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Production and characterization of recombinant antibodies against prostaglandin obtained by phage display technology
（ファージディスプレイ法を用いた抗プロスタノイドリコンビナント抗体の作製と特質）

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論文内容要旨

[Introduction]

Antibodies are proteins generated to bind with high degree of selectivity and affinity for their ligands known as antigens. Production of monoclonal antibodies (mAbs) was a breakthrough in the generation of specific antibody against a wide variety of antigen and provided significant advances in the field of Immunology.

Development of mAbs against low molecular weight haptens has a great interest because it allows a simple and specific measurement of these compounds by immunoassay in the diagnostic field.

The phage display technology is a breakthrough technology in the production of mAbs after hybridoma technology and presents the advantage that antibody genes could be manipulated. Initially, immunoglobulin genes from an immunized or a non-immunized donor were amplified by PCR with specific primers. Then the antibody fragment library is constructed on an appropriate vector and the antibody fragment is displayed on the surface of the bacteriophage for the selection and enrichment of the library with functional clones. This technology has been shown to isolate antibody fragment for any target molecule. However, the production of specific antibody fragments against low molecular weight compounds with this technology has shown some problems compared to antibody fragments against high molecular weight compounds such as proteins. It could be cited the difficulty of preparation of a large antibody fragment library and isolation of high affinity antibody fragments, and the occurrence of recombination/deletion of antibody fragment gene after several rounds of antibody fragment selection. Some efforts for these problems have been reported but further efforts should be developed to increase the efficiency of this technology.

A strategy was elaborated for the production and the recovery of high affinity anti-hapten antibody fragments, and the scheme is shown in the Fig. 1. Some modifications were introduced to optimize the yield. One modification was the design of new primers with the introduction of additional restriction enzyme rarely found in the light chain (LC) or heavy chain (HC) immunoglobulin genes that allow the discrimination of vector containing immunoglobulin gene by single digestion followed by the concentration of these vectors by the gel-purification method. The other modification was on the process called panning corresponding to the affinity selection of the phage displaying antibody fragment. The MACS (magnetic cell sorting)-panning system was introduced for this goal. Biotinylated antigen is labeled with streptavidin microbeads and coated on the column under a magnetic field. The phage displaying antibody fragment binds to the antigen and the elution occurs when the column is removed from the magnetic field.

The target model-compound chosen in this study was a prostanoid, 11-dehydro-thromboxane B2 (11D-TX), because it has various derivatives and each one has a specialized physiological activity. Development of specific anti-hapten antibody fragments against 11D-TX serves as parameter for the production of other anti-hapten antibodies.

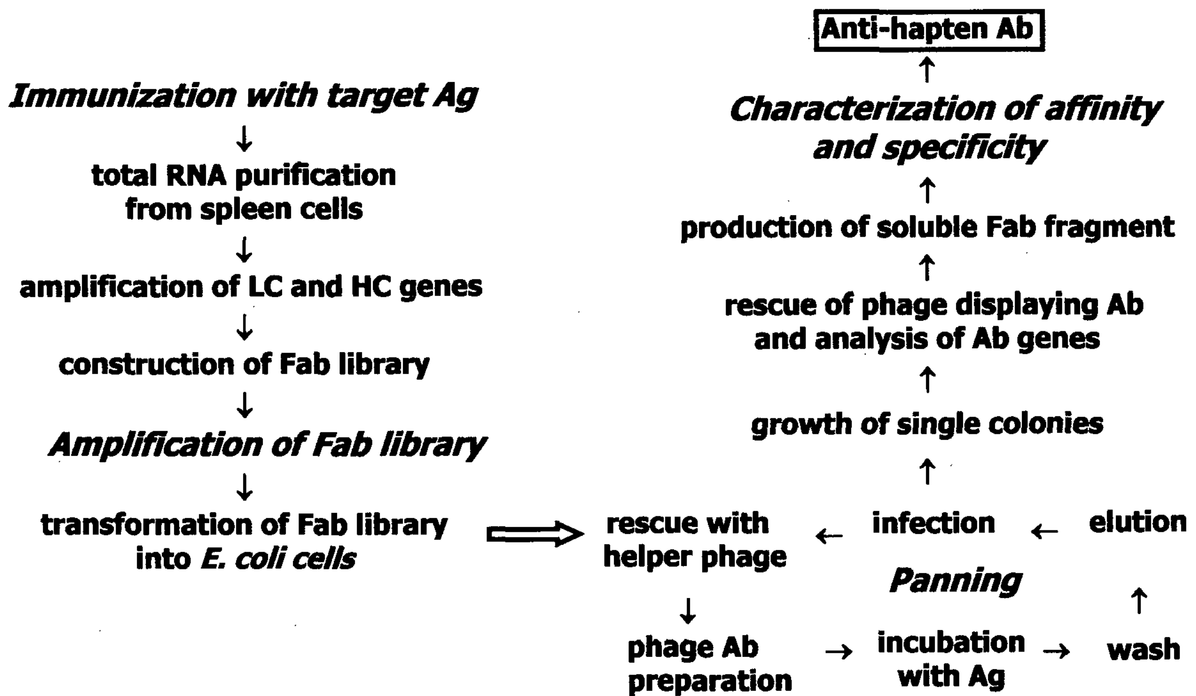

Figure 1. Flow chart showing the strategy for the production of anti-hapten antibody by the pha display technology. (Ag: antigen; Ab: antibody)

[Methdology]

The Fab fragment library was constructed on a pComb3 phage-display vector DNA from repertories of LC and HC derived from the total RNA o f spleen cells from mice immunized with 11D-TX-KLH (11D-TX conjugated-keyhole limpet haemocyanin). Primers were designed for the amplification of immunoglobulin genes and restriction enzyme site (SacII for γ_1 and *Mlu*I for κ chain) was added to reverse primers to monitor the library construction. The choice of restriction enzymes was based on the search of Gene bank database, and restriction enzymes rarely present in the immunoglobulin gene sequence were selected. The Fab fragment library was constructed and then the MACS-panning system was introduced to select anti-11D-TX because the standard method using microtiter plate panning has failed. Biotinylation of 11D-TX-BSA (11D-TX conjugated-bovine serum albumin) was performed through free thiol groups on BSA using 1-biotinamido-4-[4'-(maleimidomethyl) cyclohexanecarboxamido] butane (Biotin-BMCC). BSA was chosen for biotinylation because shows one free thiol group (Cys34) at outside position among 35 Cys present in the molecule of BSA. Another reason is that other functional groups of BSA conjugate are poorly available for biotinylation. Specific antibody fragments for anti-11D-TX were obtained with the MACS-panning system against biotinylated 11D-TX-BSA. From the fourth and fifth panning round, 24 individual phage clones were chosen randomly to evaluate the binding affinity. Then plasmid DNAs were obtained to characterize the selected clones and sequencing was performed for positive clones. Selected clones were converted to the soluble Fab expression vector by a treatment with *Spe*I/*Nhe*I restriction enzymes for excision of the gene III (gIII) because the HC is expressed as a fusion protein of gIII protein on the phage surface. The vector was religated and soluble Fab

fragments were obtained by IPTG induction protocol. The affinity for 11D-TX-BSA of these Fab fragments and the specificity for some inhibitors were characterized by ELISA.

[Results and Discussion]

Analysis of antiserum obtained from mice immunized with 11D-TX-KLH has shown high specificity for hapten (11D-TX) and low specificity for other inhibitors.

Immunoglobulin genes were amplified by PCR using the total RNA of spleen cells from immunized mice. The Fab fragment library was constructed by the successive ligation of LC and HC on a pComb3 vector. After insertion of each fragment, the vector DNA was digested with the respective new restriction enzyme introduced in the primer and vectors containing the inserts were gel-purified and religated. The library was enriched with functional vectors by this procedure. Fingerprinting analysis was performed and showed the presence of various patterns of LC and HC inserts in the library. Thus the use of new primers allowed the construction of an adequate library with great variety and showed to be reasonable.

Then phage display was performed by the infection with VCSM13 helper phage followed by the affinity bio-panning to enrich the phage display library with anti-11D-TX antibody fragments. MACS-panning system was introduced as an alternative method and 5 rounds of panning were performed. According to the phagemid titer before and after each panning round, there was panning enrichment until the fourth panning round. The binding activity of 24 individual clones selected randomly after the fourth and fifth round of panning was verified by phage ELISA for 11D-TX-BSA and BSA alone. Phage particles rescued from the fifth panning round had poor affinity for 11D-TX-BSA (4 clones in a total of 24 clones - 17% of binders), while all clones at the fourth panning showed affinity for 11D-TX-BSA and low affinity for BSA alone.

Plasmid DNAs of clones showing affinity for 11D-TX-BSA were obtained from the fifth panning round and it was observed that they had deletions or recombination-deletion in the HC gene region on pComb3 DNA and were non-binding. The result indicates that there was an overgrowth of non-binding clones and clones displaying functional antibody fragment have decreased. Plasmid DNAs were also obtained from the fourth panning and the majority (22 clones in a total of 24 clones - 92% of binders) showed both LC and HC inserts. Fingerprinting analyses of positive binders by digestion with *Bst*NI restriction enzyme showed only one pattern while sequencing showed 2 distinct patterns for the LC of anti-11D-TX Fab fragments which accession numbers are AB089650 (clone 7) and AB089651 (clone 13), and one sequence pattern for HC (AB089649). Comparing the complementary-determining region (CDR) sequences of LC, CDR1 is identical in both clones but CDR2 and CDR3 have some differences on the amino acid sequence. A homology search of deduced amino acid sequences was performed using BLAST program and the HC sequence showed to have higher homology to an anti-arsonate antibody (AJ229174). The LC sequence of clone 13 has the same sequence of mouse anti-potato virus antibody (X87231) while the clone 7 has a higher homology to a mouse anti-poly(dC) antibody (AF045490). Both LC sequence belong to the germ-line cr1 and the alignment for

the J-gene was shown to be IGKJ1*01. The constant region sequences were also analyzed and it was confirmed that the HC sequence belong to the γ_1 class and LC sequences to the κ class.

Binding assay of clone 7 and 13 Fab fragment obtained from *E. coli* for 11D-TX-BSA and BSA alone was performed and it was observed that they exhibited between 5 and 10-fold higher affinity for 11D-TX-BSA conjugate compared to BSA alone. The specificity was determined by inhibition ELISA using the hapten 11D-TX and only the clone 13 showed specificity in this assay. The specificity for 11D-TX-BSA was then performed and clone 13 Fab fragments showed more specificity for 11D-TX-BSA than hapten. It suggests that the epitope of this antibody fragment may be the region between the hapten and the carrier protein. Cross-reactivity for other compounds related to prostaglandin was analyzed to clone 13 Fab fragment and it was shown cross-reactivity for 2,3-dinor TXB_2 and low specificity for 11-dehydro-2,3-dinor TXB_2, 6-keto $PGF_{1\alpha}$ and linoleic acid. Another consideration was about the chemical structure configuration of 11D-TX recognized by anti-11D-TX Fab fragment. It is known that 11D-TX exists in the cyclic form in acid pH and in the open form in the neutral or basic pH. The affinity of anti-11D-TX Fab fragments for the closed and open form of 11D-TX-BSA conjugate was analyzed at pH 4, 5, 7.4 and 9. The highest affinity was verified at pH 7.4 and the following was at pH 9. Low binding at pH 9 was observed because the binding capacity of antigen was decreased in comparison to other conditions as was verified by analysis of biotinylated 11D-TX-BSA conjugate. According to the result obtained, low affinity was observed at acid pH suggesting that anti-11D-TX Fab fragments have more affinity for open form of 11D-TX than closed form.

Affinity constant (K_a) derived from equilibrium binding by ELISA was determined using Scatchard plot analysis and affinity binding curve. The K_a of the clone 13 Fab fragment was 1x 10^9 M^{-1}, demonstrating that a high affinity antibody fragment was isolated. Thus the MACS system showed to be a simpler and more efficient panning method to select high affinity antibody fragment than the conventional method using microtiter plate.

[Conclusion]

This work demonstrated it is possible to obtain high affinity antibody fragments against low molecular weight haptens by the optimization of the phage display technology. The construction of adequate antibody fragment library using immunoglobulin genes amplified by primers that allow the discrimination between vectors containing immunoglobulin genes and vectors without inserts combined with the MACS system provided the selection of Fab fragment with high affinity and specificity. Cross-reactivity for other prostanoids was also detected and anti-11D-TX Fab fragment obtained in this study could not be used for application in the present form. Meanwhile, this Fab fragment could be used to evaluate the alteration of specificity of anti-hapten antibodies using techniques of protein engineering in the future studies.

審査結果の要旨

今日，抗体は微量物質の測定に欠くことのできない機能性器材となっており，生命科学，環境科学，医療科学などにおいて幅広く用いられている。抗体の親和性，特異性は測定系の信頼度，感度に大きく影響する。蛋白質等の高分子化合物では，それ自身抗原性を持ち，抗体の調製が比較的容易である。そればかりか，抗原の分子量が大きい結果，認識部位の異なる2種類の抗体を用いる非競合型のtwo-site immunometric assayが可能であり，感度，特異性に優れる測定系の構築ができる。一方，低分子化合物では，それ自身抗原性を持たないため，抗体の調製にはあらかじめキャリアー蛋白質との複合体に導く必要がある。その結果得られる抗体の抗原に対する親和性が低く，しかも特異性がハプテンーキャリアー蛋白質の構造に大きく影響されるため，感度，特異性に優れる測定系の構築は大変に困難な課題である。本研究では，特に高い特異性の得難いプロスタノイドを取り上げ，ファージディスプレイという新らたな手法を導入して，人工抗体の調製に基礎的検討を加えたものである。

先ずモデル化合物として11-dehydro-thromboxane B2（11-DX）を取り上げ，これをkeyhole limpet haemocyanin（KLH）との複合体に導いた後免疫し，その脾細胞のRNAを精製して，light chain（LC）及びheavy chain（HC）遺伝子を増幅し，Fabフラグメントのライブラリーを作成した。次いでこれを*E. coli*にトランスフォーメーションし，抗原を用いてパンニング操作に付した。しかし，従来のパンニング操作では，期待するフラグメントが得られなかった。これは，抗原に強い親和性を持つフラグメントが溶出操作で回収されなかったためと考えられた。そこで磁気ビーズを用いるMACS-パンニング法を考案し，適用した結果，初期の目的を達成することができた。

次に目的とするクローンを可溶性Fabフラグメントに変換し，11-TX-牛血清アルブミン複合体に対する親和性と特異性を吟味した。その結果，他の同族体に対する特異性は，期待したほどではなかったが，得られたFabフラグメントは11-Txのオープンフォームに対してより親和性が高く，1×10^{-9}Mであった。

以上，本研究は低分子化合物に対する抗体の調製に，ファージディスプレイ法という新たな手法を導入することによって，野生型よりはるかに高い親和性と特異性を持つ人工抗体の入手できる可能性を示した。よって，本論文は博士（薬学）の学位論文として合格と認める。