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B cell responses to a peptide epitope. II: Multiple levels of selection during maturation of primary responses

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Summary This report analyses murine primary humoral recognition of a linear domain (MEP 17–31) within a 100 amino acid polypeptide, MEP-1. An analysis of the early primary IgM response revealed that MEP 17–31 presented at least two distinct domains for pre-immune B cell recognition represented by MEP-1 residues 19–23 and 26–28. However, subsequent maturation into an IgG response saw an exclusive selection for the anti-MEP 19–23 component with loss of all alternate specificities. The IgM response to MEP 19–23 was oligoclonal and composed of diverse paratope phenotypes as evidenced by varied heavy chains of immunoglobulin V–D–J combinations and CDR3 sequences. In contrast to the oligoclonality of IgM mAb, the mature IgG response to MEP 19–23 appeared to derive predominantly from a single progenitor. It therefore appears that maturation of primary humoral responses to polypeptide antigens involves two distinct levels of selection. While there is selection for a restricted subset of the initially induced antibody fine-specificities, progression of the response also entails a reduction in clonal heterogeneity of B cells responding to the dominant epitope.

Key words: clonal selection, peptide epitope, primary response.

Introduction

The combined realization that B cells generally recognize protein antigens in their native form and that the pre-immune B cell repertoire represents a virtually limitless library of preformed sIg specificities has led to the dogma that the entire accessible surface of a protein represents an antigenic continuum.[1] Although perhaps correct in principle, it, however, contrasts in practice with several studies documenting segregated B cell recognition.[2–6] A notable example of this is that of hen egg white lysozyme where the murine primary humoral response is entirely dominated by antibodies specific to the amino terminus of the molecule.[2] Selective murine humoral recognition of epitopes was also observed in our studies with a recombinant, designed polypeptide MEP-1.[6,7] A further exploration of this phenomenon revealed that at least part of the explanation resides in the epitopic bias which accrues during maturation of the primary response.[7]

Collectively the data described above suggest that immunogenicity of antigenic determinants presented by a polypeptide antigen is not necessarily a correlate of its accessibility for B cell recognition. Indeed these and some of our more recent results implicate the operation of immunological mechanisms that eventually select for only a restricted subset of B cell specificities, leading to hierarchical immunodominance.[7,8] It therefore appears that the induction and maturation of antibody responses to polypeptide immunogens involves a far greater degree of complexity than that against haptens, the currently employed models for studying such phenomena.[9]

An empirical approach was adopted in a preliminary study in which we examined generation and subsequent maturation of murine antibody responses to a linear segment, as a model antigenic determinant, of 100 amino acid residue recombinant, designed polypeptide, MEP-1.[6] The linear segment represents amino acid residues at positions 17–31 of MEP-1 and has been previously shown to constitute an immunodominant epitope of this antigen in the murine model.[6,7] A single immunization of mice with MEP-1 was found to generate an early primary IgM polyclonal response that included varied antibody specificities collectively recognizing a major proportion of the 17–31 amino acid sequence. Interestingly, dissection of this response into individual mAb specificities revealed that at least two distinct subsegments between positions 19–23 and 26–28 were independently recognized, although a bias in favour of the 19–23 sequence was noted. Subsequent maturation into a late primary IgG response was exclusively directed against the MEP 20–23 segment with loss of all alternate specificities. We also examined the clonal heterogeneity of anti-MEP 19–23-responder B cells in the early primary IgM and late primary IgG responses. While the IgM response was composed of antibodies collectively using a diverse array of V_H, D_H and J_H gene combinations, the late primary IgG response consisted of B cells expressing antibody molecules using identical V_H, D_H and J_H gene segments with identical V_H–D_H and D_H–J_H joinings along with a common κ light chain. It therefore appears that primary antibody responses to polypeptide determinants involves at least two distinct levels of selection, one for specificity and another that restricts clonal diversity of B cells initially induced against a common epitope.

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Materials and methods

Materials

Horseradish peroxidase (HRPO) abelled goat anti-mouse IgG (γ-specific) and IgM (μ-specific) were purchased from Sigma Chemical Company (St Louis, MO, USA). tBoc-protected amino acids for peptide synthesis were obtained from Nova Biochem (Laufelfingen, Switzerland). For multipin synthesis of peptides, both the non-cleavable kits and Fmoc amino acid derivatives were purchased from Chiron Mimotopes Inc. (Clayton, Vic., Australia).

The polypeptide MEP-1 was obtained as the expressed product of a synthetic gene in *Escherichia coli*. Its expression and purification, followed by disulfide rearrangements and characterization have been described in detail elsewhere.[10]

Peptide synthesis

The peptide MEP 17–31 was synthesized by the solid phase method on an ABI model 430A automated peptide synthesizer (Applied Biosystems, Foster City, CA, USA).[11] After synthesis, side-chain deprotection and cleavage from the resin was achieved with a 10% solution of trifluoromethanesulfonic acid in trifluoroacetic acid as previously described.[12] All peptides were purified to at least 95% purity by reverse phase HPLC on a C-18 column (μ Bondapak, 7.8 mm x 30 cm; Waters, Milford, MA, USA) using an aqueous gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. Identity of all peptides was ascertained by amino acid analysis.

All hexapeptides were synthesized by the method of Geysen using the non-cleavable multipin kits.[13] For synthesis the standard protocol recommended by the manufacturer was employed. After completion of synthesis, peptides were routinely acetylated at the amino terminus with a 50 : 5 : 1 mixture of dimethylformamide, acetic anhydride and triethylamine. Side chain deprotection was accomplished over 2 h at room temperature with a 38 : 1 : 1 (v/v/v) mixture of trifluoroacetic acid, ethanedithiol and anisole.

Animals and immunization

Female mice (6–8 weeks old) were obtained from the small animal breeding facility at the National Institute of Immunology, New Delhi, India. Immunizations were i.p. with a dose of 20 μg MEP-1 per mouse (100 μL) as an emulsion in CFA. For polyclonal sera, mice were bled from the retro-orbital plexus and sera within a group pooled.

Generation of monoclonal antibodies

For early primary IgM mAb, a group of three female BALB/c mice were immunized with MEP-1 as described above. Four days later mice were bled individually and sera was checked for anti-MEP-1 antibody titres. The mouse with the highest titre (1 : 500) was chosen for hybridoma production. The spleen was removed surgically and cells fused to give early stage primary hybridomas. Polyethylene glycol-mediated fusion to hypoxanthine-aminopterin-thymidine-sensitive myeloma derivative SP2/O-Ag 14, maintenance of derived lines and limiting dilution cloning was essentially as previously described.[14] Culture supernatants were screened for IgM antibody production against an MEP 17–31 synthetic peptide by ELISA. Nine anti-MEP 17–31-secreting IgM clones were initially obtained, though the hybridoma 608 subsequently lost the ability to secrete antibody on continuous culture.

For the mature IgG mAb, mice immunized with MEP-1 were boosted on day 28 intravenously with 20 μg of soluble antigen in PBS. Three days later the spleen from the highest responder mouse was removed and hybridomas obtained as described above. The dissociation constant of individual mAb for MEP-1 was determined using the solution phase saturation ELISA protocol of Friguet *et al.*[15] Culture supernatants were used with final antibody concentrations between 100 and 200 ng/mL.

ELISA assays for soluble antigen

Plates were coated at 2 μg of MEP 17–31 peptide or 200 ng of MEP-1 per well in 100 μL of PBS (pH 7.4) at 37°C for 3.5 h. They were then blocked with 300 μL/well of a 5% solution of fat-free dry milk powder in PBS at 37°C for 1 h and 100 μL of the appropriate dilution (or concentration) of mouse antiserum (or mAb) was added and plates were incubated at 37°C for 1 h. After washing, bound antibody was detected with HRPO-labelled secondary antibody (37°C, 1 h) followed by colour development with *o*-phenylenediamine as chromogen. Absorbance was measured at 490 nm.

For competitive ELISA experiments, 5 μL of PBS containing the appropriate amounts of competitor peptide was added to 250 μL of mAb diluted to 50% of its titre. These were then incubated for 10 min at room temperature prior to the addition to duplicate wells (100 μL/well). The rest of the procedure was as described above.

ELISA assays for pin-bound hexapeptides

Hexapeptide sets synthesized on pins were also evaluated for antibody cross-reactivity by ELISA. The protocol recommended by the manufacturer was strictly followed. Primary antibodies were diluted to appropriate concentrations in PBS containing 2% BSA, 0.1% (v/v) Tween 20 and 0.1% (w/v) sodium azide. Pins were incubated in 200 μL each of the antibody solution at 4°C overnight with gentle shaking. Pins were then washed and subjected to a second round of HRPO-labelled goat anti-mouse IgM or IgG at room temperature for 1 h with gentle shaking. The chromogen used for revealing bound antibody was 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium (ABTS) and absorbance was measured at 405 nm with subtraction of that at 490 nm.

Reverse transcription and amplification of Ig mRNA

Total cellular RNA was isolated from about 10^7 hybridoma cells with the R NAzol B (Wak-chemie Medical, GMBH, Germany)-based protocol with minor modifications. About 10 μg of total RNA was used for each *V* gene cDNA amplification. The first strand of cDNA was synthesized using 20 U of reverse transcriptase (Promega, Madison, WI, USA) and 800 pmol of the primer, 5′-AGACGAGGGGGAAGACATTT-3′ for Cμ, 5′-GGCCAGTGGATAGAC-3′ for Cγ and 5′-GCTCACTGGATGGTGGGAAGATG-3′ for Cκ in a final volume of 25 μL. Single-stranded cDNA was then amplified using as 5′ primer 5′-AGGT(C/G)(A/C)A(A/G)CTGCAG(G/C)AGTC(A/T)GG-3′ for V_H and 5′-GA(A/C/T)ATTGTG(A/C)T(G/C)AC(A/C)CA(A/G)(A/T)CTCCA-3′ for V_L. The primers were synthesized in the laboratory and purified prior to use. A different set of nested 3′ primers were used for amplification, 5′-CATTTGGGAAGGACTGACTC-3′ for Cμ, 5′-GGCCAGTGGATAGAC(T/C/A)GA-3′ for Cγ and 5′-GAAGATGGATACAGTTGGTGCA-3′ for Cκ. Amplification was carried out using 5 μL of cDNA and *Taq* polymerase (Stratagene, La Jolla, CA, USA) in a final volume of 100 μL. The final concentrations of reagents were 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 1.5 mmol/L $MgCl_2$, 0.01% gelatin, 200 pmol of each primer and 2.5 U of *Taq* polymerase. Polymerase chain reaction (PCR) was performed

on a Perkin Elmer thermocycler using the following programme of: one cycle at 95°C for 3 min, followed by 30 cycles at 94°C for 1 min, 58°C for 1.5 min, 72°C for 1 min and finally a 10 min incubation at 72°C. A 10 μL aliquot of the reaction was analysed on a 1.8% agarose gel.

Nucleotide sequencing of polymerase chain reaction amplified DNA

Polymerase chain reaction products of about 400 b.p. were blunt ended by polishing with the PCR polishing kit from Stratagene and subsequently cloned in the *Srf* I site of PCR-script vector of the PCR-script cloning kit from Stratagene. Positive clones were identified by restriction digestion of plasmid DNA and appropriate clones sequenced using the T7 sequencing kit purchased from Pharmacia (Uppsala, Sweden). Both strands of cloned DNA were sequenced. The immunoglobulin heavy (Ig_H) and light (Ig_L) chain variable region gene sequences were analysed using the PC Gene software and the GenBank data library.

The Ig_H nucleotide sequence data reported in this paper appears in the EMBL, GenBank and DDJB Nucleotide Sequence Databases under the following accession numbers: mAb 603, Z68168; mAb 604, Z68169; mAb 605, Z68170; mAb 609, Z68171; mAb 611, Z68172; mAb 612, Z68173; mAb 623, Z68174; mAb 624, Z68175; mAb 626, Z68176; mAb 628, Z68177; mAb 627, Z68178.

Results

Primary polyclonal responses to an immunodominant domain of MEP-1

Figure 1 gives the amino acid sequence of the polypeptide MEP-1 where the immunodominant epitope of MEP-1, residues 17–31, is highlighted. Following immunization of BALB/c mice with MEP-1, IgM class antibodies against a synthetic peptide representing the MEP 17–31 sequence could be detected as early as day 3, whereas IgG became evident by day 7, though, predictably, titres were low (< 1 : 250). With time, there was a rapid accumulation of anti-MEP 17–31 IgG and titres peaked at 1 : 30,000 by day 28 (data not shown).

A panel of overlapping hexapeptide sequences that represented single amino acid displacements over the MEP 17–31 sequence was used to probe for distribution of antibody fine specificities and results are shown in Fig. 2. Day 3 antiserum (IgM) was found to cross-react with a spectrum of overlapping hexapeptides collectively spanning MEP-1 residues 17–28 (Fig. 2a). In contrast, however, recognition by day 28 serum (IgG) was restricted to three overlapping peptides collectively representing MEP-1 residues 18–25 (Fig. 2b); suggesting that the dominant antibody specificity in this population is directed against a tetrapeptide stretch of MEP-1 residues 20–23 (sequence, DPAF).

M G T N L S V P N P L G F L P D H Q L D P A F G A 25

N S T N P D W D F N P G G M Q W N S T A L H Q A L 50

Q D P R V R G L Y L P A G G F F L L T R I L T I G 75

G C T T P A Q G N S M F P S C C C T K P T D G N C 100

Figure 1 Amino acid sequence of the polypeptide MEP-1. MEP-1 represents a polyvalent polypeptide that contains various segments derived from the envelope proteins of hepatitis B virus. Details of epitopes selected, design of polypeptide, assembly of the gene and its expression in *Escherichia coli* has been previously described.[10] The single letter code for amino acids is used and the immunodominant domain spanning residues 17–31 has been boxed.

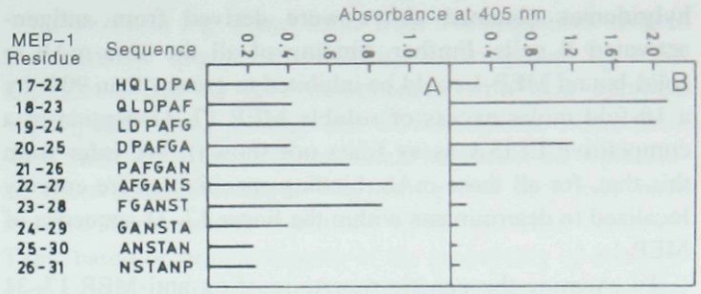

Figure 2 Localization of early and late primary polyclonal anti-MEP-1 binding sites within the MEP 17–31 sequence. Pins containing the MEP 17–31 derived overlapping hexapeptides were incubated in 200 μL each of either (A) a 1 : 100 dilution of day 3 anti-MEP-1 and bound IgM; or (B) a 1 : 6000 dilution of day 28 anti-MEP-1 and bound IgG. Values are expressed as the absorbance obtained for a given peptide after subtraction of that obtained for a negative control peptide of sequence AQGNSM that was synthesized in triplicate. The mean absorbance obtained for negative control peptide was 0.15 units with day 3 serum and 0.05 absorbance units with day 28 serum. Data are representative of three separate experiments.

Thus it would appear that, although early primary IgM recognition of the MEP 17–31 domain, in the context of MEP-1, encompasses a major proportion of the sequence, specificity of the mature primary polyclonal response is, however, far more restricted. In separate studies essentially similar results were obtained in three additional strains of mice C3H/HeJ, SJL/J and C57Bl/6 (data not shown); suggesting that the observations in Fig. 2 is not unique to a given murine H-2 haplotype.

Antibody repertoire in the MEP 17–31-specific IgM response

The early primary IgM response directed against the MEP 17–31 sequence appeared to be composed of multiple specificities. To verify this MEP 17–31-specific mAb were generated from a BALB/c mouse that had been immunized 4 days earlier with MEP-1. Selection of hybrids was specific for IgM-secreting clones to enable us to examine antibodies which represent specificities directly derived from the pre-immune B cell pool and prior to the onset of maturation events within.[9] A total of nine independently derived clones were obtained of which one (mAb 608) subsequently turned non-secretor on continuous culture. As a result only limited analysis could be performed with this antibody.

In addition, two independent fusions were also performed with spleens from non-immune BALB/c mice. While a total of 82 clones were obtained from the first fusion, the second yielded 66. Culture supernatants from all of these clones were screened for cross-reactivity against the MEP 17–31 peptide. Only a single clone from each of these sets was found to produce antibodies that cross-reacted with MEP 17–31; suggesting that at least a majority of the MEP 17–31 specific

hybridomas obtained above were derived from antigen-activated B cells. Further, binding of all the nine mAb to solid-bound MEP-1 could be inhibited to greater than 90% by a 10-fold molar excess of soluble MEP 17–31 peptide in a competitive ELISA assay (data not shown). We infer from this that, for all these mAb, binding specificities are entirely localized to determinants within the linear 17–31 sequence of MEP-1.

To examine the genetic repertoire of an anti-MEP 17–31 IgM response we determined the nucleotide sequence of the heavy chain variable regions of mAb which are shown in Fig. 3. Except for mAb 612, 613 and 614 which gave identical sequence, the remaining mAb were found to derive from distinct B cells. Diverse utilization of V_H, D_H and J_H gene combinations was evident with no apparent restriction in either length or sequence of the CDR3 region. A summary of the anti-MEP 17–31 IgM is presented in Table 1. Although four of six distinct mAb utilized V_H genes from the J558 family (Table 1), they all represented separate members (Fig. 3). The data in Fig. 3 and Table 1 would seem to indicate that the anti-MEP 17–31 IgM response is composed of phenotypically (and presumably also structurally) distinct paratopes.

Table 1 Clonal heterogeneity of the early primary IgM response to MEP 17–31

Monoclonal antibody	Light chain isotype	Heavy chain V_H	Heavy chain D_H	Heavy chain J_H	CDR3 sequence (amino acid)
603	κ	Q52	Q52	3	NAY
604	κ	J558	FL16.2	1	IPITTATNWYDFV
605	κ	J558	SP2.2	4	STMITTVYAMDY
608	κ	ND	ND	ND	—
609	κ	J558	Q52-c	4	SQAMDY
611	κ	7183	SP2.5	2	QYGNYKDYFDY
612–614	κ	J558	?	2	GGVY

This table represents a summary of the IgM mAb sequences depicted in Fig. 3. Light chain isotypes were determined by ELISA using polyclonal goat anti-mouse κ antibodies. V_H gene families were identified with the help of the GeneBank database whereas D_H and J_H segments were identified by comparison with published sequences.[18] The D_H gene used by mAb 612–614 could not be identified by comparison with known murine *D* gene sequences. ND, not determined.

```
mAb

                                                  10                                  20
603   cag gtg cag ctg cag gag tct gga cct ggc cta gtg cag ccc tca cag agc ctg tcc atc acc tgc aca gtc
604   cag gtg cag ctc cag gag tca gga cct gag ctg gtg aag cct ggg gct tca gtg aag ata tcc tgc aag act
605   cag gtc aag ctg cag cag tca ggg act gtg ctg gca agg cct ggg gct tca gtg aaa atg tcc tgc aag gct
609   cag gtc aag ctg cag cag tca gga cct gag ctg gtg aag cct ggg gct tca gtg aag ata tcc tgc aag gct
611   cag gtc caa ctg cag gag tca ggg gga ggc tta gtg aag cct gga ggg tcc ctg aaa ctc tcc tgt gca gcc
612   cag gtc aag ctg cag gag tca ggg tct gag ctg gtg agg cct gga gct tca gtg aag ctg tcc tgc aag gct

                          30                                  40
603   tct ggt ttc tca tta act agc tat ggt gta cac tgg gtt cgc cag tct cca gga aag ggt ctg gag tgg ctg
604   tct gga tac aca ttc act gaa tac acc atg cac tgg gtg aag cag agc cat gga aag agc ctt gag tgg att
605   tct ggc tac acc ttt acc agc tac tgg atg cac tgg gta aaa cag agg cct gga cag ggt ctg gaa tgg att
609   tct ggt tac tca ttt act ggc tac ttt atg aac tgg gtg aag cag agc cat gga aag agc ctt gag tgg att
611   tct gga ttc act ttc agt agc tat gcc atc tct tgg gtt cgc cag act ccg gag aag agg ctg gag tgg gtc
612   tct ggc tac aca ttc acc agc tac tgg atg cac tgg gtg aag cag agg cct gga caa ggc ctt gag tgg gtt
                                CDR 1

          50                                  60                                  70
603   gga gtg ata tgg  -  agt ggt gga agc aca gac tat aat gca gct ttc ata tcc aga ctg agc atc agc aag
604   gga ggt att aat cct aac aat ggt ggt act agc tac aac cag aag ttc aag ggc aag gcc aca ttg act gta
605   ggc gct att tat cct gga aat agt gat act agc tac aac cag aag ttc aag ggc aag gcc aaa ctg act gca
609   gga cgt att aat cct tac aat ggt gat act ttc tac aac cag aag ttc aag ggc aag gcc aca ttg act gta
611   gca acc att agt agt ggt ggt agt tac acc tac tat cca gac agt gtg aag ggt cga ttc acc atc tcc aga
612   gga aat att tat cct ggt agt gtt agt act aac tac gat gag aag ttc aag agc aag gcc aca ctg act gta
                                CDR 2

                                    80                                  90
603   gac aat tcc aag agc caa gtt ttc ttt aaa atg aac agt ctg caa gct aat gac aca gcc ata tat tac tgt
604   gac aag tcc tcc agc aca gcc tac atg gac gtc cgc agc ctg aca tct gag gat tct gca gtc tat tac tgt
605   gtc aca tcc acc agc act gcc tac atg gag ctc agc agc ctg aca aat gag gac tct gcg gtc tat tac tgt
609   gac aaa tcc tct agc aca gcc cac atg gag ctc ctg agc ctg aca tct gag gac tct gca gtc tat tat tgt
611   gac aat gcc aag aac acc ctg tac ctg caa atg agc agt ctg agg tct gag gac acg gcc atg tat tac tgt
612   gac aca tcc tcc agc aca gcc tac atg cag ctc agc agc ctg aca tct gag gac tca gcg gtc tat tac tgt

                                100                                                 110
603   gcc aga aac  -   -   -   -   -   -   -   -   -   -  gct tac tgg ggc caa ggg act ctg gtc act gtc
604   gca aga atc ccc att act acg gct acg aac tgg tac ttc gat gtc tgg ggc gca ggg acc acg gtc act gtc
605   aca aga tct act atg att acg acg gtc  -  tat gct atg gac tac tgg ggt caa gga acc tca gtc acc gtc
609   gga aga agc caa  -   -   -   -   -   -  gct atg gac tac tgg ggt caa gga acc tca gtc acc gtc
611   gca aga cag tat ggt aac tac aag gac  -   -  tac ttt gac tac tgg ggc caa ggc acc act ctc aca gtc
612   aca aga gga ggg gtc  -   -   -   -   -   -   -   -  tac tgg ggc caa ggc acc act c-c aca gtc
                                CDR 3

603   tct gca
604   tcc tca
605   tcc tca
609   tcc tca
611   tcc tca
612   tcc tca
```

Figure 3 Nucleotide sequences of the heavy chain variable regions of early primary anti-MEP 17–31 IgM mAb were determined. Identical sequences were obtained for mAb 612, 613 and 614 as a result of which only that of mAb 612 is shown. The CDR regions are underlined. Numbering of codons is according to Kabat *et al.*[18]

Distribution of epitope fine-specificities of IgM mAb

Given the diverse array of IgM paratope configurations that are induced we next analysed epitope fine-specificities for each of these mAb. This was achieved by examining individual mAb cross-reactivities against the overlapping hexapeptide panel described in Fig. 2. The majority of the mAb, on subjection to this kind of analysis, reacted with a limited set of overlapping peptides that permitted easy interpretation. Monoclonal antibodies 612, 613 and 614, however, reacted with multiple hexapeptides suggestive of polyreactivity.

Table 2 summarizes the results for all the mAb along with dissociation constant values for MEP-1. As is evident, the distribution of IgM mAb specificities suggests that the MEP 17–31 presents at least two distinct determinants for pre-immune B cell recognition (Table 2). Thus while mAb 603–605 and 611 display unique fine-specificities they nevertheless focus within a pentapeptide stretch, MEP 19–23. Monoclonal antibodies 608 and 609 collectively define a second distinct determinant within MEP residues 26–28. Notably, however, the IgM response appears to be biased in favour of the MEP 19–23 segment with four of the nine obtained mAb being directed against it.

IgG class mAb to MEP 17–31 are restricted in epitope fine-specificity and phenotype diversity

It was intriguing that even as small an antigenic determinant as the MEP 19–23 segment of MEP-1 could induce pre-immune B cells with sIg receptors that are diverse both in genetic make-up and paratope structure (as presumed from the variable Ig_H CDR3). This was in turn reflected at the level of antigen-antibody interactions where marked affinity differences were observed. We were therefore interested in determining the level of antibody heterogeneity in the mature IgG response. For this a BALB/c mouse that had been immunized with MEP-1 4 weeks earlier (day 28) was boosted with soluble antigen and anti-MEP 17–31 IgG secreting hybridomas derived from a fusion that was performed 3 days later. A total of five clones could be stabilized, all of which secreted antibodies which were found to map exclusively against the MEP 20–23 segment (data not shown but identical to Fig. 2b). These were subsequently used to determine the sequence of the IgH variable region.

Table 2 Binding properties of early primary anti-MEP 17–31 IgM mAb

Monoclonal antibody	K_d (mol/L)	Minimum binding domain
603	1.3×10^{-7}	LDPA
604	1.1×10^{-8}	DPAF
605	7.5×10^{-8}	DPA
608	ND	NST
609	2.5×10^{-8}	NST
611	1.5×10^{-8}	DPA
612–614	2.8×10^{-8}	Polyreactive

Dissociation constants were determined by the method of Friguet *et al.*[15] and are the mean of two separate determinations. The minimum binding domain and anchor residues were identified as described in the text. ND, not determined.

As shown in Fig. 4 and Table 3 all five sequences showed a remarkable degree of similarity utilizing the V_H J558, D_H FL16.1 and J_H gene combination with near identical V_H–D_H and D_H–J_H joinings and CDR3 regions. While there were minor differences in nucleotide sequence, all represented transversional changes, suggesting that they may have resulted from somatic mutations within germinal centres.[16] Thus, based on prior estimates of the probability of independent identity at the VDJ junction, it is likely that the five IgG mAb were obtained from B cells representing somatically mutated variants derived from a common progenitor.[17] This was further substantiated by obtaining the nucleotide sequence of all five light chains. Again nearly identical sequences (V_L from group V, and J_L1) were obtained barring minor differences that could also be accounted for by invoking somatic mutations (Fig. 5). A sequence comparison of IgM and IgG mAb revealed that our IgM mAb panel did not contain Ig_H sequences that were homologous to heavy chain variable regions of the IgG mAb.

Discussion

To date, generation and maturation of antibody responses have been principally studied using a variety of haptens as surrogate antigens. Such model systems have provided valuable insights into the mechanics of B cell responses. However, several unanswered questions remain, particularly with respect to more 'natural' antigens such as polypeptides. Given the inherent conformational flexibility and varied micro-environments, due to localized variations in amino acid composition, even a relatively small peptide is expected to provide a multiple array of determinants for B cell recognition. Thus, resolution of humoral responses against such antigens is likely to involve a greater degree of complexity than that against haptens. For example, immunization with polypeptide immunogens is expected to activate pre-immune B cells that are diverse not only in terms of clonality but also epitope fine-specificities. This raises the intriguing question of whether clonal selection of B cells directed against independent specificities operates in an autonomous fashion or whether it also includes interspecific competitive selection. In this connection the underlying basis for the observations of restricted immunodominance among the epitope repertoire presented by protein antigens continues to remain an enigma.

To probe some of these issues a designed, recombinant polypeptide MEP-1 was used. Prior studies had established that a segment representing residues 17–31 of the polypeptide constituted an immunodominant region of this molecule, accounting for at least 90% of the total primary antibody response to MEP-1. The MEP 17–31 segment therefore appeared to represent a good model determinant for study.

Immunization of mouse strains of a variety of H-2 haplotypes with a single dose of MEP-1 induced an early primary IgM response that was multispecific with respect to the MEP 17–31 segment. Dissection of the BALB/c IgM response into individual mAb revealed the presence of at least two distinct antigenic sub-segments, MEP 19–23 and MEP 26–28, within MEP 17–31 in the context of MEP-1. Interestingly, subsequent maturation was exclusively restricted to the MEP 19–23 region with loss of all alternate specificities. The fact that qualitatively similar results were obtained in mice of all

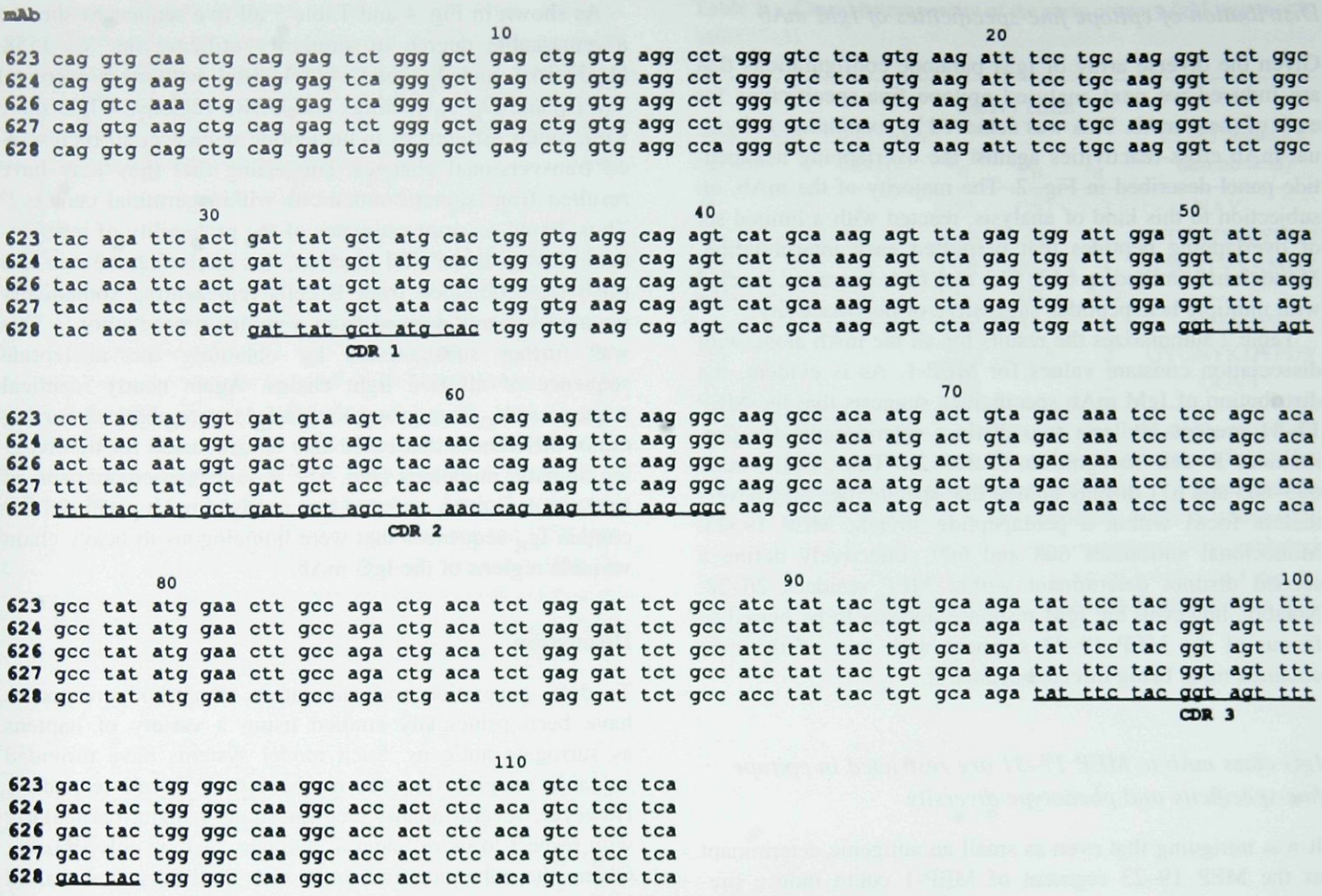

Figure 4 Nucleotide sequence of immunoglobulin heavy chain variable regions of mature anti-MEP 17–31 IgG mAb. The CDR regions are underlined. Numbering of codons is according to Kabat *et al.*[18]

Table 3 Clonal diversity of mature anti-MEP 17–31 IgG mAb

Monoclonal antibody	Antibody isotype		Heavy chain			CDR3 sequence (amino acid) of		K_d(mol/L)
	Ig_H	Ig_L	V_H	D_H	J_H	Ig_H	Ig_L	
623	2b	κ	J558	FL16.1	2	YSYGSFDY	QQGQSYPRT	4.5×10^{-9}
624	2b	κ	J558	FL16.1	2	YYYGSFDY	LQGQSYPRT	2.1×10^{-9}
626	1	κ	J558	FL16.1	2	YSYGSFDY	LQGQSYPRT	5.1×10^{-9}
627	2a	κ	J558	FL16.1	2	YFYGSFDY	QQGQIYPRT	9.2×10^{-9}
628	2a	κ	J558	FL16.1	2	YFYGSFDY	QQGQIYPRT	1.8×10^{-9}

IgG heavy chain isotypes were determined with the mouse monoclonal isotyping kit from Serotec (Oxford, UK). Light chain isotypes were determined by ELISA using appropriate antibodies. Dissociation constants were estimated using the procedure of Friguet *et al.*[15] and anchor residues determined as described in the text. Heavy chain V, D, J and CDR3 sequences are derived from Fig. 4.

four haplotypes suggests that MEP 19–23 dominance is independent of the genetic background of the mouse strain used.

The results described above clearly indicate that recognition by pre-immune B cells alone is insufficient to render an antigenic segment immunodominant. Thus for example, though the MEP 26–28 region does induce early primary IgM antibodies, it becomes cryptic in the later stages of the response. It is presently not clear as to why the early MEP 26–28 induced B cells are unable to continue in the response. A priori, however, their elimination could result from either competitive or non-competitive processes. We recently reported that mutation of the proline residue at position 21 (a residue critical for the MEP 19–23 epitope) for glycine completely abrogated the primary antibody response to MEP 19–23, with an overall 30-fold reduction in anti-MEP-1 titre.[7] This would seem to imply that loss of anti-MEP 26–28 specificities is not a consequence of competitive suppression by the anti-MEP 19–23 specific B cells, but rather they are autonomously regulated.

The existence of yet another level of selection became apparent when we examined the clonal heterogeneity of B cells participating in the early and late stages of the anti-MEP 19–23 response. The early IgM component was found to derive from an oligoclonal population of B cells as evidenced from the varied Ig_H V–D–J combinations obtained for the individual mAb. Interestingly, the divergent heavy chain

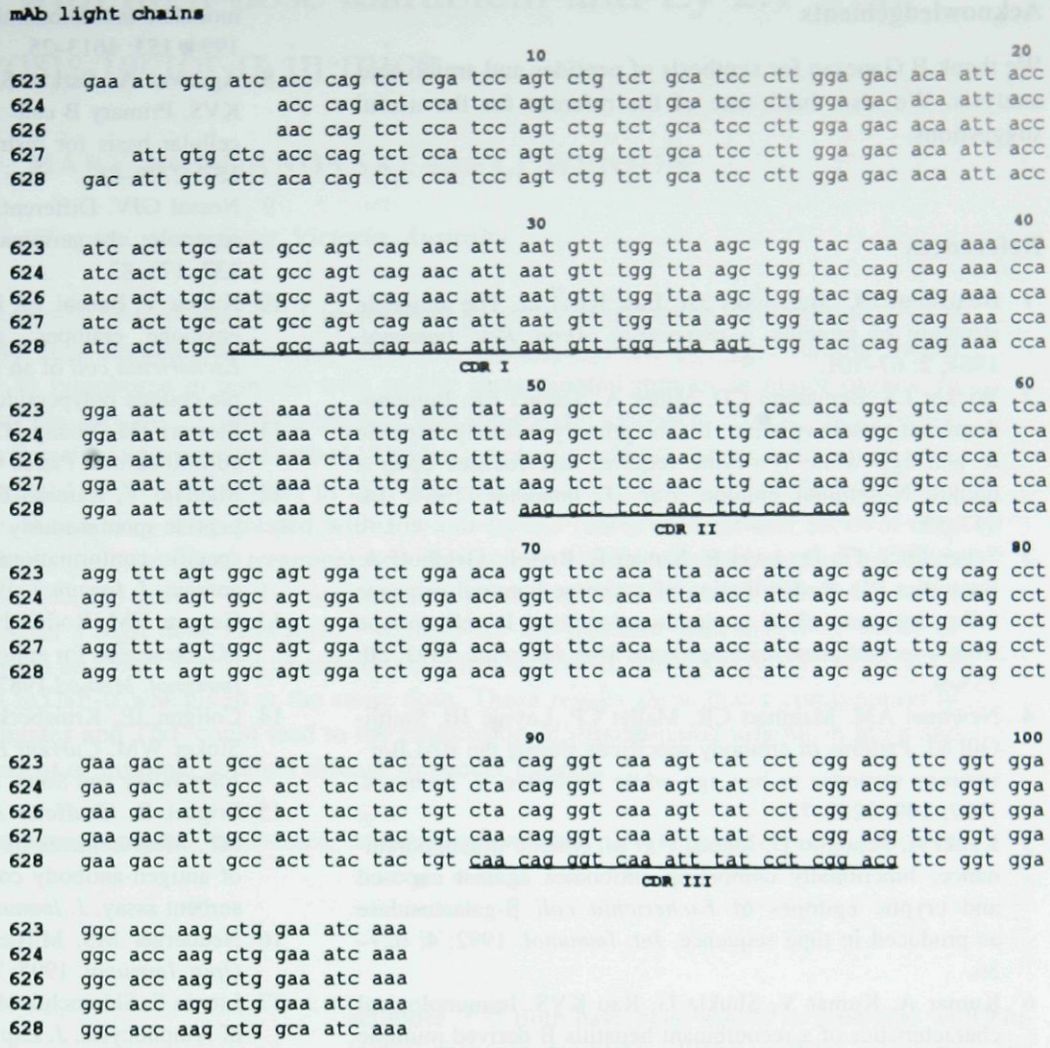

Figure 5 Nucleotide sequences of the light chains of each of the five IgG mAb obtained against MEP-1. The CDR regions are underlined. Numbering of codons is according to Kabat *et al.*[18]

CDR3 sequences obtained for these mAb were also indicative of phenotypic diversity of antibody paratopes; perhaps a reflection of some degree of conformational flexibility of MEP 19–23 segment in the context of MEP-1. In stark contrast, all five late stage IgG mAb obtained were found to utilize identical V_H, D_H and J_H gene segments with near identical V–D and D–J joinings. Furthermore, this identity also extended to the Ig light chains utilized by these mAb. These results thus provide evidence for an additional level of selection which is strictly intraspecific and seeks to restrict heterogeneity of the epitope-specific responder population during progression of the humoral response.

It is possible, though not certain, that clonotypes producing the five IgG mAb were derived from a common progenitor where minor differences in nucleotide sequence are a result of somatic mutations. This presumption is supported by the following observations: (i) prior estimates of the probability of independent identity at the VDJ junction[17] strongly suggest that these five IgG mAb are unlikely to have been a product of independently derived clonotypes; (ii) the consistent absence of *N* region additions at the V_H–D_H and D_H–J_H junctions is also indicative of clonal relatedness, though the origin of these cells needs to be ascertained; and (iii) finally, utilization of common light chain by all of these five mAb further argues against independent identity. Nevertheless, we caution that these are only inferential arguments and definitive evidence is presently lacking. If indeed these five IgG mAb-producing B cells share a common precursor then the diversity of antibody isotypes obtained is also intriguing. A plausible explanation for this could be that these five clones represent divergence from a common IgM producing progenitor with subsequent isotype switch occurring in an autonomous fashion.

To summarize, studies described here suggest that immunogenicity of a B cell determinant, at least at a functional level, is not solely dictated by its accessibility for B cell recognition. Rather, following the initial recognition event, immunological parameters come into play which restrict the antibody fine-specificities during subsequent maturation. Qualitatively, our results are consistent with prior observations of Newman *et al.*[4] In their studies on evolution of murine antibody responses to hen egg-white lysozyme they observed that the early response is not characterized by any particular specificity pattern but rather appeared to represent a random sampling from a continuum of antigenic determinants. In contrast, the late response was functionally grouped into discrete antigenic regions.[4] In addition, our data also provide evidence for a reduction in clonal heterogeneity of epitope-specific antibodies as the response progresses. What determines specificity selection during progression of a humoral response remains a question of considerable interest. Our own earlier results seem to suggest that the ability of individual antigen-activated B cells to recruit T cell help defines at least one of the discriminatory criteria.[7,8] Whether similar factors also regulate clonal heterogeneity of determinant-specific responses remains to be ascertained.

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