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# Peptide Epitopes Recognized by a Human Anti-Cryptococcal Glucuronoxylomannan Antibody

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***Cryptococcus neoformans*** **causes meningitis in 6 to 8% of individuals with AIDS. Recently, immunotherapeutic modalities including antibody therapy have been proposed for the treatment of cryptococcal meningitis in AIDS patients. This is a rational approach because existing antifungal agents fail to eradicate the infection in the setting of profound immunosuppression. Both murine and human antibodies elicited by the investigational cryptococcal capsular polysaccharide vaccine glucuronoxylomannan-tetanus toxoid (GXM-TT) have been shown to be biologically functional in different model systems. The human immunoglobulin M (λ) GXM monoclonal antibody (MAb) 2E9 expresses idiotypes that are also found in naturally occurring anti-GXM antibodies and opsonic GXM-TT sera. However, the specificity of human anti-GXM antibodies and their possible role in protection against cryptococcosis are not known. In an effort to discover epitopes that are recognized by human anti-GXM antibodies, we screened a random decapeptide phage display library with the human anti-GXM MAb 2E9. An enzyme-linked immunosorbent assay (ELISA)-based screening method led to the selection of phages with peptide inserts that bound 2E9 and inhibited 2E9-GXM binding. Analysis of the amino acid sequences of these phages revealed an increased frequency of combinations of QTGLD residues. Inhibition ELISAs demonstrated that phages with QTG/TL/D motifs inhibited 2E9-GXM binding better than phages with different motifs. A peptide synthesized from one of the inhibitory phages, peptide 13 (GMDGT QLDRW), inhibited GXM binding to solid-phase 2E9 and 2E9 binding to solid-phase GXM. Peptide 13 also inhibited the GXM binding of GXM-TT immune sera and naturally occurring serum antibodies from human immunodeficiency virus (HIV)-negative, but not HIV-positive, individuals. Taken together, our data indicate that the peptide epitopes selected by 2E9 mimic GXM epitopes and that peptide 13 may be a mimotope of a GXM epitope that is recognized by human anti-GXM antibodies.**

*Cryptococcus neoformans* is an opportunistic pathogen that causes meningoencephalitis in 6 to 8% of HIV-infected (HIV+) individuals (9). Immunotherapeutic approaches including antibody therapy have been proposed for treatment of *C. neoformans* infections because antibodies that bind the glucuronoxylomannan (GXM) capsule can enhance effector cell function against the organism (7, 30). This has been demonstrated for both murine and human antibodies (23–25, 39) elicited by an investigational GXM-tetanus toxoid (GXM-TT) conjugate vaccine (13). The sera of both HIV+ and HIV− individuals contain naturally occurring human antibodies that bind GXM (11, 14, 17). A subset of these antibodies might be protective in those with intact immunity, because immunocompetent individuals rarely develop cryptococcosis (4, 15). For murine anti-GXM monoclonal antibodies (MAbs), protective and nonprotective antibodies can be distinguished by their isotypes and fine specificities (6, 30). Although opsonization of *C. neoformans* by human GXM-TT sera is correlated with immunoglobulin G2 (IgG2) subclass titers (39), the fine specificity of human anti-GXM antibodies is unknown. Characterization of the epitopes that are recognized by human anti-GXM antibodies would help to identify protective GXM epitopes and perhaps facilitate the development of antibody-based therapies for human cryptococcosis.

The human IgM(λ) MAb 2E9 binds *C. neoformans* serotype A, B, and D GXM and expresses idiotypic determinants that are also expressed by preimmune and immune GXM-TT antibodies (31). Human antibodies that bind the capsular polysaccharides of pathogenic organisms often express restricted idiotypes and isotypes (3, 19, 30), although the mechanism for this phenomenon remains elusive. Determining whether restricted antibody responses produce protective antibodies is important for understanding mechanisms of antibody-mediated protection against capsular pathogens. The latter requires the identification of protective polysaccharide epitopes. Unfortunately, as is the case for GXM, oligosaccharide epitopes to probe the fine specificity of most antipolysaccharide antibodies are not available. Recently, applications of a technology that displays random peptide epitopes on filamentous phages have led to the identification of peptide mimotopes of a wide variety of molecules, including carbohydrates (16, 18, 32, 36, 37). In an effort to gain insight into the specificity of human anti-GXM antibodies, we screened a random decapeptide phage display library with 2E9. This paper describes the sequences and specificities of the peptide epitopes that bind this human anti-GXM antibody.

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## MATERIALS AND METHODS

**Peptide library, control phage, and host bacteria.** The decapeptide library that we screened was kindly provided by P. Valadon (Albert Einstein College of Medicine). Based on the fUSE5 vector (10), it has peptide inserts at the N-terminal end of the pIII coat protein of the tetracycline-resistant phage fd (28). The generation of the library is described elsewhere (36). The phage used for a control, tetracycline-resistant phage 33, contains no peptide insert. After phage infection, the host bacterium, kanamycin-resistant strain K91Kan, grows with resistance to both kanamycin (100 μg/ml) and tetracycline (40 μg/ml). Phage 33 and strain K91kan were provided by P. Valadon.

**Growth and purification of phages.** Two sublibraries were mixed, yielding 400 million phage for screening. Amplification of the library was carried out as follows. A single colony of K91Kan was transferred into 2 ml of Luria-Bertani (LB) medium (Difco Laboratories, Detroit, Mich.) with 100 μg of kanamycin (Gibco, Grand Island, N.Y.) per ml and shaken overnight at 37°C. A 100-μl aliquot of the solution was then added to 10 ml of Terrific broth (Difco) with 100 μg of kanamycin per ml and incubated with vigorous shaking for 3 to 4 h at 37°C until the optical density at 600 nm ($OD_{600}$) was 0.1 to 0.2 at a 1/10 dilution. Phage virions from the decapeptide library ($10^{10}$ to $10^{11}$) were mixed with K91Kan for 10 min at room temperature (RT). The mixture was transferred to 20 ml of LB medium with 0.2 μg of tetracycline (Sigma Chemical Corporation, St. Louis, Mo.) per ml and 100 μg of kanamycin (Sigma) per ml. After shaking for 30 min at 37°C, tetracycline was added to a final concentration of 20 μg/ml. The infected K91Kan was grown with vigorous shaking for 12 to 16 h at 37°C. The mixture was then centrifuged for 10 min, and the supernatant was transferred to a tube containing 0.15 volume of polyethylene glycol 8000 (PEG 8000)-NaCl (Sigma), mixed well, placed on ice for at least 4 h, and recentrifuged for 30 min collect the phage precipitate. The phage pellets were dissolved in 1 ml of $TBS_{50}$ (50 mM Tris, 150 mM NaCl, pH 7.5), precipitated twice in PEG 8000-NaCl and dissolved in 400 μl of $TBS_{50}$. The phage concentration was calculated from the $OD_{269}$ of the solution: $6.7 \times 10^{12}$ virions/ml = 1 OD unit. Phage transducing units (TU) were determined by counting the number of phage-infected K91Kan colonies grown on LB agar plates.

**Biopanning of the peptide library with the human GXM MAb 2E9.** The amplified library was screened with the IgM(λ) MAb 2E9 (31). BioMag Streptavidin Dynabeads (Dynal, Oslo, Norway) were used for four rounds of biopanning (see Table 1). For the first round, 50 μl of beads (5 mg/ml) was prepared according to the manufacturer's instructions and incubated with biotinylated goat anti-human IgM (Zymed, South San Francisco, Calif.) for 1 h at RT, washed three times with TBS, and then incubated with 1 μM 2E9 and $4 \times 10^{10}$ TU of phage for 4 h at RT. The 2E9-bound phage were separated from unbound phage with an MPC-E magnet (Dynal). For subsequent rounds, decreasing concentrations of 2E9 (0.1 μM for the second round and 10 nM for the third and fourth rounds) were incubated with $10^9$ TU of phage for 1 h at RT. After incubation, the biotinylated secondary antibodies were added, and the bound phages were separated from unbound phages with the Dynal magnet. Bound phages were washed seven times with TBS, eluted in 100 μl of 0.1 M glycine-HCl (pH 2.2), and neutralized with 15 μl of 2 M Tris-HCl (pH 8.0).

**ELISA screening of the library.** The 2E9-binding phages from the second- and third-round libraries were rescreened by an enzyme-linked immunosorbent assay (ELISA) method that was devised in an attempt to select higher-affinity 2E9 peptide epitopes (see below). Polystyrene ELISA plates (Corning Glass Works, Corning, N.Y.) were coated with 5 μg of 2E9 per ml and incubated for 1 h at 37°C. The plates were washed three times with $TBS_{10}$ (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) after being blocked with 1% bovine serum albumin (BSA)–TBS for 1 h at 37°C. Phage ($10^8$ TU) were added to the 2E9-coated wells and incubated for 10 min to 3 h following the addition of 5 μg of GXM per ml, which was added as a competitive inhibitor of the 2E9-binding phages (see Table 2). The bound phages were eluted in 100 μl of 0.1 M glycine-HCl (pH 2.2). A second round of ELISA screening was performed with GXM incubation times as indicated in Table 2. To assess the 2E9 binding of the eluted phages, they were grown on LB agar plates, the colonies were transferred by plaque lifts to nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom), and the enhanced chemiluminescence (ECL) assay (Amersham) was performed as described below.

**Determination of phage binding by ECL.** The ECL kit (Amersham) was used according to the manufacturer's instructions to detect 2E9 binding of the eluted phages. Strain K91Kan was infected with the eluted phages, spread on LB agar plates (Difco), and incubated overnight. The colonies on the plates were counted, transferred to Hybond nitrocellulose membranes (Amersham), and washed with TBS plus 0.05% Tween 20 (TBS-t). The membranes were blocked with TBS-t plus 20% fetal bovine serum (Bioproducts, Indianapolis, Ind.), washed in TBS-t, incubated with 2E9 for 2 h at RT, and incubated with horseradish peroxidase-labeled goat anti-human IgM (Southern Biotechnology, Inc.). The membranes were washed, treated with the ECL detection reagents for 1 min according to the manufacturer's instructions, and exposed to X-ray film. The number of phage with high-density signals was counted, and the percentage of bound phage was defined as number of high density colonies/number of colonies plated × 100.

**Sequencing of phage insert DNA.** The sequencing primer used was based on the fUSE5 sequence (10), 5′-CCCTCATAGTTAGCGTAACG-3′. The primer was synthesized by the Oligonucleotide Synthesis Facility of Albert Einstein College of Medicine. Eluted phages were amplified, and a single colony was used to infect K91Kan in 1.7 ml of LB medium with 20 μg of tetracycline per ml and 100 μg of kanamycin per ml. After vigorous shaking for 16 to 24 h at 37°C, the mixture was centrifuged for 10 min. One milliliter of the supernatant was microcentrifuged and transferred to a new tube containing 0.15 volume of PEG 8000-NaCl. After two cycles of PEG precipitation, the pellets were dissolved in 100 μl of 0.15 M NaCl, and 11.1 μl of 1 M acetic acid was added to the phage solution. The tube was then incubated for 10 min at RT and for 20 min on ice and microcentrifuged to precipitate the phages. The pellets were dissolved in 500 μl of TBS with 0.02% $NaN_3$ and extracted with phenol-chloroform-isoamyl alcohol (25/24/1) once and then with chloroform-isoamyl alcohol (24/1). The phage DNA was then precipitated in cold ethanol and dissolved in distilled water for the DNA-sequencing reactions. These reactions were performed according to the manufacturer's instructions from the Sequenase version 2.0 T7 DNA Polymerase Sequencing kit (United States Biotechnology, Cleveland, Ohio). The nucleic acid sequences were translated into amino acid sequences by using the Genetics Computer Group (University of Wisconsin) sequence analysis package (12).

**Phage and inhibition ELISAs.** For the ELISAs described here, unless otherwise stated, all plates were blocked with TBS–1% BSA, incubations were for 1 h at 37°C, plates were washed five times in phosphate-buffered saline (PBS)–0.01% Tween 20, and binding was detected by absorbance at 405 nm ($A_{405}$) in a Ceres 900 ELISA Reader (Bio Tek Instruments, Inc, Winooski, Vt.) after the addition of *p*-nitrophenyl phosphate substrate (Sigma). For phage ELISAs, 96-well polystyrene plates (Corning) were coated overnight at 4°C with ECL-positive phages at a concentration of $4 \times 10^{10}$ virions/ml. After being blocked, the plates were incubated with dilutions of 2E9 from 1 to $1 \times 10^{-5}$ μg/ml. After being washed, the plates were reincubated with alkaline phosphatase-labeled goat anti-human IgM (Fisher). 2E9 binding was detected after the addition of the substrate as described above. ELISAs were also performed to detect phage binding to 2E9 by using plates coated with 1 μg of 2E9 per ml. For these experiments, the plates were blocked, washed, and incubated with serial dilutions of ECL-positive phages starting at a concentration of $5 \times 10^{11}$ virions/ml. After being washed, the plates were incubated with biotinylated sheep anti-M13 antibody (5′-3′, Boulder, Colo.), washed, and reincubated with streptavidin-labeled alkaline phosphatase (Fisher). Phage binding was detected after the addition of the substrate as described above. The binding of the ECL-positive phages to a human IgM myeloma (Calbiochem, La Jolla, Calif.) and the IgM MAb RC2 (provided by A. Davidson, Albert Einstein College of Medicine) was also determined by this method.

To confirm the 2E9 specificity of the ECL-positive phages, an inhibition ELISA was performed. Plates were coated overnight at 4°C with 5 μg of serotype A GXM (strain 371) per ml, which was purified from *C. neoformans* (provided by A. Casadevall, Albert Einstein College of Medicine) as described previously (8). The plates were incubated with solutions containing 1 μg of 2E9 per ml and serial dilutions of phage supernatants. The plates were reincubated with alkaline phosphatase-labeled goat anti-human IgM (Fisher) to detect 2E9 binding. Controls for the ECL-positive phages used in the inhibition experiments were TBS–1% BSA, a phage without a peptide insert (phage 33), and a phage with inserts that did not bind 2E9 by ELISA or ECL (phage P-N). Inhibition of 2E9 binding by the phages was calculated as a percentage of total 2E9 binding: $[(A_{405}$ without inhibitor $- A_{405}$ with inhibitor$)/A_{405}$ without inhibitor$] \times 100$. ELISA results were plotted graphically by using Harvard Graphics for Windows software (Software Publishing Company, Santa Clara, Calif.).

The peptide insert of phage 13, GMDGTQLDRW, was synthesized by the Laboratory for Macromolecular Analysis of the Albert Einstein College of Medicine. Peptide 13 inhibition of GXM binding to 2E9 was determined by incubating ELISA plates coated with 1 μg of 2E9 per ml with mixtures containing 0.25 μg of GXM per ml and dilutions of peptide 13 starting at a concentration of 10 mM. GXM binding was detected by using the murine GXM MAb 2H1 (provided by A. Casadevall) (22) and alkaline phosphatase-labeled goat anti-mouse IgG1 (Fisher) as described previously (11). The reverse experiment was also performed by incubating GXM-coated ELISA plates with mixtures containing 0.25 μg of 2E9 per ml and serial dilutions of peptide 13 starting at a concentration of 10 mM. MAb 2E9 binding was detected with alkaline phosphatase-labeled goat anti-human IgM (Fisher). Peptide 13 binding to additional IgM antibodies was determined by a direct ELISA. Immulon II ELISA plates (Dynatech; Fisher catalog) coated with 25 mM peptide 13 in 0.1 M $H_2CO_3$ (pH 9.5) were incubated with 2E9, RC2, and BOR. RC2 and BOR are human IgM rheumatoid factor MAbs that do not bind GXM (provided by A. Davidson). Peptide 13 binding to the IgM antibodies was detected by incubation with biotin-labeled goat anti-human IgM (Fisher) followed by reincubation with streptavidin-alkaline phosphatase (Fisher).

Peptide 13 inhibition of serum antibody binding to GXM was studied with three groups of sera: (i) sera from HIV+ individuals that were obtained from a serum bank at the Bronx Municipal Hospital Center, (ii) sera from HIV− individuals that were collected with informed consent of the donors, and (iii) sera from volunteer GXM-TT vaccinees who were immunized at the National Institutes of Health, Bethesda, Md. (provided by R. Schneerson, National Institutes of Health). Clinical information about the donors of the HIV+ sera could not be obtained. All sera were heat inactivated for 30 min at 56°C prior to use. For the inhibition ELISAs with sera from HIV+ and HIV− individuals, the plates were coated with 5 μg of serotype A GXM (strain 371) per ml and incubated for 1 h at 37°C with a solution containing heat-inactivated sera used at a dilution of 1:10 and 5 mM peptide 13, a control peptide, or PBS–1% BSA. The plates were then washed, reincubated with goat anti-human IgM (Fisher), and developed as detailed above. For the experiments with GXM-TT sera, the samples were first serially diluted on GXM coated plates. GXM IgM and IgG antibody binding was detected with alkaline phosphatase-labeled goat anti-human IgM (Fisher) for IgM and alkaline phosphatase-labeled goat anti-human IgG (Fisher) for IgG. The serum dilution that produced 50% of the maximum $A_{405}$ for each isotype was used for inhibition ELISAs. For these experiments, the preimmune ($n = 3$) and immune ($n = 7$) sera were mixed with 5 mM peptide 13, 5 mM control peptides, or 1% BSA–PBS, and the mixtures were incubated with GXM-coated plates. GXM binding of IgM and IgG antibodies was detected separately with

TABLE 1. Results of screening the decapeptide phage library with MAb 2E9 by the magnetic bead method

| Round of screening | Concn (nM) of MAb 2E9 | % Phage recovered[a] | % Phage MAb 2E9 positive by ECL[b] |
|---|---|---|---|
| 1 | 1,000 | 0.0017 | 1.4 |
| 2 | 100 | 2.8 | 9.5 |
| 3 | 10 | 0.17 | 94 |
| 4 | 10 | 11 | 98 |

[a] The percentage of phage recovered following each round of screening was determined by dividing the number of phage eluted after MAb selection by the number of input phage.

[b] The percentage of eluted phage that were specific for MAb 2E9 was determined by dividing the number of eluted phage by the number of phage that yielded high-intensity signals by ECL.

alkaline phosphatase-labeled goat anti-human IgM (Fisher) for IgM and alkaline phosphatase-labeled goat anti-human IgG (Fisher) for IgG. The following control peptides were used: C1 (DQPQNLEEI) for the studies with GXM-TT sera, C2 (IEGRRGYVYQGL) for the studies with HIV+ and HIV− sera, C3 (GRA ATILSLWNT) for additional studies with HIV+ and HIV− sera, and C4 (WT RGYVYQGL) for peptide 13 inhibition of 2E9-GXM binding. C1, C2, and C4 were provided by Dr. Nathenson (Albert Einstein College of Medicine), and C3 was provided by A. Davidson. For the inhibition experiments, the percent inhibition was determined as follows: $\{[A_{405}$ without inhibitor $- A_{405}$ with inhibitor (peptide 13 or the control peptide)$]/A_{405}$ without inhibitor$\} \times 100$.

**Determination of the frequencies of amino acid usage in the bead- and ELISA-selected libraries.** The frequencies of given amino acids in the ELISA-selected versus bead-selected phage were determined by chi-square analysis. The usage of each of the 20 amino acids was determined for the 22 randomly chosen peptide inserts from the second screening of the ELISA-selected 60-min library (220 amino acids) (see Fig. 1), for the first ELISA screening of the second- and third-round bead libraries (140 amino acids) (see Fig. 1), and for 13 randomly chosen peptide inserts from the third-round bead-selected library (130 amino acids) (data not shown).

**Statistical analysis.** Statistical analyses were performed by the Student *t* test with Statistica for Windows 4.3 software (StatSoft, Inc.). Chi-square analyses were performed with EpiInfo6 software (Centers for Disease Control, Atlanta, Ga.).

## RESULTS

**Biopanning and ELISA screening.** When we screened the decapeptide library with 2E9 by the magnetic bead method, we identified 2E9-binding phages (Table 1). The percentage of phage that bound 2E9 was defined as the number of TU of phage eluted after each round of magnetic bead selection divided by the number of input phage. ECL was used to determine the percentage of 2E9-binding phage from the total phage eluted. Phages that produced a strong ECL signal 10 min after exposure to the ECL reagents were characterized as high-affinity binders. The phages selected by 2E9 were amplified after each round of screening such that nearly 95% were ECL positive following the third and fourth rounds (Table 1). Nonspecific binding to the biotinylated goat anti-human IgM reagent was determined for the second, third, and fourth rounds of screening, and the percentages of input phage eluted were 0.0012, 0.0023, and 0.0071%, respectively. Thirty ECL positive phages from the third- and fourth-round libraries bound to 2E9-coated plates (data not shown).

In an effort to isolate phages that had higher-affinity 2E9 binding, phages from the intermediate second- and third-round libraries were rescreened by the ELISA method detailed above. For these experiments, GXM was introduced as a competitor at different time points during the reaction between the phage and 2E9 (Table 2). The addition of GXM produced a marked reduction in the total number of phage selected by 2E9 (Table 2): 1 h after the addition of GXM, 0.047% of input phage were recovered from the third-round library and 92% of the eluted phage were ECL positive, whereas 27% of input phage were recovered when no competitor was added but none were ECL positive. The phages eluted from the 60-min GXM competition were rescreened with increased times of GXM

TABLE 2. ELISA selection of MAb 2E9 binding phages from decapeptide library[a]

| Screening | Time of incubation with competitor (min) | % Phage recovered (eluted/input) | % Phage MAb 2E9 positive by ECL |
|---|---|---|---|
| Third-round bead library | —[b] | 27 | 0 |
| | 0 | 31 | 0 |
| | 10 | 0.086 | 86.5 |
| | 20 | 0.063 | 91 |
| | 60 | 0.047 | 92 |
| | 120 | 0 | 0 |
| 60-min ELISA-selected library | — | 31 | 60 |
| | 60 | 5.1 | 88 |
| | 120 | 3.5 | 94 |
| | 180 | 2.7 | 95 |

[a] Results from two rounds of selection of MAb 2E9 binding phages by the ELISA method are shown. The method is detailed in the text. The 60-min ELISA selected library was derived from the third-round library. For the ELISA selections, GXM was used as a competitor at a concentration of 5 μg/ml at the times indicated.

[b] —, no competitor.

ELISA Screening- 2nd round (bead) library: 1st screening

```
2                  G R W G G V Q K F G
8                  K R W F L I G D R M
5              R I D R W G V P F E
1    A E F S F S V T K W
6        D R Y D M R N W M A (+ X Aromatic)
3          A L G R L K H T S R
9            L S L E K T D N S V
10     F M N S G E T R L G
```

ELISA Screening-3rd round (bead) library: 1st screening

```
1    G I G F Q T G L R F
2                  K R W F L I G D R M
5                  L R W D N F T S K D
6                P L K Y W I Q V N M
7      N D L E S L D R T E
```

ELISA Screening-3rd round library: 60 minute library

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4-27 G I G F Q T G L R F       (10 clones)
[13  W R D L Q T G D M G]      (3'-5' sequence)
13   G M D G T Q L D R W       (5'-3' sequence)
9,21   R A Y Q T T L L T L     (2 clones)
10-23    P E S W H T R W D E   (4 clones)
3      T R G F S T D R N S     (+ X Aromatic)
2-26   P P K V P P D L M S     (3 clones)
29     L W N K R T A E F K     (Aromatic X +)
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FIG. 1. Amino acid sequences of ELISA-selected MAb 2E9 binding phages. The amino acid alignments of the 2E9-selected phage inserts are based on common motifs and/or specific amino acids (e.g., charged or aromatic residues). The latter are in boldface. The sequence of phage 13 is shown in both the 3′-5′ and 5′-3′ orientations for the purposes of alignment. All of the other sequences are in the 5′-3′ orientation.

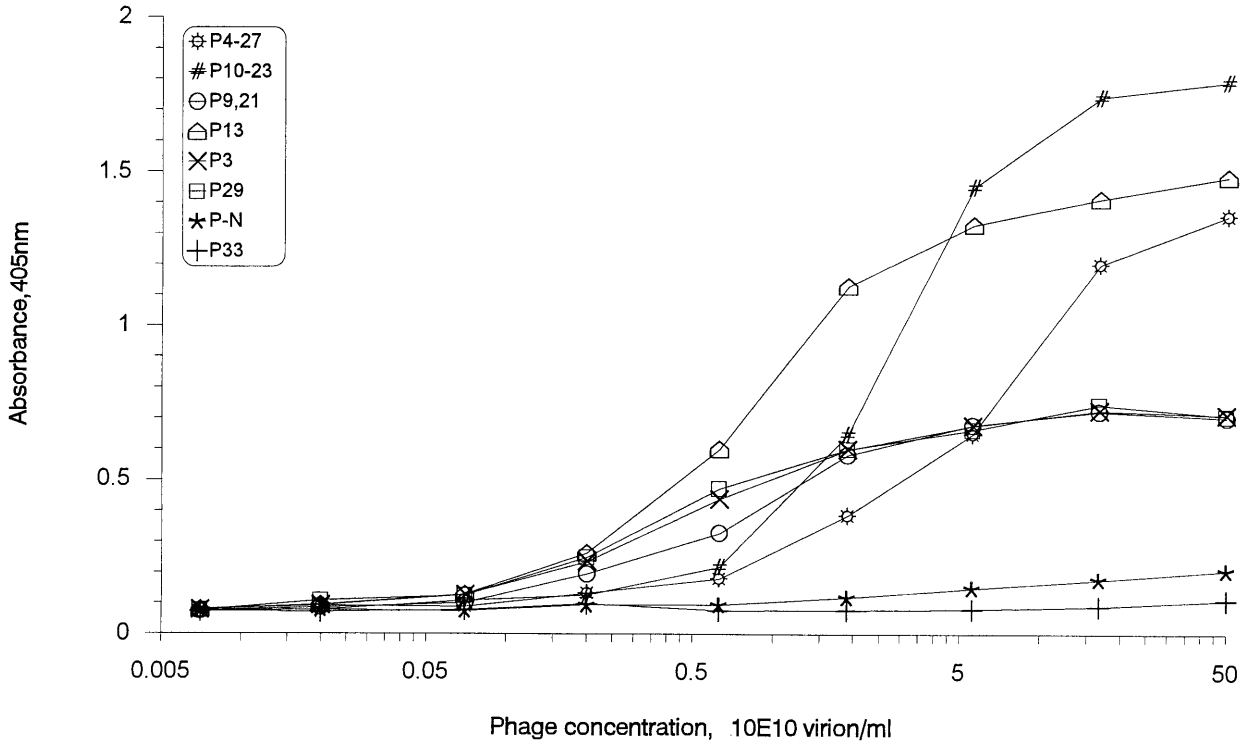


FIG. 2. Binding of ELISA-selected phages to 2E9. The phages shown were from the 60-min ELISA-selected library (Table 2). ELISA plates coated with MAb 2E9 were incubated with serial dilutions of the phage particles, and binding was detected with a biotinylated sheep anti-M13 antibody as described in the text. MAb 2E9 binding for each dilution of phage depicted on the *x* axis is plotted on the *y* axis as the $A_{405}$. Phage 33 lacks a peptide insert, and P-N is an ELISA-selected phage that did not produce an ECL signal.

competition from 1 to 3 h. After 3 h, 95.2% of the 2E9-binding phage gave high-density ECL signals (Table 2).

**Amino acid sequences of the MAb 2E9-binding phage.** Thirty-two phages from the third- and fourth-round magnetic bead libraries were amplified and purified, and sequences of the inserts were determined. The amino acid sequences did not reveal a common motif (sequences not shown). Sequence analysis of 35 phage inserts obtained from the ELISA screening method led to the identification of some phages that had similar motifs. Of the 35 phages, 11 contained motifs with positively charged amino acids and aromatic residues: 8 had amino acids with a positive charge followed by aromatic residues (RW or KY), and 3 had amino acids with positive charges followed by another amino acid and then an aromatic residue (RXW or KXW) (Fig. 1). One phage insert, phage 10-23, contained a motif with the negatively charged amino acid D followed by RW (DRW) (Fig. 1). From the second screening of the third-round library, 10 inserts were identical and contained the motif QTGL, 2 were identical with QTTL, and 1 contained QTGD (Fig. 1).

The frequency of each of the 20 amino acids did not exceed random usage for the bead-selected phage inserts. Valine and alanine were selected against among the ELISA-selected peptides ($P = 0.03$ by Fisher's exact test), but the frequencies of all of the other individual amino acids reflected random use. The combined frequencies of QTGLD residues exceeded random usage for the phage selected by the second screening of the 60-min ELISA-selected library ($P = 0.02$ by Yate's corrected test) but not for those of any of the other libraries, including the first ELISA screening of the second- and third-round bead libraries.

**Specificity of the MAb 2E9-binding phages.** The ECL-positive phages bound to 2E9-coated ELISA plates (Fig. 2), and 2E9 bound to phage-coated ELISA plates (data not shown). Control phages without an insert (phage 33) or that did not produce an ECL signal (phage P-N) did not bind 2E9 (Fig. 2). The ECL-positive phages were also tested for binding to ELISA plates coated with an IgM myeloma protein (Calbiochem) and the human IgM mAb RC2 (provided by A. Davidson, see above). The phages bound these antibodies minimally, requiring at least $5 \times 10^{10}$ more phage than 2E9 to produce a signal (data not shown).

The phage selected by 2E9 with the ELISA method inhibited 2E9 binding to GXM by ELISA. Figure 3 shows the concentrations of each of eight phages that produced a 50% reduction in 2E9 binding to GXM. Phages with QTG/TL/D motifs (QTGL, phage 4-27; QTGD, phage 13; and QTTL, phage 9,21) or one or more prolines (phages 10-23 and 2-26) were the best inhibitors in comparison to phages with KRW or other motifs ($P < 10^{-4}$) (Fig. 3). Inhibition by phage 10-23, which had a 5′ proline, an RW motif, and a net 2− charge, was equivalent to that by phages 9,21 and 13 (Fig. 3). The least effective inhibitor was phage 8(2nd), which had a KRW motif and a net 2+ charge.

A 5 mM solution of synthesized peptide 13 inhibited 90% ($n = 2$; 93.7 and 86%) of 2.5-μg/ml GXM binding to 5-μg/ml solid-phase 2E9, whereas 5 mM C3 peptide inhibited 0.5% ($n = 2$; 0 and 1%) of 2E9 binding (data not shown). The 2E9 specificity of peptide 13 was confirmed by testing the binding of 2E9 and two additional human IgM rheumatoid factor MAbs to peptide 13 by ELISA. MAb 2E9 was the only antibody that bound peptide 13 (Fig. 4).

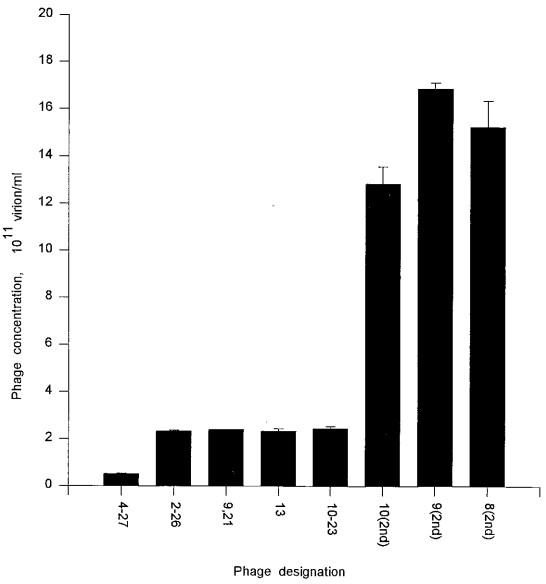


FIG. 3. Phage inhibition of the GXM binding of human 2E9. The phages shown were from the 60-min and the second-round bead ELISA selected libraries (Table 2). Phage 4-27 is the same as phage 1 from the third-round bead ELISA-selected library. ELISA plates coated with GXM were incubated with MAb 2E9 alone and with mixtures of serial dilutions of the phage (in quadruplicate) with 1 μg of 2E9 per ml. Binding of 2E9 was detected with alkaline phosphatase-labeled goat anti-human IgM as described in the text. The phage concentration that inhibited 50% of 2E9 binding is plotted on the *y* axis for each of the phages designated on the *x* axis. The parentheses used to designate some of the phage represent the library from which that phage was isolated (Table 2). The inhibitory concentrations of phages 2-26, 9,21, 13, 10-23, 10(2nd), 9(2nd), and 8(2nd) were all greater than that of phage 4-27 ($P < 1.2 \times 10^{-4}$), and the inhibitory concentrations of phages 9(2nd) and 8(2nd) were greater than that of phage 10(2nd) ($P < 0.014$ by the two-tailed Student *t* test).

**Inhibition of human serum GXM binding by a MAb 2E9-binding peptide.** Peptide 13 inhibited 32.12 ± 5.44% (26.9 to 40.7%) of the GXM binding of HIV− sera ($n = 5$) and −8.28 ± 19.43% (−41.1 to 8.9%) of the GXM binding of HIV+ sera ($n = 5$) ($P = 0.0078$ (Table 3). The mean peptide 13 inhibition was −8.64 ± 22.04% (−44 to 17%) for five additional HIV+ sera (C3 was used as a control for these samples) (data not shown). The C2 and C3 control peptides did not inhibit serum GXM binding, but in some instances the C2 peptide enhanced GXM binding (Table 3). For sera from GXM-TT vaccinees, peptide 13 inhibited the GXM binding of 40 to 60% of preimmune serum IgM (Fig. 5a), 70 to 85% of immune serum IgM (Fig. 5a and c), and 25 to 50% of immune serum IgG (Fig. 5b and c) antibodies. The C1 peptide inhibited a maximum of 5% of the IgM and 10% or less of the IgG GXM binding (Fig. 5a and b).

## DISCUSSION

This paper reports the isolation of peptide ligands that bind the human GXM MAb 2E9 from a random decapeptide phage library. The epitopes reported here represent the first characterization of epitopes that bind human anti-GXM antibodies. Biopanning of the library with 2E9 in solution led to the selection of phages without shared amino acid motifs (Table 1) that manifested randomly selected amino acid sequences (see results of chi-square analysis above). Monospecific antibodies can select unrelated mimotopes (29). However, the avidity of the phages (27) and the multivalent IgM MAb could have contributed to the selection of phages with nonspecific binding. The solid-phase ELISA selection method was developed to select higher-affinity phages from the intermediate libraries. The technique was adapted from an off-rate screening method for high-affinity single-chain Fv molecules (21). We hypothesized that the highest-affinity phage epitopes might have 2E9 binding affinities equivalent to that of GXM for 2E9 and that they would bind 2E9 in the presence of GXM. Our data supports the hypothesis that the phages that were selected bind in or in close proximity to the 2E9 binding site, because (i) both the phages (Fig. 3) and peptide 13 (see above) can inhibit 2E9-GXM binding, (ii) peptide 13 is specific for 2E9 (Fig. 4), and (iii) peptide 13 can inhibit the GXM binding of serum GXM-TT antibodies (Fig. 5). Peptide 13 might bind an idiotypic determinant expressed by both IgM and IgG anti-GXM antibodies; although the inhibition data (Fig. 5) suggests that such an idiotope is part of the GXM antibody binding site, this issue cannot be resolved without crystallography.

The 2E9-selected epitopes represent a diverse, nearly random collection of amino acid sequences. An increased frequency of aromatic amino acids has been reported for some anticarbohydrate antibody epitopes, including a murine anti-GXM MAb (16, 36, 37). However, aromatic amino acids are neither overrepresented among the epitopes selected by 2E9 nor specific for anticarbohydrate antibodies (1). Antibody (or peptide ligand) binding to carbohydrates can be facilitated by aromatic and hydrophobic interactions (5, 16, 32) and polar residue side chain hydrogen bonding to sugar hydroxyls (5) and proline residues. The latter can confer structures that expose residues that are necessary for carbohydrate binding (16, 37). The best inhibitors of 2E9 GXM binding had three or four hydrophobic amino acids separated by three or four polar residues, except phage 2-26, which has six hydrophobic residues and four prolines (Fig. 1). The polar motif QTG/TL/D was found in three of five of the phage clones that inhibited 2E9-GXM binding the most (phages 4-27, 13, and 9,21) (Fig. 3). Epitopes without QTGDL residues that have negative charges [compare phage 10-23 with phage 8(2nd) (Fig. 1 and 3)] could inhibit 2E9-GXM binding more because GXM is a polar, acidic polysaccharide (35, 36).

IgM antibodies, a predominant isotype among anticarbohydrate antibodies, often have low binding affinities. These could be similar to peptide ligands, which manifest monovalent binding. In this regard, lectin and peptide ligands of concanavalin A have similar affinities (27), and similar amounts of a peptide mimotope and a lectin ligand inhibit serum Gal(α 1-3)Gal binding (18). The peptide 13 concentration that inhibits 2E9-

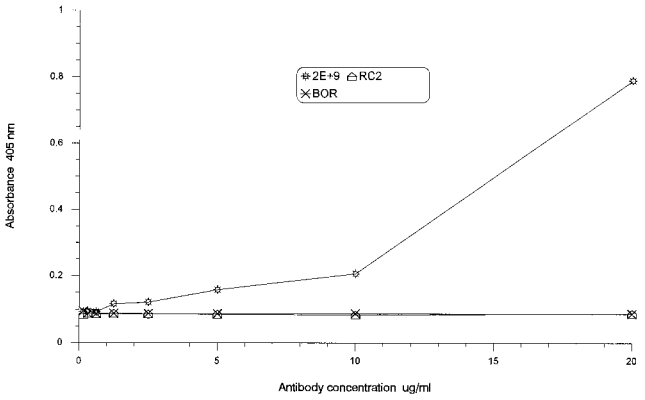


FIG. 4. Peptide 13 reactivity of human IgM antibodies. MAb 2E9 and two other IgM MAbs were incubated with peptide 13-coated ELISA plates, and antibody binding was detected as described in the text. The absorbances of the MAbs are shown on the *y* axis for each antibody concentration shown on the *x* axis.

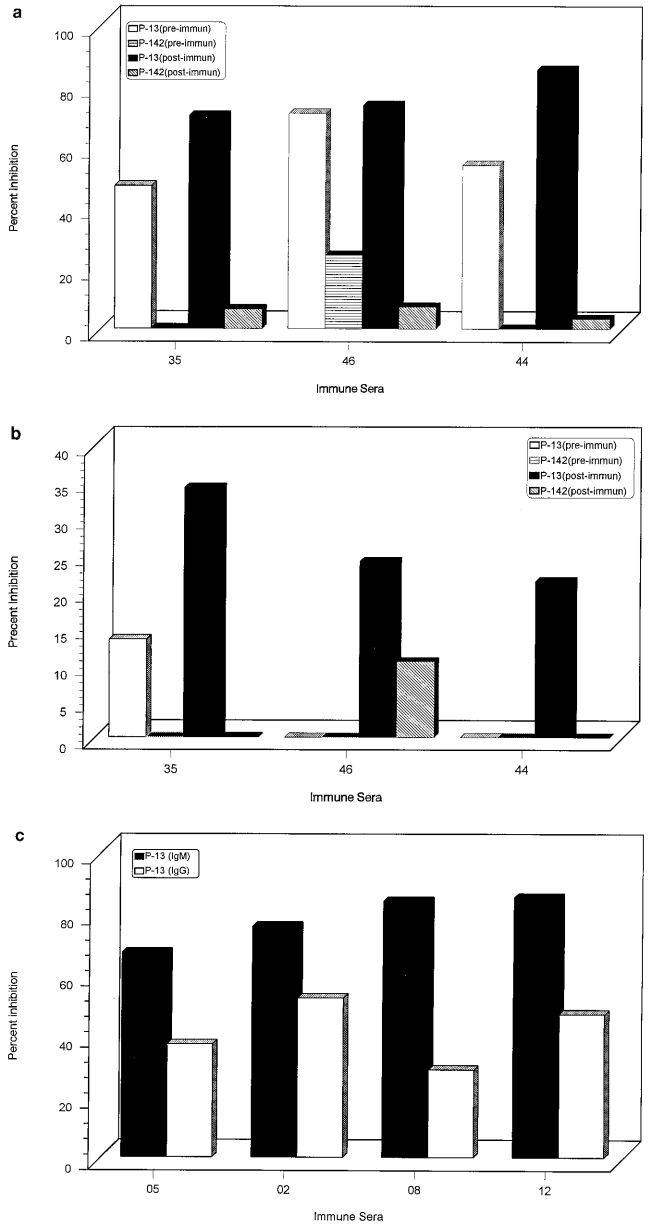


FIG. 5. Peptide 13 inhibition of GXM binding of GXM-TT sera. Serum mixtures containing 5 mM peptide 13 or the control peptide were incubated with GXM-coated plates. GXM IgM and IgG antibody binding were determined with isotype-specific alkaline phosphatase-labeled antibodies as detailed in the text. (a) Peptide 13 inhibition of GXM binding of preimmune and immune IgM from subjects 35, 46, and 44. (b) Peptide 13 inhibition of GXM binding of preimmune and immune IgG from the same subjects as in panel a (note the difference in the *y* axis scale). All of the subjects had preimmune IgG (data not shown). The flat bars for subjects 46 and 44 indicate that neither peptide inhibited the GXM binding of the preimmune IgG. (c) Peptide 13 inhibition of GXM binding of immune IgM and IgG from subjects 5, 2, 8, and 12 (no control peptide). The serum samples used are designated by numbers on the *x* axis. The *y* axis shows the percent inhibition of serum-GXM binding that is inhibited by peptide 13. The derivation of the percent inhibition is described in the text.

GXM binding is in the range for peptide ligands of lectins (18, 27) and naturally occurring antibodies (34). A difference between our work and that of others who have sought to identify carbohydrate mimotopes (16, 36) is that we used an IgM MAb for epitope selection. Polyclonal human IgM antibodies selected a TNF epitope (phage T9) with a PPLKPP motif (34), which is nearly identical to the PPKNPP motif of phage 2-26 (Fig. 1). Therefore, these PPXXPP motifs bind naturally occurring human antibodies (Table 3) (34), although the antigens that they mimic are a polysaccharide and a protein. This suggests that peptide epitopes that can bind IgM binding sites are not predictable solely on the basis of antibody specificity. Over a decade ago, the varied specificities found among IgM antibodies raised against a single polysaccharide determinant were attributed to the shapes of IgM antibody binding sites (26, 33). Several lines of evidence support the concept that restriction of anticarbohydrate antibodies (30) is not due to a limited repertoire of antigen-binding motifs but that it results from B-cell regulation and selection: (i) monospecific antibodies can select unrelated peptides that have similar binding affinities (29), (ii) crystallographic analyses have revealed diverse carbohydrate antibody structures (38), and (iii) 2E9-selected peptide mimics that have a variety of amino acid motifs (compare phages 2-26, 9,21, and 10-23 in Fig. 3) can inhibit polysaccharide (GXM) binding of an antibody (2E9).

Peptide 13 inhibits GXM binding of GXM-TT-elicited antibodies and naturally occurring antibodies from HIV− but not HIV+ individuals (Fig. 5 and Table 3). The reduced peptide 13 reactivity of HIV+ sera (Table 3) suggests that anti-GXM antibodies from HIV+ individuals have different epitope specificities. This conclusion is also supported by the enhancement of HIV+ serum binding to GXM by peptide 13 (Table 3), although the explanation for this phenomenon is unknown. Peptide 13 inhibition of GXM binding is greatest for preimmune IgM (naturally occurring) and GXM-TT IgM (Table 3 and Fig. 5a and c). This supports the hypothesis that GXM-TT elicits restricted IgM antibodies and reinforces our work demonstrating that GXM-TT IgM antibodies express shared idiotypes (31). Unlike murine GXM IgM and IgG antibodies, which can both bind the same GXM mimotopes (36), human preimmune IgG and GXM-TT IgG antibodies have reduced peptide 13 reactivity (Fig. 5b and c). This could be attributable to higher antigen affinity and/or broadened fine specificity of the anti-GXM IgG antibodies generated by affinity maturation (20) or to the possibility that human GXM IgM and IgG antibodies arise from different precursors in the antibody repertoire (2). Understanding the specificity of human anti-GXM

TABLE 3. Inhibition of GXM binding of human sera by peptide 13[a]

| HIV status | Serum | $A^b$ with: | | | % Inhibition[c] |
|---|---|---|---|---|---|
| | | C2 (control peptide) | Peptide 13 | No peptide | |
| − | 1 | 0.353 | 0.188 | 0.287 | 34 |
| | 2 | 1.057 | 0.603 | 0.825 | 26.9 |
| | 3 | 0.613 | 0.348 | 0.587 | 40.7 |
| | 4 | 1.672 | 0.941 | 1.33 | 29.3 |
| | 5 | 0.927 | 0.557 | 0.818 | 29.7 |
| + | 7 | 0.755 | 0.558 | 0.612 | 8.9 |
| | 8 | 1.897 | 1.625 | 1.626 | 0 |
| | 9 | 1.143 | 0.961 | 0.963 | 0 |
| | 10 | 2.358 | 1.751 | 1.604 | −9.2 |
| | 11 | 0.823 | 0.933 | 0.661 | −41.1 |

[a] An inhibition ELISA was used to determine whether peptide 13 inhibited the GXM binding of sera from HIV+ and HIV− individuals (see text).

[b] Mean absorbance for paired duplicates for each sample.

[c] Defined as (OD without peptide 13 − OD with peptide 13)/OD without peptide 13. The mean percent inhibition and standard deviation for the HIV− group was 32.12 ± 5.44, and that for the HIV+ group was 8.28 ± 19.43. The difference between these groups was significant ($P = 0.007$ by Student's *t* test).

IgG antibodies will require the identification of the epitopes that they recognize.

Our studies of the peptide mimotopes that were selected by 2E9 support the following conclusions: (i) preimmune and anti-GXM-TT IgM antibodies manifest shared epitope specificities, and (ii) peptide 13 may mimic a GXM epitope with important biological significance, because sera from HIV+ individuals, who are at high risk for cryptococcal infections, have limited peptide 13 reactivity. The following hypotheses are based on these conclusions: (i) peptide 13 might mimic a protective GXM epitope, because serum peptide 13 reactivity appears to differentiate those who are at risk for the disease from those who rarely become infected, and (ii) HIV+ individuals may require novel immunotherapeutic reagents that can circumvent antibody repertoire defects if these defects result in an inability to produce capsular antibodies against protective epitopes. Future studies will examine these hypotheses in a continuation of our effort to characterize the nature of human anti-GXM antibodies in HIV+ and HIV− anti-GXM antibody repertoires.

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