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Construction and preliminary panning of Fab phage display antibody library against respiratory syncytial virus

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Abstract: **Objective** To construct a human phage display antibody library, which will help to develop new drugs and vaccines against respiratory syncytial virus (RSV) and solve many of the issues that have limited the progression and application of murine monoclonal antibodies (McAbs) in the clinic. This can provide a platform for human antibody preparation and diagnosis, prophylaxis and therapy of RSV infection in children. **Methods** Peripheral blood lymphocytes were isolated from 52 children with RSV infection. cDNA was synthesized from the total RNA of lymphocytes. The light and heavy chain Fd (VH-CH1) fragments of immunoglobulin gene were amplified by RT-PCR. The amplified products were cloned into phagemid vector pComb3x and the clone samples were electrotransformed into competent *E. coli* XL1-Blue. The transformed cells were then infected with M13K07 helper phage to yield recombinant phage antibody of Fabs. The plasmids extracted from amplified *E. coli* were digested with restriction endonucleases Sac I, Xba I, Spe I and Xho I to monitor the insertion of the light or heavy chain Fd genes. RSV virions were utilized as antigens to screen Fab antibodies. **Results** By recombination of light and heavy chain genes, an immune Fab phage display antibody library against RSV containing 2.08×10^7 different clones was constructed, in which 70% clones had light chains and heavy chain Fd genes. The capacity of Fab phage antibody gene library was 1.46×10^7 and the titre of the original Fab antibody library was about 1.06×10^{12} pfu/mL. The antibody library gained an enrichment in different degrees after the preliminary panning. **Conclusions** Utilizing the technology of phage display, an immune Fab phage display antibody library against RSV was successfully constructed in this study, which laid a valuable experimental foundation for further study and created favorable conditions for preparing human McAbs. This may also contribute to the improvement in the diagnosis, therapy and prophylaxis of RSV infection in children. [**Chin J Contemp Pediatr**, **2008**, **10** (**6**):**681 – 685**]

Key words: Antibody library; Phage display technology; Fab antibody; Respiratory syncytial virus; Child

抗呼吸道合胞病毒 Fab 噬菌体抗体库的构建及初步筛选

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[**摘 要**] **目的** 该研究旨在利用噬菌体展示技术,构建小儿呼吸道合胞病毒感染患者人源性噬菌体抗体库,搭建人源性抗体制备的技术平台,解决鼠源性单克隆抗体临床应用的不足,为小儿呼吸道合胞病毒感染发病机制的研究、诊断、治疗和预防提供新的有效途径。**方法** 从 52 例呼吸道合胞病毒感染患儿外周血淋巴细胞中提取总 RNA,并逆转录为 cDNA。用 PCR 扩增轻链和重链 Fd 段(即重链的可变区和第一恒定区)基因,并将扩增的轻链和重链基因片段克隆于 pComb3x 噬粒载体,电转化 XL1-Blue 大肠杆菌,经辅助噬菌体 M13K07 超感染后构建成 Fab 段噬菌体抗体库。对此抗体库双酶切进行鉴定,并用呼吸道合胞病毒颗粒作抗原进行初步筛选。**结果** 经过重轻链基因的重组,成功构建一免疫噬菌体抗体基因库,共有 2.08×10^7 个不同的克隆菌,其中 70% 的克隆均含有轻链和重链 Fd 基因。因此,所构建的噬菌体抗体库的库容量为 1.46×10^7,经过滴定,原始抗体库的滴度为 1.06×10^{12} pfu/mL。经初步筛选,抗体库得到了不同程度的富集。**结论** 利用基因重组技术和噬菌体展示技术,成功构建了小儿呼吸道合胞病毒感染患者人源性 Fab 噬菌体抗体库,为人源性单克隆抗体的制备提供了良好的条件,为进一步的研究奠定了基础,亦将有益于小儿呼吸道合胞病毒感染的诊断、治疗和预防。

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[**关 键 词**] 抗体库; 噬菌体展示技术; Fab 抗体; 呼吸道合胞病毒; 儿童

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Respiratory syncytial virus (RSV) infection is very common in young children and so diagnosis, therapy and prevention of RSV infection are always a focus of concern for pediatricians. In 1891, Emil von Behring cured a little girl with diphtheria using antitoxinum diphthericum. It was the first case in history using an antibody as a drug for infectious diseases and laid the foundations for the development of antibodies based on infectious diseases therapy[1]. In 1975, the invention of hybridoma technology that allowed production of monoclonal antibodies (McAbs) facilitated the development of pediatrics and other subjects. McAbs revealed favorable prospects when they were widely used in diagnosis, therapy and prevention. But for human therapy, the murine McAbs were greatly limited because of human anti-mouse antibody (HAMA) response and other unexpected side effects[2]. The development of molecular biology and genetically engineered antibody technology[3], particularly the development of phage antibody library technology[4], provided a good and new approach for preparation of all kinds of humanized or human McAbs. This has become a revolutionary progress in the antibody engineering field[5]. This study used peripheral blood lymphocytes which were obtained from 52 children with RSV infection as gene sources and successfully constructed and preliminarily screened an immune Fab phage antibody library. This may be helpful in developing new drugs and vaccines against RSV in solving many issues that have limited the progression and application of murine McAbs in clinical practice.

Materials and methods

Materials

To obtain peripheral blood lymphocytes, 2 mL venous blood were collected from each of the 52 children with positive anti-RSV IgM and/or IgG proved by ELISA.

Phagemid vector pComb3x (containing ampicillin resistance gene), *E. coli* XL1-Blue (containing tetracycline resistance gene) and helper phage M13K07 (containing kanamycin resistance gene) were kindly presented by Doctor LI Yu (Fourth Military Medical University). Diethylpyrocarbonate, Trizol, RNA reverse transcription kit, Taq DNA polymerase, T4 DNA ligase, restriction endonucleases Spe I, Xho I, Sac I and Xba I were provided by AMRESCO, Invitrogen, Fermentas, TaKaRa and Promega company respectively. PCR primers were synthesized by Generay Biotech Company.

Methods

Lymphocyte separation and total RNA extraction

The lymphocytes in 100 mL peripheral venous blood were isolated by lymphocyte isolation agent. Then the total RNA was extracted immediately by Trizol and trichloromethane. Meanwhile, the integrity of RNA was checked by alkaline denaturing agarose gel electrophoresis and optical density (OD) measurement.

Light chain and heavy chain Fd genes amplification

A set of PCR primers for light chain and heavy chain Fd genes of antibody were used according to the reference [6] and experience. The cDNA was synthesized from the total RNA of lymphocytes according to the manual of RNA reverse transcription kit and used as templates for the followed PCR performance. PCR was performed with the primers under the conditions: 94℃ ×50 s, 55℃ ×50 s, and 72℃ ×1 min, with 30 cycles. The light chain and the heavy chain Fd fragments of antibody genes were amplified by RT-PCR. The restriction endonucleases cut situs Sac I + Xba I and Xho I + Spe I were introduced at the same time. All the antibody gene products were recovered by agarose gel DNA purification kit.

Construction and preliminary identification of light chain and heavy chain Fab antibody gene library

The amplified light chain gene products were digested with restriction endonucleases Sac I + Xba I and inserted into phagemid vector pComb3x and the clone samples were electroporated into competent *E. coli* XL1-Blue. These were then seeded on the SOBAG medium plate (containing 100 mg/L ampicillin and 20 g/L glucose) at 30℃ for 24 hrs to yield recombinant phage antibody gene library of light chains. The transformation efficiency was detected to calculate the capacity of light chain gene library. Ten positive colonies were picked up randomly for the restriction enzyme analysis by Sac I + Xba I to identify recombinant rate. To select the macromolecule fragments, the plasmids were extracted from all the positive colonies and cut with endonucleases Xho I + Spe I. The macromolecule fragments and Fd fragments cut with the same endonucleases were ligated by T4 ligase. The ligation products were electroporated into competent *E. coli* XL1-Blue to yield recombinant phage antibody gene library of Fab. The next steps were similar to the construction of light chain gene library.

Construction and preliminary screening of Fab phage antibody library

The products of the Fab antibody gene library were inoculated into SB-A^+T^+ liquid medium (containing 100 mg/L ampicillin and 10 mg/L tetracycline) and

incubated for 2 hours at 37℃ with shaking. Then helper phage M13K07 (10^{12} pfu/100 mL) and kanamycin (70 mg/L) were added. After an overnight culture in SB-$A^+T^+K^+$ liquid medium (containing 100 mg/L ampicillin, 10 mg/L tetracycline and 70 mg/L kanamycin), it was centrifuged (4 000 r/min × 15 min, at 4℃) and the phage antibodies in supernatants were precipitated by 40 g/L PEG8000 and re-suspended in PBS (containing 1% BSA and 10% glycerin). After a transient centrifugation, the ultimate supernatants were obtained and the original Fab phage antibody library was constructed. The original Fab phage antibody library was titrated by top agar. Purified RSV virions were coated in 96-well plate overnight at 4℃. Next day after being washed with PBS they were blocked with 3% BSA-PBS and incubated for 1 hour at 37℃. Phagemid populations above were added to the plate, and after incubation for 1 hour at room temperature, the plate was emptied and washed with 0.05% PBST (containing 0.05% Tween20) for 10 times then a further 10 times with PBS. Then log phase *E. coli* XL1-Blue and M13K07 were added. The phages were re-infected and rescued after 2 hours of incubation at 37℃. The output library was titrated. The panning process was repeated four times. To determine the concentration of phage antibodies against RSV in each round of panning, an ELISA was performed as follows: 100 μL RSV was coated in 96-well plates overnight at 4℃. Then 10 μL phage of each round was added after washing plates with PBS, and blocking with 2% MPBS (containing 2% non-fat milk) for 1 hour. After 2 hours incubation at room temperature, 100 μL HRP-antiM13 antibody was added in each well. One hour later, the plates were washed with 0.05% PBST for 5 times then ABTS were added. After 30 minutes incubation at room temperature, A value (optical density) of each well was measured with ELISA reader.

Results

Lymphocyte separation and total RNA extraction

The results of denaturing gel electrophoresis demonstrated that total RNA was integral. The ratio of OD260/OD280 was 2.03.

Light chain and heavy chain Fd genes amplification

The gel electrophoresis results showed that light chain and heavy chain Fd gene fragments amplified was about 700 bp which was accorded with anticipated size (Figure 1).

Construction and preliminary identification of light chain and heavy chain Fd gene library

E. coli XL1-Blue containing light chain genes (0.2 μL) was diluted and inoculated into agar plate and then 235 clones were obtained. So the transformation clones were $235 \times 26 \times 5 \times 10^3 = 3.06 \times 10^7$. Seven of ten random clones can release 700 bp light chain DNA fragments from the plasmids digested by Sac I + Xba I (Figure 2). Thus the recombinant rate of light chain gene library was 70% and the capacity was $3.06 \times 10^7 \times 0.7 = 2.14 \times 10^7$. Similarly, the transformation clones, recombinant rate and capacity of heavy chain Fab library were obtained and they were 2.08×10^7, 9/13 (about 70%) (Figure 3) and $2.08 \times 10^7 \times 0.7 = 1.46 \times 10^7$ respectively.

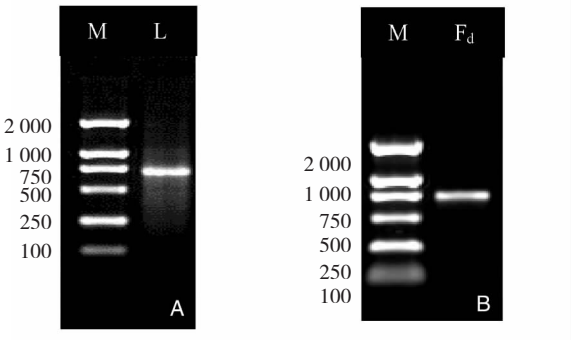

Figure 1 Gel electrophoresis of PCR products

A: Light chain genes; **B**: Heavy chain Fd genes.
M: Standard molecular markers DL2000; L: PCR product of light chain genes; Fd: PCR product of Fd fragments genes

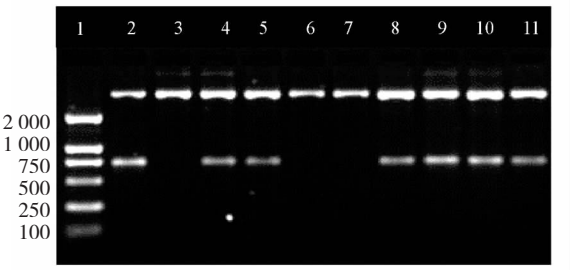

Figure 2 Recombination rate of the light chain gene library

1 represents standard molecular markers DL2000. Phage clones are named as 2-11, and seven of ten random clones can release 700 bp light chain DNA fragments from the plasmids digested by Sac I + Xba I.

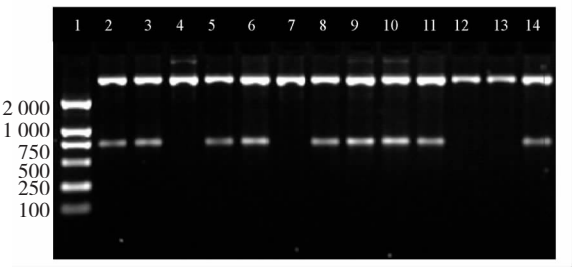

Figure 3 Recombination rate of the Fab phage antibody gene library

1 represents standard molecular markers DL2000. Phage clones are named as 2-14, and nine of thirteen random clones can release 700 bp Fd fragments from the plasmids digested by Spe I + XhoI.

Construction and preliminary screening of Fab phage antibody library

The specific phage antibodies were enriched as evidence by the increasing phage titre and A value from the 1st panning to the 4th panning (Table 1).

Table 1 Titre and A value of phage antibody library after panning

	1st panning	2nd panning	3rd panning	4th panning
Titre(pfu)	1.2×10^5	1.8×10^6	9.4×10^6	3.2×10^7
A value	0.23	0.56	1.05	1.45

Discussion

RSV is a major etiological agent of lower respiratory tract infection in infants. It is estimated that about 40% of children with RSV infection will develop a lower respiratory tract infection. About 60% of children are subjected to RSV infection in the first year of life, and 90% by age 2 years [5]. Immunosuppressed patients with RSV infection may develop progressive and serious pneumonia. Children with congenital heart disease with increased pulmonary blood flow, children with chronic lung disease, and premature infants under age 6 months are also at high risk for high mortality RSV infection [7]. Asthma may also be developed in some children with repeat RSV infection[8]. It is true that RSV infection often distresses and disturbs the children and their parents. The pediatricians and the children's parents ardently expect favorable antiviral drugs. However the effects of various available antiviral drugs are usually not satisfactory, and there is no effectual RSV vaccine up to now. According to our previous study[9], the specific anti-RSV immunoglobulin, intravenous immunoglobulin (IVIG) and murine McAbs Palivizumab are effective in the treatment of RSV infection. However, IVIG and specific anti-RSV immunoglobulin as human blood products, their quantity is limited, their price is costly and there are potential dangers such as HIV and HBV infections. Besides, the efficacy of murine McAbs appears to be limited by many unexpected hurdles, including the induction of HAMA response. It is thus essential to study and develop genetically engineered antibodies. This is the objective of this research.

With the development of molecular biology, the birth of phage antibody library technology provided a spectacular way in the attempts to gain completely human McAbs for the first time. Phage display is a molecular diversity technology that allows the presentation of large peptide and protein libraries on the surface of filamentous phage[10]. Phage display libraries permit the selection of peptides and proteins, including antibodies, with high affinity and specificity for almost any target, and provide access to a vast untapped pool of human McAbs for the treatment of tumor and viral infections[11,12]. In comparison to the time-consuming and labor-intensive cell screening processes of hybridoma production, antibody genes can be cloned directly from lymphocytes using rapid recombinant DNA methods. Having stable genetic sources, phage display recombinant antibodies can be produced quickly and economically and can be used as *in vivo* diagnostic or therapeutic reagents because of the elimination of animal immunization[13].

High affinity antibodies can be obtained by two ways. One is construction of large capacity native phage antibody library; the other is immune library[14]. Compared to the antibodies derived from native repertories, the antibodies from immunized ones are subjected to *in vivo* selection and thus are more likely to selectively recognize a given antigen without cross-reactivity to another antigen. The specific antibody gene rearranged and soma hypermutated after antigen stimulation. The corresponding antibody gene rate is so high that the expected high affinity antibodies can easily be obtained[15]. Thus, this study chose lymphocytes of patients with RSV infection as gene sources to construct an immune Fab phage antibody library. Though this method may reduce the diversity of antibodies against the target antigen, it may increase the proportion of specific antibodies and enhance the possibility of obtaining high affinity antibodies in a relatively small-size library. In addition, transforming efficiency is a critical factor affecting the capacity of antibody library. Accordingly, in the construction of antibody library, electroporation was adopted. For the higher transforming efficiency, the conditions for electroporation including concentration of competence, amount of DNA and electric field intensity were optimized. These measures ensured the diversity of the phage antibody library.

It has been shown that when the diversity of an antibody phage display library reaches 1×10^7 of individual clones, it will contain 99% of all antibody molecules. The size of an antibody library that has been constructed so far generally ranges between 10^6 and 10^8 clones [16]. The size of Fab phage antibody gene library constructed in this study was 1.46×10^7 and the titre of the original Fab antibody library was about 1.06×10^{12} pfu/mL, which was of medium size. The restriction endonucle-

ases identification showed that 70% clones had light and heavy chain genes in the original antibody library and the specific Fab phage antibodies were enriched by about 270 folds after following four rounds of panning with RSV. ELISA analysis showed that the phage display Fab had significant binding activity with antigens associated with RSV.

In sum, an immune Fab phage display antibody library was constructed successfully in this study, which provides a valuable experimental foundation for further study and may also contribute to the improvement in the diagnosis, therapy and prophylaxis of RSV infection in children. Further research, however, is needed including the selection of single positive clone, the expeted expression of the McAb and the bioactivity of the Fab.

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