学校编码：10384　　分类号＿＿＿密级 秘密

学号：21620110153946　　UDC ＿＿＿＿＿＿＿＿

厦门大学

博　士　学　位　论　文

H5 亚型流感病毒抗体 13D4 的 hCDR3 变构结合受体作用位点和广谱中和作用的结构基础

Structural basis for the wide-spectrum neutralization of H5N1 influenza virus by an antibody 13D4 with hCDR3 adaptively fitted to receptor-binding site

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论文提交日期：

论文答辩时间：

学位授予日期：

答辩委员会主席：＿＿＿＿＿＿

评　　阅　　人：＿＿＿＿＿＿

2015 年　12　月

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目录

Contents

H5 亚型流感病毒抗体 13D4 的 hCDR3 变构结合受体作用位点和广谱中和作用的结构基础

摘要

H5N1 病毒自 1997 年以来造成人类社会巨大的经济损失和社会影响，并且呈现持续性的感染与危害。目前，有效的防治药物有小分子抑制药物与预防性疫苗，但均存在着病毒逃逸现象及保护范围较小等缺陷。因此，开发新型广谱药物是一项长期且重要的研究热点。已知的，流感病毒主要抗原区是在病毒表面包膜蛋白 HA 和 NA 上，其中 HA 被认为是新药物开发的关键靶标。由于越来越多的跨型及型内广谱中和抗体被分离出来，人们将注意力转移至研究血凝素蛋白的广谱表位，以期设计出广谱通用型流感疫苗。

在本实验室前期研究工作中，从病毒免疫的小鼠体内分离出一株 H5 型特异性广谱单抗 13D4，经过血凝抑制实验和细胞中和实验等方法，鉴定出其具备识别 H5 亚型各类别的血凝素蛋白的特性，并在动物实验中呈现较好的病毒感染保护作用。再结合一系列理化分析手段，验证 13D4 抗体主要作用于血凝素的受体结合区域。因此，对 13D4 抗体作用的结构研究，将进一步阐明 H5 亚型流感病毒上的保守中和表位及抗体的中和机制。在应用方面，也可以指导疫苗的设计和研发及小分子多肽药物的开发。

本论文通过 X-ray 晶体衍射技术解析 13D4 抗体与 VN1194 血凝素蛋白复合物和单独 13D4 抗体 Fab 的晶体结构，找寻关键作用位点，再经过丙氨酸扫描法对相关位点进行突变和结合各种生物物理及生物化学手段进行关键位点的分析和验证，并基于结构分析病毒逃逸机制和探索 13D4 识别 HA 的变构作用机制，为深入了解 13D4 抗体中和作用机制及其应用于药物开发方面提供结构依据。

首先，在研究中通过昆虫细胞-杆状病毒表达系统，成功地在外源重组表达血凝素三聚体蛋白，利用 SDS-PAGE、ELISA、HPSEC 和分析超离等手段，验证其三聚体结构的形成。经过两步聚的蛋白酶切实验（Thrombin 和 Trypsin），去除血凝素外源基因，并将 HA0 蛋白酶解成 HA1 和 HA2 两部分亚基，以更好地模拟天然成熟病毒的血凝素结构。研究发现，去除三聚化后，血凝素三聚体解聚成

单体分子。但在后续的功能分析研究中，鉴定出血凝素单体分子与三聚体分子具有同样的 13D4 抗体结合活性。因此，仍选用血凝素蛋白单体进行后续的结构研究。利用晶体筛选试剂盒，优化 13D4 Fab 及其与重组血凝素蛋白的复合物晶体培养条件，而后，分别获得两个大小合适，能用于衍射的晶体。

其次，将获得的 13D4 Fab 及其与重组血凝素蛋白的复合物两种晶体进行 X-ray 衍射，分别收获 13D4 Fab (2.0 Å)和 13D4-HA 复合物（2.33 Å）两套晶体衍射数据，并利用 HKL2000、PHENIX 和 COOT 等软件上解析对应的晶体结构。利用解析后的复合物结构进行抗原抗体相互作用分析，共找出 13D4 抗体作用的 16 个关键位点，并基于结构发现 13D4 抗体主要是以重链 CDR3 伸入受体结合区凹槽与血凝素蛋白相互作用，而其他重链 CDR 区辅助的结合特征。对 13D4 抗体序列的比较和分析，表明 13D4 抗体重链区来源于 VH1-9 基因系，有 16 个氨基酸的重链 CDR3。而对血凝素上中和位点的比较和分析，表明 H5 亚型血凝素蛋白上 16 个 13D4 抗体识别位点的型内高保守性。

最后，利用位点突变，验证 13D4 抗体作用的 16 个关键位点在抗体结合抗原上的重要性。结果发现四个氨基酸的变异直接导致 13D4 抗体无法识别血凝素蛋白，分别是 W153、K156、K193 和 L194。同时，基于晶体结构进行如下相关的分析研究：1. 推测 K193 位点的变异可能是影响新 H5 亚型病毒逃逸的关键因素。2.对比受体类似物的结合方式，发现 13D4 抗体也是采用与其相似的方式与血凝素蛋白作用。3.比较 13D4 Fab 晶体与复合物结构，发现抗体重链 CDR3 可发生变构现象，通过进一步的动力学模建，构建出平均 RMSD 为 2.946 Å 的 13D4 Fab 的模建结构，说明本论文较好地模拟出 13D4 抗体的变构现象。以上研究均说明 13D4 抗体作用的特殊机制。

综上所述，本论文从 13D4 抗体与血凝素蛋白复合物晶体的结构上，研究 13D4 抗体的中和作用机制，阐明血凝素蛋白上的关键表位，在分子水平上揭示出 H5 型内广谱抗体的结构基础。这为现有广谱中和抗体的作用机理提供更多的理论数据，同时对研究 H5 亚型病毒的分子进化、逃逸机制及预防诊治提供结构信息和理论基础，也为今后的小分子多肽药物的设计开拓研究思路。

关键词：H5 型流感病毒血凝素；亚型广谱中和单抗； X-ray 衍射技术

Structural basis for the wide-spectrum neutralization of H5N1 influenza virus by an antibody 13D4 with hCDR3 adaptively fitted to receptor-binding site

Abstract

Since 1997, H5N1 virus has caused great economic loss and social influence in human society, and has been showing sustained infection and damage. At present, Drugs for effective prevention and control of H5 subtype virus are a small molecule inhibitory drug or prophylactic vaccines, however, there are some shortcomings including recognition of mutant virus and smaller range of protection. Therefore, the development of new broad-spectrum drugs is a long-term and important research hotspot. The major antigenic regions of the influenza virus are known to be harbored by HA and NA on the surface of the virus, in which HA is considered to be a key target for the development of new drugs. Because more and more cross-subtype specific and subtype specific broadly neutralizing antibodies were isolated, people pay more attention on the study of the broad-spectrum epitopes on hemagglutinin protein, hopeful to design a broad-spectrum universal flu vaccine.

In the previous research of our laboratory, we isolated an H5 subtype-specific broad-spectrum mAb 13D4 from the mice immuned with flu virus by hemagglutination inhibition experiments and in vitro neutralization methods, identified it was almost able to recognition of hemagglutinin protein from all clades of H5 subtype viruses, and proved it showed a good protective effect on flu virus infection in animal experiments. By means of a series of physical and chemical analysis, the receptor binding domain on the hemagglutinin was identified to be the effect region of 13D4 antibody. Studies on the structure of the 13D4 antibody will further clarify the conservative neutralizing epitope on H5 subtype influenza virus and the neutralization mechanism of this antibody. In the application, it can also guide the design and development of vaccine and the development of small molecule peptide

drugs.

The main contents of this research include: 1. the crystal structure determination of 13D4 antibody and VN1194 hemagglutinin immune complex through X-ray crystal diffraction; 2. To find the key functional sites; 3. mutation analysis of these functional sites through alanine scanning method; 4. fuctional analysis and verification of these key sites combined with various biophysical and biochemical methods; 5. analysis of viral escape mechanism based on the crystal structure. All these work was done for in-depth understanding of antibody effect mechanism and providing structural basis to its application in drug development.

First of all, in the study the recombinant trimeric hemagglutinin protein was expressed using baculovirus insect cell expression system. According SDS-PAGE, ELISA, HPSEC and AUC analysis, the the trimer structure of recHA was validated. After two step proteases treatment (thrombin and trypsin), the trimerization sequence was removed off design HA expression gene and HA0 was cleaved into HA1 and HA2 two part subunits, in order to better simulate the natural mature structure of virus hemagglutinin. In the study we found that, after removal of trimerization sequence, hemagglutinin trimer was depolymerized into monomer molecules. However, in the subsequent functional analysis, the same bindint activity of the monomer HA0 to 13D4 antibody was identified compared with trimer HA0. Therefore, we still chose the monomer to study the subsequent structure determinantion. Using crystal screening kits and optimizing the culture conditions of 13D4 Fab and its complex with recombinant hemagglutinin protein, we obtained two right-size crystals, which could be used for X-ray diffraction.

Secondly, the two crystals were sent to SSRF for X-ray diffraction, and two sets of diffraction data were collected for 13D4 Fab (2.0 Å) and 13D4-HA complex (2.33 Å). The crystal data was dissolved by the HKL2000 and COOT software. The antigen-antibody interaction analysis of immune complex was carried out, and 16 key sites were identified. The characterization of complex was well elaborated, in which the 13D4 antibody was mainly to interact hemagglutinin by extend its heavy chain CDR3 into the groove of the receptor binding region. The following analysis of

neutralization site of 13D4 antibody and the sequence of hemagglutinin further explained the characteristics and subtype-specific recognition of 13D4 antibody.

Finally, the site mutations were carried out to verify the effect of 16 key sites on 13D4 antibody functionally binding to hemagglutinin. Results showed that variation of four amino acids (respectively W153 and K156, K193 and L194) led directly to the loss recognition of 13D4 antibody to hemagglutinin protein. And we did more analysis on the virus escape phenomenon, receptor mimicry and heavy chain CDR allosteric simulation fit phenomenon etc. based on the crystal structure, to clarify the special action mechanism of 13D4 antibody.

In summary, this study was based on the structure determination of 13D4 antibody and hemagglutinin immune complexes crystal. The neutralizing mechanism of 13D4 antibody was clarified and the key epitopes on hemagglutinin were identified. At the molecular level we revealed the structural basis of the broad-spectrum recognition of H5 subtype virus by 13D4 antibody. This will provide more theoretical data for the mechanism of the effect of broad-spectrum neutralizing antibodies, and more structural information and theoretical basis for the study of molecular evolution, escape mechanism and prevention and treatment of H5 subtype viruses. It also enpanded the design thinking of anti-H5 subtype virus small molecular polypeptides.

Keywords: H5 subtype influenza virus hemagglutinin; Subtype-specific neutralizing antibody; X-ray diffraction