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Characterization of Phage-Displayed Recombinant Anti-Idiotypic Antibody Fragments Against Coronavirus-Neutralizing Monoclonal Antibodies

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ABSTRACT

Murine coronaviruses provide useful animal models for human neurological disorders such as multiple sclerosis. In an effort to better understand the mechanisms involved in protection from coronavirus infection, we are studying the role of the idiotypic network in the modulation of viral infectivity. We have explored the feasibility of using single-chain antibodies displayed on phage surfaces for the isolation of recombinant anti-idiotypic antibodies (anti-Ids) with antigen-mimicking properties, which has proven to be difficult with conventional hybridoma approaches. A phage-display library containing more than 10^8 different antibody specificities was screened for the presence of anti-Ids by successive rounds of panning with three different *in vitro* neutralizing and *in vivo* protective antiviral monoclonal antibodies. After five rounds of panning, between 32% and 84% of all individual clones tested showed antibody-binding in an enzyme-linked immunosorbent assay (ELISA). Although several clones showed identical antibody sequences, a number of different clones were identified and further characterized. None of the selected clones induced the production of antiviral or neutralizing antibodies or conferred reproducible protection from viral challenge in BALB/c and C57BL/6 mice. These results demonstrate that anti-Ids can be isolated from a phage-display library, although high-affinity antigen-mimicking phages with antiviral protective capacities were apparently not represented in this library. This argues for the development of more diverse phage-display libraries.

INTRODUCTION

Murine hepatitis viruses (MHV) are members of the *Coronaviridae*, a family of enveloped positive-stranded RNA viruses responsible for a number of human and animal respiratory, neurological, and gastrointestinal infections (19,23). The neurotropic A59 strain of MHV causes encephalitis and demyelination in mice and rats and is used in our laboratory as an animal model for multiple sclerosis. The virion of MHV-A59 is composed of three major structural proteins (19): the nucleocapsid protein, the membrane glycoprotein, and the spike glycoprotein (S). The S protein constitutes a major target of the immune re-

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sponse during viral infection and is responsible for binding to the viral receptor on the surface of target cells (3–5,24) and for virus-induced cell fusion (3). In order to better understand the mechanisms involved in protection from coronavirus infection, we are studying the role of the idiotypic network in modulation of viral infectivity.

The concept of the immune network proposed by Jerne in 1974 (10) describes the immune system as a set of variable domains (idiotypes) that interact. Certain types of anti-idiotypic antibodies (anti-Ids) have the property of bearing the internal image of an antigen and such anti-Ids have been successfully used as surrogate antigens in experimental vaccines against parasites (8,17), bacteria (14,20) and viruses (1,11,16,22). We have previously shown that polyclonal anti-Ids could induce the production of MHV-A59-specific anti-anti-idiotypic antibodies (Ab3s) that could mimic the idiotype bearing antibody (Ab1) and neutralize viral infection *in vitro* and protect mice from MHV-A59 infection (12,25). Further characterization of the idiotypic network in coronavirus infection requires the use of more defined monoclonal reagents. Unfortunately, the isolation of biologically active monoclonal anti-Ids has proven to be difficult using conventional experimental approaches (25). In the present study, we report the utilization of a phage-display library for the isolation of human single-chain anti-Ids to evaluate the feasibility of using this technology as an alternative to hybridoma production for the characterization of the vaccinating and/or epitope-mimicking potential of anti-Ids.

MATERIALS AND METHODS

Selection of the library. The construction of the phage-display library of human scFv fragments was described elsewhere (15). Immunotubes (Nunc, Roskilde, Denmark) were coated by overnight incubation at room temperature with each of three following monoclonal antibodies (mAb): 7-10A and 4-11G (4) and 5B19 (3,5) at 100 μg/mL in phosphate-buffered saline (PBS). The next day, the tubes were washed three times with PBS and blocked with PBS containing 2% (w/v) skim milk powder (PBS-M) and incubated at 37°C for 2 hours. The tubes were similarly washed and 10^{12} to 10^{13} transforming units (TU) of phages from the library in PBS-M were added and the tubes were incubated at room temperature for 2 hours. After 20 washes with PBS containing 0.1% (v/v) Tween 20 (PBS-T) and 20 washes with PBS alone, the bound phages were eluted by a 10-minute incubation with a 100 mM solution of triethylamine and immediately neutralized with 1.0 M Tris-HCl, pH 7.4. Exponentially growing *Escherichia coli* TG1 cells were infected with the eluted phages by incubation in a 37°C water bath for 30 minutes. Infected cells were centrifuged, resuspended in 2xTY broth and plated on one 9 × 9 inch Bio-Assay dish (Nunc) of 2xTY containing 100 μg/mL ampicillin and 1% (w/v) glucose (2xTY-amp-glu). Plates were incubated overnight at 37°C. Five milliliters of 2xTY was added on top of the dish and cells were loosened with a glass spreader. About 10^8 bacteria were added to 2xTY-amp-glu and grown until the OD at 600 nm was 0.5, at which time 10^{12} M13-K07 helper phages (Stratagene, La Jolla, CA) were added and incubated for 30 minutes in a 37°C water bath. The cells were pelleted and resuspended in 2xTY containing 100 μg/mL ampicillin and 25 μg/mL kanamycin (2xTY-amp-kan) and incubated at 30°C overnight. The culture was centrifuged and the supernatant was precipitated twice with polyethylene glycol 8000, 2.5 M and the phages were resuspended in water. The selection procedure was repeated five times before isolated clones were tested by enzyme-linked immunosorbent assay (ELISA).

Phage ELISA. Isolated colonies from the fifth round of panning were grown in 96-well plates containing 2xTY-amp-glu and infected for 90 minutes with M13-K07 helper phage. Cells were centrifuged and resuspended in 2xTY-amp-kan and grown overnight at 30°C. Each well of 96-well microtiter plates was coated with 1 μg of the appropriate monoclonal antibody or KLH (Sigma-Aldrich Canada, Ltd., Mississauga, Ontario, Canada) in PBS. After overnight incubation, the remaining binding sites in the wells were blocked with PBS-M for 90 minutes. One hundred microliters of the phage-containing supernatant or polyclonal polyethylene glycol (PEG)-precipitated phages were added and incubated for 90 minutes. The wells were washed 5 times with PBS-T and peroxidase-labeled anti-M13 antiserum (Pharmacia Canada Inc., Baie-d'Urfé, Québec, Canada) was then added and the plates incubated for another 90 minutes. The plates were washed five times with PBS-T and the bound peroxidase revealed by incubation with *O*-phenylenediamine

(Sigma) and hydrogen peroxide. The reaction was stopped with 1 N HCl and the absorbance read at 492 nm using an SLT EAR 400 AT plate reader.

Nucleotide sequencing of selected clones and sequence alignments. Precipitated phage particles from positive clones identified in the phage-ELISA described above were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 mixture) and single-stranded DNA was precipitated with ethanol. Pellets were washed with 70% (v/v) ethanol, dried and resuspended in water. Nucleotide sequencing was performed by the dideoxynucleotide chain terminating method (18) using the LINK-Seq primer (5′-CGATCCGC-CACCGCCAGAG-3′) (9), T7 DNA polymerase (Pharmacia) and [α-^{35}S]dATP (ICN Pharmaceuticals Canada Ltd, Montréal, Québec, Canada) according to the manufacturer's instructions (Pharmacia). Sequence alignments of CDR3 and V_H segments with that of the S glycoprotein of MHV-A59 (13) were performed with version 2.45 of the GeneWorks® software (Intelligenetics Inc, Campbell, CA).

Immunization of mice with selected clones. Groups of MHV-seronegative BALB/c or C57BL/6 mice (Charles River, St-Constant, Québec, Canada) were injected intraperitoneally with 2 mg of PEG-precipitated phage emulsified 1:1 in complete Freund's adjuvant. Three booster injections of the same quantity of phage in incomplete Freund's adjuvant were given at biweekly intervals. Mice were bled 7 days after each injection and the sera were tested for the presence of antiviral antibodies by ELISA and Western immunoblotting and for virus neutralization activity as described previously (12). Immunized mice were challenged 10 days after the last booster injection by intracerebral injection with 10 LD_{50} (5×10^5 PFU for BALB/c and 6×10^4 PFU for C57BL/6) of MHV-A59.

RESULTS

The phage-display library of human scFv antibody fragments was subjected to five rounds of selection by panning against three different neutralizing anti-MHV-A59 mAbs. To verify if the panning procedure had enriched the antibody-binding phage population, polyclonal populations of phages were assayed for binding to the different antibodies by ELISA. Figure 1 shows the binding curves of the three polyclonal phage populations to mAb 7-10A or to KLH after five rounds of selection with either one of three mAbs. A greater proportion of the 7-10A-selected phages reacted with 7-10A compared with phages selected with

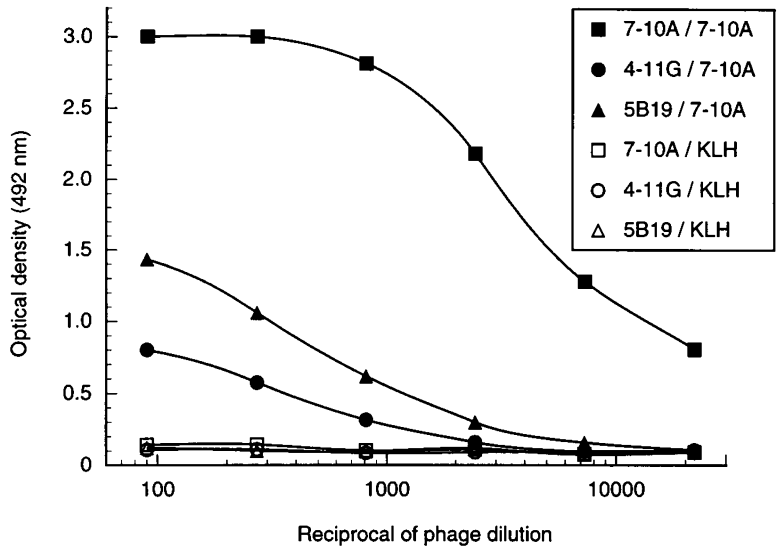

FIG. 1. Binding of polyclonal phage populations to mAb 7-10A or KLH. Microtiter plates were coated with 1 μg/well of mAb 7-10A (filled symbols) or KLH (open symbols). The binding of threefold dilutions of polyclonal phage populations after 5 rounds of selection for mAb 7-10A (■), 4-11G (●) or 5B19 (▲) was detected using horseradish peroxidase-labeled anti-M13 antibodies.

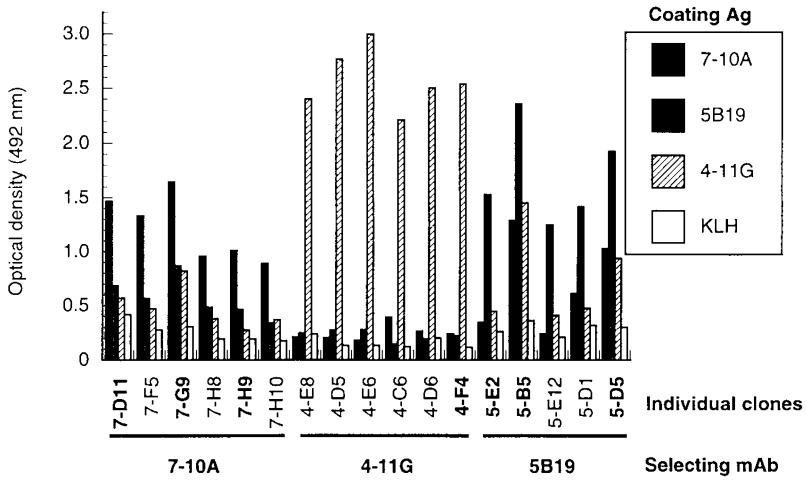

FIG. 2. Binding of individual phage clones after five rounds of selection for three antiviral mAbs. Microtiter plates were coated with 1 μg/well of mAb 7-10A, 4-11G, 5B19 or an irrelevant Ag (KLH) and the binding of individual clones selected by five rounds of panning against the three antibodies was detected using horseradish peroxidase-labeled anti-M13 antibodies. Individual clones shown in bold represent phages that were selected for *in vivo* characterization.

the other two mAbs, demonstrating the effectiveness of the selection procedure. In addition, the polyclonal mAb-selected phages did not show any reactivity with KLH, indicating that the panning protocol did not amplify phage populations with specificities for unrelated proteins. A slight cross-reactivity with the two other selecting antibodies was observed, which probably indicates the presence of phages with binding specificities for constant regions common to all three antibodies. Similar binding curves were obtained for the other two selecting antibodies (data not shown).

Individual phage clones were screened for binding to the selecting antibodies by ELISA using supernatants from infected bacteria. Figure 2 shows the reactivity of some of the antibody-selected clones against the different anti-MHV-A59 mAbs or to KLH. After five rounds of panning, positive clones were identified for all three antibodies used for selection, in proportions ranging from 32% to 84%. Again, some cross-reactivity was observed with a few clones, which possibly indicates that these phages express antibody fragments that recognize similar structures present on the other antibodies.

The variable regions of the heavy chains of ELISA-positive clones specific for each antibody were then sequenced. A number of clones showed identical V_H sequences and only clones with unique sequences were selected for further characterization and are presented in Table 1. Out of six different anti-7-10A anti-idiotypic clones sequenced, three had sequences identical to clone 7H9, with exactly the same CDR3 sequence

TABLE 1. CDR3 SEQUENCES AND V_H SEGMENTS OF THE SELECTED ANTI-IDIOTYPIC CLONES ISOLATED FROM THE PHAGE-DISPLAY LIBRARY

Clones	*Specificity*	*CDR3 sequences*	*V_H segment*[a]
7G9	7-10A	GKLVK	DP47
7D11	7-10A	MKANGRYD	DP44-45
7H9	7-10A	YIDVPFFYESPR	DP38
T32	5B19	VMKGKGTI	DP25
5B5	5B19	NKGNSNQL	DP32
5D5	5B19	YYAVRPIMN	DP32
4F4	4-11G	ETCDGVHL	DP54

[a]Nomenclature of the heavy chain germline segments as reported (21).

and DP38 V_H segment as defined previously (21). Similarly, out of five anti-5B19 clones sequenced, three had sequences identical to clone 5D5 whereas clone 5B5 used the same DP32 V_H segment with a different CDR3 sequence. Finally, all six anti-4-11G clones sequenced had identical V_H segments and CDR3 sequences.

Seven clones selected for biological characterization were concentrated from supernatant of infected cells by PEG precipitation and 2 mg of concentrated phages in adjuvant were used to immunize BALB/c and C57BL/6 mice that also received three booster injections at biweekly intervals. Mice were bled 7 days after each injection and the sera were tested for the presence of antiviral antibodies by ELISA and Western immunoblotting and for virus neutralization. No significant antiviral or neutralizing antibodies were detected in these animals, whereas antiphage antibody titers were high (data not shown). Finally, all animals were challenged with a lethal dose of MHV-A59. All BALB/c mice immunized with the wild-type phage (pHEN) died within 5 days (Fig. 3A), which is similar to the expected mortality rate of unprotected animals (12). A small delay in mortality was observed with mice immunized with the 5E2 clone and 10% of animals immunized with the 7G9 and 5D5 clones survived. However, 20% of C57BL/6 mice immunized

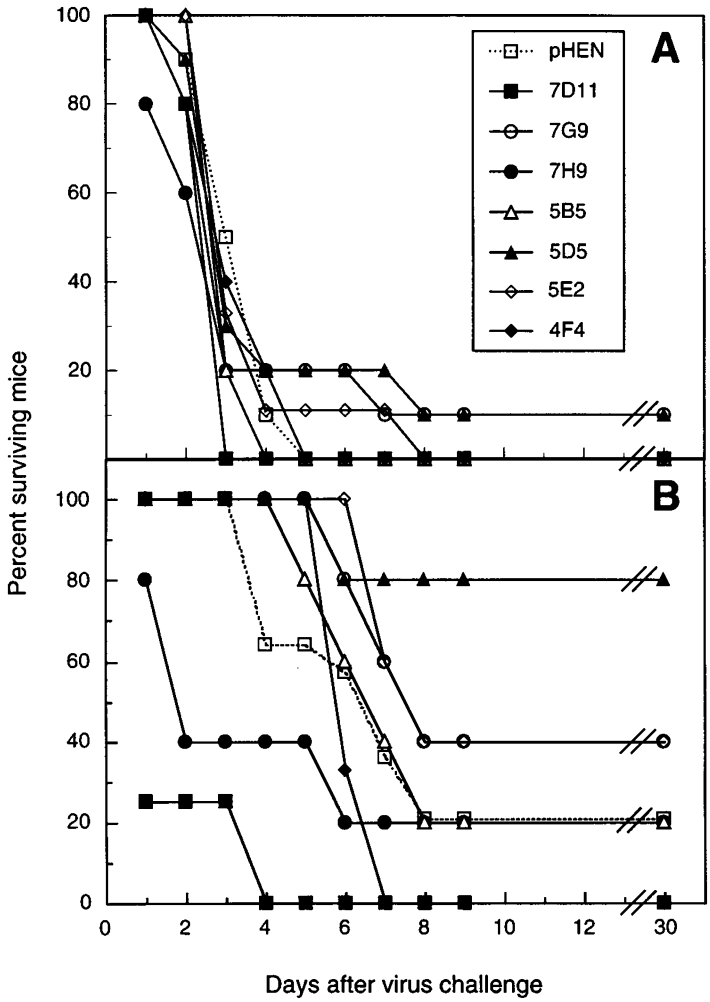

FIG. 3. Survival profiles of virus-challenged BALB/c or C57BL/6 mice previously immunized with anti-idiotypic antibody fragments displayed on phage surfaces. Groups of 5 to 14 BALB/c (A) or C57BL/6 (B) mice were immunized with phages expressing anti-idiotypic antibody fragments selected from the phage-display library by five rounds of panning against one of three different anti-MHV-A59 monoclonal antibodies and challenged with 10 LD_{50} of MHV-A59. Clone pHEN (□) represents the wild-type phage used as a negative control.

with the wild-type phage survived the viral infection. Because this strain of mice is more sensitive to MHV-A59 infection (26), the LD_{50} is reduced compared with BALB/c mice and small variations between different virus aliquots would have a greater impact on the actual virus dose given, which could explain why some control animal survived. Nevertheless, the same two clones that partially protected BALB/c mice, also partially protected C57BL/6 mice above control levels (Fig. 3B) and partial protection was also observed with clone 5E2, which induced a slight delay of mortality of BALB/c mice (Fig. 3A). However, repetition of these vaccination experiments with new batches of phage clones 5D5 and 7G9 in both strains of mice yielded mortality profiles similar to control pHEN-immunized mice.

DISCUSSION

We have used a library of human scFv fragments displayed on the surface of filamentous phage particles built from a bank of 50 cloned human V_H gene segments and random nucleotide sequences encoding CDR3 lengths of 4 to 12 residues (15). This library contains more than 10^8 clones of different specificities, which is comparable to the size of the natural primary immunoglobulin repertoire of the mouse (7).

The phage-display library was subjected to five rounds of selection by panning against three neutralizing anti-MHV-A59 mAbs and antibody-specific phage clones were identified by ELISA for all three antibodies (Fig. 2). Some of these clones showed cross-reactivity with the other two mAbs used for selection, suggesting that they recognize similar structures on different antibody molecules. This is consistent with the possibility that they could have lower affinities than more specific phages.

Seven clones selected for biological characterization were used to immunize BALB/c and C57BL/6 mice. We have previously shown that BALB/c mice can produce an anti-MHV-A59 protective immune response upon anti-idiotypic vaccination (12). However, we also selected C57BL/6 mice because previous immunogenicity experiments of repeat regions of the circumsporozoite protein of *Plasmodium falciparum* cloned into the pIII protein gene of a filamentous phage suggested that the pIII protein might not bear helper T-cell epitopes recognized on the $H\text{-}2^d$ genetic background of the BALB/c mouse, but should bear epitopes recognized on the $H\text{-}2^b$ genetic background of the C57BL/10 or C57BL/6 mice (6). Indeed, we have recently confirmed that C57BL/6 mice produce a more efficient immune response to phage-displayed antigens than BALB/c mice (M.W.N. Yu, J.K. Scott and P.J. Talbot, manuscript in preparation). Two clones induced a partial but not reproducible protection of C57BL/6 mice. This may be the result of inherent experimental variability, a relatively high viral challenge dose, or batch variations in phage production. Interestingly, four out of the five anti-5B19 clones sequenced had the same V_H segment as clone 5D5 and three of them had the same CDR3 sequence (Table 1). This V_H usage could possibly produce an antibody fragment with a higher affinity for mAb 5B19 because clones bearing this V_H segment were more frequently isolated from the library. Thus, it is likely that this particular clone had a higher affinity for the antibody, but apparently not sufficient for a reproducible antiviral activity to be demonstrated.

We postulate that the recombinant anti-Ids isolated from this small phage library were probably of relatively low affinity and thus did not induce an antiviral immune response sufficiently strong to be detected and to reproducibly protect immunized animals. Indeed, it has been reported (7) that the affinities of Fab fragments expressed on phages isolated from a small portion (10^7) of a larger library (6.5×10^{10}) were lower (0.8 to 12 μM) compared with the ones of clones isolated from the entire library (3.8 to 217 nM).

Antigen mimicry at the primary structure level has been proposed as a possible mechanism for the induction of an antigen-specific immune response by anti-Ids. The observation of an homology between a monoclonal anti-Ids and the hemagglutinin of the mammalian reovirus type 3 reinforced this hypothesis (2). A recent study confirmed that a virus-specific immune response can be induced by an irrelevant immunoglobulin engineered to bear a viral epitope within its variable region (27). To verify if a similar structural mimicry could be found in our selected recombinant anti-Ids, amino acid sequence comparisons between the heavy chain variable region of the selected clones and the S glycoprotein of MHV-A59 were undertaken. No significant regions of amino acid sequence homology were identified for any of the selected clones, even when the selecting antibody was 5B19, which recognizes a linear epitope (5). This absence of

structural mimicry may have played a role in the observed the lack of reproducible biological activity, although we did previously report that polyclonal rabbit anti-Ids induced a protective immune response without any apparent antigenic mimicry (12,25).

The present study demonstrates the feasibility of isolating monoclonal anti-Id fragments from phage-display libraries, but also makes a strong point for the need to develop more diverse libraries in order to select for biologically active anti-Ids.

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