检查显示7例异常,其中脑积水2例、脑梗死4例、脑膜明显增厚强化7例、基底池渗出1例;MRI检查显示7例异常,其中脑膜增厚强化6例、软化灶形成1例。脑脊液墨汁染色和阿利新蓝染色均呈阴性。经抗结核药物治疗后,发病≤14 d组好转者8例、未治愈2例;15～30 d组好转者14例、未治愈3例;>30 d组好转者2例、未治愈1例。

2. 非结核性颅内感染组(对照组) 病例来源均

为2011年1月–2012年6月在我院神经内科住院治疗的中枢神经系统感染性疾病患者,共计30例,男性22例,女性8例;年龄15~75岁,平均(37.20±8.47)岁;发病时间3~30 d,其中≤14 d 24例、15~30 d 6例,>30 d 0例。根据相应诊断标准,病毒性脑膜炎14例、化脓性脑膜炎15例、新型隐球菌性脑膜炎1例。头部CT检查显示9例异常,其中脑膜炎症性改变4例、脑梗死5例;18例接受MRI检查11例异常,6例呈现炎症性改变、5例为脱髓鞘改变。经抗炎、对症治疗,20例治愈、8例好转、2例未治愈。

　　二、研究方法

　　1.仪器与试剂　(1)主要仪器:S.SW-CJ-2F超净工作台购自上海跃进医疗器械技术有限公司。HF90/HF240二氧化碳培养箱为上海力康生物医疗科技控股有限公司产品。(2)主要试剂:T-SPOT.TB试剂盒购自深圳达科为生物技术有限公司,含冲洗缓冲液(50×),10 ml稀释缓冲液R(1×)×2,100 μl生物素标记的IgGⅡ抗单克隆抗体以稀释缓冲液R(1×)稀释(1∶100),100 μl辣根过氧化物酶(HRP)标记的链霉菌卵白素以稀释缓冲液R(1×)稀释(1∶100),3-氨基-9-乙基卡唑(AEC)显色液[10 ml AEC稀释液、500 μl AEC溶液Ⅰ(20×)、500 μl AEC溶液Ⅱ(20×)、50 μl AEC溶液Ⅲ(200×)]使用时按照180∶10∶10∶1比例混匀,淋巴细胞生长刺激分裂素植物凝集素(PHA)冻干粉,预包被聚偏二氟乙烯(PVDF)板1块(含IgGⅠ抗单克隆抗体),300 μl结核杆菌特异性混合多肽A和多肽B。淋巴细胞分离液由挪威Fresenius Kabi Norge公司提供。RPMI-1640培养基购自美国Gibco公司。无血清培养基购自深圳达科为生物技术有限公司。杜氏磷酸盐缓冲液(DPBS)购自美国Hyclone公司。

　　2.样本采集和预处理　(1)外周血单个核细胞的分离和冻存:采集外周静脉血5~10 ml,置肝素抗凝管即刻混匀,室温3~4 h分离提取外周血单个核细胞,于静脉血中加入等量RPMI-1640液混匀,取预先加入5 ml淋巴细胞分离液的离心管,将混匀的细胞悬液缓慢加入淋巴细胞分离液上方,800×g高速离心25 min,加样器汲取呈云雾状的单个核细胞层至15 ml离心管,加入RPMI-1640液至15 ml,400×g离心6 min,弃上清,洗涤2次,以无血清培养基或小牛血清混悬细胞,调整细胞密度至2.50×10⁶/ml,滴加0.10 ml二甲基亚砜(DMSO)冻存备用。(2)脑脊液单个核细胞分离和冻存:留取新鲜脑脊液5~10 ml,
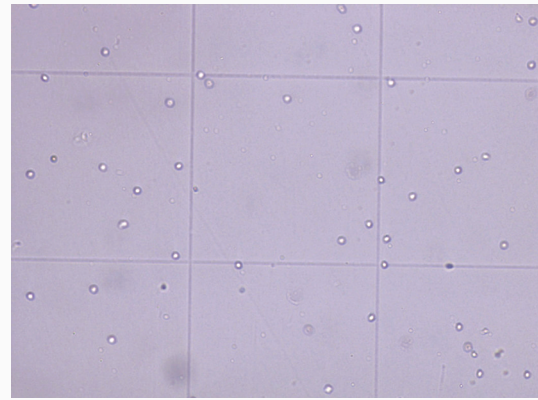

图1　经37~40℃温水快速复苏后光学显微镜计数外周血单个核细胞数目　体积分数为2%台盼蓝染色　×20

Figure 1　The number of mononuclear cells in PB was counted under optical microscope after rapid thawing in 37-40 ℃ water　Trypan blue (2% volume fraction) staining　×20

室温3~4 h加入等量RPMI-1640液混匀,400×g离心6 min,弃上清液,洗涤2次,无血清培养基混悬细胞,调整细胞密度至0.10×10⁶/ml,滴加0.10 ml DMSO冻存备用。

　　3.ELISPOT试验　(1)检测方法:样本置37~40℃温水中快速解冻,于细胞悬液中加入10倍的RPMI-1640液、混匀,400×g离心6 min,重悬于无血清培养基或RPMI-1640液行细胞计数,并取0.10 ml解冻的细胞悬液进行细胞存活率检测(存活率>90%,图1)。于预包被抗原的微孔板内滴加100 μl样本细胞混悬液,每一测试样本检测4孔,其中阴性对照孔加无血清培养基、阳性对照孔加植物凝集素,其余两孔分别加入抗原A[早期分泌抗原靶-6(ESAT-6)]和抗原B[培养分泌蛋白-10(CFP-10)],37℃、体积分数为5%的二氧化碳培养箱培养16~20 h,加冰冷去离子水,4℃冰箱放置10 min低渗裂解细胞,冲洗缓冲液冲洗5次;每孔滴加100 μl检测抗体(Ⅱ抗),37℃孵育1 h,冲洗缓冲液洗板5次,每孔滴加100 μl辣根过氧化物酶标记的链酶菌卵白素,37℃孵育1 h,冲洗缓冲液冲洗5次,每孔滴加100 μl AEC显色液,室温避光静置15~45 min,斑点生长至合适大小后,去离子水洗涤2次,终止显色,解剖显微镜(×25)下记录斑点参数。(2)阳性结果判定:依据试剂盒提供的阳性结果判定标准,斑点数为0~5个计为阴性;样本A和B斑点数–阴性对照孔斑点数≥6个为阳性,如果阴性对照孔斑点数≥6个,检测孔斑点数≥2倍的阴性对照孔斑点数,亦

Group	N	Positive	Negative
CSF	30	28 (93.33)	2 (6.67)
PB	30	23 (76.67)	7 (23.33)

*Fisher exact probability test, $P = 0.254$
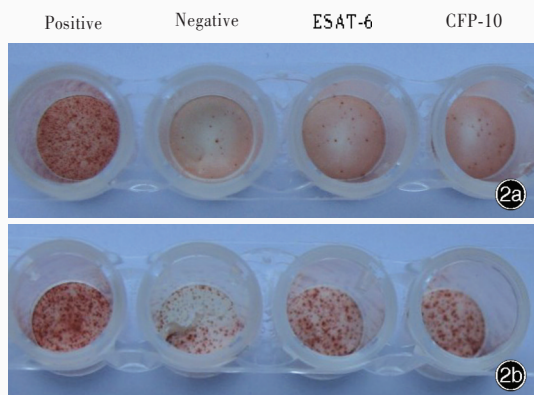
图2 结核性脑膜炎T-SPOT.TB检测所见　2a　脑脊液ELISPOT试验显示,阴性对照孔斑点数为3~10个,样本A和B的斑点数为2倍的阴性对照孔斑点数　2b　外周血ELISPOT试验显示,阴性对照孔斑点数为20~100个,样本A和B的斑点数为2倍的阴性对照孔斑点数
Figure 2 T-SPOT.TB findings in TBM patients. CSF ELISPOT assay showed the dots in negative control were 3–10, while the dots in test sample A and B were two times more than that (Panel 2a). PB ELISPOT assay showed that the dots in negative control were 20–100, while the dots in test sample A and B were two times more than that (Panel 2b)

为阳性。

4. 统计分析方法　采用SPSS 12.0统计软件进行数据计算与分析。计数资料以率(%)或相对数构成比(%)表示,检测结果以灵敏度、特异度、假阳性率、假阴性率、阳性似然比、阴性似然比表示,行Fisher确切概率法。以$P ≤ 0.05$为差异具有统计学意义。

结　果

一、脑脊液检测

脑脊液ELISPOT试验显示,TBM组30例患者中28例被诊断为结核性脑膜炎、2例为非结核性脑膜炎;对照组30例患者中仅1例被诊断为结核性脑膜炎、29例为非结核性脑膜炎。脑脊液ELISPOT试验检测灵敏度为93.33%(28/30),特异度为96.67%(29/30),假阳性率为3.33%(1/30),假阴性率为

6.67%(2/30);阳性似然比28.33,阴性似然比0.07(表1,图2)。

二、外周血检测

血清ELISPOT试验显示,TBM组30例患者中23例被诊断为结核性脑膜炎、7例为非结核性脑膜炎;对照组30例患者中4例被诊断为结核性脑膜炎、26例为非结核性脑膜炎。血清ELISPOT试验对结核性脑膜炎的检测灵敏度为76.67%(23/30),特异度为86.67%(26/30),假阳性率为13.33%(4/30),假阴性率为23.33%(7/30);阳性似然比5.79,阴性似然比0.15(表1,图2)。

脑脊液单个核细胞ELISPOT试验检测敏感性(93.33%)高于外周血单个核细胞(76.67%),但差异无统计学意义(Fisher确切概率法:$P = 0.254$;表1)。

讨　论

T-SPOT.TB技术是利用结核杆菌感染患者外周血或脑脊液单个核细胞中特异性活化T细胞,这些单个核T细胞在受到结核杆菌特异性抗原刺激后分泌干扰素-γ而设计的T细胞斑点试验[8],通过计数斑点数可以推测体内是否存在对结核杆菌反应的T细胞,从而对结核杆菌感染进行辅助诊断。此项技术可以从$60 × 10^3$个分泌干扰素-γ的T细胞中检测到一个细胞[15],具有相当高的敏感性,无论患者是否有临床症状,检测效应T细胞在外周血或脑脊液中的表达水平均可以作为机体被感染的指标,同时也能够反映机体是否清除病原体。

Codecasa等[16]发现一段结核杆菌存在RD1基因序列。但卡介苗菌株和大部分环境中的分枝杆菌则缺乏"RD1"基因序列,后者可编码两种蛋白质,分别为ESAT-6和CFP-10,ESAT-6/CFP-10融合蛋白综合了单一抗原优势,可针对外周血或脑脊液单个核细胞上的不同表位进行刺激,产生干扰素-γ,具有更强的活化能力,比单一抗原更为灵敏。因此,ESAT-6/CFP-10融合蛋白作为特异性刺激抗原具备更高的检测敏感性和特异性,从而对临床症状不典型、细胞涂片、结核杆菌培养均呈阴性的疑似结核杆菌感染和隐性结核杆菌感染患者提供有效的诊断方法[15]。

本研究2例结核性脑膜炎患者脑脊液T-SPOT.TB检测呈阴性反应,均于抗结核药物治疗1个月以上时采集脑脊液或外周血标本,据文献报道,效应T细胞存活期极短,一般病原体消灭后即消失[17]。鉴

于此,我们推测此2例患者可能经治疗后其体内的特异性T细胞数目即迅速减少,故T-SPOT.TB检测呈阴性反应;对照组仅一例被诊断为结核性脑膜炎,该患者同时合并糖尿病、脑梗死、肺癌术后,是否存在结核杆菌潜在感染尚不能排除。本研究结果显示,外周血单个核细胞ELISPOT试验检测结核性脑膜炎的灵敏度为76.67%,脑脊液为93.33%,有外周血检测敏感性明显低于脑脊液的趋势。Goletti等[18]经临床观察发现,部分肺结核患者体内RD1肽反应更低。提示不同疾病状态或不同宿主体内RD1肽T细胞反应受到影响,低抗原分布可以解释结核性脑膜炎患者较低的外周血ELISPOT试验检测结果。至于是否与各种肺外结核病临床征象不同而呈现不同的ELISPOT试验结果有关,尚待进一步研究加以证实。

　　活动性结核病的感染位置聚集着大量的结核杆菌特异性T细胞[10-11,19-28],从逻辑上说,通过ELISPOT试验检测受感染组织中的T细胞反应因子,能够提高活动性肺结核诊断的特异性。然而本研究结果显示,脑脊液单个核细胞ELISPOT试验检测结核性脑膜炎的特异度为96.67%,而外周血单个核细胞特异度则为86.67%,表明受感染组织的ELISPOT试验较单纯外周血检测对诊断活动性结核病有更具特异性的趋势。对于脑脊液ELISPOT试验,一般要求对所分离出的单个核细胞须在4小时内施行检测,因为脑脊液样本存放时间过长,可导致细胞计数出现虚假降低[20],因此脑脊液细胞存活率对ELISPOT试验敏感性有一定影响。

　　虽然本研究结果显示ELISPOT试验对诊断结核性脑膜炎具有较好的敏感性和特异性,但仍存在一些局限性。首先,判断患者为结核性脑膜炎,主要依据为脑脊液特点、神经影像学检查,以及患者对抗结核药物治疗的反应性,传统的结核杆菌培养、PCR反应和脑脊液细胞涂片等方法多因敏感性较差而致部分患者缺乏微生物学证据[12,18]。因此,我们以临床分类作为诊断标准,尚需联合结核杆菌培养、抗酸染色、PCR反应、临床症状、神经影像学、患者对抗结核药物治疗反应等[10-13,21]。其次,纳入的部分患者多种疾病共同存在,如糖尿病、肝病、脑梗死、中枢神经系统肿瘤等,导致机体免疫力低下,对检测结果是否存在影响尚无明确报道。总之,本研究结果表明,外周血和脑脊液单个核细胞ELISPOT试验可以作为当前诊断结核性脑膜炎的辅助方法,且有脑脊液单个核细胞ELISPOT试验较外周血检测效率更高的趋势。

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（收稿日期：2012-11-15）

·小词典·

中英文对照名词词汇（三）

抗心磷脂抗体　anti-cardiolipin（ACA）

抗中性粒细胞胞质抗体
　anti-neutrophil cytoplasmic antibody（ANCA）

颗粒溶素　granulysin（GNLY）

可提取性核抗原　extractable nuclear antigen（ENA）

辣根过氧化物酶　horseradish peroxidase（HRP）

类风湿因子　rheumatoid factor（RF）

离子型谷氨酸受体　intropic glutamate receptor（iGluR）

良性多发性硬化　benign multiple sclerosis（BMS）

临床孤立综合征　clinically isotated syndrome（CIS）

磷酸盐缓冲液　phosphate-buffered saline（PBS）

路易体痴呆　dementia with Lewy body（DLB）

卵泡刺激素　follicle stimulating hormone（FSH）

迈-格-姬染色
　May-Grunwald-Giemsa staining（MGG staining）

梅毒螺旋体　treponema pallidum（TP）

酶联免疫斑点试验
　enzyme-linked immunospot assay（ELISPOT）

酶联免疫吸附试验
　enzyme-linked immunosorbent assay（ELISA）

美国多发性硬化治疗评价小组
　Multiple Sclerosis Therapy Consensus Group（MSTCG）

美国风湿病协会　American Rheumatism Association（ARA）

美国国家过敏性疾病与感染病研究所联合抗病毒研究组
　National Institute of Allergy and Infectious Diseases
　Collaborative Antiviral Study Group（NIAID-CASG）

美国国立卫生研究院　National Institute of Health（NIH）

美国食品与药品管理局
　Food and Drug Administration（FDA）

免疫荧光染色　immunofluorescence assay（IFA）

免疫荧光细胞染色　cell-based assay（CBA）

耐甲氧西林金黄色葡萄球菌
　methecillin-resistant staphlococcus aureus（MRSA）

耐甲氧西林凝固酶阴性葡萄球菌
　methicillin-resistant coagulase negative staphylococcus
　（MRCNS）

耐甲氧西林葡萄球菌
　methicillin-resistant staphylococcus（MRS）