Expression of a human monoclonal anti-(rhesus D) Fab fragment in *Escherichia coli* with the use of bacteriophage λ vectors

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A human anti-(rhesus D) antibody (IgG1 λ) Fab fragment was cloned from an Epstein–Barr-virus-transformed cell line and expressed in *Escherichia coli* with the use of bacteriophage λ vectors. The cloned protein is active in binding to human erythrocytes and permits the development of a recombinant reagent for the prevention of haemolytic disease of the newborn. The method offers a rapid and effective means of rescuing human Fabs from potentially unstable cell lines secreting human antibodies.

INTRODUCTION

A novel bacteriophage λ vector system has previously been described for the expression, in *Escherichia coli*, of a combinatorial library of the mouse antibody repertoire [1]. The system was used to clone monoclonal Fab fragments of defined specificity from the repertoire with the use of RNA from a hyperimmunized spleen. It is, though, the cloning of the human immune repertoire that will ultimately yield the most therapeutically useful immunoglobulins. At present there are available a number of human hybridomas and Epstein–Barr-virus (EBV)-transformed cell lines, most notably producing antibodies against rhesus-blood-group antigens [2–6] and human-immunodeficiency-virus (HIV) proteins [7]. Such cell lines can, however, be unstable and cease secreting antibody [8]. They may also be of an unsuitable isotype, e.g. Epstein–Barr-virus-transformed cells often secrete IgM [9], which does not trigger many cytotoxic mechanisms.

In this paper we describe the application of the combinatorial bacteriophage λ system to extract from an Epstein–Barr-virus-transformed cell line and introduce into a bacterial expression system a human anti-(rhesus D) (anti-RhD) Fab fragment. The Fab can now be linked to the desired effector function in a eukaryotic vector for stable expression in existing non-human mammalian cell lines, thus by-passing fears, associated with human cell lines, of human viral and DNA contamination of the secreted antibody.

MATERIALS AND METHODS

Design of primers

The heavy-chain 5' primers, VH1a and VH3a, for γ -chain amplification are designed to maximize sequence identity with the VH1 and VH3 subgroup families respectively (all sequences taken from ref. [10] and GenBank), although considerable cross-priming is expected. The 3' primer for the γ -chain, CG1z, corresponds to part of the hinge region. A 5' control primer, CONGa, corresponds to the 5'-end of the CH1 domain. The 5' primers for amplification of the λ -chain are designed to maximize sequence identity with known λ -chain sequences (GenBank) and are termed VL1 to VL6. CL2 is a 3' primer corresponding to the

3'-end of known λ -chains. The 5' control primer CONL1 corresponds to the 5'-end of known C λ 1 sequences.

Library construction

Total RNA was isolated [11] from 10⁸ cells of the Epstein–Barr-virus-transformed cell line H1121GS secreting a human IgG1 λ anti-RhD antibody (kindly provided by Dr. Tom Setcavage, Ortho Diagnostics, Rareton, NJ, U.S.A.). A 20 μ g portion of the RNA was hybridized to 60 pmol of either γ 1-chain or λ -chain 3' primers (Table 1) at 70 °C for 10 min. The mixture was then used in a 50 μ l reverse-transcription reaction buffer (BRL, Bethesda, MD, U.S.A.) containing 200 μ M each of dATP, dCTP, dGTP and dTTP and 600 units of reverse transcriptase (SuperScript; BRL) that was incubated at 37 °C for 1 h. Samples (3 μ l) of the reaction mixtures were used in PCR reactions (buffer containing 10 mM-MgCl₂; Promega, Madison, WI, U.S.A.) containing all four dNTPs at 60 μ M, 5 units of *Thermus aquaticus* polymerase (Promega) and 60 pmol of the appropriate 3' primer (Table 1). The reaction mixtures were then subjected to 35 rounds of amplification (Perkin–Elmer Cetus thermal cycler) at 52 °C for 2 min, 72 °C for 1.5 min and 91 °C for 2 min, followed by a final incubation at 72 °C for 10 min. A sample of the reaction material was subjected to electrophoresis on a 2% agarose gel. After successful amplification (denoted by a band of approx. 650 bp) the remainder of the RNA·cDNA was amplified, and the products of a common 3' primer were pooled, extracted with phenol/chloroform and precipitated with ethanol. The DNA was then digested with the appropriate restriction enzymes, extracted and precipitated again before being subjected to electrophoresis on a 2% agarose gel. The desired band was purified by using GeneClean (Bio101, La Jolla, CA, U.S.A.), and DNA was ligated into the phosphatase-treated vector arms λ HC1 or λ LC1 [1] in roughly equimolar amounts before packaging with Gigapack Gold Plus II (Stratagene, La Jolla, CA, U.S.A.). The resulting libraries were titred and assayed for the expression of heavy chain (anti-decapeptide [1]) or light chain (anti- λ -chain; Southern Biotech, Birmingham, AL, U.S.A.). These libraries were amplified and bacteriophage DNA was purified by a glycerol-step-gradient method. Approx. 10 μ g of this DNA was digested with the appropriate restriction enzymes, treated with phosphatase, digested with *Eco*RI, re-ligated and packaged as described previously [1]. The resulting combinatorial

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Table 1. Primers used for amplification of human γ 1-chain (Fd) and λ -chain sequences

VH1a and VH3a are 5' primers for the amplification of the γ -chain. The *Xho*I site for cloning into the heavy-chain vector λ HC2 is shown underlined. CG1z is the 3' primer for the γ -chain. The *Spe*I site for cloning into λ HC2 is underlined. CONGa is a 5' control primer. VL1 to VL6 are 5' primers for amplification of λ -chain sequences. The *Sac*I site for cloning into the λ LC1 is underlined. CL2 is a 3' primer for the λ -chains; the *Xba*I site is underlined. CONL1 is a 5' control primer.

Primer	Sequence
γ -Chain (Fd):	
VH1a	5'-CAGGTGCAGCTCGAGCAGTCTGGG-3'
VH3a	5'-GAGGTGCAGCTCGAGGAGTCTGGG-3'
CG1z	5'-GCATGTACTAGTTTTGTCAAGATTGGG-3'
CONGa	5'-TCCACCAAGGGCCCCATCG-3'
λ -Chain:	
VL1	5'-AATTTTGAGCTCACTCAGCCCCAC-3'
VL2	5'-TCTGCCGAGCTCCAGCCTGCCTCCGTG-3'
VL3	5'-TCTGTGGAGCTCCAGCCGCCCTCAGTG-3'
VL4	5'-TCTGAAAGAGCTCCAGGACCCTGTTGTGTCTGTG-3'
VL5	5'-CAGTCTGAGCTCACGCAGCCGCC-3'
VL6	5'-CAGACTGAGCTCACTCAGGAGCCC-3'
CL2	5'-CGCCGTCTAGAAGTATGAACATTCTGTAGG-3'
CONL1	5'-AAGGCTGCCCCACGGTCACTCTG-3'

Fig. 1. Agarose-gel electrophoresis of PCR amplification products of (a) γ 1-chains and (b) λ -chains from an Epstein-Barr-virus-transformed cell line secreting a human anti-RhD antibody

PCR amplification indicated VH3a and VL2 to be the most effective heavy-chain and light-chain 5' primers respectively as shown.

library was examined for co-expression of heavy chains and light chains before amplification and storage at 4 °C in 0.3% chloroform.

Antigen-binding properties of the cloned protein

The ability of the cloned Fab to bind to RhD(+) and RhD(−) erythrocytes was examined by flow cytometry. A number of plaques found to co-express both the light chains and the heavy chains were cored, and the plasmids were excised, with the aid of the helper bacteriophage R408 (Stratagene), and infected into XL1 Blue (Stratagene). Cultures of the infected cells were induced overnight at room temperature with 5 mM-isopropyl β -D-thiogalactoside. Concentrated supernatants were purified over a goat anti-[human F(ab')] antibody (Pierce Chemical Co., Rockford, IL, U.S.A.) affinity column. The isolated Fab was incubated with both RhD(+) and RhD(−) erythrocytes. A second fluorescein isothiocyanate-conjugated anti-[human F(ab')] antibody (Pierce Chemical Co.) was added and the cells were analysed by flow cytometry (Fig. 2).

RESULTS

Library construction

Heavy-chain and light-chain sequences were amplified using the primers listed in Table 1. Yields given by the various primer pairings were examined by agarose-gel electrophoresis. Successful amplification was denoted by a single band at 650 bp (Fig. 1). Several amplifications were performed with the effective primers, and the individual products were pooled and digested with the appropriate restriction enzymes, before ligation into the vector arms. This gave a γ 1-chain library of 2×10^6 members and a λ -chain library of 4×10^6 members. Bacteriophage DNA prepared from these libraries was digested with the appropriate enzymes [1] and ligated together, creating a combinatorial library of 10^5 members, 88% of which expressed both γ 1-chains and λ -chains.

Antigen-binding properties of the cloned protein

The 18 bacteriophages co-expressing both chains were plaque-purified, and plasmids were excised (by co-infection with helper

Fig. 2. Antigen-binding properties of the cloned protein

The ability of the cloned Fab to bind to RhD(+) and RhD(-) erythrocytes was examined by flow cytometry as illustrated.

bacteriophage) and used to transform *E. coli*. Protein expression was induced, and five cell supernatants were affinity-purified and examined by SDS/PAGE (results not shown). Under non-reducing conditions a single band at 50 kDa was observed, and this yielded a doublet at 25 kDa under reducing conditions. The isolated Fabs were incubated with both RhD(+) and RhD(-) erythrocytes and analysed by flow cytometry. All five clones were shown to be positive; an example is illustrated in Fig. 2.

Studies indicate (R. A. Williamson, M. A. A. Persson & D. R. Burton, unpublished work) that the recombinant Fab has a binding constant within an order of magnitude (10^7 M^{-1}) of that of the Fab isolated by papain digestion of the native IgG. Additional, as a control, the same binding assay was performed with a human IgG Fab generated by the same methodology and specific for thyroglobulin. This antibody displayed no affinity for the RhD(+) or RhD(-) erythrocytes (results not shown).

DISCUSSION

Haemolytic disease of the newborn due to the production of maternal anti-D antibody can be prevented by antenatal and post-partum administration of anti-D immunoglobulin to non-immunized RhD(-) mothers who have delivered RhD(+) children [12]. With rising demand for antenatal administration and a diminishing group of Rh-immunized donors because of the success of prophylaxis and increasing concern over transmissible infectious agents associated with blood products, alternative sources of human anti-D antibodies are sought. The results reported in this paper suggest a route to the development of a

laboratory-based anti-D reagent. Further anti-D antibodies expressed in bacteria in quantity can be used as reagents for routine blood typing. Cloning of anti-A and anti-B antibodies from existing cell lines could also yield, within the context of well-characterized expression systems [13], cost-effective typing reagents.

The greatest potential of human repertoire cloning is in the generation of monoclonal antibodies with desired specificity. At present, though, the technology is limited by the screening methods employed, which are restricting in both the type of antigen that can be screened for and the numbers that can be processed. Here we show it is possible to take advantage of existing human cell lines to extract Fab for expression in bacteria, thus readily permitting manipulations such as the addition of new constant regions for stable expression in non-human cell lines and random mutagenesis to increase affinity.

The bacteriophage system permits analysis of correct expression of light chains and heavy chains at an early stage in cloning. Where there is a fusion partner, the presence of non-productive antibody genes at the mRNA level will lead to the amplification of sequences encoding non-functional proteins. In mouse hybridomas there has been a frequency of positives as low as 1 in 1000 (L. Sastry, personal communication). By screening at the bacteriophage level, which is possible with the combinatorial approach, low-frequency positives can be identified.

We are most grateful to Richard Lerner for his continued support and encouragement and to Diane Schloeder for technical assistance. We thank James O'Connell and Thomas Setcavage for supplying the cell line. We acknowledge the financial support of Johnson and Johnson, of Birthright and of the Sven and Dagmar Salen Foundation. D.R.B. is a Jenner Fellow of the Lister Institute of Preventive Medicine.

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