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Two Human Neonatal IgM Antibodies Encoded by Different Variable-Region Genes Bind the Same Linear Peptide: Evidence for a Stereotyped Repertoire of Epitope Recognition¹

Bradley T. Messmer,* James J. Sullivan,* Nicholas Chiorazzi,[†] Toby C. Rodman,* and David S. Thaler^{2*}

Two monoclonal IgM Abs have been produced from lymphocytes isolated from two human umbilical cord bloods. These mAbs recognize a conformational epitope present in a CNBr digestion fraction of lactoferrin. Linear epitopes recognized by each mAb were selected from several phage display peptide libraries. In each case, phages displaying a peptide with a motif defined by [WF]_HG₁[EQS]_HN were recovered. Phages displaying that motif bound equally well to either mAb but did not bind to control IgM. A peptide bearing this motif competed with the phage-displayed peptides for binding to either mAb. The same peptide also competes with a component of the CNBr digestion fraction of lactoferrin for Ab binding in ELISA. The Abs use different families of V_H, J_H, and V_K gene cassettes but use the same J_K cassette. All segments are virtually identical to their germline gene counterparts. This work provides further evidence that certain innate specificities are stereotyped among individuals. *The Journal of Immunology*, 1999, 162: 2184–2192.

Human serum contains Abs that have not arisen as a result of direct immunization with foreign Ag or pathogenic exposure (1). These Abs have been called “natural” (2) or “innate” (3). Several diverse roles for these naturally occurring Abs have been proposed, including a homeostatic role as part of a connectivity scheme (4, 5); a primary defensive role (6), perhaps in conjunction with the complement system (7); and a role (through their loss or incorrect activation) in autoimmune disease (1). It has also been suggested that natural Abs may have acquired several of these functions during the course of the immune system’s evolution (8). Natural Abs are often, though not exclusively (9), IgM class, and some cross-react with a variety of self and nonself Ags (10). IgM class Abs do not cross the placenta in significant amounts during normal pregnancy (11), presumably due to their large pentameric structure and the lack of a specific transporter mechanism as for IgG (12). Therefore, the IgM Abs circulating in the umbilical cord and neonate are predominantly the product of neonatal lymphocytes (although 1 in 10⁴–10⁵ nucleated cells are of maternal origin (13–15)). Therefore, in a normal, noninfected neonate the IgM Abs are predominately naturally occurring in that they were not stimulated by foreign Ags (excepting the possibility of Abs developed against maternally transferred Ags).

Several lines of evidence support the possibility that the naturally occurring Ab repertoire in human neonates contains certain stereotyped specificities. Homogenous tissue extracts separated on a one-dimensional gel and subsequently immunostained with umbilical cord blood sera have a pattern of reactivities conserved among cord blood samples but variable in adult sera (16–19). Furthermore, cord blood sera from many individuals contain significant titers of IgM reactive with a specific peptide epitope found on protamine and HIV Tat protein (20, 21). Ig variable-region gene usage bias has been noted and proposed as another factor potentially limiting the neonatal repertoire (22–25).

Here, we report another type of evidence that individuals have stereotypical natural Ab specificities. Two cord blood B cell hybridomas from different individuals were constructed. Each hybridoma secreted IgM Abs reactive with a conformational epitope of lactoferrin inferred to be present on sperm heads (26). We used the method of screening phage-displayed peptides (27–30) to identify linear peptides that bind to the Abs. In both cases, the Abs bound to phages displaying a specific peptide motif, indicating that the two hybridomas secrete Abs with near identical specificity. Furthermore, the peptide motif competed with the conformational epitope of lactoferrin for Ab binding. The sequences of the expressed rearranged heavy (H)³ and light (L) chain variable-region genes used by each hybridoma have revealed that there are virtually no somatic mutations and that the two hybridoma lines use different germline V_H, J_H, and V_K genes to achieve this same specificity.

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Materials and Methods

Libraries and Phage

Three phage-displayed peptide libraries were purchased from New England Biolabs (Beverly, MA). The random peptide is displayed at the N terminus of the gene III protein (pIII). There is a small leader sequence

³ Abbreviations used in this paper: H, heavy; L, light; pIII, gene III peptide; FR, framework region; OPD, *o*-phenylenediamine dihydrochloride; CDR, complementarity determining region.

separating the peptide from the protein as shown: Ph.D.7, NH₂-XXXXXXXX-GGGS-pIII; Ph.D.12, NH₂-XXXXXXXXXXXXX-GGGS-pIII; Ph.D.C7C, NH₂-ACXXXXXXXXX-GGGS-pIII. These libraries were built in M13 mp18 and as such contain the *lacZα* fragment. They will not form blue plaques on S26*lacZ*⁻ (31) when plated on media containing Xgal. All phage stocks were grown on strain K91, an S26 derivative that has been cured of λ.

Strain cclac⁺ f1 is an f1 phage containing a *lacZ* insertion. This phage forms blue plaques when plated on a *lacZ*⁻ strain on media containing Xgal. This phage was a gift from Dr. C. Cupples and is described elsewhere (32). All phages discussed here that result from a panning will be identified by a label as follows: φ(target Ab)-(library)x, where x is a letter unique to that phage clone. For example, a candidate from a Ph.D.7 panning against mAbRWL1 would appear as φRWL1-7e. Where appropriate, the amino acid sequence of the displayed peptide will also be given.

Production of hybridomas and characterization of mAbRWL1 and mAbRWL2

mAbRWL1 was produced from a cord blood B cell hybridoma and originally characterized as binding to a fraction of human lactoferrin as previously described (26). mAbRWL2 was produced by the same methodology, described briefly here. Mononuclear cells were isolated from cord blood (from different individuals for mAbRWL1 and mAbRWL2) by density gradient centrifugation and, after transformation with EBV, fused with parental cell line HMMA by the method of Chiorazzi et al. (33, 34). Monoclonal hybridoma cell lines RWL1 and RWL2 secreted IgM reactive with fraction 7B of lactoferrin determined by ELISA as described (26). IgM was purified from cell culture supernatants of both cell lines as follows. Whole-cell culture supernatant was concentrated to 1/100 original volume and dialyzed overnight in ddH₂O buffered with HBSS, pH 7.2. The remaining culture supernatant was then passed through a centricon C100 column to remove all proteins <100 kDa. A sephacryl S100 fast flow column was prepared as per manufacture instructions (Pharmacia, Bridgewater, NJ) and washed with 10× volume of pH 7.2 PBS without CaCl₂ or MgCl₂. Then, 5 ml of concentrated culture supernatant was applied to the column bed and eluted at 1 ml/h for ~62 h. Fractions were read spectrophotometrically at 280 nm, revealing a sharp peak within eluants from 36–48 h. This peak was then pooled and reconcentrated to a volume of 2 ml and reappplied to the column at a flow rate of 2 ml/h. Fractions were read as above, revealing a strong peak in the eluants from 20–24 h. SDS-PAGE with 2-ME confirmed the presence of a 28-kDa L chain and 72-kDa H chain band.

mAbRWL1 and mAbRWL2 were tested by described methods (9) against a panel of Ags consisting of porcine insulin, porcine thyroglobulin, porcine actin, single-stranded calf thymus DNA, phosphocholine, human IgG (Sigma, St. Louis, MO), and fraction 7B of lactoferrin (26). The two mAbs only gave a positive signal against fraction 7B of lactoferrin (data not shown) and are thus considered to be monospecific.

Rearranged Ig variable-region sequencing

Total RNA was isolated using Ultraspec RNA (Biotech Laboratories, Houston, TX) according to the manufacturer's instructions. First, 1 μg of RNA was reverse transcribed to cDNA using 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY), 1 U of RNase inhibitor (5 Prime 3 Prime, Boulder, CO) and 20 pmol of oligo dT primer in a total volume of 20 μl. The reaction mixture was incubated at 42°C for 1 h, heated at 65°C for 10 min to stop the reactions, and then diluted to a final volume of 100 μl.

To determine the V_H and V_L gene families used by the cell lines, 2 μl of cDNA were amplified using a sense V_H or V_L family-specific framework region (FR) primer in conjunction with the appropriate antisense C_H or C_L primer (35). The reactions were conducted in 50 μl using 20 pmol of each primer and cycled with a 9600 GeneAmp System (Perkin-Elmer Cetus, Emeryville, CA) as follows: denaturation at 94°C for 45 s; annealing at 65°C for 45 s; and extension at 72°C for 45 s. After 35 cycles, extension was continued at 72°C for an additional 10 min.

The DNA sequence of the V_H and V_L genes were then determined by reamplifying 5 μl of the original cDNA using the appropriate V_H or V_L leader and C_H or C_L primers. These reactions were conducted as follows: denaturation at 94°C for 45 s; annealing at 62°C for 30 s; and extension at 72°C for 45 s. After 30–32 cycles, extension was continued at 72°C for an additional 10 min. PCR products were sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI) using an automated sequenator (Applied Biosystems, Foster City, CA). cDNAs were prepared and amplified from at least two different samples of each cell line. Each PCR product was subject to three separate sequencing runs. Sequences were compared with those in the V BASE sequence directory (36) using MacVector software, version 6.0 (Eastman Kodak, New Haven, CT).

Panning

For each target/library combination, a separate well of a 96-well high-capacity ELISA plate (catalogue number 25805-96, Corning, Corning, NY) was coated with 1–5 μg of target Ab in 100 μl PBS, pH 7.4. Plates were left at 4°C overnight. Coating solution was removed, and 200 μl blocking buffer (0.5% BSA) was added. Plates were left at room temperature for at least 1 h. Blocking buffer was removed, and the wells were washed six times with 200 μl PBS plus 0.1% Tween 20. A total of 10¹¹ library phages in 100 μl PBS were added, and the plates were left on the bench for at least 1 h. Wells were washed 10 times with PBS plus 0.1% Tween 20. 100 μl of elution buffer (0.2 M glycine-HCl, pH 2.2) was added and removed after 20 min then neutralized with 15 μl Tris-HCl, pH 9.6. The eluant was titered and 50 μl was added to 100 μl of a fresh overnight K91 culture. After at least 15 min on the bench, the infected culture was added to 5 ml Luria-Bertani medium and grown at 37°C with shaking for ~10 h. The following morning the cultures were spun at 5000 × g for 5 min, and the supernatant filter was sterilized by passage through a 0.45-μm syringe tip filter. Then, 1.0 ml of supernatant was mixed with 250 μl polyethylene glycol (PEG) solution (30% PEG m.w. 8000, 1.6 M NaCl) in a small centrifuge tube, left at room temperature for at least 20 min, and spun at 14,000 × g for 5 min. The supernatant was removed, and the tube was respun for 30 s. The last trace of PEG solution was removed by pipet, and the cloudy phage pellet was resuspended in 100 μl PBS. Then, 10 μl of this concentrated phage was used for the next round of panning. In the third round, 10 μl of cclac⁺ f1 phage (10¹²/ml) were added with the library phage, thus giving a roughly 1:1 ratio between library phage and cclac⁺ f1. The third round eluant was titrated on K38*lacZ*⁻ with Xgal and isopropyl β-D-thiogalactoside. Library phages form white plaques while the cclac⁺ phages form blue plaques. A white:blue plaque ratio greater than 100:1 was usually indicative of a successful panning.

Candidate phage sequencing and analysis by eluant phage titer

A dilution series of the final-round eluant was plated on a lawn of K91. Individual plaques were picked with a pasteur pipet from a plate with well dispersed plaques, vortexed in 5 ml of Luria Bertani medium, and incubated with shaking at 37°C for 10–18 h. The phage containing supernatant was filtered as above. Between 1.5 and 3.0 ml was used to prepare ssDNA with a Qiagen (Valencia, CA) qiaspin kit as per manufacturer instructions, and this was submitted for sequencing at the Rockefeller University Sequencing Facility.

Individual phage candidates were tested for binding specificity as follows. First, 1 ml of the filtered phage was concentrated as above. Concentrated phages were mixed with roughly equal numbers of cclac⁺ phages (which form blue plaques when plated with Xgal) in PBS and panned against mAbRWL1, mAbRWL2, human IgM (myeloma; purchased from Jackson Immunoresearch Laboratories, West Grove PA), and a well with no target by the procedure above. Successful candidates showed a large white:blue enrichment in the eluant from the specific mAb-coated well but not from either control well when plated on media containing Xgal (20 μg/ml).

Candidate phage analysis by ELISA

Individual wells of a 96-well high-capacity ELISA plate were coated with 100 μl of ~10¹⁰/ml phage candidates and left at 4°C overnight. The following day, the wells were blocked with 200 μl blocking buffer (5% BSA) and left at room temperature for 2 h. The plates were washed, and 100 μl of a 100 ng/ml Ab solution was placed in each well. After 1 h at room temperature, the plates were washed, and 100 μl of a 1:1000 dilution of anti-human IgM-Peroxidase (Sigma) was added to each well. After 1 h, the plates were washed, and 200 μl of *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigmafast tablets as per instructions) was added. Development proceeded for 30 min before the ODs were measured on a plate reader (E_{max} Multi Plate Reader, Molecular Devices, Palo Alto, CA) at 450 nm.

Peptides

All peptides were purchased from Biosynthesis (Lewisville, TX) at the "discovery" scale. Peptides were analyzed by mass spectrometry by the manufacturer and the spectra provided. Table I shows the amino acid sequence of the various peptides used in this study.

Table I. Peptide sequences

Peptide	Amino Acid Sequence
D017-1	WGENNYTSQIRP
D017-2	FKDPTDSGNRGF
D058-1	GPRPPLNHALHS
D059-1	NLRAPAWYQVAQ
D093-1	LHSTTFWGGGS
D118	SHLAHNLPFRPG
D531-1	PRIQSTYNNNEGW
C977	FGRNGSDTPDKF

Results

Ig variable-region sequence analysis of hybridoma cell line RWL1 and RWL2

cDNA was prepared from cloned hybridoma cell lines RWL1 and RWL2 by the RT-PCR technique described in *Materials and Methods*, and the rearranged variable regions of the H and L chains were sequenced. The two cell lines express different rearranged V_H genes derived from separate V_H families (RWL1, V_H4-39 (previously DP79); and RWL2, V_H3-09 (previously DP31)) and consequently are significantly different from each other in both nucleotide (Fig. 1) and amino acid sequence (Fig. 2). However, when compared with the appropriate ancestral germline gene counterpart, only rare differences are noted (Fig. 1). These could represent heretofore unidentified allelic polymorphisms or somatic mutations. Similarly, the two lines express different V_K genes (RWL1, PPK21; and RWL2, DPK24) derived from different V_K families (V_KIII vs V_KIV , respectively), that are also very similar to the germline counterparts (Fig. 1). Although the J_H segments in the two lines differed, they both used identical J_K4 genes with identical amino acid sequences. In both instances, there was insufficient similarity to the described D genes to make a D segment assignment by the method of Corbett (37) for either rearranged V_HDJ_H .

Thus, the expressed rearranged Ig V genes of these two cell lines are very different based primarily on the use of distinct V gene segments and not on the basis of somatic alterations. The amino acid sequences are also very different, particularly in the complementarity determining region (CDR) (Fig. 2). Therefore, differences in the specificities of the Abs coded by these two sets of genes would not be surprising.

Epitope definition by phage display

Three phage-displayed peptide libraries obtained from New England Biolabs (Ph.D.-7, Ph.D.-12, and Ph.D.-C7C) were independently "panned" (see *Materials and Methods*) against each mAb. After three rounds of panning, individual plaques were used to grow phage cultures for sequencing and testing. In total, six independent pannings were conducted (3 libraries \times 2 mAbs). The displayed peptide sequences derived from the nucleotide sequences of the phage candidates are shown in Fig. 3.

The motif [WF],G,[EQS],N clearly dominates the population of recovered phages. The conserved motif most often appears at the N terminus of the displayed peptide. The N terminus of the peptide is the N terminus of the pIII in the Ph.D. 7 and Ph.D. 12 libraries. In the cysteine-constrained library, the N terminus of the peptide is followed by a cysteine and an N-terminal alanine. Thus in either case the displayed motif shows a bias for the most N-terminal position possible. A likely explanation is that N-terminal sequences are further from the phage body and thus more accessible for interaction with the Ab.

Tryptophan (W) and phenylalanine (F) both contain aromatic rings although tryptophan is also polar. Their apparent interchang-

ability is most likely due to that aromatic ring. The different residues that occupy the third position of the motif show some similarity as well. Glutamine (Q) and glutamic acid (E) have side chains with acidic groups, but serine (S) has a polar hydroxy group. There may be influences from those residues outside the core motif, but testing such a hypothesis was beyond the scope of this work.

Phages bearing the consensus motif are specific for mAbRWL1 and mAbRWL2

Six phage candidates, one from each panning, were tested for specific binding to mAbRWL1 and mAbRWL2 (Fig. 4A). Each phage candidate displays a unique peptide that contains the consensus motif, as shown in Fig. 4. Candidate phages were panned (see *Materials and Methods*) against mAbRWL1, mAbRWL2, IgM myeloma, and blank (no Ab) wells. A roughly equal number of $cclac^+$ f1 were included as an indicator of the background level of nonspecific binding. The titer of the eluant from wells coated with either mAb was at least 100-fold greater than that from the control wells for all six candidate phages. This indicates that phage-displayed peptide sequences selected as binding to one of the mAbs also bind the other but do not bind to unrelated IgM. The level of nonspecific phage binding, indicated by the eluant titer of $cclac^+$ f1, was the same regardless of the presence of Ab on the well, suggesting that the greater eluant titer from the mAb-coated wells is due to a specific interaction with the displayed peptides.

A second assay confirms and extends the results above. While demonstrating the specificity of the phage candidates, the results above do not unequivocally show that the phages are interacting with the mAbs and not some other component of the coating solution (i.e., a contaminant in the Ab preparation). This possibility was addressed by an ELISA in which the mAbs and IgM myeloma were each used as a primary reagent with the phages as targets (Fig. 4B). Ab binding was detected with α IgM-horseradish peroxidase as described in *Materials and Methods*. Two control phages were included: $cclac^+$ f1, which has no displayed peptide, and ϕ CLL57-7a, which displays a peptide selected in an unrelated experiment. Each of the six candidate phages gave a positive signal when mAbRWL1 or mAbRWL2 was used as primary reagent, but gave no signal above background when the IgM myeloma was used. Furthermore, none of the Abs gave a signal above background against $cclac^+$ or ϕ CLL57-7a, reaffirming the specificity of mAbRWL1 and mAbRWL2. The congruent results of these two experimental approaches strongly support the interpretation that these phages are directly and specifically interacting with these mAbs.

Phage-displayed peptide motif functions as a free peptide

It is possible that the phage-displayed peptide motif requires some structural contribution from the phage body. If such were the case, a small peptide containing the motif would lack the activity of the phage-displayed motif. Several peptides (Table I) were tested for their ability to inhibit consensus peptide-bearing phage ϕ RWL1-C7b (with displayed peptide sequence **FGENTGY**; consensus motif in bold) binding to the mAbs over a wide concentration range. Peptide D017-1 (sequence **WGENNYTSQIRP**) contains the consensus motif, while peptide D058-1 (sequence GPRPPLNHALHS) has an unrelated sequence and is a control. As shown in Fig. 5A, peptide D017-1 inhibited phage-displayed peptide binding in a concentration-dependent manner, while the peptide D058-1 had no effect at any concentration. Neither peptide had any effect on the level of nonspecific binding as indicated by the constant level of $cclac^+$ f1 phage binding.

A

Heavy Chain

mAbRWL1 CAGCTGCAGCTGCAGGAGTGGGGCCAGGACTGGTGAAGCCTTCGGAGAACCTTCCCTCACCCTGCACCTGTCTCTGGTGGCTCCATCAGCAGTAGTAGTTACTACTGGGG
 4-39

mAbRWL1 CTGGATCCGCCAGCCCCAGGGAAGGGGCTGCAGTGGATTGGGAGTATCATATATAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGTCCGAGTACCATAATCCGTAG
 4-39

mAbRWL1 ACACGTCCAGAACCCAGTCTCCCTGAAGCTGAGCTCTGTGACCCGCCAGACACGGCTGGTATTAATCTGTCCAGACCTTGGACTATCTGGTCTTCGATCTCTGGG
 4-39A
 CTA...A..... JH2

mAbRWL1 CCGTGGCACCCCTGGTCACTGTCTCCTCAG
 JH2

Light Chain

mAbRWL1 GAAATAGTGAATGACCCAGTCTCCAGCCACCCCTGTCTGTGCTCTCCAGGGGAAGAGCCACCCCTCTCCTGCAGGGCCAGTCAGAGTGTAGCAGCAACTAGCCCTGGTACCA
 DPK21

mAbRWL1 GCAGAAACCTGGCCAGGCTCCAGGCTCCTCACTATAGTGGCAATCCACAGGGCCACTGGTATCCAGCCAGGTTGAGTGGCAGTGGGCTTGGGACAGAGTCACTCTCA
 DPK21

mAbRWL1 CCATCAGCAGCCTGCAGTCTGAAAGATTTGCAAGTTTATTAATCTGTGAGCAGTATTAATTAATCTGGCTCTGACTTTCCGGGAGGGACCAAGTGGAGATCAAAAC
 DPK21C
 G...C..... JK4

B

Heavy Chain

mAbRWL2 GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCCTGGCAGGTCCTCGAGACTCTCCTGTGTCAGCCCTCTGGATTACCTTTGATGATTATGCCATGCCTGGGT
 3-09

mAbRWL2 CCGCAAGCTCCAGGGAAGGGCTGGAGTGGGCTCAGGATATTAGTTGGAATAGTGGTAGCATAGGCTATGCGGACTCTGTGAAGGGCCGATTACCATCTCCAGAGACA
 3-09

mAbRWL2 ACGCCAAGAACTCCTGTATCTGCAAAATGAACAGTCTGAGAGCTGAGGACACGGCTTGTATTACTGTGCAAAAGCCAACCGTGGACGCTACTACGGTATGGACGTCTGG
 3-09AT.
 AITACTACTA..... JH6b

mAbRWL2 GGCCAAGGGACCAAGGTACCCGTCTCCTC
 JH6b

Light Chain

mAbRWL2 GACATCGTGAATGACCCAGTCTCCAGACTCCCTGGCTGTGCTCTGGGCGAGAGGGCCACCATCAACATGCAAGTCCAGCCAGAAATGTTTATATAGCTCCAAACAATAAGAA
 DPK24G.....C.....

mAbRWL2 CTACTTAGCTTGGTACCAGCAGAAACAGGACAGCCTCCTAAGCTGTCTCATTTACTGGGCATCTAACCGGGAATCCGGGTCCCTGACCGATTTCAGTGGCAGCGGGTCTG
 DPK24

mAbRWL2 GGACAGATTTCACTCTCACCATCAGCAGCCTGCAGGCTGAAGATGGCAGTTTATTACTGTGCAAAATATTATAGTATTCGGCTCACTTTCCGGCGAGGGACCAAGGTG
 DPK24C...T.C
 JK4

mAbRWL2 GAGATCAAAC
 JK4

FIGURE 1. Nucleotide sequences of rearranged variable regions of mAbRWL1 (A) and mAbRWL2 (B) and nearest germline gene from VBASE database. Dots indicate identical nucleotides. Where there are differences between the Ab sequence and the corresponding germline, the germline nucleotide is indicated below.

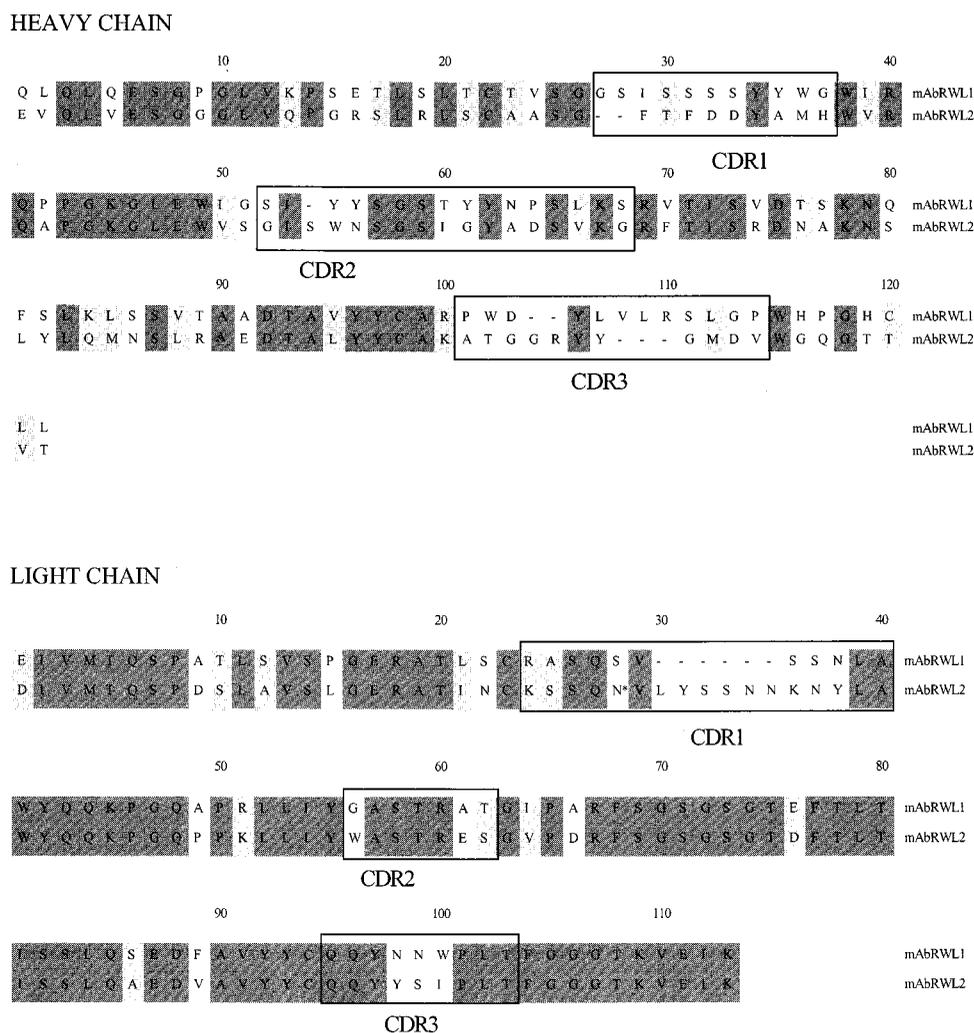


FIGURE 2. Amino acid sequences of the H and L chains of mAbRWL1 and mAbRWL2. Darkly shaded residues are identical between the two sequences, while light shading indicates that the residues are members of the same functional group. Alignment was done by the Clustal method, and the CDRs were labeled according to the Kabat scheme (38). The asterisk at position 28 of mAbRWL2 indicates the only amino acid substitution resulting from a deviation of the nucleotide sequence from germline. The germline encoded residue is a serine (S).

Peptide D017-1 also inhibited binding to mAbRWL1 by five other phages displaying different peptides, all containing the consensus motif, as shown in Fig. 5B. The recovered phage titer from the candidate testing protocol was no higher than background for each of these phages in the presence of peptide D017-1 at a concentration of 10 μ g/ml.

Peptides were also tested in a direct ELISA in which mAbRWL1 and mAbRWL2 were used as primary Abs against wells coated with various peptides (Fig. 5C). Only peptide D017-1 gave a signal above background. Furthermore, that signal was found to be dependent on the peptide concentration (data not shown). Peptide D531-1 (PRIQSTYNNEGW) has the reverse amino acid sequence of peptide D017-1 and does not give a positive signal. Thus the Ab binding is dependent on the order of amino acids in peptide D017-1 and not simply the amino acid composition.

A peptide containing the consensus motif competes with fraction 7B for binding to mAbRWL1

Competitive ELISAs were performed to determine whether peptide D017-1 could compete with the previously identified ligand of mAbRWL1 and mAbRWL2, CNBr digestion fraction 7B of lac-

toferrin (26). Peptide D017-1 blocks mAbRWL1 from binding to fraction 7B in a concentration-dependent manner (Fig. 6), while peptides D017-2, D118, and C977 have no effect. Similar results have been obtained with mAbRWL2 (data not shown). The sequence of C977 is taken from the SWISS-PROT database sequence of human lactoferrin and is the only region of lactoferrin to contain a sequence similar to the consensus motif identified here.

Discussion

Here we describe two monoclonal IgM Abs, derived from the cord blood of different individuals, that specifically recognize the same linear phage-displayed epitope. The two Abs each selected phage bearing the same consensus motif from several phage-displayed peptide libraries. The exact amino sequences of the selected peptides were not the same. This difference could reflect differences in the fine specificity of the two Abs. On the other hand, it could be a consequence of sampling in the peptide display library. The peptide library has a diversity of $\sim 10^9$. The consensus motif is four amino acids in length and is thus estimated to be present in $\sim 6 \times 10^4$ unique members of the library. A peptide sequence initially selected on phages binds specifically to both mAbs as free peptide

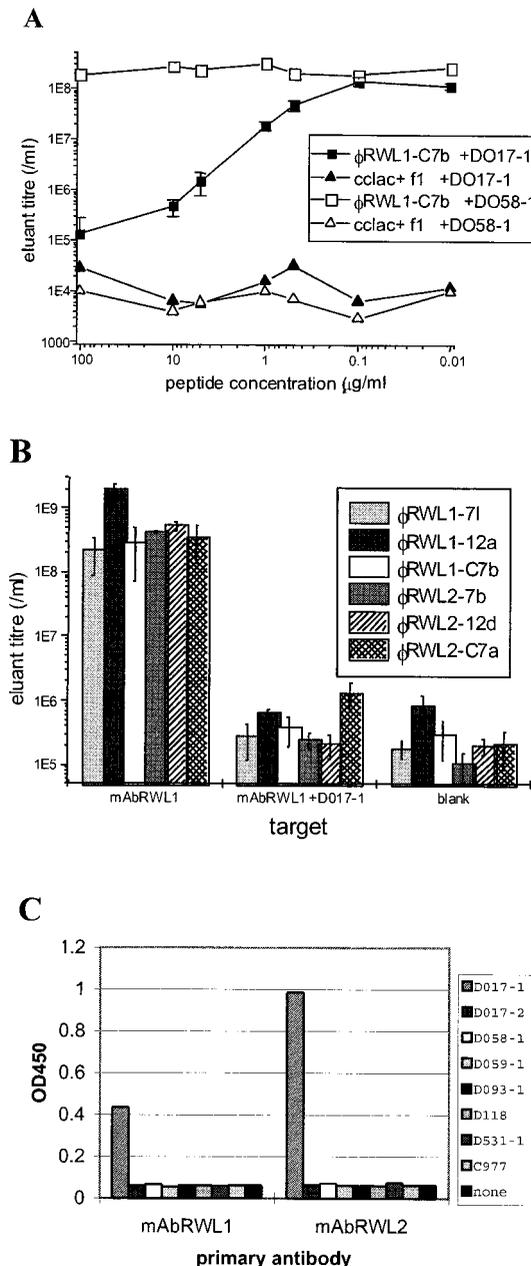


FIGURE 5. Free peptides compete with phage-displayed peptides for Ab binding. *A*, Pannings were done essentially as described in *Materials and Methods*. After blocking the coated wells with 0.5% BSA, cclac⁺ phage (10¹⁰/ml) and peptide were added in 100 µl to the wells. After 1 h, φRWL1-C7b was added to a final concentration of 10¹⁰/ml. *B*, Wells were coated with 1 µg/ml mAbRWL1 overnight and blocked with 0.5% BSA. Peptide was added as before at a concentration of 10 µg/ml. cclac⁺ phages were included at ~10¹⁰/ml and eluted at 2–5 × 10⁵/ml in all cases. Error bars are ± SD of three experiments. *C*, Wells were coated with 100 µl of 100 µg/ml peptide. After blocking, 1 µg/ml mAbRWL1 or mAbRWL2 was added. Detection was by anti-human IgM-horseradish peroxidase conjugate with OPD as the substrate.

chains may play a key role in determining the shared specificities of mAbRWL1 and mAbRWL2.

We are intrigued by the possibility that individuals are born already synthesizing an Ab repertoire that recognizes a particular set of epitopes. Several lines of evidence support such a hypothesis of stereotyped specificities but each line of evidence has significant limitations.

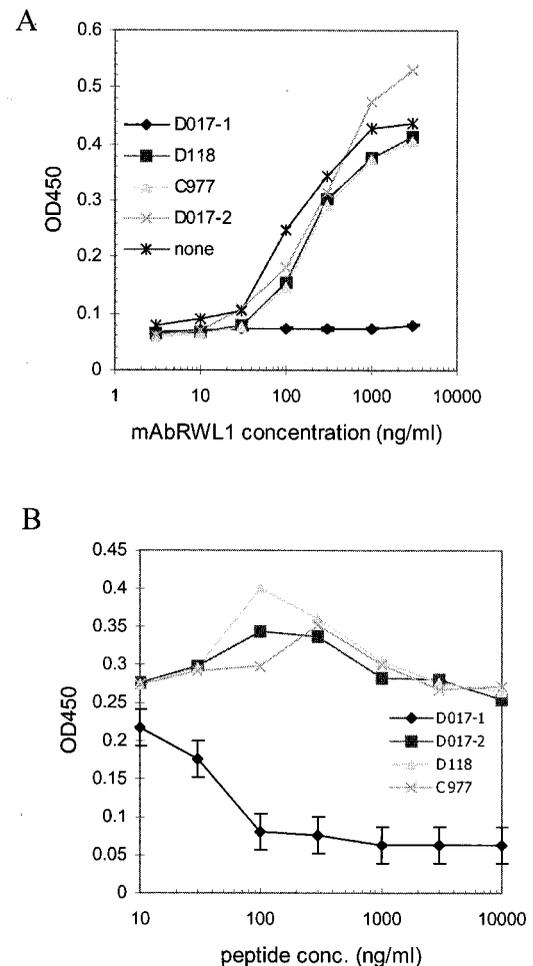


FIGURE 6. Phage-derived peptides compete with the CNBr fraction 7B of lactoferrin. *A*, Peptide DO17-1 blocks binding of mAbRWL1 to fraction 7B of lactoferrin over a wide range of Ab concentration. Wells were coated overnight with 100 µl of 0.5 µg/ml fraction 7B. Plates were blocked with 3% BSA. Dilutions of Ab were premixed with peptide at 10 µg/ml for 1 h before being put on blocked wells. Detection was by anti-human IgM-horseradish peroxidase conjugate with OPD substrate for development. *B*, Peptide DO17-1 blocks binding of mAbRWL1 to fraction 7B of lactoferrin but control peptides do not. Error bars are ± SD of three experiments.

The results presented in this paper are limited to the analysis of two mAbs that recognize a single epitope. As such it is somewhat tenuous to generalize from these findings. However, previous studies have shown that adult and cord blood serum from many individuals contain Abs that react with the CNBr digestion fraction 7B of lactoferrin (26, 48). Therefore, we propose that the cord blood of most, if not all, individuals will contain Abs reactive with this epitope. We further propose that this epitope specificity is one of many that are present in serum from most, if not all, individuals at birth. One of the strongest lines of evidence for stereotyped specificities among the cord blood IgM repertoire comes from Kazatchkine and colleagues (18). They have generated profiles of autoreactive Abs present in sera by separating homogenous tissue extracts on a polyacrylamide gel to resolve protein bands. Whole serum is then used as a primary immunostain and detected with labeled anti-IgM Abs. The resulting densitometric profiles from different individuals can be compared. They find that IgM profiles generated from cord blood serum are essentially invariant from individual to individual. The primary limitation of this approach is that it is not known what Ags or epitopes react with the serum Abs

to generate the signals. The method does not distinguish whether many monoreactive or a few polyreactive Abs are responsible for the various peaks in their profiles.

Numerous studies have addressed the possibility that the fetal and neonatal repertoires could be restricted as part of a developmental program of gene usage bias. Preferential use of J_H proximal V_H genes (22) in mouse pre-B cells and the prevalence of diversity segment D_HQ52 , as well as significantly restricted V_H gene usage among second trimester fetal humans (23), have been noted. However, the restrictions placed on the early human fetal repertoire are not as apparent in the cord blood (24, 25), although the cord blood κ repertoire has been found to overuse a small subset of V_K genes (49).

Neonatal B cells are mostly $CD5^+$, whereas the adult contains a greater proportion of $CD5^-$ B cells (50). When V_H gene usage by adult $CD5^+$ and $CD5^-$ peripheral B cells was examined, there was not a significant difference between the two populations. In both cases, the V_H3 family genes were found at a slightly greater frequency than expected by random selection (51). However, the $CD5^-$ population contains the "sister" cells (B1b) of the $CD5^+$ (B1a) class as well as the conventional B2 cells (9). When the $CD5^-$ population was separated into B2 and B1b populations (by the level of CD45RA expression and CD5 mRNA levels) and compared with the $CD5^+$ B1a population, it was found that the B2 population was more restricted to V_H3 family genes whereas the B1a and B1b populations use both V_H3 and V_H4 family genes (52). EBV-transformed B cell clones that secreted polyspecific Abs were not found to have significant V_H or V_L gene usage pattern differences when compared with clones of unknown specificity (53). Analysis of B cells at different stages of ontogeny has suggested that there may be sequential mechanisms responsible for some of these observed biases (54). It will most likely be some time before there is a complete understanding of the mechanistic origins and patterns of variable-region gene use bias.

There is a significant limitation to analysis of Ig variable-region gene sequences when one is concerned with the repertoire of Ab specificities. It is not currently possible to predict the Ag specificity of an Ab by looking at its variable-region sequence. Even if the protein-folding problem were solved, it would still not be likely that predictions of epitope specificity could always be made from variable-region gene sequences. Variable-region sequences and epitope specificities do not exist as discreet linked pairs. There is not a strict one-to-one correspondence between an Ab and a single epitope. On the one hand, a single variable-region sequence may be capable of binding several diverse Ags as is the case for polyspecific Abs (1). On the other hand, the data presented here and in the lysozyme example discussed above (42) imply that Abs that differ in variable-region gene usage and the amino acid sequence of the CDRs can share a near identical epitope specificity. Thus the number of different variable-region sequences present may not accurately reflect the number of different epitope specificities embodied therein, and the error could be in either direction. These complications lie at a key juncture in our understanding of immune responses to pathogens as well as the tendency to fall prey to deleterious autoreactive Abs.

In conclusion, we have presented further evidence that human neonates have naturally occurring Abs of defined specificity. Furthermore, our evidence from two mAbs derived from different individuals' cord blood indicates that such defined specificities can be generated by multiple combinations of essentially germline configuration genes. Elucidating the physiological relevance of this redundantly encoded specificity remains a challenging problem.

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