Expression and Characterization of Recombinant Anti-Rh(D) Antibodies on Filamentous Phage: A Model System for Isolating Human Red Blood Cell Antibodies by Repertoire Cloning

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The production of human anti-red blood cell (RBC) IgGs in vitro from immunized individuals would greatly facilitate the genetic analysis of the human immune response to RBC antigens and also provide useful serologic reagents. Technical difficulties inherent in human B-cell immortalization have led to the development of molecular approaches that bypass the need for cell transformation. By cloning human Ig gene segments into bacterial expression vectors, libraries are created of filamentous phage particles displaying Fab fragments on their surfaces. Libraries have been screened with purified, soluble antigen and selected clones genetically manipulated in Escherichia coli to produce soluble Fab fragments. Our goal has been to adapt this technique to the study of RBC autoantibodies and alloantibodies that have specificities against unpurifiable membrane-bound antigens. To test the feasibility of this approach, two sets of phage were created, one set expressing a human anti-Rh(D) Ig and the other expressing a human anti-tetanus toxoid Ig. After verifying the presence of functional phage-displayed Fabs through biochemical, flow cytometric, and electron microscopic analyses, a model library was constructed comprising one anti-Rh(D)-expressing phage per 10^4 antitetanus toxoid-expressing phage. A method was developed for screening the library with intact Rh(D)-positive RBCs. After four rounds of panning, anti-Rh(D) specificity was enriched more than 10,000-fold to a final frequency of approximately 100%. Plasmid DNA derived from anti-Rh(D) phage was used to produce milligram quantities of soluble recombinant anti-Rh(D) Fabs purified by nitrogen cavitation and nickel-chelation affinity chromatography. The authenticity of the Fabs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, which showed bands with molecular weights of approximately 50 kD and 26 kD under nonreducing and reducing conditions, respectively. Binding of recombinant anti-Rh(D) Fabs to Rh(D)-positive RBCs was demonstrated by flow cytometry and by an agglutination assay. Our results suggest that repertoire cloning by cell-surface enrichment may have broad application to the study of the human immune response to erythroid antigens in addition to membrane-bound antigens present on other hematopoietic cells.

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The ability to generate red blood cell (RBC)-specific human monoclonal antibodies would greatly facilitate the genetic analysis of the human autoimmune and alloimmune response to erythrocyte antigens as well as provide an endless supply of useful serologic reagents. Despite the development of murine hybridoma technology nearly 20 years ago, attempts to produce human RBC antibodies in vitro have been limited by the absence of reliable, effective procedures for immortalizing human B lymphocytes. Protocols using Epstein-Barr virus (EBV) transformation result in relatively low transformation efficiencies (~1% to 3% of B cells under optimal conditions), and those methods that require cell fusion display relatively low fusion frequencies (~0.1% depending on conditions and fusion partner) when compared with the murine system. Furthermore, EBV-transformed human B cells can undergo a decline in antibody production or cell growth that may necessitate their "rescue" through fusion with mouse myeloma cell lines to create human/mouse heterohybridomas. Subsequently, such hybrid cell lines can become unstable because of the progressive loss of human chromosomes.

To avoid these pitfalls, molecular approaches have been developed that bypass the need for cell transformation. Conceptually, these techniques known as repertoire cloning, immortalize Ig genes rather than the cells from which they were derived. Briefly, cDNA is prepared from the mRNA of the rearranged Ig genes from donor B cells, and heavy and light chain gene segments are amplified with the polymerase chain reaction (PCR). The heavy and light chain cDNA are cloned into one of a number of recently described phagemid vectors that, after transfection into Escherichia coli, results in the accumulation of Ig Fab fragments in the periplasmic space. Upon coinfection with M13 helper phage, phagemid particles are generated that contain plasmid DNA and that incorporate copies of the Fab fragment as part of their coat protein. Thus, a physical association is established between each antibody molecule and the DNA that encodes that antibody's specificity. Libraries of such Ig-displaying phagemid particles can be incubated with immobilized antigen to adsorb phage bearing antibody specificities of interest. Adsorbed phage can then be eluted with acid, allowed to reinfest E. coli cultures, and further propagated and enriched through subsequent rounds of "panning." Once monospecific phage are isolated, further molecular manipulations of the plasmid DNA can unl ink the Fab molecules from the phage surface and result in the bacterial production of soluble Fab fragments.

Repertoire cloning has been used to isolate human antibodies directed at a number of soluble, purified antigens, including tetanus toxoid, human immunodeficiency virus...
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(HIV) gp120, hepatitis B surface antigen, and respiratory syncytial virus F glycoprotein. Our goal has been to develop a method by which this approach could be used to isolate and study RBC-specific antibodies from phage display libraries prepared from immunized donors. The majority of RBC antigens of interest are serologically defined only and their precise biochemical structures are either unknown (as in the case recognized by warm-reactive autoantibodies) or are not purifiable in a nondenatured state that retains their antigenic properties (as in the case of the Rhesus antigens). Therefore, in most cases, purified RBC antigen cannot be prepared and bound to a solid phase to be used to screen for immunoreactive phage particles. In the current study, a method is presented for screening RBC-reactive phage display libraries with membrane-bound antigens using intact RBCs of defined phenotype. To establish the method and prove its feasibility, two sets of phage were created—one set expressing a monoclonal human anti-Rh(D) alloantobody (derived from an unstable human heterohybridoma cell line) and the other set expressing a human antitetanus antibody (derived from an unstable human heterohybridoma cell line) and the other set expressing a human antitetanus antibody (derived from an unstable human heterohybridoma cell line). An artificial library consisting of one anti-Rh(D)—expressing phage particle per 10,000 antitetanus—expressing phage particles was constructed and conditions were found that facilitated the selection of anti-Rh(D)—bearing phage by panning with Rh(D)-positive cells. These results show that anti-Rh(D) specificity could be enriched more than 10,000-fold after four rounds of panning to a final frequency approaching 100%. It is further demonstrated how plasmid DNA isolated from antigen-selected phage particles can be used to express milligram quantities of soluble anti-Rh(D) Fab molecules that can be purified to near homogeneity by nickel-chelation affinity chromatography and used to antigen type Rh(D)-positive RBCs using conventional indirect agglutination techniques.

MATERIALS AND METHODS

Expressing Anti-Rh(D) and Antitetanus on M13 Phagemids

The phagemid vector used in these studies was pComb3 (kindly provided by Dr Carlos Barbas, Scripps Research Institute, La Jolla, CA), which is engineered to facilitate the expression of Fab molecules on the surface of M13 filamentous phage. Specifically, a dicistronic message is transcribed that encodes full-length light chain and the Fv fragment of heavy chain (Fv denotes the framework 1 of variable region through Cμ1 domain of constant region) fused to a segment of phage gene III coat protein. As provided, the vector already contained the heavy and light chain DNA for a human IgGκ antigen type Rh(D) toxoid antibody previously isolated by repertoire cloning. To prepare pComb3 with heavy and light chain cDNA encoding anti-Rh(D) specificity, total cell RNA was prepared from 10⁶ cells of an unstable IgGlκ anti-Rh(D)—secerting heterohybridoma (H1121GS; kindly provided by Dr Tom Setcavage, Ortho Diagnostics, Raritan, NJ) and cDNA synthesis and PCR amplification of heavy and light chain segments was performed essentially as described. After gel purification on 3% NuSieve (FMC BioProducts, Rockland, ME), the heavy and light chain products were digested with the appropriate restriction enzymes, gel purified again, and then ligated sequentially into a gel-purified, phosphatase-treated preparation of pComb3 vector from which the antitetanus cDNA had been previously excised.

To generate phagemid particles displaying antitetanus or anti-Rh(D) on their surface, 2-μg aliquots of pComb3 containing the appropriate heavy/light chain pairs were electroporated into E coli and subsequently coinfected with VCSM13 helper phage (10¹² pfu; Stratagene, La Jolla, CA) and grown overnight at 30°C as described, except that SURE cells (Stratagene) were used for all steps in DNA cloning and phagemid preparation rather than the XL1-Blue strain of E coli. SURE cells were used because of the lack of stability of phagemid libraries produced in XL1-Blue resulting from the loss of sections of the pComb3/heavy and light chain construct in subsequent generations (unpublished results). We hypothesized that this was caused by genetic recombination of the plasmid caused by the presence of two identical promoter/leader sequences upstream from the heavy and light chain insert (see Barbas et al for map of pComb3). We have found that exclusive use of SURE cells that lack the relevant recombine enzymes maintains the integrity of the pComb3 DNA (data not shown). To compensate for the slower growth characteristics of SURE cells in LB or SB media as compared with XL1-Blue, a highly enriched media (HEM) was designed consisting of 24 g/L tryptone, 48 g/L yeast extract, 10 g/L MOPS, pH 7.0. In addition, bacterial plates were made using HEM rather than LB medium. Phagemid suspensions were purified from the bacterial supernatants by two rounds of polyethylene glycol-8000 precipitation, resuspended in phosphate-buffered saline (PBS) to a final volume of ~1 mL, and dialyzed versus 1 L of PBS overnight at 4°C. Phagemid titers were determined as described (typically ~10¹⁴/mL) and the resulting bacterial colonies were used to verify production of recombinant Fab/gene III fusion protein by performing colony lift assays as described below. The presence of recombinant Fab molecules on the phage surface was verified by immunoblotting (see Results).

Flow Cytometric Analysis of Phagemid Preparations

Aliquots of anti-Rh(D) or antitetanus-bearing phagemid particles (~10¹¹ pfu) were incubated with 5 μL of a 3% suspension of Rh(D)-positive or -negative RBCs (Panel Twenty; Gamma Biologicals Inc., Houston, TX) at 37°C for 3 hours on a laboratory rotator in a final volume of 100 μL of PBS containing 3% bovine serum albumin (BSA; Sigma A-7030, fatty-acid free, Sigma, St Louis, MO). The cells were pelleted for 4 seconds at full speed in a microcentrifuge and washed 10 times at 4°C with 200-μL volumes of 3% BSA/PBS, allowing the cells to incubate in each wash solution for 5 minutes. To the final washed cell/phage pellet, 200 μL of a 1:120 dilution of biotinylated sheep anti-M13 phage antibody (5’→3’ Inc, Boulder, CO) in 3% BSA/PBS was added and the suspension was rotated for 60 minutes at 4°C. After three 200-μL washes at 4°C with incubation buffer, 200 μL of a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated streptavidin (Tago Immunologicals, Camarillo, TX) was added, followed by 30 minutes of incubation at 4°C. The suspension was washed three times as above, to 500 μL of PBS, and analyzed by a FACScan microfluorometer equipped with Lysis II (Ver 1.1) software (Becton Dickinson, Mountain View, CA). Monoclonal anti-Kell antibodies used as controls in inhibition studies (see Results) were derived from the supernatant of an IgGκ-secreting lymphoblastoid cell line (MID11G4; kindly provided by Dr Dominique Goossens, INTS, Paris, France).

Panning Using Intact RBCs

For each round of panning, ~10¹¹ pfu of phage were added to 3 μL of packed Rh(D)-positive RBCs in a total volume of 200 μL of PBS containing 2% BSA/1% nonfat dry milk. After 3 hours of incubation at 37°C on a laboratory rotator, the cells were pelleted and washed 10 times at 4°C (5 minutes each) with 200-μL volumes of the BSA/milk/PBS incubation medium. The final cell pellet with bound phage was incubated with 200 μL of elution buffer (0.1 mol/
L. HCl adjusted to pH 2.2 with solid glycine, containing 1 mg/mL BSA \(^{28}\) for 10 minutes at room temperature. The cell debris was pelleted for 30 seconds at full speed, the supernatant was neutralized with 12 \(\mu\)L of 2 mol/L Tris base, and the resultant phage eluate added to 2 mL of (OD\(_{260}\) = 1) SURE cells. After 15 minutes of incubation at room temperature, 8 mL of prewarmed (37°C) HEM containing 20 \(\mu\)g/mL carbenicillin and 10 \(\mu\)g/mL tetracycline was added. Twenty microliters was removed for titering to determine the number of phage contained in the eluate, and the balance of the 10 mL culture was incubated at 37°C for 1 hour at 300 RPM. VCSM13 helper phage (10\(^{10}\) pfu) was then added and, after 15 minutes of incubation at 37°C, the suspension was added to 100 mL of media containing carbenicillin and tetracycline (for final concentrations of 50 \(\mu\)g/mL and 10 \(\mu\)g/mL, respectively) and shaken overnight at 300 RPM and 30°C. Phagemid preparations were then purified from bacterial supernatants as described above and titered to determine the concentration of phage so that a volume calculated to contain \(\sim\)10\(^{11}\) pfu could be added to RBCs for the next round of panning.

**Colony Screening**

Bacterial clones from each round of panning were taken from the phage titering plates and restreaked in duplicate (see Results) on fresh HEM/carbenicillin (100 \(\mu\)g/mL) plates and processed essentially as described, \(^{28}\) except for the final detection method. Briefly, bacterial streaks were grown for 4 hours at 37°C, after which the plates were overlaid with nitrocellulose filters (82 mm, 0.2-µm pore size; Schleicher and Schuell, Keene, NH) presoaked (and dried) in 5 mmol/L isopropyl-\(\beta\)-d-thiogalactopyranoside (IPTG) and incubated overnight at 30°C. The filters were removed, incubated in a chloroform chamber for 15 minutes, transferred to a lysozyme/Tris buffer (25 mL/filter containing 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 5 mmol/mL MgCl\(_2\), 5% BSA, 400 \(\mu\)g/mL lysozyme, and 1 U/mL Nuclease I) and rocked for 1 hour at room temperature. Buffer was removed and fresh lysozyme/Tris buffer was added for an additional hour. After removing adherent bacterial debris, filters were blocked in 3% gelatin in TBS (20 mmol/L Tris, pH 7.5, 500 mmol/L NaCl) for 1 hour at 30°C with gentle rocking. After two 5-minute washes in TTBS (TBS with 0.05% Tween 20), one filter in each pair was incubated for 3 hours at 30°C in 1% gelatin/TTBS containing horseradish peroxidase (HRP)-conjugated goat antihuman \(\lambda\) light chain antibody (1:2,000 dilution; Tago Immunologicals) for the detection of anti-Rh(D) clones or HRP-conjugated goat antihuman \(\kappa\) light chain antibody (1:1,500 dilution; Sigma) for the detection of anti-\(\kappa\) clones. Filters were then washed three times for 10 minutes each in TTBS (50 mL/filter) followed by a brief rinse in TBS before development with a nickel-enhanced diaminobenzidine substrate solution (800 \(\mu\)g/mL diaminobenzidine, 400 \(\mu\)g/mL NiCl\(_2\), 0.009% H\(_2\)O\(_2\) in 100 mmol/L Tris, pH 7.5).

**Preparation and Characterization of Soluble Fab Molecules**

Production of recombinant protein. Bacterial colonies positive for anti-Rh(D)/gene III production (as determined by colony screening positivity with anti-\(\lambda\) antibody; see Results) were grown overnight in 100-ml cultures of HEM containing 50 \(\mu\)g/mL carbenicillin and 10 \(\mu\)g/mL tetracycline. Phagemid DNA was purified (Plasmid Maxi Kit; Qiagen Inc, Chatsworth, CA), digested with Spe I and Nhe I, and self-ligated (Spe I and Nhe I produce compatible cohesive ends) to form plasmids lacking the M13 gene III coat protein sequence. \(^{28}\) Ligated DNA (2 \(\mu\)g) was electroporated into SURE cells as described \(^{28}\) and shaken at 120 RPM at 37°C for 1 hour in 1 L of HEM media (500 mL per 2-L fluted flask; Nalgene Co, Rochester, NY) containing 50 \(\mu\)g/mL carbenicillin and 20 mmol/L MgCl\(_2\) until an OD\(_{260}\) of \(-0.4\) was reached. IPTG was added to 0.5 mmol/L, the temperature was reduced to 30°C, and Fab expression was induced for \sim\)20 hours.

**Harvesting recombinant protein by nitrogen cavitation.** Bacteria containing expressed Fab molecules in the periplasmic space were pelleted at 4,000g for 20 minutes and resuspended in 40 mL of 300 mmol/L NaCl/50 mmol/L NaPO\(_4\)/pH 8.0 (extraction buffer). Lysozyme was added to 100 \(\mu\)g/mL along with a protease inhibitor cocktail comprising phenylmethylsulfonyl fluoride, leupeptin, and pepstatin for final concentrations of 0.2 mmol/L, 0.5 \(\mu\)mol/mL, and 0.7 \(\mu\)mol/mL, respectively. The bacterial suspension was placed into a nitrogen cavitation chamber (Parr Cell Disruption Bomb Model 4639; Parr Instrument Co, Moline, IL), pressurized to 1,250 psi for 30 minutes at room temperature, and decompressed into a centrifuge tube in which cellular debris was subsequently removed for 30 minutes at 27,200g. The viscous supernatant was then further clarified of bacterial chromosomal material by centrifugation at 270,000g for 60 minutes.

**Purification of recombinant Fab molecules by nickel-chelation affinity chromatography.** Recombinant Fab molecules, encoded by Nhe I/Spe I digested pComb3 (see above), contain 6 histidine residues on the carboxy terminus of the heavy chain (see Results) that facilitate their binding to nickel-conjugated agarose (Ni\(^{2+}\)-NTA resin; Qiagen) and elution with imidazole at neutral pH. To determine the appropriate imidazole concentrations for affinity purification of Fab molecules, preliminary studies were performed in which a 100-µL volume of crude bacterial extract was adsorbed with a 25-µL aliquot of nickel-agarose as described in the product literature. The gel suspension was washed batch-wise with increasing concentrations of imidazole (Sigma I-0250, fluorescence-depleted) in extraction buffer and eluates were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels under nonreducing and reducing conditions for protein staining with Coomassie blue and immunoblotting with HRP-conjugated goat antihuman F(ab')\(_2\); antibody. From these pilot studies, it was determined that a prewash with 10 mmol/L imidazole would quantitatively remove bound contaminants and 250 mmol/L imidazole would elute pure recombinant Fab molecules. Therefore, the balance of the bacterial extract (~40 mL) was loaded at 30 mL/h onto a 2.5-mL Ni\(^{2+}\)-NTA agrose column (prewashed in extraction buffer) and washed with extraction buffer until a baseline OD\(_{260}\) of ~0.01 was achieved (~150 mL). Contaminants were eluted with 10 mmol/L imidazole (flow rate of 10 mL/h, ~75 mL) and recombinant Fab molecules were eluted with ~30 mL of 250 mmol/L imidazole. The 10 mmol/L and 250 mmol/L imidazole washes were concentrated and dialyzed to 400 µL of PBS by vacuum dialysis (Micro-ProDicon membrane, 10,000 molecular weight cutoff; Spectrum Medical Industries Inc, Los Angeles, CA), measured for protein concentration (BCA protein assay; Pierce Chemical Co, Rockford, IL), and analyzed as described below and in the Results.

**Protein electrophoresis and immunoblotting.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% separation/6% stacking gels according to Laemmli. \(^{28}\) For immunoblotting, protein bands were electrophoretically transferred to 0.2-µm pore size nitrocellulose (Schleicher and Schuell) at 500 mA constant current for 5 hours at 15°C in a miniature transfer unit (Hoefer Scientific Instruments, San Francisco, CA) filled with 193 mmol/L glycine/25 mmol/L Tris/20% methanol containing 0.05% SDS. Filters were blocked and incubated with HRP-conjugated antibodies and HRP substrate as described above for colony lifts.

**Flow cytometric analysis of recombinant Fab molecules.** Aliquots of recombinant Fab preparations (300 ng) were incubated with 5 µL of a 3% suspension of Rh(D)-positive or -negative RBCs at 37°C for 1 hour on a laboratory rotator in a final volume of 100 µL of PBS containing 1.5% BSA. The cells were washed three times with 100-µL volumes of incubation buffer at 4°C followed by 60 minutes of incubation at 4°C with a 1:50 dilution of R-phycocer-
Production of Anti-Rh(D) and Antitetanus Phage Particles

Human antibody without RBC binding specificity (anti-Rh(D) and anti-tetanus), was produced by selecting phage particles expressing Fab or F(ab')2 domains. The cells were washed three times with PBS and analyzed through a FACScan microfluorimeter as described above for phagemid analysis.

Agglutination reactions with recombinant Fab molecules. Aliquots of purified recombinant Fab (70 μg) were incubated with 25 μL of a 3% suspension of Rh(D)-positive or -negative RBCs at 37°C for 1 hour in a final volume of 75 μL of PBS containing 1.5% BSA. The cells were washed three times with 200 μL of incubation buffer at 4°C and the pellets resuspended in 50 μL of a 36 μg/mL solution of goat anti-human F(ab')2 antibody (Pierce) in 10 mm potassium phosphate buffer (pH 7.4). The cell buttons were gently resuspended and examined for agglutination using standard blood typing techniques.

Electron Microscopy

For examination of phage-coated RBC preparations, samples were adsorbed to Formvar-coated 300-mesh grids, stained with 1% phosphotungstic acid, and viewed through a JEOL 100CXII transmission electron microscope (JEOL USA Inc, Peabody, MA) at a beam acceleration voltage of 80 kV. For scanning electron microscopy of RBC agglutinates, samples were fixed with 2% glutaraldehyde/PBS overnight at 4°C on a laboratory rotator, sedimented at 100g for 4 minutes, washed three times with PBS, postfixed with 1% OsO₄ in distilled water, and cyto spun onto poly-L-lysine-coated glass coverslips (Sigma). Samples were dehydrated through a series of ethanol solutions (70%, 80%, 95%, 100%) for 15 minutes each, followed by a final wash in Freon 113 (Electron Microscopy Sciences, Fort Washington, PA). RBC-coated coverslips were critically point dried from liquid CO₂, sputter coated with 100 Å of gold, and examined in a JEOL T330A scanning electron microscope at a beam acceleration voltage of 20 kV.

RESULTS

Production of Anti-Rh(D) and Antitetanus Phage Particles

Our initial goal was to produce two sets of antibody-bearing phage particles—one set expressing a human anti-RBC antibody [anti-Rh(D)] and the other set expressing a human antibody without RBC binding specificity (antitetanus toxoid). Dilution of the anti-Rh(D) phage 10,000-fold into the antitetanus phage would provide a model system by which conditions could be developed for isolating and enriching for RBC-specific phage through panning on intact antigen-positive RBCs. By exploiting the fact that the anti-Rh(D) antibody contains a λ light chain, whereas the antitetanus antibody contains a κ light chain, immunologic techniques could be used to distinguish between bacterial clones producing anti-Rh(D) or antitetanus.

Plasmid constructs containing anti-Rh(D) or antitetanus DNA were introduced into E. coli by electroporation, and production of recombinant antibody was verified by performing nitrocellulose lifts and immunoblots on lysed bacterial colonies. Only bacteria transformed with pComb3/anti-Rh(D) stained with anti-λ antibody, whereas only those transformed with the antitetanus construct stained with anti-κ antibody (data not shown).

Functional Characterization of Phage-Displayed Antibodies

A flow cytometric assay was developed to demonstrate that the anti-Rh(D)—expressing phagemid particles retained their binding specificity. Figure 1A shows that phagemid particles bearing anti-Rh(D) Fab molecules bound to Rh(D)-positive RBCs. In contrast, they bound to Rh(D)-negative RBCs at the same background level as antitetanus-expressing phage bound to either Rh(D)-negative or -positive cells. That anti-Rh(D) phage were indeed binding to Rh(D) antigens present on the Rh(D)-positive cells is shown in Fig 1B, in which anti-Rh(D) phage binding was blocked when RBCs were preincubated with the parent IgG anti-Rh(D) heterohybridoma supernatant. As a control, binding of anti-Rh(D) phage was not inhibited when Rh(D)-positive cells were preincubated with an α-Kell monoclonal IgG preparation. (Kell is an unrelated RBC alloantigen that was also present on the Rh(D)-positive cells chosen for this experiment.) Furthermore, anti-Rh(D)—bearing phage were incubated as above with a panel of RBCs comprising over a dozen Rh(D)-
negative and -positive cells (as is done for determining the specificity of RBC alloantibodies in patient specimens31), and the recombinant phage antibodies bound only to cells carrying the Rh(D) antigen (data not shown).

Morphology of Phage/RBC Interaction

To visualize the interaction of RBC-specific filamentous phage particles with the erythrocyte surface, Rh(D)-positive and Rh(D)-negative cells were incubated with anti-Rh(D)—bearing phage as above and examined by transmission electron microscopy. The electron micrograph in Fig 2 shows Rh(D)-positive RBCs decorated with filamentous structures approximately 0.3 to 0.6 μm in length, characteristic of filamentous bacteriophage.52,53 Of particular significance is the observation that phage appear to attach to the surface only at their tips, which is the precise location where the gene III/Fab fusion protein should reside.16 Extensive examination of Rh(D)-negative RBC anti-Rh(D) phage preparations did not show the presence of phage on RBC surfaces or elsewhere within the microscopic field (data not shown).

Panning Phage Libraries for RBC Reactive Phage

Having verified the expression of anti-Rh(D) and antitetanus Ig on the surface of their respective phage particles, an artificial library was constructed containing one anti-Rh(D)—expressing phagemid per 10,000 that express antitetanus. The ability to efficiently enrich for anti-Rh(D)—specific phage was tested by conducting rounds of panning using Rh(D)-positive cells under an extensive array of incubation, washing, and elution conditions. From these studies, an optimized panning protocol was developed, the results of which are presented in Figs 3 and 4.

As a qualitative assessment of enrichment for anti-Rh(D)—expressing phage, aliquots of phage from the initial library and from each of four rounds of panning were spotted in duplicate onto nitrocellulose and probed with HRP-conjugated anti-α or anti-κ antibody. As shown in Fig 3A, initially the library comprised essentially all anti-κ (ie, antitetanus) —reacting phage, the level of which decreased in the third round of panning as anti-λ [ie, anti-Rh(D)] reactivity increased. The dramatic increases in enrichment that begin at the third round of panning are similar to those seen when panning with soluble, purified antigen.26-28 Phage binding experiments in which phage after each round of panning were incubated with Rh(D)-negative or -positive RBCs and then eluted and titered showed essentially equal, background binding to either cell phenotype after each of the first two rounds of panning but showed increased binding to antigen-positive cells beginning during the third round (Fig 3B). A more quantitative assessment of the composition of the panned phage preparations is shown in Fig 4, in which colonies from bacterial cultures reinfected with phage from each round of panning were streaked in duplicate and probed for anti-Rh(D) or antitetanus production. After 3 rounds of panning, 28 of 31 colonies (90%) were positive for anti-Rh(D) and, after an additional round, 31 of 31 colonies (100%) were anti-Rh(D) positive. Therefore, collectively, these studies show that phage displaying a clinically relevant human RBC alloantibody initially present at a frequency of 1 in 10,000 can be quantitatively isolated through panning with antigen-positive RBCs.

Production of Soluble, Recombinant Anti-Rh(D) Fab Molecules

Bacterial colonies producing anti-Rh(D)/gene III were used to express soluble anti-Rh(D) Fab molecules for the purpose of developing efficient methods for their isolation, purification, and characterization. This allowed testing of the practical utility of bacterially produced RBC antibodies as potential serologic typing reagents. pComb3 DNA containing heavy and light chain DNA antibody segments were digested with restriction endonucleases and self-ligated to create plasmid DNA that encodes separate heavy and light chain polypeptide chains that assemble in the periplasmic space to form disulfide-bridged Fab molecules.29 In addition, the removal of the gene III coat protein segment juxtaposes a string of six histidine codons at the 3' end of the heavy chain Cα1 sequence. Such a motif expressed on the carboxy terminus of the heavy chain Fd fragment facilitates affinity purification of Fab molecules on nickel-conjugated agarose columns.34

Bacterial cultures containing Nhe I/Spe I-digested anti-Rh(D) pComb3 plasmid DNA lacking the gene III sequence were induced with IPTG. Several techniques for the isolation of assembled Fab molecules from the bacterial periplasmic space were evaluated. Nitrogen cavitation was superior to cell disruption by sonication,28 freeze-thawing,35 and osmotic shock37,38 in terms of its speed, reproducibility, yield of recombinant protein, and retention of antibody/antigen binding.
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Fig 3. (A) Enrichment for anti-Rh(D)-expressing phage through panning on Rh(D)-positive RBCs. In the positions indicated, 1-μL samples of phagemids (2 × 10^8 pfu) from the starting anti-Rh(D)/antitetanus library ("S") and after each of 4 rounds of panning ("1st" through "4th") were spotted in duplicate onto strips of nitrocellulose and developed with HRP-conjugated goat anti-human λ light chain antibody (to detect recombinant anti-Rh(D), upper panel) or HRP-conjugated goat anti-human κ light chain antibody (to detect recombinant antitetanus toxoid; lower panel). (B) Binding of panned phage preparations to Rh(D)-positive versus -negative RBCs. Phage (~10^11 pfu) from each round of panning were incubated with Rh(D)-negative or -positive RBCs, unbound phage were removed by washing, and bound phage were eluted and titrated as described in Materials and Methods.

(see below). Methods to affinity purify the Fab protein from the bacterial debris included the use of immobilized goat anti-human Fab antibody as well as the nickel-chelation affinity chromatography. In preliminary, small-scale preparations, the first method proved unsatisfactory as the acid treatment used to elute bound Fab molecules irreversibly denatured them (data not shown). The second method, in which Fab molecules bound to nickel are eluted at neutral pH with imidazole, did not have this drawback. A second advantage of the nickel-chelation technique is that the resulting Fab preparations contain only full-length heavy chain polypeptides because lower molecular weight heavy (and light) chain proteolytic fragments or partially translated product do not contain the heavy chain carboxy terminus and are not bound by the column in contrast to the anti-Fab antibody columns.

Nickel-agarose columns loaded with our crude bacterial extract and eluted with imidazole yielded a molecular weight (M_r) ~50-kD band (unreduced) or M_r ~ 26-kD band (reduced) on Coomassie-stained and immunoblotted SDS-polyacrylamide gels (Fig 5A and B, lanes e and j). In addition, by immunoblotting under nonreducing conditions, a lightly staining doublet with M_r ~26 to 28 kD was shown (Fig 5B, lane e) in which the upper band migrated at the position of

Fig 4. Quantification of panning efficiency. Thirty-one randomly chosen colonies from bacterial cultures reinfeected with phage derived from the initial anti-Rh(D)/antitetanus mix (top row) and from the third and fourth rounds of panning (middle and bottom rows) were streaked in duplicate and stained for anti-Rh(D) production (left column) or antitetanus production (right column) as described in Materials and Methods. One-microliter spots of λ and κ human light chain standards were applied as controls to each filter in the positions indicated.
reduced light chain polypeptide (Fig 5B, lane j). We hypothesize that this doublet represents a small fraction of light chain that does not undergo disulfide bridge formation with heavy chain, yet is retained by the nickel column through noncovalent associations with heavy chain. On exposure to SDS, it dissociates from heavy chain (Fig 5B, lane e). Its appearance as a doublet may represent two light chain populations, one with and one without intra-light chain disulfide bonds, because the two fractions migrate with the same electrophoretic mobility on reduction (Fig 5B, lane j). It is also of interest to note the presence of low molecular weight (M, ~18 kD) light chain fragments in the bacterial extract and column flow through (Fig 5B, lanes b, c, g, and h) that are absent from the purified Fab preparation (Fig 5B, lanes e and j), an advantage of the nickel-chelation chromatography technique over immunologically based methods, as noted above. Unfortunately, the presence of the heavy chain F₄ cannot be explicitly identified on immunoblots because of the commercial unavailability of antibodies directed against human IgG F₄ fragment. However, the binding of recombinant protein to the nickel column (presumably mediated by the heavy chain histidine tail), the precise comigration of unreduced and reduced recombinant Fab with authentic chemically produced human IgG Fab standard (Fig 5, lanes a and f), and the binding of recombinant antibody to antigen (see below) provide strong evidence for the presence of heavy chain polypeptide.

In summary, from a 2-L bacterial culture, 135.0 mg of bacterial extract were obtained (Fig 5, lanes b and g), 131.4 mg (~97%) of which did not bind to the column (lanes c and h). Low imidazole eluted 1.4 mg (~1.0% of load) of non-Fab material (mostly polypeptides with M, ~70 to 100 kD; Fig 5, lanes d and j) and higher concentrations of imidazole released 2.2 mg (~2.0% of load) of highly purified anti-Rh(D) Fab protein (lanes e and j).

Ability of Recombinant Anti-Rh(D) Fab Molecules to Bind to RBCs

Equal nanogram amounts of column fractions isolated above were incubated with Rh(D)-negative or -positive RBCs and binding was assessed by flow cytometry. As shown in Fig 6, the high imidazole column eluate displayed strong binding to Rh(D)-positive cells, whereas the column load and low imidazole eluate contained significantly less anti-Rh(D) per nanogram of protein. The column flow through bound to Rh(D)-positive cells at the same background level as incubation buffer alone, indicating that the nickel column was efficient in adsorbing all anti-Rh(D) activity. That the purified anti-Rh(D) recombinant Fab molecules could discriminate between Rh(D)-negative and -positive cells was shown by flow cytometry (Fig 6, inset) and by an indirect agglutination assay (Fig 7).

DISCUSSION

Recent advances in molecular biology have facilitated the isolation and in vitro production of antibodies by creating libraries of recombinant Ig Fab fragments expressed on the surface of bacteriophage particles. These approaches have been particularly valuable to the study of human antibodies, because cell immortalization techniques for human lymphocytes are fraught with technical difficulties. Furthermore, the use of phage display technology may offer additional advantages over cell culture methods as access is provided to populations of terminally differentiated B cells that are rich in mRNA but are not able to fuse. To date, repertoire cloning has been successfully used to study the immune response to a variety of soluble, purified autoantigens and alloantigens and to produce Ig molecules with potential therapeutic and/or diagnostic utility. These molecular approaches could have broad application...
Fig 6. Binding of recombinant anti-Rh(D) Fab fragment preparations to human RBCs. Equal nanogram amounts of column fractions analyzed in Fig 5 were incubated with RBCs that were then washed, incubated with R-phycocerythrin–conjugated goat antihuman F(ab')2 antibody, and analyzed by flow cytometry (see Materials and Methods). Curves a through e all represent incubations with Rh(D)-positive cells. Samples are BSA/PBS incubation buffer alone (a), column load (b), column flow through (c), low imidazole column eluate (d), and high imidazole column eluate (e). (Inset) High imidazole column eluate [purified recombinant anti-Rh(D) Fab] incubated with Rh(D)-negative (−) or -positive (+) cells. (Axes scales are identical on both cytograms.)

in the areas of basic immunohematology and transfusion medicine. For example, a major obstacle in studying the pathogenesis of warm autoimmune hemolytic anemia has been the inability to obtain sufficient quantities of autoantibodies that are homogeneous in structure and specificity. Consequently, an understanding of the biochemistry of the relevant autoantigen(s) and the nature of the corresponding pathogenic immune response have been limited by serologic observations and the analyses of minute quantities of polyclonal Ig molecules eluted off of the surface of patient RBCs. Autoreactive phage derived from phage display libraries made from patients with warm autoimmune hemolytic anemia would produce both the gene segments as well as the expressed autoantibodies that can be used to analyze

Fig 7. Agglutination of Rh(D)-positive RBCs with recombinant anti-Rh(D) antibody. Rh(D)-negative (left column) or -positive (right column) RBCs were incubated with purified recombinant anti-Rh(D) Fab fragment (top row) or anti-Rh(D) IgG-containing H11216S hybridoma supernatant (bottom row, see Materials and Methods). RBCs were washed, incubated with goat antihuman F(ab')2 antibody, and prepared for scanning electron microscopy. (Magnification × 1,200.)
the clonality and genetic origin of the pathologic autoantibodies and the relevant autoantigenic structures. Libaries produced from patients alloimmunized to RBCs through transfusion or pregnancy could serve as model systems to study the human immune response to foreign antigens as well as to show the immune repertoires directed against specific RBC antigens of clinical significance. For example, analyses of the immune response to the Rh(D) antigen could lead to an understanding of the immunologic composition of Rh(D)-immune globulin and aid in the design of studies to dissect the mechanisms by which Rh(D)-immune globulin exerts its immunomodulatory effect. Furthermore, heavy and light chain "swapping" among specific subsets of RBC antibodies could show the relative contributions of heavy and light chain polypeptides to the binding of similar yet serologically distinct structures such as the A and B or I and i blood group antigens. From a practical standpoint, error-prone PCR or CDR3 randomization of existing RBC monoclonals cloned into phagemid vectors could alter their fine specificity or improve their affinity. For example, an anti-Rh(D) antibody could be made into one capable of detecting Rh(D)*, a weakened form of Rh(D). Additionally, alloantibodies isolated from phage display libraries could provide an economical source of blood bank typing reagents that would resolve current ethical concerns regarding the hyperimmunization of human donors with foreign RBCs. As a prerequisite to the application of repertoire cloning to the study of human RBC antibodies, methods needed to be established for retrieving phage specific for unpurifiable, membrane-bound antigens. In the current study, a model library comprising one anti-Rh(D)—expressing phage particle per 10^4 irrelevant antitetanus-specific particles was used to develop the appropriate conditions for isolating and characterizing anti-Rh(D)—specific clones using intact RBCs. In actual practice, phage libraries constructed from immunized individuals may contain a number of different RBC specificities and one or more rounds of "negative panning" on panels of antigen-negative cells may be required. This would deplete libraries of undesired specificities or "pan-reactive" phage before positive selection on antigen-positive cells. Alternatively, selective elution methods can be used during panning in which patient sera, depleted of undesired specificities through adsorption with antigen-negative cells, can be used instead of acid glycine solutions. By competing with RBC-bound phage, specific populations of phage can be displaced and subsequently propagated. Selective elution techniques using patient sera containing RBC autoantibodies would be of particular value when attempting to isolate phage bearing pan-reactive warm auto-specificities. In addition, the efficiency of panning phage libraries with intact RBCs can be increased by exploiting the extensive knowledge base provided by RBC serologists through the use of enzyme-treated or chemically modified erythrocytes. In this regard, anti-Rh(D)—expressing phage behave similarly to authentic serum anti-Rh(D) antibodies because they demonstrate enhanced binding to ficin-treated Rh(D)-positive cells versus untreated Rh(D)-positive cells (data not shown). For diagnostic purposes, blood bank typing reagents could be developed and used in indirect agglutination assays (Fig 7) or heavy and light chain DNA could be cloned into pComb8, a phagemid vector that couples Fab fragments to bacteriophage gene VIII coat protein. Gene VIII coat protein is present in hundreds of copies per phagemid (versus 1 to 3 copies of gene III) so that anti-RBC antibodies expressed on pComb8 would serve as polyvalent direct agglutinating reagents that are self-replicative.

The reason for choosing a 1:10^4 ratio of specific to nonspecific phage in the model library relates to what was estimated to be an underapproximation of the expected prevalence of antigen-specific phage present in an actual phage library. In such a library, the precise frequency of phage with a given specificity is a function of both the number of B cells encoding that specificity and the degree of B-cell activation (ie, amount of Ig mRNA per cell). Before immunization, it has been estimated that ~1 in 10^7 B cells recognize a particular epitope with an affinity great enough to stimulate B-cell proliferation. After immunization, the proportion of B cells in the immune repertoire secreting a defined specificity is difficult to quantify; however, data from hybridomas made from mice immunized with p-azophenylarsonate—conjugated protein showed that 1 of 73 hybridomas secreted antibodies specific for the hapten. In humans, it is of interest to note that the serum concentration of anti-Rh(D) alloantibodies can be as high as 500 μg/mL, which indicates that, in alloimmunized patients, ~1 of every 25 serum IgG antibodies can have specificity for Rh(D). Although a direct correlation cannot be made between the serum concentration of antibodies with a given specificity and the frequency of phage particles displaying that specificity in a library derived from that individual, the data suggest that a model system that assumes a frequency of 1:10^4 is a reasonable one even when taking into account the random shuffling of heavy and light chain PCR products that occurs during the library’s construction. In actuality, the sensitivity of our approach is most likely more than 1:10^4, because a 10,000-fold enrichment was nearly achieved after three rounds of panning, and an additional round resulted in retrieval of 100% anti-Rh(D) clones (Fig 4). With additional rounds of panning and further refinements in technique, the ability to isolate specific phage by panning on intact cells should approach sensitivities of 1:10^6 to 1:10^8, as estimated for panning against purified antigen.

In addition to its utility in RBC immunology described above, repertoire cloning by cell-surface enrichment may prove useful for the analyses of the human immune response to nonerythroid, membrane-bound antigens that are structurally uncharacterized, unpurifiable in a nondenatured state, or are components of macromolecular complexes that lose their antigenicity when disassembled.

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Expression and characterization of recombinant anti-Rh(D) antibodies on filamentous phage: a model system for isolating human red blood cell antibodies by repertoire cloning

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