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B Cell Responses to a Peptide Epitope. X. Epitope Selection in a Primary Response Is Thermodynamically Regulated[1]

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We examine the etiological basis of hierarchical immunodominance of B cell epitopes on a multideterminant Ag. A model T-dependant immunogen, containing a single immunodominant B cell epitope, was used. The primary IgM response to this peptide included Abs directed against diverse determinants presented by the peptide. Interestingly, affinity of individual monomeric IgM Abs segregated around epitope recognized and was independent of their clonal origins. Furthermore, affinity of Abs directed against the immunodominant epitope were markedly higher than that of the alternate specificities. These studies suggested that the affinity of an epitope-specific primary response, and variations therein, may be determined by the chemical composition of epitope. This inference was supported by thermodynamic analyses of monomer IgM binding to Ag, which revealed that this interaction occurs at the expense of unfavorable entropy changes. Permissible binding required compensation by net enthalpic changes. Finally, the correlation between chemical composition of an epitope, the resultant affinity of the early primary humoral response, and its eventual influence on relative immunogenicity could be experimentally verified. This was achieved by examining the effect of various amino-terminal substitutions on immunogenicity of a, hitherto cryptic, amino-terminal determinant. Such experiments permitted delineation of a hierarchy of individual amino acid residues based on their influence; which correlated well with calculated Gibbs-free energy changes that individual residue side chains were expected to contribute in a binding interaction. Thus, maturation of a T-dependant humoral response is initiated by a step that is under thermodynamic control. ***The Journal of Immunology,*** **2000, 164: 5615–5625.**

The stochastic processes which drive both gene segment rearrangements and subsequent pairing in the generation of Ag receptors equip the host with a preimmune B cell repertoire that is collectively capable of recognizing a virtually limitless array of antigenic determinants (1, 2). It was this realization, along with the fact that B cells generally recognize protein Ags in their native form, that has led to the proposition that the entire accessible surface of a protein potentially represents an antigenic continuum (3).

Although the above notion represents a logical derivative of our understanding of parameters regulating B cell responses, it, however, appears to be inconsistent with experimental observations. Characterization of immune responses to a variety of protein Ags has revealed that only a fraction of the repertoire of B cell epitopes presented by such Ags is functionally capable of eliciting Abs (4–13). Some insight into this apparent discrepancy was provided by our own studies employing synthetic peptides as model T-dependent Ags (13–15). These investigations revealed that the primary IgM response was indeed consistent with expectations in that Abs were produced against all accessible domains on Ag (14). Interestingly however, these early activated clonotypes were soon subjected to a stringent selection process which ensured continued survival of only those induced B cells with a high enough affinity for Ag (14). This selection process preceded initiation of the germinal center (GC)[3] reaction, and was enforced by the need for the diverse Ag-activated B cells to compete for a limiting pool of Ag-primed Th cells (14). Although the precise mechanism is unclear, affinity for Ag was found to be crucial in defining ability of the individual clonotypes to recruit T cell help in a competitive milieu (14). Only those Ag-activated B cells selected at this stage were shown to be permitted to populate GCs, thereby influencing the character of secondary responses (16, 17). These observations could also be complemented, at the cellular level, by our demonstration that differential Th cell thresholds operate during Ag-driven activation of preimmune B cells and the subsequent selection step that restricts clonotype seeding of GCs (17, 18).

It is obvious, therefore, that the pre-GC phase selection process outlined above constitutes the first active step initiating maturation of T-dependent humoral responses to multideterminant Ags. However, although cellular interactions that drive this process were becoming apparent, there was one important facet that remained unresolved. This related to the question of whether there were any underlying factors regulating the affinity of Abs recruited from a pool of stochastically generated receptors. This issue became particularly relevant in light of our observations of an intriguing parallel wherein Ag-affinity dependent pre-GC phase selection also simultaneously resulted in a "filtering" of epitopes recognized on Ag (14, 17). Furthermore, relative immunodominance of an individual epitope subsequently proved to be resistant to a variety of predictable variables such as mouse H-2 haplotype, nature of flanking domains, positional effects, varying degrees of conformational flexibility, as well as any restriction at the level of B cell repertoires induced against it (13–15, 19). These and related results suggested, to us, that the affinity of early primary responses may be epitope dependent. In other words, the chemical composition of a

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[3] Abbreviations used in this paper: GC, germinal centers; s, surface; CD, circular dichroism; NMR, nuclear magnetic resonance.

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given epitope may constitute at least one parameter that dictates the affinity of primary Ab responses elicited against it.

The present set of investigations was, therefore, undertaken with the objective of verifying the above possibility. Results presented here, indeed, argue in favor of such a correlation. Furthermore, they also establish the functional significance of such distinctions in epitope-specific early primary Ab affinities, by demonstrating that they impinge upon the hierarchy of epitopic dominance observed in the later stages of humoral responses.

Materials and Methods

Materials

Heavy chain-specific, HRP-labeled, goat anti-mouse IgM and IgG were purchased from Sigma (St. Louis, MO). Anti-B7-2 (clone GL1) was purchased from PharMingen (San Diego, CA). For multipin synthesis of peptides, both the noncleavable kits and F-moc amino acid derivatives were obtained from Chiron Mimotopes (Victoria, Australia).

Peptide synthesis

Peptide PS1CT3 and all its analogues used in this study were synthesized by the solid-phase method (20, 21) using F-moc chemistry (22) on a Milligen 9050 automated peptide synthesizer (Millipore, Bedford, MA). Crude peptides were purified to at least 95% purity by reverse-phase HPLC on a C_{18} column (15μ δ pak, 19 $\times$ 300 mm; Waters, Milford, MA) using an aqueous gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The identities of all peptides were ascertained by mass spectrometry.

Overlapping hexapeptide panels were synthesized according to the method of Geysen et al. (23) on noncleavable multipin kits. For synthesis, the standard protocol recommended by the manufacturer was adhered to. After completion of synthesis, all peptides were routinely acetylated at the amino terminus with a 50:5:1 (v/v/v) mixture of dimethylformamide, acetic anhydride, and triethylamine. Side chain deprotection was accomplished over a 2-h period at room temperature with a 38:1:1 (v/v/v) mixture of trifluoroacetic acid, ethanedithiol, and anisole.

Animals and immunizations

Female BALB/c mice (6–8 wk old) were obtained from the small animal facility at the National Institute of Nutrition (Hyderabad, India). Except where stated, immunizations were generally given i.p. at a dose of 50 μg/mouse as an emulsion in CFA. For polyclonal sera, mice were bled from the retro-orbital plexus, and sera within a group were pooled. In all cases, the IgM and IgG fractions were separated by passing over a protein G-Sepharose column (Pharmacia, Uppsala, Sweden). The flow through contained IgM, whereas bound IgG, after thorough washing of the column, was eluted with glycine-HCl buffer (pH 2.7). The eluate was immediately neutralized, concentrated, and then dialyzed against PBS before use.

Affinity purification of Abs

All IgM mAbs described were obtained as ascites from hybridomas that had been generated earlier (14, 15). These mAbs represent primary IgM responses to the PS1 segment at 4 days after a primary immunization of BALB/c mice (14, 15). Each of the IgM preparations was purified by affinity chromatography over an Affi-Gel 10 Gel (Bio-Rad, Hercules, CA) column coupled to peptide PS1CT3 at 10 mg/ml of gel. The ascites preparation was incubated with the affinity matrix overnight at 4°C on a rotating shaker. After this, the flow through was removed and the column was washed with 10 column volumes of PBS (pH 7.2), followed by 10 column volumes of phosphate buffer containing 1 M NaCl, and finally again with 5 column volumes of PBS. Bound IgM was then eluted in glycine-HCl buffer (pH 2.7) and then immediately neutralized with 1 M Tris buffer (pH 8.0). The neutralized eluate was subjected to buffer exchange into 0.5 M Tris buffer containing 2 mM EDTA (pH 8.0) and concentrated to a final IgM concentration of 10 mg/ml. Purity of the IgM fraction was ascertained by SDS-PAGE.

For peptide-specific polyclonal IgM, sera were first depleted of IgG Abs by two rounds of incubation with protein G-Sepharose. The flow through fraction was further enriched for IgM by chromatography over DEAE-cellulose (Sigma). The IgM-containing fraction was then purified by affinity chromatography for Abs specific either for peptide PS1CT3 or, as required, its analogues as described above.

Preparation of monomeric IgMs

This was essentially achieved using a standard protocol (24). Briefly, affinity-purified IgM Abs obtained above were reduced with a final concentration of 1 mM DTT at room temperature for 1 h with gentle shaking. After this, reduced IgM was alkylated with a final concentration (added in one-tenth the volume) of 2.1 mM iodoacetamide in the dark at 4°C for 30 min. At the end of this period, the mixture was resolved over a Sephadex G-200 column (Sigma; column dimensions, 25 cm $\times$ 1.5 cm; load volume, 0.5 ml) using blue dextran as a marker for the void volume. Fractions containing monomeric IgM, which elutes immediately following the void volume, were pooled and concentrated to between 10 and 40 mg/ml in PBS containing 0.05% Tween 20. Ab concentrations were determined spectrophotometrically by measuring absorbance at 280 nm. An average molecular mass of 200 kDa was taken for monomeric IgM (24).

Analysis of monomeric IgM binding to peptide PS1CT3

Monomeric mAb binding to peptide PS1CT3 was examined according to the technique of surface plasmon resonance on an Iasys Auto$^+$ instrument (Affinity Sensors, Cambridge, U.K.). Peptide was immobilized onto carboxymethyl dextran cuvettes using the protocol recommended by the manufacturer. Ab binding to immobilized peptide was monitored at multiple Ab concentrations, ranging from 5-fold below to at least 5-fold above preliminary estimates of equilibrium dissociation constants (K_d) for each mAb. All reactions were conducted at 25°C. Association was monitored over a period of 7–10 min, whereas dissociation was monitored for 5 min. Kinetic analysis was performed using the FASTfit software (Affinity Sensors, Cambridge, U.K.) provided by the manufacturer, which provided the values of k_{on} and k_{diss} at each Ab concentration. Second-order association rate constants (k_{ass}) were then obtained from a linear regression plot of k_{on} vs Ab concentration, again using the FASTfit software. Equilibrium affinity constants (K_a) were then derived, for each mAb, from the equation: $K_a = k_{ass}/k_{diss}$.

For a thermodynamic analysis of peptide-mAb association, monomer Abs were used at a single concentration that was 5-fold above its K_d value. The association reaction for each mAb was studied, as described above, at four different temperatures of 20, 25, 30, and 35°C. The corresponding k_{on} values were obtained, from which the apparent association rate constant for each Ab at each temperature was obtained by dividing it with Ab concentration.

ELISAs

Plates were coated with 2 μg/ml of peptide per well in 100 μl of PBS (pH 7.2) at 37°C for 3.5 h. Subsequently, they were blocked with 300 μl/well of a 5% solution of fat-free dry milk powder in PBS at 37°C for 1 h. Then 100 μl of the appropriate dilution of mouse antiserum was added and incubated at 37°C for 1 h. After washing, bound Ab was detected with HRP-labeled secondary Ab (37°C, 1 h), followed by color development with *o*-phenylenediamine as chromogen. Absorbance was measured at 490 nm, and background absorbance obtained for preimmune serum at the corresponding dilution was subtracted.

For competitive ELISA experiments, the procedure described earlier was followed (14, 19). Briefly, the purified IgG fraction, at concentrations 2-fold that of the 50% titer value, was mixed with appropriated concentrations of competitor peptide in equal volumes and incubated for 10 min at room temperature. This was then added to duplicate wells at 100 μl/well. The remaining procedure is as described above.

ELISAs for pin-bound hexapeptides

Ab cross-reactivity with pin-bound hexapeptides was also evaluated by ELISA. For this, the protocol recommended by the manufacturer was strictly followed. Primary Abs were diluted appropriately in PBS containing 2% BSA, 0.1% Tween 29, and 0.1% sodium azide. Pins were incubated in 200 μl each of the Ab solution at 4°C overnight with gentle shaking. Subsequently, they were washed and subjected to a second round of incubation with the appropriate dilution of HRPO-labeled goat anti-mouse IgG at room temperature for 1 h, again with gentle shaking. The chromogen used for detecting bound Ab was 2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid)diammonium, and the absorbance was measured at 405 nm after subtracting the background at 490 nm.

Calculation of amino acid side chain ΔG values

Specific side chain contribution from residues in interacting domains derives from two distinct types of interactions: hydrophobic and electrostatic. If one assumes that side chain entropy changes, when engaged in an interaction, are negligible, the Gibbs-free energy changes (ΔG) accompanying idealized interactions for a given residue side chain can be described as, $\Delta G_{total} = \Delta G_{HB} + \Delta G_{EL}$ (25). Here, ΔG_{HB} represents the energy released through hydrophobic interactions and has been estimated to be -25 cal/

HQLDPAFGANSTNPDGGDIEKKIAKMEKASSVFNVVNS

FIGURE 1. Amino acid sequence of peptide PS1CT3. The primary amino acid sequence, in the single-letter code, is given for the model peptide PS1CT3. The bars above and below the PS1 segment (residues 1–15) represent individual domains recognized by the IgM mAbs listed in Table I.

mol/A^2 of surface area (25, 26). Since side chain area for all individual residues are known (26), ΔG_{HB} is easily calculated. ΔG_{EL} represents the enthalpy change due to electrostatic interactions. For the present case, where we have assumed idealized interactions, the maximal value of -5.0 kcal/mol was taken for each salt-bridging interaction (27). Thus, from the values of ΔG_{HB} and ΔG_{EL}, ΔG_{total} was easily calculated for each individual amino acid residue side chain.

Results

Characterization of primary IgM responses to peptide PS1CT3

We continued to employ the model peptide PS1CT3 for our current studies. As previously described (14), this peptide represents a T-dependent immunogen and is comprised of a well-characterized B (residues 1–15, segment PS1) and a T (residues 18–38, segment CT3) cell epitope. Separating these two domains is a spacer of two glycine residues at positions 16 and 17 (Fig. 1). Our earlier results have demonstrated that the murine primary IgM response to this peptide was composed of individual Ab specificities that collectively spanned the entire PS1 segment (14). Interestingly, however, subsequent class switch to IgG was restricted to only those Ab subsets directed against a unique tetrapeptide epitope between positions 4 and 7 of the PS1 segment (sequence: DPAF, Fig. 1) (14). This apparent monospecificity, which derived from an oligoclonal B cell population, was also shown to be retained in the secondary IgG response (14, 19, 28).

In the course of our earlier characterization of the murine primary immune responses to the PS1 segment, we had generated a panel of IgM class mAbs derived from fusions performed at 4 days after a primary immunization of BALB/c mice (14, 15). Subsequent epitope-mapping studies had confirmed that these mAbs constituted a heterogenous set, including specificities for a diverse set of determinants within the PS1 segment as shown in Fig. 1. As an initial exploration, we asked whether these individual mAbs displayed any qualitative differences in binding to their respective epitopes on peptide PS1CT3. To do this, however, we first performed a reduction reaction on each of these IgM mAbs to obtain monomeric IgMs (see *Materials and Methods*). The objective in doing this was 2-fold. At one level it was expected that a bivalent IgM molecule would allow for a greater degree of resolution of differences in Ag-binding properties that might normally be masked due to the increased valency of pentameric IgM. More importantly though, we reasoned that monomeric IgM would also be reminiscent of the surface (s) IgM receptor on preimmune B cells, thereby providing an insight into Ag recognition by preimmune B cells.

Each of the individual monomeric IgM preparations obtained were subsequently analyzed for their concentration-dependent binding to peptide PS1CT3. For this, we employed the technique of surface plasmon resonance (see *Materials and Methods*), and representative data for one such monomeric IgM preparation is shown in Fig. 2. Analyses of these data yielded values for the association (k_{ass}) and dissociation (k_{diss}) rate constants, from which the equilibrium affinity constant (K_a) could readily be determined for each mAb as described in *Materials and Methods*. The monomeric IgM preparations analyzed, their corresponding epitope specificities, as well as their Ag-binding properties are listed in Table I. At the first glance, it is interesting to note that this monomer mAb panel represents a wide distribution of affinities with K_a values ranging from 10^4 to 10^6 M^{-1} (Table I). Considering that these mAbs were derived from B cells actively participating in a primary response (14, 15), these results would then suggest that Ag-binding affinity constants of as low as 10^4 M^{-1} are sufficient to induce preimmune B cells into a primary Ag-specific IgM response. This is entirely consistent with our previous demonstration that induction of a primary IgM response involves low activation thresholds (18).

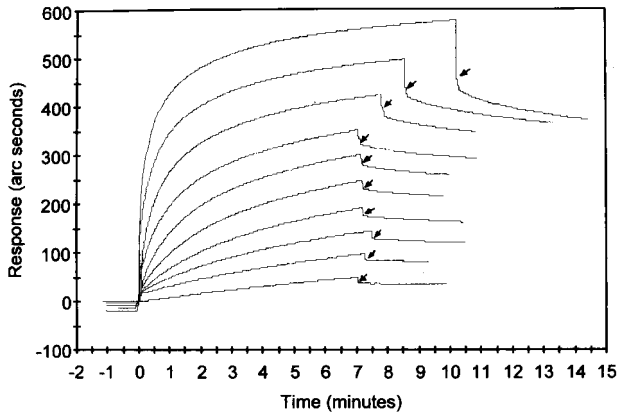

FIGURE 2. Binding profiles of a monomeric IgM mAb to peptide PS1CT3. Depicted are the association and dissociation curves for the interaction of a representative monomeric IgM mAb (7bM) to immobilized peptide PS1CT3 over a concentration range of 20–2000 nM. The arrows for each plot mark commencement of the dissociation reaction. For experimental details refer to *Materials and Methods*.

A particularly intriguing feature of the data in Table I is the fact that Ab affinities were not randomly distributed across the various

Table I. *Binding properties of primary monomeric IgM mAbs to peptide PS1CT3*[a]

mAb	Epitope Specificity	$k_{ass} \times 10^{-3}$ ($M^{-1}s^{-1}$)	$k_{diss} \times 10^{3}$ (s^{-1})	$K_a \times 10^{-5}$ (M^{-1})
605	LDPA	2.40	21.00	1.10
601	DPA	0.74	8.47	0.87
603	DPA	0.88	8.96	1.02
606	DPA	0.78	12.00	0.65
7bM	DPAF	4.80	1.50	32.00
7cM	DPAF	18.00	7.10	25.40
604	DPAF	18.20	9.20	19.80
16b	PAF	0.27	8.00	0.34
6aM	FG	0.13	3.00	0.42
613	FG	0.80	22.00	0.36
19M	FG	0.85	17.90	0.47
609	NST	0.30	9.20	0.33
42M	NSTN	2.10	17.00	1.20
8aM	NSTN	0.19	1.40	1.36
49M	NSTN	1.90	8.10	2.30

[a] Listed are the individual monomeric IgM preparations analyzed for peptide PS1CT3 binding by the technique of surface plasmon resonance as described in *Materials and Methods*. Fine specificity of these mAbs was determined by mapping against an overlapping PS1-derived hexapeptide panel as described previously (14). Values given for k_{ass} and k_{diss} (*Materials and Methods*) are the mean of two separate determinations, where the variation was <30% in all cases. The equilibrium affinity constant (K_a) was obtained by dividing k_{ass} with the value for k_{diss} (*Materials and Methods*).

epitopes but, rather, were segregated around the epitopes recognized. Thus, for example, all anti-DPAF monomer IgM mAbs displayed comparable affinities for epitopes, which was markedly higher than that of those directed against alternate determinants within the PS1 segment (Table I). Similarly, the various NSTN-specific mAbs were also comparable in their affinity for the peptide (Table I). It must be noted here that the anti-DPAF mAbs 7bM, 7cM, and 604 derive from clonally distinct B cell precursors as revealed by nucleotide sequencing of the Ig heavy chain variable region genes. Thus, the predicted amino acid sequences of the heavy chain CDR3 regions for mAbs 7bM, 7cM, and 604, respectively, were as follows: QRTIGTPGAY, WAWFAY, and IPITT ATNWYDFV (Ref. 15 and B. P. Nayak and K. V. S. Rao, unpublished results). This was also true of the anti-NSTN mAbs 42M, 49M, and 8aM where, again, nonidentical IgH CDR3 sequences were obtained (B. P. Nayak and K. V. S. Rao, unpublished results). Thus, the comparable affinities displayed by mAbs directed against a common epitope is not necessarily a consequence of clonal relatedness of the B cells from which they were derived.

Association between Ag and monomeric IgMs is driven by favorable enthalpy changes

The overall energetics and, therefore, the affinity of a binding interaction is modulated by net changes in two thermodynamic parameters, namely, enthalpy and entropy. Although the enthalpy term generally describes heat changes that take place as a consequence of interactions which occur at the binding interface, entropy changes largely represent net conformational/stereochemical/structural perturbations that occur either within the interacting entities or in the surrounding solvent molecules (29, 30). Therefore, to understand the basis of epitope-dependent distinction in monomer IgM affinities seen in Table I, we examined the relative roles of enthalpy and entropy in regulating association between Ab and Ag.

All mAbs listed in Table I were extensively analyzed for contribution of enthalpic and entropic parameters in binding to peptide PS1CT3. Even though the detailed results will be presented elsewhere (V. Manivel, P. Nakra, N. Sahoo, and K. V. S. Rao, manuscript in preparation), we discuss here results obtained for the association phase of the reaction with four representative monomeric IgM mAbs. For this, Ab association with peptide was studied at various temperatures and the k_{ass} value at each temperature was determined (see *Materials and Methods*). Fig. 3 gives the Arrhenius plot for the temperature dependence of k_{ass} for the four individual Ab preparations. The slope of each plot yielded the activation energy (Ea) for each of the mAbs. This then permitted calculation of the individual activation parameters for association using equations 1–3 given below (see Ref. 31):

$$\Delta H = Ea - RT \quad (1)$$

$$\ln (k_{ass}/T) = -\Delta H_{ass}/RT + S_{ass}/R + \ln (k'/h) \quad (2)$$

$$\Delta G_{ass} = \Delta H_{ass} - T\Delta S_{ass} \quad (3)$$

In the above equations, k_{ass} represents the association rate constants obtained at 25°C, T is the temperature in degrees Kelvin, k' is the Boltzmann constant, and h is the Planck constant. Results from such analyses for the four representative monomer IgM mAbs are presented in Table II. It is evident from these data that, in all cases, association of monomeric IgM with peptide is accompanied by large unfavorable changes in entropy as indicated by the large negative values obtained for ΔS_{ass} (Table II). In contrast, the negative ΔH_{ass} values clearly identify that enthalpy changes accompanying Ab-Ag association were highly favorable in all cases (Table II). Similar results were obtained for all of the monomeric IgM mAbs listed in Table I (data not shown). Consequently, the driving force for Ag-monomeric IgM association appears to derive from stabilizing interactions at the paratope-epitope interface, and can occur only if these forces can compensate for the unfavorable entropy changes that such an interaction necessitates. Because these studies employed monomeric IgM mAbs, this interpretation could also, in principle, be extended to apply to the sIgM receptor on preimmune B cells.

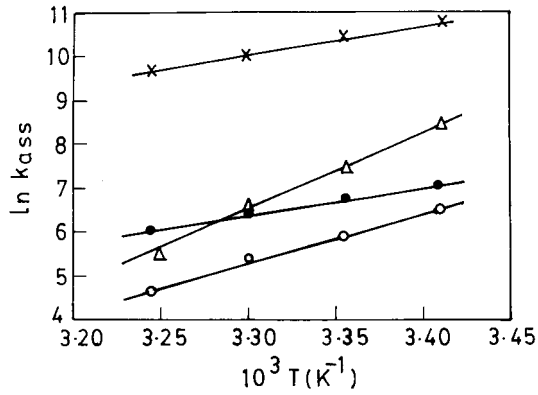

FIGURE 3. Arrhenius plots of the association reaction of the interaction between monomeric IgM mAbs and peptide PS1CT3. Natural log values of association rate constants of monomeric IgM mAbs 7bM (x), 42M (●), 8aM (○), and 19M (△) at various temperatures (see *Materials and Methods*) are plotted against $1/T \times 10^{-3}$. T represents the temperature in degrees Kelvin. All rate constants are the mean values of three independent runs. Slopes yield the Ea for the association step for each monomeric IgM mAb.

Thus, although the data in Table I reveal the existence of a relationship between affinity of primary recognition of Ag and the chemical nature of the epitope recognized, results summarized in Table II rationalize this observation by implying that this may derive from differential epitope-dependent enthalpic contributions.

Distinctions in affinity of preimmune recognition influence relative immunodominance in the later stages of the response

The influence of affinity of primary epitope-specific humoral responses on the eventual hierarchy of relative immunodominance, in the context of multideterminancy, was suggested from our earlier studies employing single residue glycine substitutions with the DPAF epitope of peptide PS1CT3 (14). To further confirm that the former does, indeed, impact upon the latter, we synthesized three additional analogues of peptide PS1CT3. In one, Asp (4) was replaced with an Asn residue (peptide PS1CT3N31), whereas Phe (7) was substituted for Leu in another (peptide PS1CT3L34). The

Table II. *Thermodynamic parameters regulating association of antigen with monomeric IgM*[a]

mAb	ΔH_{ass} (kJM^{-1})	ΔS_{ass} ($JK^{-1}M^{-1}$)	ΔG_{ass} (kJM^{-1})
7bM	−33.98	−286.53	50.91
42M	−27.68	−272.07	53.40
8aM	−48.68	−361.37	59.00
19M	−78.06	−450.75	56.26

[a] Ea for the association of each of the monomeric IgM mAbs listed here were determined as the slopes of the Arrhenius plots shown in Fig. 3. From this, the enthalpy (ΔH_{ass}), entropy (ΔS_{ass}), and Gibbs-free energy (ΔG_{ass}) changes accompanying the association reaction were calculated using equations 1 to 3 described in the text.

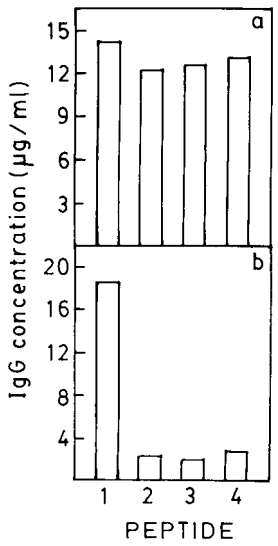

FIGURE 4. Relative immunodominance of peptides PS1CT3 and its DPAF-substituted analogues. Groups of five mice each were immunized with 10 nmol/mouse each of either peptide PS1CT3 (peptide1), PS1CT3N31 (peptide 2), PS1CT3L34 (peptide 3), or PS1CT3N31L34 (peptide 4). Four weeks later, the mice were bled, sera within a group were pooled, and peptide-specific IgG titers were estimated by quantitative ELISA (*a*). A parallel group of mice was also immunized with a mixture containing 10 nmol/mouse of each of the above peptides, followed by determination of individual peptide-specific IgG titers 4 wk later (*b*). Data are from one of two separate experiments.

third analogue represented a doubly substituted peptide where both Asp (4) and Phe (7) were substituted with Asn and Leu residues, respectively (peptide PS1CT3N31L34). The rationale for such substitutions was to specifically alter the chemical nature of the corresponding residue side chains, but without significantly affecting their steric bulk. The side chain areas for Asp and Asn are 106 A^2 and 113 A^2, respectively, whereas those for Phe and Leu are 175 A^2 and 137 A^2 (25).

In preliminary experiments, separate groups of mice were immunized with the individual peptides and the consequent peptide-specific IgM Abs were isolated from sera collected 7 days later. Monomeric derivatives were then prepared (see *Materials and Methods*), and relative avidity for the homologous peptide was determined by competitive inhibition ELISA experiments. These experiments yielded IC_{50} values of 14 ± 3 μM, 72 ± 8 μM, 64 ± 5 μM, and 88 ± 9 μM for anti-PS1CT3, anti-PS1CT3N31, anti-PS1CT3L34, and anti-PS1CT3N31L34 monomeric IgM preparations, respectively. Thus, the avidity of primary recognition is indeed influenced by the amino acid composition of Ag.

We next immunized mice with an equimolar mixture of peptide PS1CT3 and the three analogues described above. Since all of these peptides encode a common T cell epitope (segment CT3), immunization with the mixture was expected to result in activation of diverse B cells directed against determinants presented by the individual peptides, but all requiring help from a common pool of CT3-specific Th cells. In other words, such a system would simulate the situation obtained with more complex multideterminant Ags presenting a wide array of B cell epitopes. For comparative purposes, additional groups of mice were also immunized with the individual peptides. The subsequent IgG responses, obtained 4 wk later, were then analyzed and the data are presented in Fig. 4. An intriguing dichotomy can be noted in immunogenicity of the analogue peptides when immunized individually, as opposed to immunization in a mixture (Fig. 4). Individual immunizations yielded comparable IgG levels for all four peptides (Fig. 4*a*). In contrast, immunogenicity of these analogues was markedly suppressed, relative to that of PS1CT3, in the mixture (Fig. 4*b*). This attenuation of immunogenicity of the analogues, in a mixture, is well in keeping with the poorer avidity of monomeric IgM responses elicited by them in comparison to that against the parent peptide PS1CT3.

The results described so far, therefore, cumulatively argue for the existence of a correlation between the chemical composition of an epitope, the avidity of its recognition in an early primary response, and the resultant effect on relative immunodominance, in the context of multideterminancy, in the later stages of the response.

Hierarchical contribution of individual amino acid residues toward epitopic dominance

We also adopted an alternate strategy to further confirm the existence of a relationship between chemical composition of an epitope on a multideterminant Ag and its position in the hierarchy of relative immunodominance in a humoral response. For this, we synthesized analogues of peptide PS1CT3 where the amino-terminal His residue was independently replaced with each of the 19 remaining naturally occurring α amino acid residues. As shown earlier (14, 19, 28), the amino-terminal PS1-derived hexapeptide normally represents a cryptic determinant in the primary anti-PS1CT3 IgG response. Our objective, therefore, was to assess the efficacy of each of the various substitutions in creating a novel amino-terminal epitope. It was anticipated that such an approach would also provide information as to whether individual residues do in fact contribute variably toward immunogenicity of a given epitope on a multideterminant Ag.

Subsequent to synthesis of these N-terminal substituted analogues of peptide PS1CT3, we first ensured that these changes did not lead to any secondary structural changes in the resulting molecule. This was achieved by circular dichroism (CD) spectroscopy. As earlier described, peptide PS1CT3 exists in a random distribution of conformations in solution (14, 27). This "randomness" was found to be unaffected in all analogues synthesized, and CD spectra of representatives are shown in Fig. 5. Although CD studies do not provide information on subtle changes in structure, these results nevertheless confirm that the substitutions performed did not lead to imposition of any gross structural constraint.

The amino-terminal substituted analogues of peptide PS1CT3 were separately immunized into BALB/c mice, and the resulting IgG responses were analyzed for distribution of epitope-fine specificities. This was achieved by screening against appropriate overlapping hexapeptide panels derived either from PS1 or its analogous segment. Fortunately for us, in all cases, the polyclonal IgG response was found to be restricted toward the amino-terminal half of the PS1 segment. There was no appearance of any reactivity toward the carboxyl-terminal half of the sequence, the only variable between the analogues being the extent of cross-reactivity with the amino-terminal hexapeptide. Where detected, the latter reactivity always depended on the presence of the appropriate, homologous, residue at the amino terminus, as substitution with any other completely abrogated this reactivity (data not shown). Following this initial screen, the relative proportion of DPAF-specific vs amino-terminal hexapeptide-specific Abs in the sera were estimated by saturation inhibition ELISA experiments with homologous and the parent PS1CT3 peptide.

A representative analysis for three separate substitutions is shown in Fig. 6. It is evident from the data presented that the

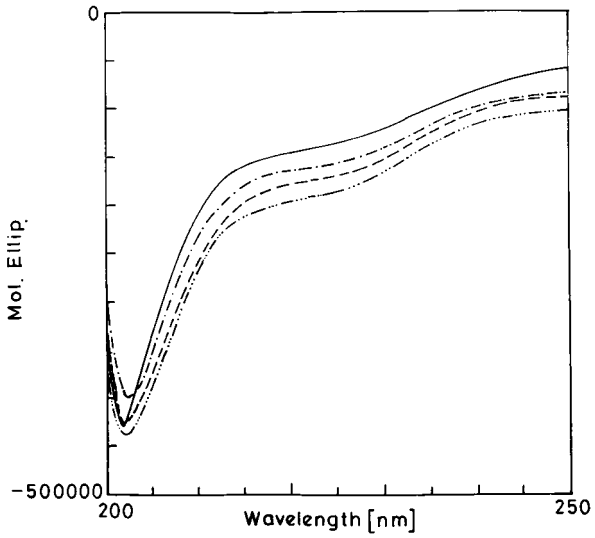

FIGURE 5. CD spectra of representative amino-terminal-substituted analogues of peptide PS1CT3. CD spectra, obtained at 6 μM concentrations in PBS, of peptide PS1CT3 (—) or its analogues containing amino-terminal substitutions with either Arg (– · –), Trp (– ··· –), or Gly (–) are shown. Spectra were recorded on a Jasco model J710 spectropolarimeter (Jasco, Tokyo, Japan) over a wavelength range of 200–250 nm. A step resolution of 0.1 nm and a scan speed of 200 nm/min were employed. Spectra shown are those averaged over a total of 30 accumulations.

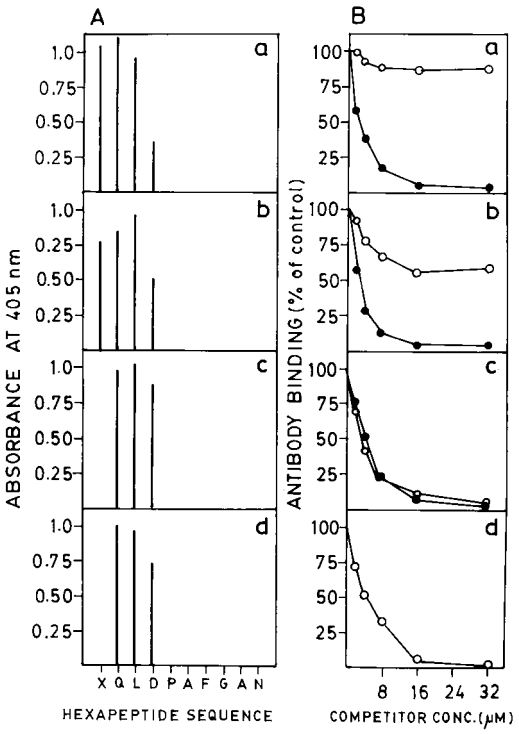

FIGURE 6. Characterization of late-stage primary IgG responses to representative amino-terminal-substituted analogue peptides. *A*, An analysis for distribution of epitope-fine specificities by screening against the homologous overlapping hexapeptide panel by ELISA (see *Materials and Methods*). Sera used were obtained at day 42 after a primary immunization of mice with each peptide. The *x*-axis denotes each hexapeptide as its N-terminal residue, except in the first position (identified as X) which represents the appropriate amino-terminal substitution in each of these cases. *B*, The results of a saturation inhibition ELISA for the above serum preparation using either the homologous analogue peptide (●) or peptide PS1CT3 (○) as the competitive inhibitor (see *Materials and Methods*). Results shown are from one of three experiments from independent immunizations. Analogues for which data are shown here are those containing amino-terminal substitutions of either Arg (*a*), Trp (*b*), or Gly (*c*). Data for the parent peptide, PS1CT3, are shown in *d*.

proportion of amino-terminal reactivity obtained is, indeed, dependent on the nature of substitution performed at the N-terminal position. Thus, the presence of an Arg residue at this position was most conducive for a maximal shift in immunodominance away from the DPAF epitope and toward the amino terminus (Fig. 6). In contrast, substitution for a Gly residue at this position had no significant effect (Fig. 6).

To rationalize the distinctions observed in Fig. 6, and in light of the observations in Tables I and II, we reasoned that any variable effects of individual amino acid residues would probably emanate from nonidentical contributions of their side chains, toward energetically stabilizing complexation with the corresponding Ab. In principle, this argument could also be extended to binding of an epitope with the sIg receptors on specific B cells. Therefore, to explore for such a possible correlation, we also calculated the total potential free energy that each of the 20 various amino acid residue side chains can contribute in a binding interaction (see *Materials and Methods*). The cumulative comparison of values thus obtained for each residue, with its effect on relative immunodominance of the amino-terminal hexapeptide segment is shown in Table III. A general correlation between the potential energetic contribution of side chain of a given residue toward binding and the proportion of shift in immune recognition toward the amino terminus is distinctly evident (Table III). All residues with side chain ΔG values of negativity $\geq$5.0 induced a significant shift in Ab specificity toward amino-terminal recognition. In contrast, those with ΔG values of negativity $\leq$4.0 proved ineffective in this regard (Table III).

To confirm that the observed differences in Table III do correlate with differences in avidity of primary recognition, primary IgM responses were obtained against a representative set of peptides described in Table III. These preparations were then resolved for Abs specific for the amino-terminal segment vs those directed against the remainder of the sequence by sequential affinity chromatography. Resulting IgM fractions were reduced to their monomeric forms, and the relative avidity of these preparations for the homologous peptide was determined by competitive inhibition ELISA. Results from such experiments are given in Table IV. It is clear that those substitutions which yielded a significant shift in epitopic immunodominance toward the amino terminus in Table III were also those that produced amino-terminal-specific monomeric IgM Abs of an avidity that was significantly higher than that obtained against the rest of the sequence (Table IV). In contrast, substitutions that had a minimal effect on epitope specificity of the IgG response elicited cognate monomeric IgM Abs of avidities that were indistinguishable from that generated against the rest of the sequence (Table IV).

The influence of amino acid composition of epitope is limited by accessibility of its side chains

A notable discrepancy with the hierarchy shown in Table III was that of the parent peptide PS1CT3. Although results presented both here and earlier (14, 19, 28) demonstrated that the amino-terminal segment constituted a cryptic determinant in primary responses, this was incompatible with the estimated value of ΔG_{total} of -6.275 for the His side chain (Table III). To explore the basis for this, we examined the ^{1}H-nuclear magnetic resonance (NMR) spectrum of peptide PS1CT3. Intriguingly, we observed here that

Table III. *Effect of N-terminal substitutions on relative immunogenicity of the amino-terminal determinant*[a]

Substituted Residue	N-terminal Reactivity (% of total)	Side Chain ΔG_{total} (kcal/mol)
Arg	85 ± 4	−9.900
Lys	80 ± 7	−9.175
Glu	76 ± 5	−8.450
Asp	66 ± 8	−7.825
His[b]	54 ± 6	−6.275
Trp	55 ± 6	−6.125
Tyr	43 ± 9	−5.675
Phe	38 ± 7	−5.075
Met	11 ± 3	−4.000
Ile	13 ± 4	−3.500
Gln	10 ± 4	−3.450
Leu	12 ± 3	−3.425
Val	11 ± 3	−2.925
Asn	ND[c]	−2.825
Pro	ND[c]	−2.625
Cys	ND[c]	−2.600
Thr	ND[c]	−2.550
Ser	ND[c]	−2.000
Ala	ND[c]	−1.675
Gly	ND[c]	0.0

[a] As described in the text, N-terminal-substituted analogues of peptide PS1CT3 were immunized in BALB/c mice and pooled sera were collected at day 42 for subsequent analysis as shown in Fig. 6. The extent of inhibition with the parent peptide PS1CT3 was taken to represent the proportion of Abs directed against amino-terminal-independent epitopes. By subtraction of this, the relative proportion of Abs directed to the amino-terminal epitope (expressed as percentage of total Abs) was then calculated. Data for the His residue were obtained from immunization with the acetylated peptide PS1CT3. Values are the means (±SD) of determinations with at least three independently derived serum preparations from groups of five mice each. Side chain ΔG_{total} values were calculated as described in *Materials and Methods.*

[b] N-terminal reactivity results shown are for the acetylated derivative of peptide PS1CT3 (see text).

[c] ND, Not detectable.

Table IV. *Relative avidities of N-terminal-specific primary monomeric IgM Abs in representative analogues*[a]

N-Terminal Substitution	IC_{50} of Monomeric IgMs Against (μM)	
	N terminus	Others[b]
Arg	2.1 ± 1.2	28.0 ± 8.0
Asp	4.2 ± 2.3	22.0 ± 6.4
Trp	9.4 ± 3.2	34.0 ± 8.3
Gln	32 ± 5.0	32 ± 6.8
Ser	31 ± 3.6	24 ± 5.0
Ala	28 ± 4.0	26 ± 6.0

[a] Groups of 20 BALB/c mice each were individually immunized with each of the amino-terminal-substituted analogues of peptide PS1CT3 listed here. One week later, blood was collected and sera within a group were pooled. Each serum preparation was then depleted of any IgG Abs using protein G-Sepharose as described in *Materials and Methods.* After this, Abs directed against determinants common to the parent PS1CT3 sequence were first purified by two rounds of affinity chromatography over a matrix coupled to peptide PS1CT3 (*Materials and Methods*). Subsequently, amino-terminal-specific IgM Abs were also purified from the flow through fraction by two rounds of affinity chromatography over a matrix coupled to the homologous peptide (*Materials and Methods*). The two individual IgM fractions, for each peptide, were concentrated and reduced to monomeric IgM as described in *Materials and Methods.* Relative avidities of the monomer IgM fractions for the homologous peptide were determined by competitive inhibition ELISA, with preparations normalized for an Ab concentration of 2.0 μg/ml (*Materials and Methods*). Results shown are a representative of two independent experiments, and values for IC_{50} are the means (±SE) of triplicate determinations.

[b] Indicates Abs directed against determinants common to the parent peptide PS1CT3 (i.e., that IgM fraction which bound to a peptide PS1CT3-containing column).

signals for the protons at C2 and C4 positions of the imidazole ring of His were shifted significantly downfield from the expected values (32). As shown in Fig. 7*A*, signal for the proton at the C2 position appears at 8.73 ppm, whereas that for the C4 proton appears at 7.36 ppm. These signals are downfield of the corresponding shifts obtained for these same protons even when His residues are present in a highly acidic environment (32). Thus, the deshielding of ring protons seen in Fig. 7*A* reveals a significantly decreased electronegative character for the side chain imidazole ring of His in peptide PS1CT3.

Although a variety of interactions could account for the observed deshielding of the His side chain, a detailed analysis by CD spectroscopy revealed that the PS1 segment in peptide PS1CT3 was resistant to imposition of any structural constraints, even in the presence of high (up to 50%) concentrations of the secondary structure enhancing solvent trifluoroethanol (data not shown, but see Ref. 33). Although not conclusive, these results, nevertheless, suggested to us that this deshielding was probably not due to long-range interactions. Another possible explanation that was considered was that of short-range hydrogen-bonding interactions between the protonated α amine group of the N-terminal His and the nitrogen atoms of the imidazole ring. We tested this possibility by synthesizing a derivative of peptide PS1CT3 where the terminal amino group was acetylated. This capping of the α amino functionality of the terminal His residue was expected to disrupt any such intramolecular interactions.

Amino-terminal acetylation of peptide PS1CT3 did, in fact, result in an upfield shift of both the imidazole protons at the C2 and C4 positions, as revealed by ^{1}H-NMR spectroscopy (Fig. 7, *C* and *D*). Thus, although the precise mode remains unclear, amino-terminal acetylation of peptide PS1CT3 results in an increased electronegative character for the His side chain. Parallel groups of mice were then immunized either with the parent peptide PS1CT3 or its acetylated derivative. Sera obtained 6 wk later were subsequently analyzed for both distribution of epitope specificities and relative proportion of N-terminal-reactive Abs as described for Fig. 6. The native peptide, as expected (14, 19, 28), elicited an anti-DPAF monospecific response. In contrast, however, the polyclonal IgG derived against the acetylated derivative displayed significant amino-terminal reactivity, both by epitope-mapping and saturation inhibition protocols (Fig. 8). As shown in Table III, the relative proportion of N-terminal reactivity in the acetylated peptide was well in keeping with the calculated ΔG value for the His side chain.

Discussion

Antigenicity of individual determinants on a polypeptide Ag is generally thought to constitute a surface static property of the Ag (35, 36). Such a notion stems from the joint realization of the plasticity of the preimmune B cell repertoire and the fact that B cells generally recognize Ags in their native form. Consequently, attempts at ab initio identification of B cell epitopes have primarily focused on structural aspects of polypeptide Ags. These analyses rely on assessment of individual domains either for their surface accessibility, flexibility, or structural propensity (37–41). However, although both repertoire plasticity and a requirement for epitope accessibility cannot be denied, our own earlier results indicate that the spectrum of epitopes recognized in an Ag-specific humoral response constitutes only a fraction of that which may be anticipated (13–15). These studies have demonstrated that, subsequent to the initial recognition of Ag, immunological parameters are soon brought into play to restrict the range of epitope-specific responses that are retained (13–18). Of the diverse clonotypes activated upon first exposure to Ag, these selection processes ensure that only those B cells with highest affinity for Ag are selected for seeding of GCs and consequent retention in the response (14).

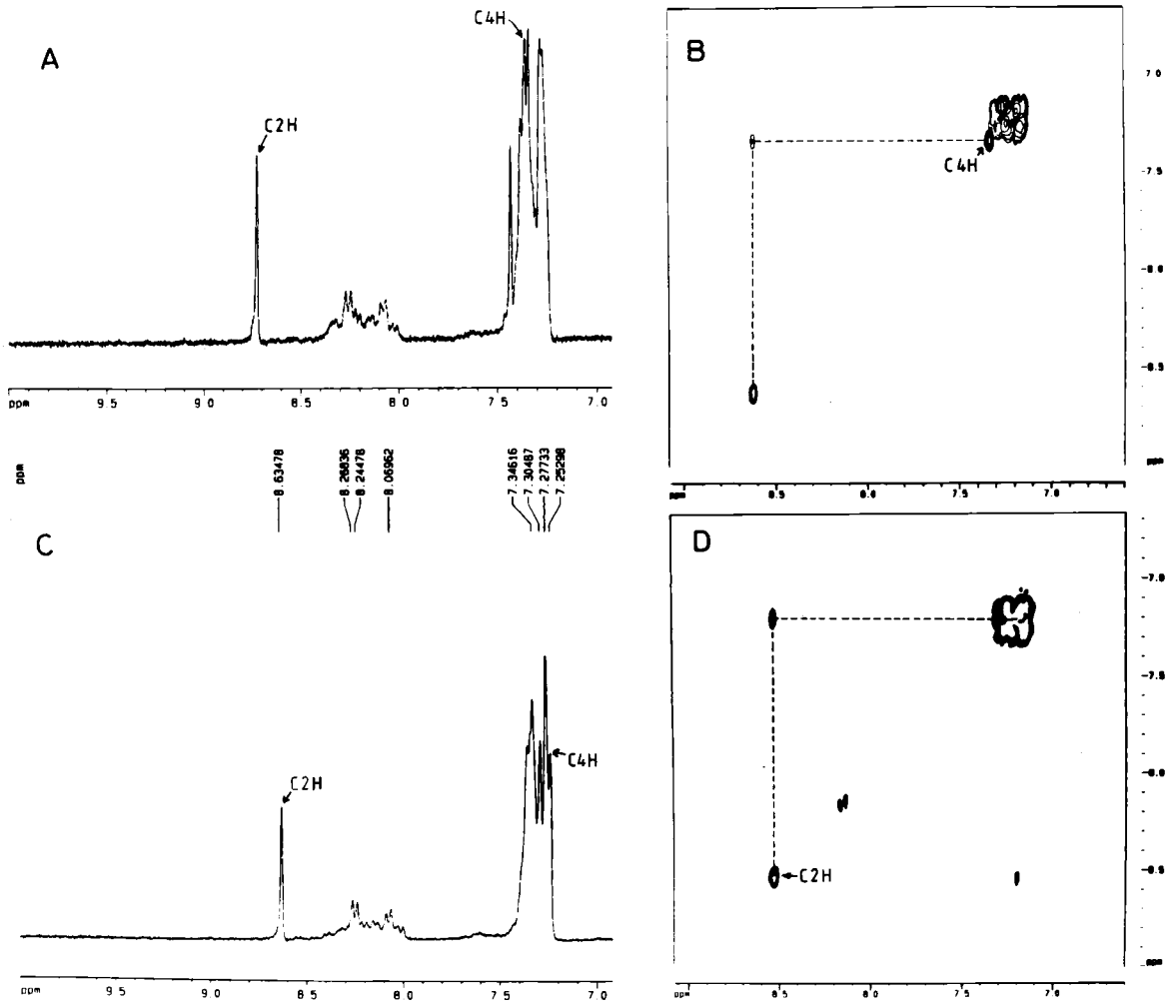

FIGURE 7. One-dimensional and two-dimensional proton NMR spectra of peptide PS1CT3 and its acetylated analogue. Three hundred MHz proton NMR spectra of peptide PS1CT3 (*A* and *B*) and its amino-terminal acetylated derivative (*C* and *D*) were acquired in D_2O at a final concentration of 10 mM at 25°C, after exchange of labile amide protons. *A* and *C*, The region between 7 and 9 ppm in the one-dimensional spectra. Chemical shifts were measured relative to the internal standard 3-(trimethylsilyl)propionic acid, sodium salt (DSS). Positions of C2 and C4 protons of the imidazole ring of His side chain are indicated. *B* and *D*, The 300-MHz ^{1}H COSY (2D correlation spectroscopy) spectrum of the corresponding regions shown in *A* and *C*. While the C2H of the His side chain is characteristic (32), C4H was identified by cross-peak connectivities (shown by the dashed line) between the distinct diagonal peaks.

Thus, the pre-GC phase selection appears to constitute the first and, perhaps critical, filtering step in the pathway that defines sequential progression of humoral responses.

An intriguing corollary noted in these above studies was that clonotype selection in the pre-GC phase also translates into restriction of epitope specificities. Thus, for example, the early primary response to peptide PS1CT3 always resulted in exclusive selection for B cells directed against a single tetrapeptide epitope of sequence DPAF (14). The consequences of this selection was exercised upon subsequent stages where both late primary IgG and the Ag-specific memory B cell pool were monospecific for the DPAF epitope (17). At least in the context of peptide PS1CT3, these findings suggested that the affinity/avidity of preimmune B cell responses to individual epitopes may not be stochastically generated but, perhaps, could be associated with at least some degree of determinism. However, the factors, if any, that controlled such an outcome proved especially enigmatic since a variety of predictable parameters such as surface accessibility, mouse H-2 haplotype, secondary structural propensities, and positional influences could be ruled out as probable causes (13–15, 19). Interestingly though, single residue glycine substitutions within the DPAF epitope was found to result in a redistribution of epitope specificities in favor of alternate determinants within the PS1 segment, although the avidity of the resulting responses were markedly reduced. These latter results provided a possible clue by implicating that the affinity/avidity of a primary response to a multideterminant Ag may be epitope dependent.

Although somewhat unusual, the above proposal is not altogether untenable if one considers the fact that the affinity of interaction of Ag with either Ab or sIgM receptor on B cells directly relates to the Gibbs-free energy of complexation (ΔG) by the equation, $\Delta G = -RT\ln K_d$. Here K_d represents the equilibrium dissociation constant. In this context, it is pertinent to recall that, as demonstrated for a variety of systems (17, 42–44), Ag-Ab interactions in the early primary response do not involve idealized surface complementarity. Rather, this is subsequently achieved by the combined processes of somatic mutation and positive selection within GCs (45–47). Consequently, the role of the chemical composition of an epitope in defining the energetics and thereby the affinity of its interaction with a stochastically generated receptor on preimmune B cells could not be excluded.

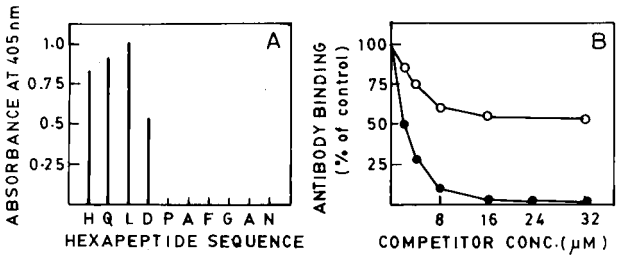

FIGURE 8. Primary IgG response to the acetylated derivative of peptide PS1CT3. Groups of mice were immunized with the amino-terminal acetylated derivative of peptide PS1CT3 (see text), and sera were collected 42 days later. The separated IgG fraction from this serum was then analyzed for distribution of epitope specificities against the PS1-derived hexapeptide panel as described for Fig. 6. Results are shown in *A*. *B*, The results of a saturation inhibition ELISA performed for the same IgG preparation using either the homologous acetylated peptide (●) or peptide G28CT3 (○) as the competitive inhibitor. Peptide G28CT3 represents a nonacetylated analogue of PS1CT3 where the amino-terminal His was substituted for Gly. As shown earlier (28), this peptide competes as effectively as the native nonacetylated peptide PS1CT3 for anti-PS1CT3 Abs, with near identical IC_{50} values.

Support for such a possibility was afforded by our examination of monomeric IgM mAbs derived against the PS1 segment of peptide PS1CT3. As already indicated, our choice of monomeric IgMs was based on the expectation that they would be reminiscent of the sIgM receptor on preimmune B cells from which they were derived. At one level, it was interesting to note that a diverse range of affinity constants were obtained, with some Abs yielding values for K_a as low as 10^4 M^{-1}. Although this confirms our earlier proposal that Ag-driven preimmune B cell activation involves low thresholds (18), they further add by revealing that Ag-binding affinity constants of as low as 10^4 M^{-1} suffice to induce preimmune B cells into a primary response. Another important highlight of these results was the comparable affinities displayed by Abs directed against a given epitope that, at least in some instances, was independent of their clonal origins. Consequently, the hierarchy of Ab affinities obtained also distinguished between independent epitopes recognized on the PS1 segment; implicating epitope dependency of monomeric IgM Ab affinity.

A rationale for the above findings was provided by our subsequent thermodynamic analysis of the association process between Ag and monomeric IgMs. Regardless of either clonal origin or epitope specificity of Ab, these studies identified that the driving force for this process derived exclusively from net favorable changes in enthalpy and occurred at the expense of an overall decrease in the entropy of the system. Although the entropy changes probably reflect conformational readjustments within the paratope of Ab, the negative enthalpy changes clearly point to the dominant contributions from epitope-paratope interface interactions in rendering this association permissible. Thus, a synthesis of these two lines of evidence permits the inference that, by influencing the nature of interface interactions, the chemical composition of an epitope represents at least one parameter that defines the affinity of its binding to either its corresponding monomeric IgM mAb or, by extension, the sIgM receptor on cognate preimmune B cells.

Although these studies provided an insight into the physicochemical forces driving Ag recognition by preimmune B cells and distinctions arising therein, the functional relevance of these distinctions remained to be validated. One approach to resolving this employed isosteric substitutions for chemically dissimilar residues within the DPAF epitope of peptide PS1CT3. This permitted generation of analogues that, upon immunization, yielded primary monomeric IgM Abs of markedly reduced avidities relative to that obtained against the native peptide. Interestingly, immunization with equimolar mixtures of these analogue peptides and peptide PS1CT3 resulted in a marked suppression in immunogenicity of the analogues relative to the parent peptide. These results provided empirical evidence in favor of a causal link between avidity of primary recognition and immunogenicity in the later stages of the response.

The dependence of immunogenicity of an epitope on its amino acid composition could also be unequivocally demonstrated with analogues of peptide PS1CT3 incorporating various substitutions at the amino terminus. Amino-terminal substitutions were preferred on the basis that alteration at this position was least likely to impose any structural influences. Thus, depending upon whether, for example, the amino-terminal residue was Gly or Arg, the resulting primary IgG response displayed the extreme situations from a cryptic amino-terminal segment to one where this domain was the most immunodominant. Furthermore, by using the anti-DPAF response as an internal comparative standard, we were also able to delineate a hierarchy for individual amino acid residues in terms of their relative ability to render the amino-terminal segment immunogenic. Interestingly, in addition to underscoring the nonidentical contributions from individual residues, this hierarchy was found to correlate well with the potential energetic contribution of each amino acid side chain, defined as calculated Gibbs-free energy changes, toward a binding interaction. Thus, while the experimental data in Table III clarify a role for amino acid constituents of epitope in defining its relative immunogenicity, the correlation with side chain ΔG values supports that this role derives from energetic considerations. Experimental support for this correlation could be provided, at least for some of the analogues, by comparing avidities of primary monomeric IgMs obtained against the amino-terminal epitope vs that obtained against the remainder of the sequence. Whereas those substitutions that enhanced immunogenicity of the amino-terminal epitope also yielded amino-terminal-specific monomer IgMs of higher relative avidity, this was not true of those that had no significant effect.

More recently, we have also examined the frequency of occurrence of individual amino acid residues as anchor residues within epitopes described in the literature (V. Manivel and K. V. S. Rao, unpublished results). Such a frequency analysis revealed an overwhelming preference, as anchor residues, for amino acid residues with side chain ΔG values of negativity >5.0 (as defined in Table III) (V. Manivel and K. V. S. Rao, unpublished results). These results further support the proposal that delineation of functional B cell epitopes from the array presented by a multideterminant Ag is driven by thermodynamic considerations.

Thus, in summary, divergent lines of evidence presented in this report highlight some important operating principles governing the early stages of a humoral response to T-dependent multideterminant Ags. First, the initial encounter with Ag results in activation of an array of B cell clonotypes against diverse epitopes and with varying affinities for Ag. Although this was not unexpected, what was surprising, however, was the near complete dependence of affinity on epitope recognized, as also its independence from the clonal origins of Ab. This dependence appears to derive from thermodynamic considerations, an analysis of which revealed yet another interesting facet of Ag recognition by preimmune B cells. At least for peptide PS1CT3, binding to the B cell receptor on preimmune B cells was found to involve large conformational changes within the paratope of receptor. As a result, association

was allowed only if the gain resulting from paratope-epitope interface interactions could sufficiently compensate for this. It is pertinent to recall here that Ag recognition by preimmune B cells differs from bindings observed for classical receptor-ligand pairs that regulate biological responses. Although the latter generally represents systems where both partners have coevolved for idealized complementarity over biological time, the former constitutes a search for the best available fit by scanning through a library of "imperfect" receptors. Similarly, Ag recognition in a primary response is also distinct from that in a secondary IgG response, the latter not requiring unfavorable changes in paratope conformation (V. Manivel, N. Samoo, and K. V. S. Rao, manuscript in preparation). Thus, in a situation where the feasibility of an induced fit is defined by the enthalpy of the reaction, it is not surprising that the chemical composition of an epitope plays an important role.

Epitope-dependent distinctions in Ab affinity in the early primary response are functionally significant in that they impact upon the hierarchy of epitopic dominance observed in the later stages of the response. This is presumably achieved by mechanisms elucidated earlier (14, 16–18), wherein seeding of GCs is restricted to the highest affinity B cell subsets which, consequently, also leads to a restriction in epitope specificities. We emphasize here that these studies are not intended to permit prediction of functional B cell epitopes on multideterminant Ags. Indeed, given that the three-dimensional structure of most Ags is unknown, as also the fact that the influence of chemical composition of epitope is limited by accessibility of its side chains for interaction with Ab, any prediction is clearly not possible at this stage. Nonetheless, the relevance of our present investigations lie in elucidating mechanisms that govern the initiating step in Ag-driven optimization of T-dependent humoral responses.

In previous studies, we had shown that, at least in the context of peptide epitopes, Ab optimization for epitope within GCs was driven in favor of increased on-rates of Ag binding (19). Interestingly, this kinetic control of clonotype selection in GCs was found to correlate with increased Ag specificity because it facilitated discrimination between conformational variants of the same epitope (19). These earlier results, in conjunction with the present data, therefore reveal that maturation of Ag-specific T-dependent humoral responses occurs in two discrete sequential stages. The first, which is under thermodynamic control, constitutes a selection for the best of available fits between the spectrum of epitopes on Ag and the repertoire of receptors on preimmune B cells. This is then followed by a further optimization, within GCs, of this selected subset for the kinetics of Ag/epitope binding. It is probably due to the concerted action of these two processes, that the twin imposing demands of high affinity and exquisite fidelity are so rapidly achieved in T-dependent humoral responses.

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