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厦门大学

硕 士 学 位 论 文

基于酶联免疫斑点技术的 I 型单纯疱疹病毒中和抗体检测方法的建立

Development of an Enzyme-Linked Immunosorbent Spot Assay to Measure Serum Neutralizing Antibodies against Type I Herpes Simplex Virus

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摘 要

单纯疱疹病毒（Herpes Simplex Virus，HSV）属于疱疹病毒科 a 病毒亚科，根据血清型和抗原性的差异，单纯疱疹病毒分为 I 型和 II 型（HSV-1、HSV-2）。其中 HSV-1 病毒主要引起口唇性疱疹，HSV-2 病毒主要引起生殖器疱疹。HSV-1 病毒相比于 HSV-2 病毒危害性较小，80%以上的中国人都曾经感染过 HSV-1 病毒。

恶性肿瘤严重威胁着人类生命健康，其发生率呈逐年上升趋势。据世界卫生组织 2010 年统计结果显示目前肿瘤引起的死亡数目已经超越心脑血管疾病引起的死亡数目而跃居首位。随着肿瘤诊疗技术水平的不断提高，早期肿瘤患者的整体生存率显著提高，但是中晚期肿瘤患者生存率仍相对较低。目前缺乏针对中晚期肿瘤有效的治疗手段。因此亟需探索研究一种新型有效的肿瘤治疗手段。近年来研究证实基因工程改造的 HSV-1 型溶瘤病毒能够特异性的在肿瘤细胞中复制而杀伤肿瘤细胞，而对正常细胞几乎不损伤，能显著抑制肿瘤的生长。溶瘤病毒作为一种新型有效的肿瘤治疗手段，同时具有安全、高效、副作用小等优点，在肿瘤治疗的临床开发研究上具有广阔的应用前景。目前多种 HSV-1 型溶瘤病毒已经进入临床试验阶段，美国安进公司研发的 HSV-1 型溶瘤疱疹病毒 OncoVex$^{GM\text{-}CSF}$ 已经完成了在晚期恶性黑色素瘤患者治疗的 III 期临床试验，并取得了较为理想的临床治疗效果，表明 HSV-1 是一个优良的肿瘤治疗病毒载体。然而，HSV-1 在人群中感染十分普遍，流行病学调查研究表明全球自然人群中 HSV-1 病毒抗体阳性率为 45%-90%，我们推测血清中可能存在不同程度的针对 HSV-1 的中和抗体，高滴度的中和抗体势必会影响 HSV-1 型溶瘤病毒体内的治疗效果。

传统检测血清中针对 HSV-1 中和抗体滴度主要是利用空斑减少中和试验法（Plaque Reduction Neutralization Test, PRNT），该方法工艺较为复杂、需要投入大量人力物力且耗时较长（约 7-10 天），难于满足临床上快速、准确、高通量检测需求，因此亟需建立一种稳定、简便、高通量的检测手段。

本论文旨在建立一种基于酶联免疫斑点法（The Enzyme-Linked ImmunoSpot Assay, ELISPOT）的快速、准确、高通量检测 HSV-1 中和抗体的检测平台，并在此基础上评估健康人群血清中 HSV-1 中和抗体的水平，为 HSV-1 型溶瘤病毒的临床治疗提供依据和理论指导。

本研究利用 HSV-1 病毒（KOS strain）免疫小鼠获得 12 株对 HSV-1 反应性良好的单克隆抗体。利用 ELISPOT 法我们筛选出一株针对 HSV-1 灵敏度、特异性较高的抗体 2G5，该抗体与病毒反应的斑点数与感染的病毒量之间具有很好的相关性，且检测的线性范围较宽。我们进一步利用非还原蛋白印迹法和免疫荧光法证实 2G5 能特异性的识别 HSV-1 gD 糖蛋白，与 HSV-1 其它糖蛋白没有交叉反应，并鉴定其为识别 gD 糖蛋白构象表位的单克隆抗体。利用 2G5 抗体我们尝试建立一种基于 ELISPOT 法检测 HSV-1 中和抗体的检测平台。在确定最佳病毒感染剂量和感染时间的实验中，我们发现 ELISPOT 斑点检测的最佳线性范围为每孔感染 2000~20000pfu 病毒，最佳检测时间为病毒感染后 14hr（<18hr 病毒感染处于停滞期）。在病毒斑点检测线性范围内人血清中中和抗体的滴度不受病毒感染剂量因素的影响。我们分别利用新建立的 ELISPOT 法和传统的 PRNT 法测定人血清标本中 HSV-1 中和抗体水平，结果发现两种方法的检测结果一致，符合率较高且具有很高的可重复性。上述结果表明我们建立的基于 ELISPOT 法检测 HSV-1 中和抗体的方法具有良好的可靠性。此外利用该方法检测 HSV-1 中和抗体的时间仅需 1-2 天，整个检测过程可实现高通量半自动化，从而避免了传统空斑减少法的主观误差，有利于进行快速、准确检测。我们利用该平台检测了来自厦门市疾控中心 290 份不同年龄普通人群血清中 HSV-1 中和抗体水平，结果发现普通人群中和抗体平均滴度 Nt_{50} 为 1087.92 (0-11687)，无显著性性别差异，人体内 HSV-1 中和抗体水平随着年龄的增长而呈上升趋势。

综上所述，本研究通过筛选获得一株特异性识别 HSV-1 gD 糖蛋白的单克隆抗体 2G5，并利用该单抗建立了一种基于酶联免疫斑点法（ELISPOT）检测 HSV-1 中和抗体滴度的方法。相比于 PRNT 法，该方法具有简单、快速及高通量检测等明显优势，同时能够保证血清样本检测的准确率，可以为临床病人血清中 HSV-1 中和抗体的检测和 HSV-1 病毒中和表位研究提供检测技术支持，为 HSV-1 型溶瘤病毒的临床治疗提供依据和理论指导。

关键词：溶瘤病毒； HSV-1； 中和抗体； gD 特异性抗体 2G5； ELISPOT 检测

Abstract

Herpes simplex virus (HSV), also as known as human herpesvirus, belongs to the Herpesviridae family, alphaherpesvirinae subfamily. According to the serotype and antigenicity, herpes simplex virus is divided into two types: type I and II herpes simplex virus (HSV-1 and HSV-2). HSV-1 causes primarily oral infections while HSV-2 more commonly causes genital infections. HSV-2 infection causes more severe disorder than HSV-1. HSV-1 brings mild damage to human health, more than 80% percent of Chinese populations were infected with HSV-1.

Malignant cancer poses a great threat to human health, the incidence of cancer is increasing year by year. According to the statistic data of World Health Organization, the number of deaths caused by cancer has surpassed that caused by cardiovascular diseases and ranked at No. 1 place of disease mortality. With the technique improvement in both cancer diagnostics and therapy, the overall survival rate in patients with early stage has been significantly improved, however, that in patients with advanced stage (middle and end stage) is still relatively low due to lack of effective treatment for advanced cancers. Therefore, novel and effective cancer treatment strategies should be urgent to develop for combating the advanced cancer. Recent data has proved that engineered HSV-1 virus can replicate only in tumor cells but hardly influence the normal cells, which is a potential therapeutic for cancer treatment. Oncolytic virus (OV) is a novel treatment option with tremendous advantages, such as safe, effective and fewer side effects, oncolytic viotherapy has shown great promise for clinical application against cancer. Currently, several types of oncolytic HSV-1 virus have entered into clinical trails, the phase III clinical trial of OncoVexGM-CSF in advanced melanoma patients has been completed recently by U.S. Amgen company, exhibits a great success, indicating that HSV-1 is an excellent viral vector for cancer treatment. It is worth to note that HSV-1 infection is very common, worldwide rates of HSV-1 infection is between 45% and 90%. Almost all these population with HSV-1 infection show HSV-1 antibody positive in serum, so we hypothesize that there may be

varying degrees of serum neutralizing antibodies against HSV-1 in serum, high neutralizing antibody titer definitely have an impact on the efficacy of HSV-1 based oncolytic virotherapy.

Traditional assay for tittering the neutralizing antibody against HSV-1 in serum is the use of plaque reduction neutralization test (PRNT), which process is complex, labor intensive and time-consuming (about 7-10 days), it is difficult to meet the needs of clinical application for fast, accurate, high-throughput detection, therefore, it is necessary to establish a stable, simple, high-throughput testing methods for detection of neutralizing antibody against HSV-1.

This thesis aims to establish a fast, accurate and high-throughput detection platform for tittering the neutralizing antibody against HSV-1, which is based on ELISPOT (The Enzyme-Linked Immunospot Assay) technique, so we can evaluate the anti-HSV-1 neutralizing antibody titers of general population with this method. These data will provide solid basis and theoretical guidance for clinical application of oncolytic therapy.

In this study, HSV-1 virus (KOS strain) was propagated in U2OS cells and served as an immunogen for development of HSV-1 specific monoclonal antibodies (Mabs). 12 mAbs were obtained from mice immunized with HSV-1 virus with good reactivity to HSV-1 virus in the ELISPOT detection. We screened these Mabs for their specifity and sensitivity against HSV-1 virus and found 2G5 was a good candidate for detection with broad linear detection range of detection and accurate spot relevance when different viral doses were applied in this system. 2G5 was a conformational-epitope antibody for specifically recognizing HSV-1 glycoprotein D (gD) based on the results obtained from the non-reducing Western blot and immunofluorescence experiments.

We tried to establish a detection platform for tittering the neutralizing antibody against HSV-1 by virtue of 2G5 antibody. To determine the optimized dose and experimentally detection time, we set up a series of experiments and found that the best linear range of virus dose with good detection spots was 2000~20000pfu per well, optimal detection time of virus infection was 14 hr (Virus stayed in the lag phase when <18 hr). In the abovementioned detection range, the titers of neutralizing antibody

against HSV-1 were unaffected by the infection dose. The titer of neutralizing antibody against HSV-1 in serum tested by our new ELISPOT based assay was consistent with the detection results tested by classical PRNT assay, which indicated the ELISPOT based assay had good repeatability and reliability. The entire process took only 1-2 days and could be fully achieved with the characteristics of high-throughput and semi-automated procedure, thus this new assay was beneficial to reduce the subjective errors introduced by PRNT assay. We used this platform to detect the titers of neutralizing antibody in a cohort consisting of 6 ages- and sex-matched specimen of general population from Xiamen Center of Disease Control (CDC), the average titers of neutralizing antibody (Nt_{50}) was 1087.92, the level of Nt_{50} was in an upward trend with the factor of age.

In summary, this thesis identified a new HSV-1 gD-specific mAb, named 2G5, which could be employed to establish an ELISPOT based method for detecting titers of neutralizing antibody against HSV-1. Compared to the PRNT assay, this new assay had several significant advantages, such as simple, fast, and high-throughput. We found the detection accuracy of this assay was also guaranteed when clinical specimen were tested. This assay will provide solid technique support for detection of neutralizing antibodies in serum from patients either HSV-1 infected or cancer patients with OV therapy, as well as for HSV-1 related neutralizing epitope studies. These finding in clinical detection can provide useful evidence and theoretical guidance for clinical application of oncolytic therapy.

Keyword: oncolytic virus; Herpes simplex virus; neutralizing antibody; gD specific antibody 2G5; ELISPOT detection

缩略表

缩写	英文全称	中文名称
HSV-1	Human Simplex virus type 1	I型单纯疱疹病毒
OV	Oncolytic Virus	溶瘤病毒
PFU	Plaque Forming Unite	空斑形成单位
ELISA	Enzyme Linked Immuno-Sorbent Assay	酶联免疫吸附试验
ELISPOT	Enzyme-Linked Immuno-Spot Assay	酶联免疫斑点实验
IF	Immunofluorescence	免疫荧光
gD	Glycoprotein D	糖蛋白D
PRNT	Plaque Reducing Neutralization Test	空斑减少中和试验
MOI	Multiplicity of Infection	感染复数
FBS	Fetal Bovine Serum	胎牛血清
PEG	Polyethylene Glycol	聚乙二醇
DMSO	Dimethyl Sulfoxide,	二甲基亚砜
mAb	Monoclonal Antibody	单克隆抗体
DNA	Deoxyribonucleic acid,	脱氧核糖核苷酸
RNA	Ribonucleic acid	核糖核苷酸
ORF	Open Reading Fragment	开放阅读框
HRP	Horseradish Peroxidase	辣根过氧化物酶
PCR	Polymerase Chain Reaction	聚合酶链式反应
FITC	Fluorescein Isothiocyanate,	异硫氰酸荧光素
KDa	Kilo Daltons	千道尔顿
ACV	Acyclovir	阿昔洛韦
GCV	Ganciclovir	更昔洛韦
Nt_{50}	Neutralization titer 50	50%抑制时中和抗体滴度
RPM	Revolutions per minute	转/分钟
bp	Base Pair	碱基对

PBS	Phosphate Buffered Saline	磷酸盐缓冲液
ATCC	American Type Culture Collection	美国模式菌种保藏中心
WHO	World Health Organisation	世界卫生组织
SFDA	China Food and Drug Administration	国家食品药品监督管理总局