SUMMARY
Summary

The mechanism underlying B cell response has exhaustively been delineated using a variety of haptens as surrogate antigens. However several unanswered questions nevertheless remain particularly with respect to more natural antigens such as polypeptides and proteins. My interest in this area has been to examine and understand the B cell responses to a peptide epitope displayed on a polypeptide antigen with respect to (a) peptide epitope driven induction and selection of B cells; (b) the basis of antigen dependent repertoire discrimination from a pool of activated pre-immune B cells; (c) influence of secondary structure on both immunodominance and immunogenicity of an epitope and (d) relationship between memory generation and specificity maturation with kinetic optimization of an antibody.

The model antigenic determinant employed for the above study is a fifteen amino acid residue sequence (segment PSI, a linear B cell epitope) derived from the large envelope protein of Hepatitis B virus surface antigen (HBsAg) which was synthesized co-linearly with a well characterized T cell epitope derived from circumsporozoite stage of malaria parasite, Plasmodium falciparum. The other two peptides, namely AibCT3 and CysCT3, are analogues of PS1CT3, where the immunodominant epitope (DPAF) exists in an altered conformation compared to the parent peptide, PS1CT3. In peptide AibCT3, the amino acid residue at position 1 and 10, in the parent sequence were substituted with \(\alpha\)-aminoisobutarate, which confers a propensity for helix formation. And in case of CysCT3, residues at this position were substituted with cysteine and subsequently this segment was locked into a loop by oxidation to generate a disulfide bond. Thus while the immunodominant segment (sequence DPAF) exists in a disordered conformation in peptide PS1CT3 and in a \(\alpha\) helical propensity in peptide
AibCT3, the same exists in a relatively rigid cyclic loop in peptide CysCT3. In chapter II, the influence of the altered conformations on immune responses to the immunodominant epitope within the synthetic peptide immunogen was examined in mouse animal model. Though overall immunogenicity was affected, the model epitope (sequence DPAF) remained the predominant recognition site. The peptides AibCT3 and PS1CT3 did not show a major difference in their immunogenicity, but CysCT3 found to be poor immunogen. The poor anti-CysCT3 IgG antibody response was shown to result from its inability to prime T cells as effectively as peptide PS1CT3. Further more it could also be demonstrated that the attenuated T cell priming efficacy of peptide CysCT3 was purely a consequence of the altered structure of PSI segment rather than due to the nature of substitutions made. This was shown by a linearized analogue of peptide CysCT3 that was synthesized, where the cysteine-sulfhydryl groups were blocked as acetamidomethyl-derivatives (termed as peptide Cys(Acm)CT3). Immunization of BALB/c mice with peptide Cys(Acm)CT3 followed by an examination of LNC proliferative responses revealed that this analogue was far more potent at priming T cells than peptide CysCT3. Interestingly however, while primary T cell responses were sensitive to structural environment of the CT3 segment, both peptides PS1CT3 and CysCT3 proved equally competent at recalling secondary T cell responses in vitro. This result confirms the previous reports of the differences in requirements for activation of primary and secondary T cell responses. In addition, the comparable recall efficiencies of peptide PS1CT3 and CysCT3, regardless of whether LNCs were primed with peptide PS1CT3, CysCT3 or CT3, strongly points towards quantitative rather than qualitative differences in antigen presentation from two analogues. Thus the results from this study also suggests that the local environmental influence by modifying T cell priming efficacies of individual T cell epitopes, can add yet another
variable in defining the repertoire of T cell specificities generated in a primary response in to protein antigens (Nayak, B.P., 1999. FEBS.Letters, 443: 159-162) (Chapter II).

A subset of B cell clones those were initially activated and subsequently entered into the memory compartment in response to the two analogue peptides; PS1CT3 and CysCT3, where the immunodominant epitope 'DPAF segment' was either unconstrained or held within a cyclic loop, revealed a significant homology in their paratope composition. Raising mAbs for the two peptides and analyzing their variable region nucleotide sequences revealed this. On comparison of both heavy and light chain variable regions of a subset of anti-PS1CT3 and anti-CysCT3 mAbs found to have derived from a common progenitor, but with non-identical somatic mutations. However, interestingly these antibodies (mAbs) did not show any bias in terms of their relative affinities for the homologous antigen. This was shown by measurement of relative affinities of the mAbs for the two peptides. In contrast, mAb-binding on-rates clearly discriminated between peptides representing the homologous versus the heterologous conformer of the DPAF epitope. Here it has been shown that the kinetics of antigen recognition dominates over equilibrium binding criteria both in epitope-driven repertoire selection and antibody maturation during a humoral immune response (Nayak et. al. 1998. J. Immunol. 161: 3510-3519) (Chapter I).

As processes related to affinity maturation, B cell differentiation and selection into plasma or memory compartments are events of a GC reaction during a T-dependent antigen response, the role of immune complexes in both formation of germinal centers and processes that occur subsequently within was analyzed using an in vivo GC reconstitution protocol. The presence of an antigen as immune complex was not found to
constitute a limiting requirement for the initiation of a Germinal Center formation. Early studies have suggested that the formation of GCs was facilitated by the trapping of immune complexes within the network of follicular dendritic cells (FDCs) and the B cells in the lymphoid follicle (chapter III, Ref. 5,6). Such an inference has been derived from the observations that, immunization with antigen-specific immune complexes, as opposed to antigen alone, results in a more rapid induction of GCs (chapter III, Ref. 7,8). The present study shows no detrimental effects on either number or size of the resulting Germinal Centers when antigen-containing immune complexes were omitted during in vivo GC reconstitution. Thus, both recruitment and proliferation of antigen-activated B cells within Germinal Centers does not appear to be limited by antigen concentrations. In contrast, the presence of immune complexes was observed to be obligatory for the generation of antigen-specific memory B cells and this requires immune complexes to be of IgG isotype with epitope specificities that are homologous to those of the Germinal Center B cells. And the Germinal Center reaction was found to enhance the specificity of the antibodies for the homologous epitope. Although some improvement in specificity was noted in recall responses from immune complex-deficient Germinal Centers, the presence of appropriate immune complexes - however - served to further optimize the outcome. Here again, isotype and epitope-specificity of the antibody constituent in immune complexes proved to be important (communicated to J. Immunol.) (Chapter III).
LIST OF PUBLICATIONS

Publications included in thesis


Manuscripts communicated to peer reviewed journals (included in thesis)


Publications not included in thesis


B Cell Responses to a Peptide Epitope. V. Kinetic Regulation of Repertoire Discrimination and Antibody Optimization for Epitope

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The influence of imposing various conformational constraints on immune responses to a model epitope within a synthetic peptide immunogen was examined in mice. Although overall immunogenicity was affected, the model epitope (sequence DPAF) remained the predominant recognition site regardless of the conformation in which it was presented. A comparison of anti-DPAF mAbs obtained in response to two analogue peptides, PS1CT3 and CysCT3, in which the DPAF segment was either unconstrained or held within a cyclic loop, respectively, revealed a significant homology in the paratope composition. At one level a subset of anti-PS1CT3 and anti-CysCT3 mAbs was found to share a common heavy chain variable region. In addition, nucleotide sequence homology comparisons of both heavy and light chain variable regions identified the presence of anti-PS1CT3 and anti-CysCT3 mAbs that collectively appeared to derive from a common progenitor, but with nonidentical somatic mutations. Interestingly, however, no bias toward homologous Ag could be discerned on measurement of relative affinities of the mAbs for the two peptides. In contrast, mAb binding on-rates clearly discriminated between peptides representing the homologous vs the heterologous conformer of the DPAF epitope. Thus, it would appear that the kinetics of Ag recognition dominate over equilibrium binding criteria both in epitope-driven repertoire selection and Ab maturation in a humoral response. The Journal of Immunology, 1998, 161: 3510–3519.

The antigenicity of domains on protein Ags has long been suspected to result from a variety of biophysical properties, such as backbone mobility, side chain stereochemistry, solvent accessibility, shape, secondary or three-dimensional structure, and local hydrophilicity (reviewed in Ref. 1). The underlying basis for such assumptions has been founded on the fact that B cells generally recognize protein Ags in their native form (1). Given that the preimmune B cell repertoire is not limiting (2), accessibility or easy availability for B cell recognition seemed to be the only intrusive criterion in identifying a B cell epitope. As a result, it was therefore logical to seek parameters that may help determine either surface exposure or ready accessibility of domains in a folded protein as direct correlates of antigenicity (3–8).

Recent results from our laboratory, however, have identified an additional constraint that intervenes to eventually determine the immunodominance of a given B cell epitope. We have shown that while the early primary T-dependent IgM response was indeed consistent with expectations, in that Abs were produced against all accessible domains, subsequent progression entailed stringent selection for only a restricted subset from the initially induced polyclonal pool (9). Positive selection of Ab specificities was found to be regulated by the ability of individual cloneotypes to recruit the appropriate level of help, in a competitive environment, from a limiting pool of early Ag-activated Th cells (10). This, in turn, was dependent on both equilibrium (11, 12) and kinetic (13) binding properties of B cell surface Ig (sIg)3 receptor recognition of its epitope on Ag, critical prognosticators that describe the Ag-presenting efficacy of a B cell. Thus, immunologic parameters implicated in modulating immunogenicity appear to prevail over determinants of antigenicity to eventually define the functional identity of putative B cell epitopes on polypeptide Ags.

The model antigenic determinant employed for the above studies was a 15-residue sequence (segment PS1) derived from the large envelope protein of hepatitis B surface Ag (HBsAg), in conjunction with one or more well-characterized T cell epitopes (12). We observed that the murine primary IgG response to such model immunogens was always directed exclusively against a tetrapeptide sequence (sequence: DPAF) encoded between positions 4 and 7 of the PS1 domain (10–13). The immunodominance of the DPAF sequence was independent of either the nature or the number of T cell epitopes included (10, 12). It was also independent of the position of the PS1 segment within the immunogen sequence (i.e., at the amino-terminal, middle, or carboxy-terminal positions) (12). Finally, immunodominance of the DPAF epitope was also established in immunogen sequences of random structure, as determined by circular dichroism (CD) spectroscopy (12, 13), implying that accessibility for recognition was not the sole selection criterion. Such synthetic peptide antigens therefore represent good model systems to further understand both biophysical and immunologic parameters that help determine the immunodominance of a given B cell epitope.

Using analogues of a model peptide with different secondary structural propensities, we show here that immunodominance is independent of either perturbation in conformational preferences

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All nucleotide sequences described herein have been submitted to the EMBL database. Their accession numbers are from Y16445 to Y16464.

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1 Abbreviations used in this paper: sIg, surface Ig; HBsAg, hepatitis B surface Ag; CD, circular dichroism; NMR, nuclear magnetic resonance; Aib, α-aminoisobutyric acid; TFE, trifluoroethanol; GC, germinal centers.

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Peptide synthesis

Peptides were synthesized on a Milligen 9050 automated peptide synthesizer (Millipore, Bedford, MA) using F-moc chemistry (14–16). Crude peptides were purified to at least 95% purity by reverse phase HPLC on a C18 column (15 μm, 8pak, 19 × 300 mm; Waters, Milford, MA). The identities of all peptides were ascertained by amino acid analysis.

For the synthesis of peptide CysCT3, the side chain protecting group used for cysteine was the acetylimidomethyl group. Subsequent to synthesis and cleavage from the solid support, simultaneous deprotection of cysteine side chains and oxidation to form intramolecular disulfide bonds were achieved with iodine in acetic acid (17). Briefly, 70 mg of the acetylimidomethyl-ethyl-derivated peptide was dissolved in 4 ml of 50% aqueous acetic acid. To this was added 1 ml of 1 M hydrochloric acid, followed immediately by 40 ml of a 50-mM solution of iodine in 50% aqueous acetic acid. After 30 min of vigorous stirring, the reaction was quenched with 2 ml of 1 M aqueous sodium thiosulfate. This was then concentrated in vacuo, following which it was passed over Celite. The resulting solution was lyophilized to yield the crude product, which was purified by reverse phase HPLC as described above. In addition to amino acid analysis, the correct identity of peptide CysCT3 could be established by mass spectrometry (expected mass, 3965.2; experimentally obtained mass, 3964.2).

Overlapping hexapeptide panels were synthesized by the method of Geyser (18) using the multipin noncleavable kits (Chiron Mimotopes, Victoria, Australia) strictly adhering to the protocol of the manufacturer. After completion of synthesis, all peptides were routinely acetylated at the amino terminus and subsequently deprotected as previously described (12).

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). Immunizations were given i.p. at a dose of 50 μg/mouse as an emulsion in CFA. For polyonal sera, mice were bled from the retro-orbital plexus, and sera within a group were pooled.

Preparation of anti-CysCT3 IgG mAbs

A group of four BALB/c mice was immunized with a single dose of peptide CysCT3 as described above. Twenty-eight days later they were boosted with 50 μg/mouse of soluble peptide CysCT3 in PBS given i.v. Three days later, the highest responder from the group was taken for the generation of IgG-secreting hybridomas. Polyethylene glycol-mediated fusion to hypoxanthine-aminopterin-thymidine-sensitive myeloma plasmacytoma, SP2/O-Ag14, maintenance of derived cell lines, and limiting dilution cloning were essentially as previously described (12, 19). The secretion of Ab in culture supernatants was screened by ELISA against wells coated with peptide CysCT3.

ELISA

Plates were coated with 2 μg/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 horseradish peroxidase-labeled secondary Ab (37°C, 1 h), followed by color development with o-phenylenediamine. Absorbance was measured at 490 nm.

For competitive ELISA experiments, antisera was used at dilutions ranging 50% of the titer value. Twofold higher concentrations of antigen and competitor peptide were mixed in equal volume and incubated for 10 min at room temperature. This was then added to duplicate wells at 100 μl/well. The remaining procedure was as described above.

Determination of on-rates and dissociation constants

On-rates of mAb binding to either peptide PS1CT3 or CysCT3 were determined as previously described (13). Briefly, equal volumes of mAb and appropriate peptide in PBS were mixed at room temperature, and time-dependent Ab binding in terms of quenching of tryptophan fluorescence was continuously monitored over a 100-min period in a Shimadzu RF-1501 spectrofluorometer (Shimadzu, Tokyo, Japan). The excitation wavelength used was 280 nm, and emission was recorded at 330 nm. The final Ab concentration employed was between 200 and 300 nM, whereas peptide was maintained at between 10- and 25-fold in molar excess over binding sites (assuming bivalency per Ab molecule) to ensure pseudo-first-order conditions. The extent of fluorescence quenching was used to determine on-rates of mAb binding as a function of time. The log of the concentration of unbound Ab was plotted vs time, and the slope, which was obtained by linear regression analysis, was used to determine k on. The k off value was subsequently calculated by dividing k on by the peptide concentration. Values of k on presented are the mean (±SD) of determinations at three independent peptide concentrations. For dissociation constants, mAbs (final concentration between 100–150 μM) were incubated alone or with either peptide PS1CT3 or CysCT3 at concentrations ranging from 5 × 10−3 to 1 × 10−5 M at room temperature for 1 h. Subsequent to this extent of quenching of tryptophan fluorescence was determined, from which the concentration of peptide bound was calculated assuming bivalency for each IgG molecule at saturation. Kd values were subsequently obtained from a Scatchard analysis of the resulting data.

Nuclear magnetic resonance (NMR) spectroscopy

Natural abundance, proton-decoupled (Waltz 16 decoupler) 1H NMR spectra were recorded in a mixture of H2O and D2O (9:1) on a Bruker (Avance Series, DR5 300, Billerica, MA) spectrometer at 75.47 MHz. The spectral width was maintained at 19,607.84 Hz, with a time domain size of 15,000 scans were accumulated at 300 K, using 3-(tetramethylsilyle)-1-propane sulfonic acid as the reference standard.

RT and amplification of IgG mRNA

Total cellular RNA was isolated from about 107 hybridoma cells with an RNAzol (Wak-Chemie Medical, Homburg, Germany)-based protocol with minor modifications. About 10 μg of total RNA was used for each reaction. The first strand of cDNA was synthesized using random primers and the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using the following program: one cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Amplification was performed on a Perkin-Elmer thermocycler using the following program: one cycle at 95°C for 3 min, followed by 30 cycles of 1 min each at 94°C, 58°C for 1 min, 72°C for 1 min, and finally a 10-min incubation at 72°C. A 10-μl aliquot of the reaction mixture was analyzed on a 1.8% agarose gel.

Nucleotide sequencing of the PCR-amplified DNA

PCR products of about 400 bp were blunted by ending by polishing with the PCR polishing kit from Stratagene and were subsequently cloned in the SfiI site of PCR-script vector of the PCR-script cloning kit from Stratagene. Positive clones were identified, restriction digestion of plasmid DNA was performed, and appropriate clones were sequenced using the T7 sequencing kit purchased from Pharmacia (Uppsala, Sweden). Both strands of cloned DNA were sequenced.
The model parent immunogen (peptide these studies has been described previously represents a hybrid of a B cell epitope derived from the envelope protein of HBsAg within the circumsporozoite protein of the malaria parasite, \textit{Plasmodium falciparum} (12). Separating the B and T cell epitopes is a spacer of two glycine residues at positions 16 and 17. The immunodominant sequence, DPAF, in peptide PSICT3 is highlighted within a box. The double bond between the Cys residues in peptide CysCT3 is meant to denote a disulfide bond. B, CD spectra of peptides Aib-PS1 (a) and PSI (b) in aqueous and secondary structure-enhancing solvents. The CD spectra of peptide Aib-PS1 at a final concentration of 6 \, \mu \text{M} was recorded on a JASCO model J710 spectropolarimeter (JASCO, Tokyo, Japan) over a wavelength range of 200 to 250 nm with a step resolution of 0.1 nm and a scan speed of 200 nm/min. The spectra shown are those averaged over a total of 30 accumulations. a shows the CD spectra of peptide Aib-PS1 either in 0.01 M phosphate buffer (pH 7.2) alone (---) or in buffer containing 10\% (---), 20\% (---), or 50\% (---) of TFE. Panel b shows the CD spectra obtained for a peptide representing the B cell epitope segment (residues 1–15) of the parent peptide PSI (peptide PSI) under identical conditions. This has been included for comparative purposes. In the latter case the profiles obtained in buffer alone and in the presence of 10\% TFE were superimposable. As is evident, peptide Aib-PS1 shows a markedly greater helix-forming potential than peptide PSI. This is further supported by the occurrence of an isodichroic point in panel a at about 204 nm, which is indicative of TFE-dependent transition from random to a helical structure. No such isodichroic point is observed in the CD spectra of peptide PSI (b).

DNA were sequenced. Normally two independent clones were sequenced for each mAb. However, in instances where any discrepancy was noted, additional clones were sequenced to rule out artificial mutations as a result of the PCR procedure. The Ig heavy and light chain variable region gene sequences were analyzed using \textit{PC} Gene software (Oxford Molecular Group PLC, Oxford, U.K.) and the GenBank data library.

**Results**

**Peptide PSICT3 and its analogues**

The model parent immunogen (peptide PSICT3; Fig. 1A) used in these studies has been described previously (10, 12, 14). Briefly, it represents a hybrid of a B cell epitope derived from the envelope protein of HBsAg (20) and a promiscuous T cell epitope resident within the circumsporozoite protein of the malaria parasite, \textit{Plasmodium falciparum} (21). We have shown in prior studies that this peptide represents a T-dependent Ag and that immunization in BALB/c mice results in a primary and a secondary IgG response that is exclusively directed against a tetrapeptide sequence between positions 4 and 7 (sequence: DPAF) (12). Furthermore, based on a combination of results derived from an analysis of early primary IgM responses to peptide PSICT3 and the CD spectrum of peptide, it was inferred that surface accessibility alone was an insufficient criterion to explain the immunodominance of the DPAF segment within peptide PSICT3 (12).

To probe further whether altered conformational constraints of an epitope influences its immunodominance, we synthesized two additional analogues of peptide PSICT3, namely, peptides AibCT3 and CysCT3 (Fig. 1A). Peptide AibCT3 represents an analogue where the amino acid residues at positions 1 and 10 in the parent sequence were substituted with \alpha-aminoisobutyric acid (Aib), a nonnatural amino acid known to promote \alpha-helix formation when introduced into peptide sequences (22). That Aib substitution also confers a propensity for helix formation in the present instance could be confirmed with the help of a synthetic peptide representing the B cell epitope segment (positions 1–15) of peptide AibCT3. Analysis of the CD spectra of this peptide in the presence of varying concentrations of the secondary structure-enhancing solvent, trifluoroethanol (TFE) (23), revealed ready inducibility into an \alpha-helix (Fig. 1B, a). The second
analogue, peptide CysCT3, was one in which the residues at positions 1 and 10 were substituted with cysteines, subsequent to which this segment was locked into a loop by oxidation to generate a disulfide bond (Fig. 1A). Thus, while the immunodominant DPAF segment exists in a disordered conformation in peptide PS1CT3, it is conferred with an α-helical propensity in peptide Aib-PSl. In contrast, this segment was expected to be held within a relatively rigid cyclic loop in peptide CysCT3.

We were further able to verify that the substitutions performed do, in fact, variably influence conformational distributions of the DPAF epitope by NMR spectroscopy. While a detailed analysis will be published elsewhere (R. A. Vishwakarma, et al., manuscript in preparation), Figure 2 shows two relevant regions of proton-decoupled, natural abundance 13C NMR spectra of peptides PS1, Aib-PS1, and Cys-PS1. Figure 2A represents that portion of the spectrum that includes the chemical shifts for the α-carbon atoms of proline residues. In peptide PS1 (top panel), the signal for the α-carbon of the proline residue at position 14 appears at 61.1314 ppm (peak 2), whereas that for the residue within the DPAF sequence (Pro5) is further upfield at 61.0344 ppm (peak 3). Peaks 1 and 4 (66.9532 and 60.6537 ppm) correspond to the β- and γ-carbons of the threonine residue at position 12 in the PS1 sequence. A comparison with the corresponding region of the spectrum for peptide Aib-PS1 (middle panel) reveals an interesting difference with respect to the α-carbon of the proline residue within the DPAF sequence (Pro5). In the latter case an upfield shift to 60.8712 ppm was observed. Furthermore, this signal displayed multiplicity (Fig. 2A, middle panel), indicative of the existence of this carbon atom in a multiplicity of chemical environments, all of which were nonidentical with that for the corresponding carbon atom in peptide PS1. In contrast, the α-carbon of Pro5 in peptide Cys-PS1 (Fig. 2A, bottom panel) was shifted downfield to merge with the signal for the α-carbon of Pro14. While the signal for the α-carbon of Pro5 was variably shifted depending upon either Aib or Cys substitution, the signals for the corresponding carbon atoms in Pro14 and Thr12 remained invariant among the three analogues (Fig. 2A).

Figure 2B gives the aromatic region of the 13C NMR spectra of these peptides. The top panel is that for peptide PS1, where peak 1 represents the carbon at position 1 (with respect to β-carbon substitution) of the benzene ring of phenylalanine. Peaks 2 and 3 correspond to the ortho and meta aromatic carbons, respectively, whereas peak 4 represents the carbon in the para position. The remaining two signals are derived from the carbons in the imidazole ring of histidine, which are absent in peptides Aib-PS1 (middle panel) and Cys-PS1 (bottom panel). Although the distribution of signals for the aromatic carbons of Phe remain unchanged in the spectrum of peptide Aib-PS1, that for peptide Cys-PS1 reveals interesting differences (Fig. 2B, bottom panel). Signals for the aromatic carbons at both the 1 and ortho positions displayed multiplicity in the case of peptide Cys-PS1, indicative of differences in the chemical environment of the Phe residue in peptides PS1 and Cys-PS1.

Collectively, the data in Figure 2 clearly demonstrate that both Aib and Cys substitutions nonidentically perturb the stereochemical environment of the DPAF epitope with respect to that in peptide PS1.
of either a peptide coated with homologous Ag, and bound Abs were determined by immunogen (i.e., representing only the homologous B cell epitope segment (residues separate experiments.

Epitope specificity of the primary IgG response is independent of conformational constraints

We have shown earlier that the murine primary response to peptide PS1CT3 was exclusively directed against the B cell epitope segment (segment PS1) between positions 1 and 15, with no detectable Abs against the rest of the sequence (12). To confirm whether this was also true for peptides CysCT3 and AibCT3, we performed competitive inhibition ELISA experiments in which binding of day 28 IgG with either CysCT3 or AibCT3 was examined in the presence of either the homologous immunogen or a synthetic peptide representing only the homologous B cell epitope segment (residues 1–15) as inhibitor. The results from such an experiment are shown in Figure 4, where parallel data obtained for anti-PS1CT3 IgG is also included for comparison. Nearly identical inhibition profiles were obtained regardless of whether the whole homologous immunogen or only its derived B cell epitope segment was used as inhibitor, with virtually complete inhibition at higher concentrations. Further, a synthetic peptide representing the T cell epitope segment (residues 18–38) was also unable to inhibit Ab binding to any significant extent over the dose range tested in any of the cases (<10%; data not shown). Collectively, these results suggest that, similar to peptide PS1CT3, the specificity of primary IgG responses to peptides AibCT3 and CysCT3 is also at least predominately restricted to within the amino-terminal 15 residues, with little or no response against the rest of the Ag sequences.

To further localize fine specificities, we resorted to epitope mapping with a panel of overlapping hexapeptides, displaced by one residue at a time, derived from the B cell epitope segment of each of these analogues. The results from such an experiment are shown in Figure 5. The cross-reactivity profile obtained with anti-PS1CT3 IgG is entirely consistent with our earlier observation, in that only three overlapping peptides (sequences: QLDPAF, LDPAFG, and DPAFGA) were recognized (12). This was shown to result from virtual monospecificity of the IgG response against a common tetrapeptide sequence, DPAF (12). Interestingly, almost identical results were obtained for both anti-AibCT3 and anti-CysCT3 IgGs (Fig. 5). Thus, although the modifications performed influenced immunogenicity, the fine specificity of the primary IgG response was apparently unaffected.

The monoclonal IgG response to peptide CysCT3 is predominantly monospecific but is genetically diverse

To distinguish between narrow range polyspecificity and monospecificity, we selected one analogue, peptide CysCT3, for further study. mAbs of the IgG class were generated using a protocol identical with that described for peptide PS1CT3 (12). A total of 13 mAbs were obtained, which were subsequently analyzed for cross-reactivity against the overlapping hexapeptide panel described in Figure 5. In addition, we determined the nucleotide sequence of the heavy chain variable regions of these mAbs. Results from both of these studies are summarized in Table I (mAbs Cys2 to Cys23).

Of the 13 mAbs obtained, 12 were directed exclusively against the DPAF sequence, whereas the remaining one, mAb Cys2, also
required the leucine residue at position 2 for optimal binding (Table I). Analysis of the nucleotide sequences of heavy chain variable regions revealed that these mAbs derive from no less than seven distinct precursor B cells (Table I). Interestingly, however, the genetic repertoire of these mAbs appeared to be biased, with as many as seven mAbs using V\textsubscript{H} genes from the 36–60 family and a D\textsubscript{H} Q52 gene (Table I). In addition, these seven mAbs shared a highly homologous heavy chain CDR3 region of consensus sequence GGTGFXY, where X denotes the sole position of variance in these regions (Table I).

We next determined the heavy chain nucleotide sequence of the anti-PS1CT3 mAbs described previously (12), the results of which are also summarized in Table I (mAbs PC2811 to PC 289). It is obvious that the monospecific anti-PS1CT3 IgG response similarly originates from an oligoclonal B cell population, with no less than four distinct B cell precursors contributing to it (Table I). Intriguingly, heavy chain variable regions of three of the 11 anti-PS1CT3 IgG mAbs (mAbs PC287, 283, and 289) bore a resemblance to the dominant anti-CysCT3 mAb population with V\textsubscript{H} 36–60 and D\textsubscript{H} Q52 usage, and a consensus CDR3 sequence of GGTGFXY (Table I). The substitution of Thr for Ala at position X in mAb 283 vis-à-vis mAb 289 represents a single nucleotide change in the corresponding codon. It is therefore likely that this change results from a point mutation as a consequence of somatic hypermutation during affinity maturation (24, 25).

The results presented in Table I reiterate that selective immunodominance of the DPAF epitope is retained regardless of whether it is presented within a linear segment or constrained within a disulfide-held loop. Further, there is a significant sharing of the Ab paratope phenotype repertoire between the corresponding mAb populations, at least at the level of the heavy chain variable region.

**Affinity for Ag is not a criterion for repertoire distinction**

The overlap in repertoires, at least at the level of the heavy chain, between anti-PS1CT3 and anti-CysCT3 IgG mAbs was intriguing. If one assumes that fusion is a purely stochastic process, then the frequency of occurrence of a particular Ab in an mAb panel is also likely to reflect the frequency of occurrence of the parent B cell in the Ag-activated B cell pool within the host. If this is true, then the data in Table I are indicative of a shift in the profile of dominant paratope phenotypes, at least at the level of the heavy chain, invoked by peptides PS1CT3 and CysCT3. Thus, while the heavy chains used by mAbs PC281, PC282, PC284, PC285, and PC286 constitute the dominant component of the anti-PS1CT3 mAb panel, it is the heavy chain used by mAbs PC283, PC287, and PC289 that predominates in the anti-CysCT3 mAb panel, being present in as many as seven of the 13 Abs (Table I). mAbs analogous to the remaining anti-PS1CT3 mAbs were not detected in the anti-CysCT3 panel, indicative either of a relatively lower frequency of occurrence or their absence from it.

Given the altered presentation of the DPAF segment by peptides PS1CT3 and CysCT3, we expected that the Ab repertoires induced by each of these Ags should discriminate between the homologous and heterologous Ags in terms of their binding properties. To assess this, we compared the relative affinities of anti-PS1CT3 and anti-CysCT3 mAbs for both homologous and heterologous Ags by

### Table I. Characterization of anti-CysCT3 and anti-PS1CT3 IgG mAbs

<table>
<thead>
<tr>
<th>mAb</th>
<th>Heavy Chain</th>
<th>CDR3 Sequence (amino acid)</th>
<th>Minimal Binding Sequence</th>
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<tbody>
<tr>
<td>Cys-2</td>
<td>36–60</td>
<td>SP2.2</td>
<td>AGNWDY</td>
</tr>
<tr>
<td>Cys-5</td>
<td>36–60</td>
<td>SP2.3</td>
<td>DPAF</td>
</tr>
<tr>
<td>Cys-16</td>
<td>36–60</td>
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<td>Misc</td>
<td>SP2.5</td>
<td>DPAF</td>
</tr>
<tr>
<td>PC2812</td>
<td>Misc</td>
<td>SP2.5</td>
<td>DPAF</td>
</tr>
<tr>
<td>PC281</td>
<td>J558</td>
<td>Q52</td>
<td>AGNWDY</td>
</tr>
<tr>
<td>PC282</td>
<td>J558</td>
<td>Q52</td>
<td>AGNWDY</td>
</tr>
<tr>
<td>PC284</td>
<td>J558</td>
<td>Q52</td>
<td>AGNWDY</td>
</tr>
<tr>
<td>PC285</td>
<td>J558</td>
<td>Q52</td>
<td>AGNWDY</td>
</tr>
<tr>
<td>PC286</td>
<td>J558</td>
<td>Q52</td>
<td>AGNWDY</td>
</tr>
<tr>
<td>PC287</td>
<td>36–60</td>
<td>Q52</td>
<td>DPAF</td>
</tr>
<tr>
<td>PC288</td>
<td>36–60</td>
<td>Q52</td>
<td>DPAF</td>
</tr>
<tr>
<td>PC289</td>
<td>36–60</td>
<td>Q52</td>
<td>DPAF</td>
</tr>
</tbody>
</table>

* Nucleotide sequences of the heavy chain variable region genes and identification of families to which individual segments belong were determined as previously described (9). The predicted amino acid sequences of the heavy chain CDR3 region is also given. Epitope fine specificities of individual mAbs were identified as described in the text. Gene segment families were identified either with the help of the GenBank database or by comparison with published sequences (54).

* Ab fine specificity data for the anti-PS1CT3 mAbs were taken from reference 12.

* The V\textsubscript{H} genes of these mAbs could not be assigned to any known family and therefore have been identified as miscellaneous (Misc.).

---

**FIGURE 5.** Monospecificity of the polyclonal day 28 IgG response to either peptide PS1CT3 or its analogues. Day 28 sera from Figure 2 obtained in response to peptide PS1CT3 (A), CysCT3 (B), or AibCT3 (C) were screened for IgG cross-reactivity against a panel of overlapping hexapeptides derived from the homologous B cell epitope segments of residues 1 to 15 (see Fig. 1A) as previously described (12). The x-axis denotes each hexapeptide as its N-terminal residue in the parent PS1 sequence. The stars on H and N indicate that these positions were substituted with either Cys (B) or Aib (C) when used to screen for anti-CysCT3 and anti-AibCT3 cross-reactivities. Results are presented as absorbance obtained for each hexapeptide after subtracting that obtained for a negative control peptide of sequence AQGNSM. This figure is a representative of three separate experiments. The serum dilutions used were 1/100 for anti-PS1CT3 and anti-AibCT3, and 1/50 for anti-CysCT3.
Table II. Relative affinities of anti-PS1CT3 and anti-CysCT3 mAbs for homologous vs heterologous Ags

<table>
<thead>
<tr>
<th>mAb</th>
<th>Inducing Ag</th>
<th>I_{50} (μM) for Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PS1CT3</td>
</tr>
<tr>
<td>PC288</td>
<td>PS1CT3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>PC2812</td>
<td>PS1CT3</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>PC281</td>
<td>PS1CT3</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>PC282</td>
<td>PS1CT3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>PC284</td>
<td>PS1CT3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>PC285</td>
<td>PS1CT3</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>PC286</td>
<td>PS1CT3</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>PC287</td>
<td>PS1CT3</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>PC283</td>
<td>PS1CT3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>PC289</td>
<td>PS1CT3</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Cys2</td>
<td>CysCT3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Cys8</td>
<td>CysCT3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Cys18</td>
<td>CysCT3</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Cys25</td>
<td>CysCT3</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Cys4</td>
<td>CysCT3</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Cys20</td>
<td>CysCT3</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>Cys16</td>
<td>CysCT3</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>Cys24</td>
<td>CysCT3</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>Cys3</td>
<td>CysCT3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Cys7</td>
<td>CysCT3</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Cys10</td>
<td>CysCT3</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Cys11</td>
<td>CysCT3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Cys23</td>
<td>CysCT3</td>
<td>1.0 ± 0.5</td>
</tr>
</tbody>
</table>

* Relative affinities were estimated by competitive inhibition ELISA (Materials and Methods), and data are presented in terms of concentration of each peptide required to achieve 50% inhibition in Ab binding to surface-absorbed homologous Ag. Values are the mean (±SD) of three independent determinations.

It is evident that none of the mAbs displayed any significant difference in affinity for the homologous vs the heterologous peptide (Table II). This was equally true regardless of whether the mAbs were generated against peptide PS1CT3 or peptide CysCT3. Thus, affinity for Ag does not appear to constitute the discriminatory criterion for repertoire selection by peptide PS1CT3 vis-a-vis that by its homologue peptide CysCT3.

**Clonal relatedness of anti-PS1CT3 and anti-CysCT3 mAbs employing a common Ig heavy chain**

The anti-PS1CT3 and anti-CysCT3 mAbs bearing identical or near identical heavy chain CDR3 regions could be divided into two groups on the basis of heavy chain gene segment composition. The first group included mAbs PC283, PC289, Cys16, and Cys24, all of which use ιJ3 in addition to a common D_{H} segment and an identical member of the 36–60 family of V_{H} genes (Table I). The only detectable difference represented replacement point mutations, which are likely to have arisen from somatic hypermutations in the course of the GC reaction (24–28). The second group, involving mAbs PC287, Cys3, Cys7, Cys10, and Cys11, could be identified on the basis of utilization of the ι_{2} gene segment with the remaining variable region gene segment composition being identical with that in group I (Table I). However, point mutational variations leading to amino acid replacements could again be observed among the mAbs of this group (Table I).

Given that both groups included representatives from the anti-PS1CT3 and anti-CysCT3 mAb panels, it was of interest to assess the degree of clonal relatedness between the members of each group. To this end we determined the nucleotide sequence of the mAb light chains after first establishing that all the mAbs in these two groups employed a light chain of the κ isotype. The salient features derived from such an analysis for the mAbs in both groups are summarized in Table III. With respect to group I, although mAbs PC283 and PC289 appear to employ a common light chain it was, nevertheless, distinct from that present in either mAb Cys16 or Cys24 (Table III). These distinctions were readily apparent at the level of both amino acid sequence of the CDR3 region and ι_{κ} utilization (Table III). Thus, while it is possible that mAbs PC283 and PC289 represent mutational variants of a common precursor, it is clear that both mAb Cys16 and Cys24 derive from progenitors that are distinct from each other as well as from that for the anti-PS1CT3 subset. Nevertheless, given that all four mAbs in this group use Ig heavy chains with near homologous variable regions (Table I), at least some degree of structural homology may be expected at the level of paratope for all four mAbs in this group.

A comparison of light chain variable region nucleotide sequences of the group II subset of mAbs revealed that in addition to sharing derivatives of a common heavy chain variable region, all these mAbs bore light chains with, barring a few point mutations, identical variable regions (Table III). The near negligible probability of B cells deriving from independent lineages sharing a common gene segment composition for variable regions of both heavy and light chains with identical CDR3 regions (29) strongly suggests that mAbs PC287 and the anti-CysCT3 subsets in this group represent progenitors derived from a common precursor.

**Ag discrimination by the anti-CysCT3 and anti-PS1CT3 mAbs is characterized by differences in the kinetics of recognition**

The mAbs described in group I presented an interesting case of paratope discrimination by the mAbs of this group. To this end we determined the nucleotide sequence of the light chain variable region genes as described in Materials and Methods.

Table III. Light chain variable region composition of anti-PS1CT3 and anti-CysCT3 mAbs in groups I and II

<table>
<thead>
<tr>
<th>Group</th>
<th>mAb</th>
<th>Inducing Ag</th>
<th>Light Chain (α)</th>
<th>Amino Acid Sequence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>V_{H}</td>
<td>ι_{H}</td>
</tr>
<tr>
<td>I</td>
<td>PC283</td>
<td>PS1CT3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PC289</td>
<td>PS1CT3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cys16</td>
<td>CysCT3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cys24</td>
<td>CysCT3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PC287</td>
<td>PS1CT3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cys3</td>
<td>CysCT3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cys7</td>
<td>CysCT3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cys10</td>
<td>CysCT3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cys11</td>
<td>CysCT3</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* Deduced amino acid sequence of the CDR regions of the light chains utilized by the mAbs in groups I and II are given. These were derived from nucleotide sequencing of the light chain variable region genes as described in Materials and Methods.
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Table IV. Selectivity for the homologous Ag by both group I and II mAbs is evidenced at the level of Ag-binding kinetics.

<table>
<thead>
<tr>
<th>Group</th>
<th>mAb</th>
<th>Inducing Ag</th>
<th>PS1CT3</th>
<th>CysCT3</th>
<th>PS1CT3</th>
<th>CysCT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>PC283</td>
<td>PS1CT3</td>
<td>2.0 ± 0.6</td>
<td>1.4 ± 0.8</td>
<td>1.0 ± 0.32</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>PC189</td>
<td>PS1CT3</td>
<td>5.1 ± 1.2</td>
<td>4.8 ± 1.7</td>
<td>2.3 ± 0.51</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Cys16</td>
<td>CysCT3</td>
<td>3.1 ± 0.7</td>
<td>2.8 ± 0.6</td>
<td>0.60 ± 0.23</td>
<td>5.10 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>Cys24</td>
<td>CysCT3</td>
<td>3.8 ± 0.7</td>
<td>3.3 ± 0.7</td>
<td>0.07 ± 0.03</td>
<td>1.15 ± 0.35</td>
</tr>
<tr>
<td>II</td>
<td>PC287</td>
<td>PS1CT3</td>
<td>1.8 ± 0.9</td>
<td>1.5 ± 0.6</td>
<td>5.60 ± 0.50</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Cys3</td>
<td>CysCT3</td>
<td>3.2 ± 1.2</td>
<td>3.6 ± 0.8</td>
<td>1.24 ± 0.35</td>
<td>20.00 ± 3.20</td>
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<tr>
<td></td>
<td>Cys7</td>
<td>CysCT3</td>
<td>1.6 ± 0.4</td>
<td>1.9 ± 0.6</td>
<td>2.80 ± 0.42</td>
<td>43.00 ± 6.40</td>
</tr>
<tr>
<td></td>
<td>Cys10</td>
<td>CysCT3</td>
<td>2.8 ± 1.6</td>
<td>2.1 ± 0.9</td>
<td>2.40 ± 0.83</td>
<td>51.00 ± 5.30</td>
</tr>
<tr>
<td></td>
<td>Cys11</td>
<td>CysCT3</td>
<td>3.8 ± 1.4</td>
<td>2.7 ± 1.1</td>
<td>2.90 ± 0.76</td>
<td>49.40 ± 5.50</td>
</tr>
<tr>
<td></td>
<td>Cys23</td>
<td>CysCT3</td>
<td>2.4 ± 0.4</td>
<td>2.6 ± 0.5</td>
<td>0.10 ± 0.04</td>
<td>1.45 ± 0.24</td>
</tr>
</tbody>
</table>

* Determination of on-rate binding constants (kon) and data presentation are as described in Materials and Methods. Values are the mean (±SD) of determinations at three separate peptide concentrations. Dissociation constants (Kd) were also obtained as described in Materials and Methods, and values are the mean (±SD) of three independent determinations.

hand, the anti-PS1CT3 and anti-CysCT3 mAbs in group II repre­
sent diversified progeny derived from a single precursor. The oc­
currence of nonidentical replacement mutations in the variable re­
gions of the anti-PS1CT3 vs anti-CysCT3 mAbs suggested that at least some degree of differential optimization for the variably pre­

Discussion

Although surface accessibility is undoubtedly a prerequisite, recent results (9–13) now suggest that downstream, immune-mediated mechanisms will also need to be taken into consideration to ex­plain the hierarchical immunodominance of B cell epitopes on pro­tein Ags (30–38). An intriguing observation pertaining to this is­ue was our findings on the immunodominance of the DPAF sequence in segment PS1 when placed in the context of a variety of alternate sequences (9, 12). This was found to be independent of the position of the PS1 sequence in the Ag, the nature of the flan­king domains, and also the genetic background of the mouse strain employed (9–13). Indeed, regardless of the influence of such changes on the overall immunogenicity of the resulting molecules, thereby implying quantitative differences in B cell recognition, the DPAF segment was always the most immunodominant among the various epitopes presented by the Ag (12). Having previously ruled out a role for position within the immunogen or nature of flanking sequence (9, 12), in the present study we sought to explore how perturbations in the conformational degrees of freedom of the seg­ment encoding the DPAF epitope would influence its immu­nomodulativeness relative to the rest of the molecule.

An alteration in the degrees of freedom of the amino-terminal 10-residue segment was alternatively achieved by substitution at appropriate positions with Aib residues to confer a bias toward α-helix formation or with cysteine residues to enable subsequent cyclization. That such substitutions indeed influenced the local en­vironment within the DPAF epitope could be verified by an analysis of the 13C NMR spectra of these peptides. An effect on the immunodominancy of the alterations performed was also observed on immunization of mice with the resultant analogues. While peptide AibCT3 yielded primary IgG Abs that were marginally higher than those against the parent peptide PS1CT3, those against peptide CysCT3 were markedly diminished. Surprisingly, however, this effect on immunogenicity did not translate into altered profiles of relative immunodominance among the putative epitopes contained within these Ags. The apparent monospecificity of the anti­CysCT3 response could be further verified by generating mAbs, all of which were found to be confined to recognition of the DPAF sequence. Thus, in addition to factors described earlier, the relative
immunodominance of the DPAF epitope is also insensitive to perturbations in the conformational degrees of freedom available to the segment within which it is contained. Furthermore, the observations reported here and previously (12) that preponderance of the anti-DPAF Ab population was independent of overall immunogenicity strongly imply that parameters involved in defining immunogenicity of a multideterminant Ag are independent of those that influence interepitopic hierarchy on the same Ag.

The analogues described here also provided us with an opportunity to examine how variations in the conformational freedom of an epitope qualitatively influence repertoire selection and maturation from the preimmune B cell pool. This was possible since all three peptides produced, almost exclusively, an anti-DPAF response. For this purpose we selected only one of the analogues, peptide CysCT3, as it was expected that a covalent disulfide-mediated ring closure would enforce a greater degree of rigidity as opposed to Aib substitution. A comparison of the anti-PS1CT3 and anti-CysCT3 mAb panels revealed that an alteration in the conformational propensities of the DPAF epitope did not lead to complete repertoire diversification. Rather, a partial overlap between the two sets was observed on comparison of the Ig heavy chain variable regions. This suggests at least some degree of similarity between the independently derived paratope phenotypes. Although both the anti-PS1CT3 and anti-CysCT3 mAb panels also included distinct members, the stochastic nature of the fusion process does not permit an inference as to whether they represent unique Ag-specific products or simply reflect differences in clonal population sizes in vivo responses to the two Ags.

Considering the fact that peptides PS1CT3 and CysCT3 represent conformer variants of the same epitope, we had anticipated that the anti-PS1CT3 and anti-CysCT3 mAbs would display selectivity in terms of binding behavior for the homologous vis-a-vis heterologous confomer. Surprisingly, this did not hold true on comparison of the relative affinities for the two Ags. No significant differences in relative affinity could be detected for the two peptides with any of the mAbs tested. To investigate further we next selected those anti-PS1CT3 and anti-CysCT3 mAb subsets that shared a common heavy chain variable region, after accounting for point mutations as possible derivatives of the somatic hypermutation pathway in GCs. The underlying rationale for such a selection was based on the premise that a comparison between homologous paratope phenotypes would serve as a more reliable and accurate indicator of nuances in Ag selectivity if any. Such mAbs could subsequently be segregated into two groups based on the utilization of either the J43 (group I) or the J42 (group II) gene segment. By nucleotide sequencing of the light chain variable regions of mAbs in group I we were able to establish the independent clonal origins of the two anti-CysCT3 mAbs as well as their nonidentity with the anti-PS1CT3 mAbs of this group. In contrast, at least based on gene segment composition and CDR3 regions of both heavy and light chain variable regions, the mAb panel described in group II appeared to share a common precursor. Thus, the mAbs representing both groups seemed to us to provide two levels at which Ag-driven repertoire discrimination could be examined.

Based on the commonality of the heavy chain variable region but the diversity in that of the light chain, the anti-PS1CT3 and anti-CysCT3 mAbs in group I could be considered as a case of a relatively minor Ab paratope repertoire shift in response to the different DPAF conformer variants. On the other hand, the group II mAbs represented an interesting example of a single precursor Ab that had divergently adapted to optimally accommodate the variant epitope conformers presented by peptides PS1CT3 and CysCT3. The presence of nonidentical replacement mutations in the paratope components of the anti-PS1CT3 vs anti-CysCT3 mAbs strongly suggested that some degree of Ag-specific adaptation had indeed occurred.

In contrast to the lack of discrimination at the level of affinities, the facility of epitope recognition, as indicated by binding on-rates, was found to correlate well with the Ag that was used to elicit a particular mAb of either group. Thus, for example in group I, mAbs PC283 and PC289 bound the homologous peptide PS1CT3 with on-rates that were 8- to 23-fold higher than that for the heterologous peptide CysCT3. On the other hand, the reverse was true for mAbs Cys16 and Cys24 where the rate of peptide CysCT3 binding was much higher than that for peptide PS1CT3. Similarly, a pronounced bias in favor of the homologous conformer, in terms of binding on-rates, was also noted for the anti-PS1CT3 and anti-CysCT3 counterparts assigned to group II. This consistent observation for groups I and II that Ag specificity correlates with the kinetics of epitope recognition but not with the affinity of it strongly suggests that both Ag-specified discrimination between Ab repertoires and Ab optimization for epitope binding, by way of somatic mutations and subsequent positive selection in GCs, are under kinetic control. Our latter inference is entirely consistent with prior studies by Foote and Milstein (39), who have already demonstrated that intraclonal selection of mutated B lymphocytes in GCs is kinetically driven. Nevertheless, our results provide an added perspective in also suggesting that improvement of an Ab epitope fit in GCs may primarily represent a kinetic optimization.

While the high affinity of anti-PS1CT3 and anti-CysCT3 mAbs obtained in the early stages of a humoral response is surprising, it is consistent with more recent findings in other systems. Thus, for example, Zinkernagel and co-workers have shown that murine primary responses to vesicular stomatitis virus is composed of very high affinity Abs (40). More recently, Smith et al. (41) have demonstrated the presence of high affinity Ab-forming cells in primary humoral responses to the hapten (4-hydroxy-3-nitrophenyl)acetyl. Collectively, these studies suggest that optimal affinities for Ag may be achieved early, perhaps even before recruitment of B cells within germinal centers (GCs) (40, 41). Under such conditions, therefore, it may be expected that further optimization of Ag-Ab binding may be restricted to kinetic considerations.

Although our findings that optimization of the paratope-epitope fit is regulated by the kinetics of the interaction may appear to deviate from the common assumption that affinity for Ag plays the pivotal role (42–46), it is, however, consistent with our current understanding of the induction and progression of primary T-dependent humoral responses. It is now generally accepted that an Ag-activated B cell is constantly faced with the opposing choices of either positive selection for survival or death (47), the deciding factor being the ability of such B cells to recruit T cell help (10). Thus, in a competitive environment, such as that presented by GCs (24–28), where the available pool size of Th cells is limiting, rapid recruitment of T help by a given B cell is likely to confer on it an advantage for survival over alternate, competing clones. Indeed, our own recent results have demonstrated that the quantum of T cell help recruited by an Ag-activated B cell is proportional to the on-rate of Ag binding to its sIg receptor (13). This presumably relates to the rate at which such B cells can generate a high enough ligand density on the cell surface to ensure TCR triggering above the requisite threshold value (48, 49). Consequently, it is perhaps not surprising that those clonal variants in GCs kinetically optimized for epitope binding are the ones that are selected.

The revelation that Ab optimization for epitope conformation is kinetically determined would also imply that facility of an epitope fit assumes premium over the perfection of such a fit. This may explain the high frequency of occurrence of flexible domains on protein Ags as B cell epitopes (7, 50). Further, this observation
may serve to rationalize the anomalous findings of imperfect Ag-Ab interfaces in the x-ray crystal structure of a variety of immumne complexes (51, 52).

In summary, the results presented here provide evidence to support the following inferences. 1) Relative immunodominance of a peptide B cell epitope is independent of the conformational constraints imposed on it. We stress here again that our interpretation limits to varying degrees of conformational freedom as opposed to transitions from one discrete secondary structural form to another. 2) Kinetics override equilibrium binding criteria in mediating Ag-specific discrimination between alternate possible Ab repertoire subsets. 3) Positive selection following somatic hypermutation in GCs is biased in favor of a kinetic optimum. Finally, our studies reported here also highlight the utility of appropriately designed model peptide Ags as novel probes to delineate mechanisms regulating humoral responses.

Acknowledgments

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References

Differential sensitivities of primary and secondary T cell responses to antigen structure

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Received 28 September 1998

Abstract Here we examined T cell responses to two analogs of a chimeric peptide encoding a known B and a known T cell epitope. In one of the analogs, the B cell epitope existed in a random conformation whereas it was restricted within a disulfide-bonded cyclic loop in the other. Immunization of these peptides in mice revealed that the latter peptide was significantly poorer at priming T cells and our preliminary results suggest this could be the result of differential processing of the two analogs. While primary T cell responses were sensitive to the influence of conformation, secondary responses were not discriminatory for the two antigens confirming the differences in activation requirements for primary and secondary T cell responses. Further, our studies also suggest that the priming efficacy of a T cell epitope is influenced by its structural environment.

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Key words: Synthetic peptide; T cell priming; Secondary structure

1. Introduction

A viable T-dependent humoral response hinges on the ability of an antigen to appropriately prime specific B and T lymphocytes. Subsequent progression is then driven by the efficiency with which such antigen-activated B cells can recruit help from the antigen-specific T helper cell pool [1]. Antigen-activated B and T lymphocytes engage in a cognate interaction, leading to reciprocal proliferation and differentiation into effector and memory populations [2].

While activated T cells play a pivotal role in driving humoral responses, we have recently shown that primed T cells are also critical in defining the spectrum of B cell fine specificities that are positively selected from the multitude of clonotypes that are initially activated [3]. The available pool size of primary antigen-activated T cells serves as a rate-limiting determinant that enforces a competitive process to select for high affinity clones [3]. It is the successful subset that dominates the population of B cells contributing to the later stages of a primary humoral response [3]. Thus, effective priming of CD4 T cells by an antigen is critical in elaborating not only the quantitative aspects of a humoral response, but also the antibody specificities that constitute it.

The present report continues our earlier investigations on modulation of B cell responses using analogs of a chimeric peptide encoding a known B and a known T cell epitope [3-7]. We show here that the efficacy of T cell priming in such constructs can be significantly affected by the imposition of a conformational constraint on the flanking B cell epitope. Interestingly however, although T cell priming was significantly diminished, secondary T cell responses were relatively unaltered. In addition to confirming differences in requirements for primary and secondary T cell activation, our results also suggest that the efficiency or priming by individual T cell epitopes on a multi-determinant antigen may be sensitive to the structural milieu in which they occur.

2. Materials and methods

2.1. Materials

Horseradish peroxidase-labeled anti-mouse IgG (heavy chain-specific) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). F-moc amino acid derivatives were purchased from Novabiochem (Laufelfingen, Switzerland).

2.2. Peptide synthesis

Peptides were synthesized on a Milligen 9030 automated peptide synthesizer using F-moc chemistry [8]. Crude peptide was purified to at least 95% purity by reverse phase HPLC. For the synthesis of peptide CysCT3, the side chain protecting group used for cysteine was the acetamidomethyl (Acm) group. Subsequent to synthesis and cleavage from solid support, simultaneous deprotection of the cysteine side chains and oxidation to form intramolecular disulfide bonds was achieved with iodine in acetic acid [9].

The correct identity of all peptides synthesized was ascertained both by amino acid analysis and by mass spectrometry.

2.3. Animals and immunizations

Female BALB/c mice (6-8 weeks old) were obtained from the small animal facility at the National Institute of Nutrition (Hyderabad, India). Immunizations with either peptide PS1CT3 or CysCT3 were i.p. at a dose of 50 μg/mouse (CFA, base of tail) was administered 7 days prior to immunization with peptide CysCT3.

ELISA and LNC proliferation assays were essentially as described earlier [6].

3. Results

3.1. Immunogenicity of peptides PS1CT3 and CysCT3

Peptide PS1CT3 is a chimeric peptide with the sequence HQLDPAFGANSTNPDGDIEKIKIAKMEKASSVFNVV-NS. The first 15 residues of this sequence (segment PS1) represent a well characterized B cell epitope derived from the envelope protein of hepatitis B virus [10]. Residues 18–38 (segment CT3) correspond to a promiscuous T cell epitope present on the circumsporozoite protein of the malaria parasite Plasmodium falciparum [11]. Separating the B and T cell epitopes is a spacer of two glycine residues at positions 16 and 17. Prior analysis of peptide PS1CT3 by circular dichroism spectroscopy had revealed that this peptide exists in a random distribution of conformations in aqueous solutions [3]. Immu-
Possibilities we synthesized a linearized analog of peptide either due to altered secondary structure of the neighboring B not shown). Obtained with peptide CysCT3 as the challenge antigen (data monologous peptide (Fig. 2A) or a peptide representing the CT3 segment of these immunogen molecules (Fig. 2B). Further­more, challenge of CysCT3-primed LNCs with peptide CysCT3-primed LNCs yielded significant proliferative responses, those from CysCT3-primed mice were markedly diminished (Fig. 2). This was true over the entire range of time points evaluated. It would thus appear that peptide CysCT3 is markedly less immunogenic than peptide PSICT3.

3.2. Peptide CysCT3 primes T cells poorly

To examine whether the differences in immunogenicity could have resulted from differences at the level of T cell priming, proliferative responses of lymph node cells (LNCs) from mice immunized with either peptide were measured. While LNCs from peptide PSICT3-immunized mice yielded significant proliferative responses, those from CysCT3-primed mice were markedly diminished (Fig. 2). This was true regardless of whether the challenge antigen employed was the homologous peptide (Fig. 2A) or a peptide representing the CT3 segment of these immunogen molecules (Fig. 2B). Furthermore, challenge of CysCT3-primed LNCs with peptide PSICT3 resulted in stimulation index values identical to those obtained with peptide CysCT3 as the challenge antigen (data not shown).

The poor T cell priming ability of peptide CysCT3 could be due to altered secondary structure of the neighboring B cell epitope segment, or a consequence of the nature of the substitutions performed. To discriminate between these two possibilities we synthesized a linearized analog of peptide CysCT3 where the cysteine-sulphydryl groups were blocked as acetamidomethyl derivatives (peptide Cys(Acm)CT3). Immunization of BALB/c mice with peptide Cys(Acm)CT3 followed by an examination of LNC proliferative responses revealed that this analog was far more potent at priming T cells than peptide CysCT3 (Fig. 3). Thus, it would appear that the attenuated ability of peptide CysCT3 to prime specific T cells is a consequence of the altered conformation of the flanking B cell epitope.

3.3. Poor B cell immunogenicity of peptide results from inefficient T cell priming

It was of interest to ascertain whether the poor antibody response to peptide CysCT3 in Fig. 1 was due to insufficient T cell priming. For this, mice were first preprimed with peptide CT3 7 days prior to immunization with peptide CysCT3. Pre­priming with peptide CT3 has been shown to function by increasing the frequency of antigen-activated T cells available at the time of antigen exposure to naive B cells [5]. An analysis of anti-CysCT3 IgG levels in sera collected subsequently revealed that CT3 prepriming resulted in a marked improvement in anti-CysCT3 titers over those from mock-preprimed mice. This clearly suggests that the poor B cell immunogenicity of peptide CysCT3 was likely to be due to the presence of a limiting amount of T cell help. This possibility could be further validated by comparing primary anti-peptide IgG titers obtained in mice immunized with either peptide CysCT3 or Cys(Acm)CT3. In these experiments we observed that peptide Cys(Acm)CT3 was at least five-fold more immunogenic, in terms of IgG antibody levels, than peptide CysCT3 (data not shown).

3.4. Peptides PSICT3 and CysCT3 are equally competent at eliciting secondary T cell responses

Although the analogs studied here differed markedly in their abilities to prime for a T cell response, we also compared their potencies at recalling a preprimed population of antigen-specific T cells. For this, LNCs from mice primed with either peptide CT3 or PSICT3 were challenged in vitro with varying

Fig. 1. Relative immunogenicity of peptides PSICT3 and CysCT3. Groups of five mice each were immunized either with peptide PSICT3 (○) or with peptide CysCT3 (●) as described in Section 2. Blood was collected at indicated time points and sera within a group pooled. Peptide-specific IgG antibody levels were quantitated by ELISA. The figure shows a representative of five independent experiments.

Fig. 2. Primary T cell responses induced by peptide PSICT3 and its analogs. LNCs from mice primed with either peptide PSICT3 (○), peptide CysCT3 (●) or peptide Cys(Acm)CT3 (△) were cultured in vitro with the indicated concentrations of either the homologous peptide (A) or peptide CT3 (panel B). Lymphocyte proliferation was measured as counts of [3H]thymidine incorporated. Mean background counts varied between 1500 and 2000 cpm. Values are presented as the stimulation index (mean ± S.E.M. of quadruplicate sets), which represents the ratio of counts obtained at a given concentration of antigen over that obtained in the absence of any challenge antigen. The figure shows a representative of five independent experiments.
and affinity of the resulting peptides for the MHC class II on T cell activation [15-17].

Although true in principle, activation of an influence the efficacy with uptake by specific T cell is nevertheless appropriate ligand to it. Thus, for example, extent of antigen in association with MHC class II molecules [13, 14]. As a tended conformation - generated by processing of the parent result, recognition of an antigenic determinant by T cells is contrast, T cell recognition is restricted to peptides in an context in which a given antigenic determinant is present. In response can be expected to be influenced by the structural environment of the CT3 segment, both peptides PS1CT3 and CysCT3 proved equally competent at recalling secondary T cell responses in vitro. This latter result confirms a difference in requirements for activation of primary and secondary T cell responses [18].

The mechanism by which an altered conformation of the PSI segment inhibits T cell priming by peptide CysCT3 is currently unclear. However, it is likely that the difference is experienced at the level of antigen presentation, presumably by dendritic cells and/or macrophages – the principal cell types implicated in priming of CD4+ T cells [19]. Such variance could arise as a consequence of either altered antigen uptake or alterations at the level of antigen processing. Although our observations that both peptides PS1CT3 and CysCT3 elicit nearly identical recall responses may appear to rule out dramatic differences in processing of the two peptides, we cannot ignore the possibility of minor differences that exert their influence at the level of primary but not secondary T cell responses. Furthermore, the finding of comparable recall efficiencies of peptides PS1CT3 and CysCT3, regardless of whether the LNCs were primed with peptide CysCT3, PS1CT3 or CT3, strongly points towards quantitative rather than qualitative differences in antigen presentation from the two peptides.

Our demonstration here that the structural environment in the vicinity of a T cell epitope can affect its ability to prime T cells raises some interesting implications. This is particularly true with respect to the more complex multi-determinant antigens such as proteins. It is generally believed that immune responses are initiated by presentation of epitopes for CD4+

![fig. 3](image_url)

**Fig. 3.** Enhanced immunogenicity of peptide CysCT3 in CT3-preprimed mice. Groups of four mice each were either preprimed with peptide CT3 (group 2) or mock-primed with adjuvant alone (group 1). Seven days later, both groups were immunized with peptide CysCT3. Sera were collected at weekly intervals and anti-CysCT3 IgG titers measured by ELISA. Data shown here are for sera obtained from individual mice at 14 days after CT3 immunization.

concentrations of either peptide PS1CT3 or peptide CysCT3 and the resulting proliferation was measured. As shown in Fig. 4, recall proliferative responses elicited by peptide CysCT3 were comparable to those by peptide PS1CT3 both in PS1CT3- (panel A) and CT3- (panel B) primed LNCs. Thus, although peptides PS1CT3 and CysCT3 differ in their abilities to prime T cells they are equally proficient at recalling a preprimed antigen-specific T cell population.

Pretreatment of CT3-primed LNCs with chloroquine abolished recall responses to both peptides PS1CT3 and CysCT3 (stimulation index values < 2.0), confirming our earlier observations [6] that PS1CT3 and its analog peptides require processing prior to appropriate presentation in association with MHC class II molecules.

4. Discussion

A productive immune response requires optimal activation of both antigen-specific B and T lymphocytes. Recognition – a prerequisite for activation – by B lymphocytes involves a direct encounter between the B cell antigen receptor (BCR) and target antigen, the latter in its native form [12]. Consequently, both qualitative and quantitative aspects of a humoral response can be expected to be influenced by the structural context in which a given antigenic determinant is present. In contrast, T cell recognition is restricted to peptides in an extended conformation – generated by processing of the parent antigen within antigen-presenting cells (APCs), and presented in association with MHC class II molecules [13, 14]. As a result, recognition of an antigenic determinant by T cells is expected to be independent of the structural milieu of its origin. Although true in principle, activation of an antigenspecific T cell is nevertheless variably affected by factors that influence the efficacy with which an APC can present the appropriate ligand to it. Thus, for example, extent of antigen uptake by APCs, rate and specificity of antigen processing, and affinity of the resulting peptides for the MHC class II molecule have all been shown to serve as regulatory influences on T cell activation [15-17].

The present study describes a surprising effect of conformation of a flanking domain on in vivo priming by a T cell epitope. Peptide CysCT3, where the neighboring B cell epitope sequence was held in a disulfide-bonded loop, was distinctly less immunogenic than its parent peptide PSI CT3 where the PSI segment existed in a random distribution of conformations. The poor anti-CysCT3 IgG antibody response was shown to result from its inability to prime T cells as effectively as peptide PS1CT3. Furthermore, it could also be demonstrated that the attenuated T cell priming efficacy of peptide CysCT3 was purely a consequence of the altered structure of the PSI segment rather than due to the nature of the substitutions performed. Interestingly however, while primary T cell responses were sensitive to the structural environment of the CT3 segment, both peptides PS1CT3 and CysCT3 proved equally competent at recalling secondary T cell responses in vitro. This latter result confirms a difference in requirements for activation of primary and secondary T cell responses [18].

![fig. 4](image_url)

**Fig. 4.** T cell recall responses to peptides PS1CT3 and CysCT3. LNCs from mice primed with either peptide PS1CT3 (A) or peptide CT3 (B) were challenged with indicated concentrations of either peptide PS1CT3 (○) or peptide CysCT3 (●) and proliferation measured as described for Fig. 2. Data presentation is as described for Fig. 2. The figure is a representative of three separate experiments.
T cells by antigen non-specific APCs [20]. The poor efficiency of non-specific uptake mechanisms, leading to low concentrations of peptide fragments generated intracellularly, has been proposed to account for the selective immunodominance of peptide epitopes in primary CD4+ cell responses [20]. The results presented here suggest that local environmental influences, by modifying T cell priming efficacies of individual T cell epitopes, can add yet another variable in defining the repertoire of T cell specificities generated in a primary response to protein antigens. Indeed, in this connection, a recent study has identified the existence of a predictive correlation between immunodominant T cell epitopes, and structurally unstable segments in protein antigens [21].

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References

B Cell Responses to a Peptide Epitope. VII. Antigen-Dependent Modulation of the Germinal Center Reaction

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Germinal center responses to two analogous peptides, PS1CT3 and G32CT3, that differ in sequence only at one position within the B cell epitopic region were examined. In comparison with peptide PS1CT3, peptide G32CT3 elicited a poor germinal center response. By demonstrating equal facility of immune complexes with IgM and IgG Ab isotypes to seed germinal centers, we excluded differences in isotype profiles of early primary anti-PS1CT3 and anti-G32CT3 Ig as the probable cause. Quantitative differences in germinal center responses to the two peptides were also not due to either qualitative/quantitative differences in T cell priming or variation in the frequency of the early Ag-activated B cells induced. Rather, they resulted from qualitative differences in the nature of B cells primed. Analysis of early primary anti-PS1CT3 and anti-G32CT3 IgMs revealed that the latter population was of a distinctly lower affinity, implying the existence of an Ag affinity threshold that restricts germinal center recruitment of G32CT3-specific B cells. The impediment in anti-G32CT3 germinal center initiation could be overcome by making available an excess of Ag-activated Th cells at the time of immunization. This resulted in the appearance of a higher affinity population of G32CT3-specific B cells that, presumably, are now capable of seeding germinal centers. These data suggest that the strength of a germinal center reaction generated is Ag dependent. At least one regulatory parameter represents the quality of B cells that are initially primed. The Journal of Immunology, 1998, 161: 5832–5841.

It is generally believed that T-dependent Ag-induced activation of B cells first occurs in the T cell-rich extrafollicular sites. Shortly thereafter, foci of specific Ab-producing B cells (AFCs) concentrate in the periphery, adjacent to the red pulp, of the periaortiolar lymphoid sheath (PALS) (1). Within the next few days, germinal centers (GCs) can be observed to develop within the primary B cell follicles, where processes related to affinity maturation and B lymphocyte differentiation into either memory or plasmacytes are initiated (1–9).

A consensus view has emerged in the literature to account for the relationship between AFCs in the outer PALS and GCs during a primary T-dependent humoral response. The identification of clonal relatedness between cells that seed developing GCs and those within AFCs has led to the proposition that constituents of both compartments derive from a common precursor (10). As a result, it is generally believed that a subset of B cells activated in the T cell areas to form AFC also migrate into the primary follicle to initiate GC development (4). Seeding of GCs is thought to be driven by deposition of immune complexes of Ag with Ab secreted in the foci onto follicular dendritic cells (FDCs) (1–9). Although this has found general acceptance, there are, however, data in the literature that highlight inconsistencies with the overall scheme. For instance, Klinman’s group has provided evidence to suggest that B cells that nucleate early AFCs and GC reactions derive from distinct lineages and independently give rise to primary Ab and memory B cell responses (11–13). These populations were phenotypically distinguished on the basis of either high or low levels of expression of the surface marker J11D (11). It was further demonstrated that only the J11D⁺ population of B cells was capable of originating GCs (12). Recent studies also question the criticality of AFC formation as a precondition to induction of GCs. Thus, for example, a primary murine humoral response to vesicular stomatitis virus was found to occur in the absence of any detectable virus-specific AFCs, although specific GCs could readily be identified (14). More recently, Vora et al. (15) were unable to observe AFC formation in an antiarsonate response in A/J mice despite the occurrence of a vigorous GC response. It has been suggested from these studies that there are intrinsic differences in the nature of B cell clones that participate in the AFC and GC responses (15).

Regardless of whether phenotypic distinctions do, in fact, exist, it is, nevertheless, clear that there is some degree of differentiation between B cells that populate GCs and those that constitute AFCs. A variety of studies have observed that the overall clonal heterogeneity of GC B cells is markedly diminished compared with those initiated in the PALS (1–10). In other words, not all B cells activated by Ag in the PALS are eventually successful in seeding GCs. These findings clearly point toward the involvement of a selection mechanism(s) that filters the candidates vying for accommodation within the GC pathway. Processes that guide such a selection and parameters that enforce this discrimination, however, remain to be elucidated.

We have been using synthetic peptides as model Ags in an effort toward delineating clonal selection mechanisms that regulate an early primary T-dependent humoral response (16–20). In a recent report we employed a pair of analogous peptides, PS1CT3 and G32CT3, which differed in sequence only at a single amino acid residue position (19). Although both peptides were equally competent at priming T cells in vivo, the primary humoral response to peptide G32CT3 was markedly reduced compared with that against peptide PS1CT3 (19). In the present report we exploited...
Animals and immunizations

Female BALB/c mice (6–8 wk old) were obtained from the small animal breeding 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 usually pooled.

For CT3 prepriming, mice were immunized with 50 μg/mouse of a CFA emulsion of peptide CT3 (at the base of the tail) 7 days before immunization with either peptide PS1CT3 or G32CT3.

Ab isotype separation and generation of immune complexes

Immune sera collected at various time points were individually resolved for the IgM and IgG components 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. Both verification of the absence of the unwanted isotype and estimation of Ag-specific IgG and IgM were determined by quantitative ELISA.

For preparation of immune complexes, IgM, IgG, or total Ig preparations were incubated with a 10-fold excess of peptide (based on the estimated number of specific binding sites) in PBS for 1 h at 37°C with occasional shaking. The preparation was then dialyzed against PBS (three changes over 4 h) to remove unbound peptide and concentrated if necessary. Aliquots of 100 μl containing immune complexes corresponding to 500 ng of starting Ab were injected i.v. into recipients. We first established, in pilot experiments, that this was the optimum Ab concentration for use in GC reconstitution experiments.

Enrichment of B and T cells from immunized mice

After depletion of RBCs (RBC lysis buffer, Sigma) either splenocytes (for B cells) or inguinal lymph node cells (for T cells) were first separated from adherent cells by two rounds (1 h each at 37°C) of centrifugation on plastic plates. For enriched B cells, the resulting splenocyte suspension was depleted of T cells by two rounds of treatment with anti-Thy1.2-coated magnetic beads (Dynal). On the other hand, for enriched T cells, lymph node cells were deprived of B cells by two rounds of treatment with anti-B220-coated magnetic beads (Dynal). The remaining cell suspension was pelleted and resuspended in culture medium (RPMI 1640) to the appropriate cell concentration. By these procedures a cell purity between 90–95% was obtained as determined by a FACS analysis, with contaminating lymphocytes (i.e., either B or T cells) representing between 2–4% of the total population. Cell viability was determined by trypan blue exclusion.

Reconstitution of GCs in vivo

A total volume of 200 μl containing immune complexes of 500 ng of Ab and 5 × 10⁶ enriched T cells from mice primed with CT3 10 days earlier and then boosted after 1 wk was transferred (i.v.) into irradiated (550 rad) BALB/c mice. Twenty-four hours later these mice also received (i.v.) enriched B cells (1 × 10⁶ in 200 μl/mouse) derived from splenocytes of mice immunized 2 days earlier with the appropriate peptide (i.e., PS1CT3 or G32CT3). Ten days after B cell transfer, spleens were removed from the irradiated hosts, and sections prepared for detection and enumeration of Ag-specific GCs. These conditions were optimized after a series of pilot experiments.

Immunohistochemical staining of Ag-specific GCs

Six-micron-thick sections of frozen spleens were taken on a cryostat microtome and thaw-mounted on glass slides. Sections were allowed to dry briefly, after which they were fixed in ice-cold acetone for 10 min, air-dried for 10 min, and stored at −70°C until use.

When required, the frozen sections were thawed and rehydrated in PBS for 20 min. Endogenous peroxidase activity was quenched with 0.1% phenylhydrazine (Sigma). After three washes, sections were blocked for nonspecific binding with a 1/1 (v/v) solution of 3% BSA (in PBS) and mouse monoclonal serum for 1 h at 37°C. Slides were then incubated for 90 min with 20 μg/ml of PNA-biotin in HEPES (pH 7.5) and washed, followed by a second incubation with streptavidin-HRPO (5 μg/ml in PBS) for 45 min. Both incubations were performed at 37°C. Subsequent to this either Tet-PS1 or Tet-G32 was added in PBS at a concentration of 30 μg/ml, and the slides were incubated overnight at 4°C. After a wash the slides were treated with the recommended concentrations of streptavidin–alkaline phosphate conjugate in PBS for 45 min at 37°C. Bound conjugates were then visualized in a sequential manner. The HRPO conjugate was first detected by color development with the 3-amino-9-ethylcarbazole (AEC) staining kit (Vector), where a red color for PNA⁺ cells was obtained. Bound alkaline

these differences to examine Ag-dependent modulation of GC responses, with particular emphasis on the role of Ag-activated B cells. We show here that the intensity of a GC reaction obtained is dependent on Ag and, more specifically, on the quality of B cells that are initially activated. Further, our results suggest that an Ag-affinity barrier exists that enforces the selection of a limited spectrum of B cells clones for seeding the GC reaction.

Materials and Methods

Materials

HRPO-labeled secondary Abs were obtained from Sigma (St. Louis, MO). Coated magnetic beads for panning of B and T cells (Dynabeads, mouse pan T and mouse pan B) were purchased from Dynal (Oslo, Norway). Derivatized amino acids for peptide synthesis were procured from NovaBiochem (Laufelfingen, Switzerland). Biotinylated PNA, streptavidin–alkaline phosphatase, and streptavidin–HRPO conjugates were obtained from Vector (Burlingame, CA), and anti-B7-2 (clone GL1) was purchased from PharMingen (San Diego, CA).

Peptide synthesis

Peptides were synthesized on a Milligen 9050 synthesizer (Millipore, Bedford, MA) using F-moc chemistry (21). Crude peptides were purified to >95% purity by reverse phase HPLC on a C₄ column (15 μm, 8AK, Waters, Milford, MA; 19 × 300 mm) using an aqueous gradient of 0–70% acetonitrile in 0.1% TFA. The identities of all peptides were ascertained by both amino acid analysis and mass spectrometry.

For synthesis of Tet-PS1 and Tet-G32 peptides the following scheme was employed. A protected cysteine was first added to the solid support, to which was then coupled a lysine that was differentially protected at the ε-amino group with a β-butoxyacarbonyl functionality and with F-moc at the ε-amino group. After selective F-moc deprotection, a second lysine was coupled, but both the amino groups were derivatized with F-moc. F-moc on which either the tetrapeptide containing two copies of either the PSI or the AC sequence was sequentially built using a protected cysteine was first added to the solid support, to which was then coupled a lysine that was differentially protected at the ε-amino group with a β-butoxyacarbonyl functionality and with F-moc at the ε-amino group. After selective F-moc deprotection, a second lysine was coupled, but both the amino groups were derivatized with F-moc. F-moc on which either the tetrapeptide or the G32 sequence was sequentially built using a protected cysteine was first added to the solid support, to which was then coupled a lysine that was differentially protected at the ε-amino group with a β-butoxyacarbonyl functionality and with F-moc at the ε-amino group. After selective F-moc deprotection, a second lysine was coupled, but both the amino groups were derivatized with F-moc. F-moc on which either the tetrapeptide or the G32 sequence was sequentially built using a protected cysteine.
phosphatase was revealed with the blue staining kit (Vector), which detected the presence of Ag-specific cells. A minimum of 30 individual sections, spread longitudinally across the spleen, were examined from each spleen, and each experimental group included from three to five mice.

ELISAs

Plates were coated with 2 μg/ml of peptide/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 antisera was added and incubated at 37°C for 1 h. After washing, bound Ab was detected with HRPO-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, antisera were used at dilutions representing 50% of the iter value. Twofold higher concentrations of antisera and competitor peptide were mixed in equal volumes and incubated for 10 min at room temperature. This was then added to duplicate wells at 100 μl/well. The rest of the procedure was performed as described above.

ELISPOT assays

Twenty-four well culture plates (Nunc) were coated with 1 ml of a 20 μg/ml solution of either peptide PSI1CT3 or peptide G32CT3 in PBS at 4°C overnight. Nonspecific sites were then blocked with 0.5 ml/well of a 1% solution of BSA in PBS at 37°C for 1 h. Spleenocytes from mice immunized with either peptide PSI1CT3 or G32CT3 were removed at appropriate times and depleted of RBCs and adherent cells as described above. These were then suspended in RPMI 1640 medium at varying dilutions of 1 × 10^4, 1 × 10^5, and 1 × 10^6 cells/ml of viable cells. Of this, 1 ml aliquots were added to quintuplet wells, and the plates were incubated at 37°C for 2 h. After this, the wells were washed (three times) and then incubated with the appropriate dilution of a mixture of anti-mouse IgM and anti-mouse IgG conjugated to alkaline phosphatase at 4°C overnight. The plates were then washed again, and bound conjugate was revealed with 5-bromo-4-chloro-3-indolyl phosphate in 2-amino-2-methyl-1-propanol buffer. Scoring of spots was performed under a microscope at ×10 magnification.

Results

Peptides PSI1CT3 and G32CT3

The model peptide, PSI1CT3, that we used in our studies is shown in Fig. 1A. This peptide represents a chimeric sequence in which residues 1–15 (segment PSI1) correspond to a known B cell epitope within positions 28–42 of the preS1 region of hepatitis B surface Ag (22). Residues 18–38 (segment CT3) represent a well-characterized, promiscuous T cell epitope derived from the circumsporozoite protein of the malaria parasite Plasmodium falciparum (23). Separating the B and T cell epitopes is a spacer of two glycine residues at positions 16 and 17, which have been included for reasons described previously (24). Peptide G32CT3 represents a single amino acid-substituted analogue of peptide PSI1CT3 in which the proline at position 5 has been replaced with a glycine residue (Fig. 1A).

In our previous studies we have shown in mice that while the early IgM response to peptide PSI1CT3 includes Ab specificities that cumulatively recognized the entire PSI segment, a subsequent class switch was restricted to B cell specificities directed only against the tetrapeptide sequence positions 4–7 (sequence DPAF) (17). Interestingly, as described previously (19), immunodominance of the DPAF segment was independent of a variety of variables that included the H-2 haplotype of the mouse strain employed. Further, the Ab repertoire elicited against the epitope was also heterogeneous; it was composed of Abs containing a variety of VN, and VV gene segments with diverse CDR3 lengths and composition (25). More recently we have demonstrated that a single amino acid substitution within this sequence (proline to glycine, peptide G32CT3) was sufficient to render the resultant analogue virtually nonimmunogenic (19). While immunization with both peptides PSI1CT3 and G32CT3 yielded comparable levels of early primary IgM Abs, the anti-G32CT3 response was abated, in that neither class switch of Abs from IgM to the IgG isotype or of B cell differentiation into a memory population was detected (19).

This was particularly surprising considering that peptide G32CT3 was shown to be as potent as peptide PSI1CT3 at priming T cells in vivo (19). This enigma was subsequently resolved by our demonstration of a higher T-dependent activation threshold for the step coinciding with Ab isotype switch, where the affinity of B cell Slg receptor of a given B cell clonotype for Ag proved the deciding factor between positive selection and elimination at this stage (19). For reasons presently unclear, the early Abs generated against peptide G32CT3, in contrast to the anti-DPAF component in PSI1CT3, proved to be of too low an affinity to permit successful cross-over (19).

From our description above, it is obvious that peptides PSI1CT3 and G32CT3 represent analogous immunogens that, although identical with respect to induction of T cell responses, significantly differ at the level of the B cell responses elicited. Thus, these peptides provided us with a good model system to evaluate a possible role for distinctions in primary B cell activation in the efficiency of GC formation. We recall here that both peptides PSI1CT3 and G32CT3 have previously been shown to represent T-dependent Abs, even at the level of early primary IgM Ab induction (17).

Immunization with peptide G32CT3 elicits a poor GC response

To evaluate any differences in GC responses against the two analogue peptides, parallel groups of BALB/c mice were immunized with peptide PSI1CT3 or peptide G32CT3. Subsequently, spleens were removed at various time points, and sections thus obtained were stained for the immunohistochemical detection of Ag-specific GCs. A double-staining protocol for B cells that were both Ag specific and PNA- was employed (see Materials and Methods). For the detection of Ag-specific B cells, a separate, biotinylated peptide containing only the B cell epitope segment (PS1 or G32), but in a tetrimeric configuration, was designed and synthesized (peptides Tet-PS1 and Tet-G32; Fig. 1B). In preliminary experiments we observed that such a peptide provided for a higher intensity of staining, as opposed to a biotinylated monomer, presumably by increasing the avidity of binding to B cells (data not shown).

Fig. 2 depicts a representative staining obtained for GCs induced in mice immunized 7 days earlier with peptide PSI1CT3. The kinetics of GC formation against PSI1CT3 are shown in Fig. 3. GCs were first evident on day 6 and rapidly increased in number up to day 10, after which their number began to decline (Fig. 3). Surprisingly, in contrast to peptide PSI1CT3, peptide G32CT3 induced only a marginal GC response, which was also transient (Fig. 3). Since both peptides are known to be capable Ag, in contrast to peptide PSI1CT3, in which the proline at position 5 has been replaced with a glycine residue (Fig. 1A).

In our previous studies we have shown in mice that while the early IgM response to peptide PSI1CT3 includes Ab specificities that cumulatively recognized the entire PSI segment, a subsequent class switch was restricted to B cell specificities directed only against the tetrapeptide sequence positions 4–7 (sequence DPAF) (17). Interestingly, as described previously (19), immunodominance of the DPAF segment was independent of a variety of variables that included the H-2 haplotype of the mouse strain employed. Further, the Ab repertoire elicited against the epitope was also heterogeneous; it was composed of Abs containing a variety of VN, and VV gene segments with diverse CDR3 lengths and composition (25). More recently we have demonstrated that a single amino acid substitution within this sequence (proline to glycine, peptide G32CT3) was sufficient to render the resultant analogue virtually nonimmunogenic (19). While immunization with both peptides PSI1CT3 and G32CT3 yielded comparable levels of early primary IgM Abs, the anti-G32CT3 response was abated, in that neither class switch of Abs from IgM to the IgG isotype or of B cell differentiation into a memory population was detected (19).

This was particularly surprising considering that peptide G32CT3 was shown to be as potent as peptide PSI1CT3 at priming T cells in vivo (19). This enigma was subsequently resolved by our demonstration of a higher T-dependent activation threshold for the step coinciding with Ab isotype switch, where the affinity of B cell Slg receptor of a given B cell clonotype for Ag proved the deciding factor between positive selection and elimination at this stage (19). For reasons presently unclear, the early Abs generated against peptide G32CT3, in contrast to the anti-DPAF component in PSI1CT3, proved to be of too low an affinity to permit successful cross-over (19).

From our description above, it is obvious that peptides PSI1CT3 and G32CT3 represent analogous immunogens that, although identical with respect to induction of T cell responses, significantly differ at the level of the B cell responses elicited. Thus, these peptides provided us with a good model system to evaluate a possible role for distinctions in primary B cell activation in the efficiency of GC formation. We recall here that both peptides PSI1CT3 and G32CT3 have previously been shown to represent T-dependent Ags, even at the level of early primary IgM Ab induction (17).

Immunization with peptide G32CT3 elicits a poor GC response

To evaluate any differences in GC responses against the two analogue peptides, parallel groups of BALB/c mice were immunized with peptide PSI1CT3 or peptide G32CT3. Subsequently, spleens were removed at various time points, and sections thus obtained were stained for the immunohistochemical detection of Ag-specific GCs. A double-staining protocol for B cells that were both Ag specific and PNA- was employed (see Materials and Methods). For the detection of Ag-specific B cells, a separate, biotinylated peptide containing only the B cell epitope segment (PS1 or G32), but in a tetrimeric configuration, was designed and synthesized (peptides Tet-PS1 and Tet-G32; Fig. 1B). In preliminary experiments we observed that such a peptide provided for a higher intensity of staining, as opposed to a biotinylated monomer, presumably by increasing the avidity of binding to B cells (data not shown).

Fig. 2 depicts a representative staining obtained for GCs induced in mice immunized 7 days earlier with peptide PSI1CT3. The kinetics of GC formation against PSI1CT3 are shown in Fig. 3. GCs were first evident on day 6 and rapidly increased in number up to day 10, after which their number began to decline (Fig. 3). Surprisingly, in contrast to peptide PSI1CT3, peptide G32CT3 induced only a marginal GC response, which was also transient (Fig. 3). Since both peptides are known to prime T cells equally well (19), it follows then that the differences shown in Fig. 3 are likely to be a result of distinctions in the B cells that have been activated by the two immunogens.

We have shown earlier that the defect in an anti-G32CT3-promoted humoral response could be overcome by prepriming the mice with a peptide (peptide CT3) representing the T cell epitope segment (segment CT3) of the immunogen molecules (19). In other words the constraint imposed by the low affinity, for Ag, of G32CT3-activated B cells could be circumvented by increasing the frequency of available Ag-primed Th cells. To determine whether such a strategy may also ameliorate GC induction, a parallel cohort of mice was first preprimed with peptide CT3 before immunization with peptide G32CT3. As shown in Fig. 3, CT3 prepriming clearly resulted in a vigorous and sustained anti-G32CT3 GC reaction that was even more pronounced than that obtained against peptide PSI1CT3. In contrast, administration of peptide CT3 2 days after primary immunization with peptide G32CT3 (day 0) did not result in any significant improvement in the
number of GCs detected on day 7 (data not shown). Thus, the inadequacy of G32CT3-primed B cells to initiate a GC reaction could be circumvented by increasing the pool size of available Ag-activated T cells, which appear to be required in the very early stages of a primary anti-G32CT3 humoral response.

Although we have suggested that the positive effect of CT3 prepriming derives from an increased frequency of Ag-activated T cells, the possibility of differential ability of memory vs primary activated T cells in supporting development of G32CT3-specific GCs cannot be ignored at present.

Immune complexes with both IgM and IgG Abs are equally proficient at supporting GC formation

It was possible that the observed differences in the GC response to the two peptides could be due to the absence of an IgM to IgG Ab class switch in the peptide G32CT3-immunized mice (19). There is some variance in the literature regarding the relative abilities of immune complexes generated with IgG or IgM Abs to seed GCs. While earlier reports suggested that only the IgG-constituted immune complexes are functional (26), more recent studies have demonstrated the presence of IgM-constituted immune complexes on FDCs within active GCs (1). Nevertheless, given the uncertainty, a formal possibility remained that immune complexes with the IgG isotype of Ab are more efficient at promoting GC formation, thereby explaining the marginal number of GCs obtained in the anti-G32CT3 response. Consequently, by inducing an anti-G32CT3 IgM to IgG class switch (19), CT3 prepriming would be expected to restore GC initiation.

To examine any potential differences between immune complexes with either IgG or IgM Abs, we performed in vivo GC reconstitution experiments using peptide PS1CT3 as the test system. For this, irradiated BALB/c mice (550 rad) were adoptively transferred with enriched T cells from CT3-primed mice and immune complexes of peptide PS1CT3 with day 7 anti-PS1CT3

![Image of GCs](https://example.com/gcs.jpg)

**FIGURE 2.** Identification of peptide PS1CT3-specific GCs by immunohistochemical staining of spleen sections. For experimental details refer to Materials and Methods. Shown is a ×100 magnification of a representative spleen section, double stained for Ag-specific (Tet-PS1) and PNA<sup>+</sup> B cells. PNA positivity is revealed by the red color, whereas Ag-specific cells stained blue. A depicts a peptide PS1CT3-specific GC. Specificity of Ag staining is revealed by comparison with B, which shows a GC from the same spleen, but negative for Tet-PS1 reactivity. In addition, GCs in splenic sections from mice mock immunized with CFA alone or those immunized with keyhole limpet hemocyanin (KLH) also did not stain with peptide Tet-PS1.

**FIGURE 3.** Ag-dependent modulation of GC responses. For the data in A, groups of four mice each, immunized with either peptide PS1CT3 (●) or G32CT3 (○), were sacrificed at the indicated time points, spleens were removed, and sections were processed for the immunohistochemical detection peptide-specific GCs using either Tet-PS1 or Tet-G32 as appropriate (see Materials and Methods). An additional group of mice was first preprimed with peptide CT3 (50 μg/mouse in CFA, huse of tail, day −7) followed by immunization (day 0) with peptide G32CT3 (▲). At least 30 dispersed sections were examined per spleen, and the data in A are presented as the number of GC (±SD) averaged over 10 sections. The negative controls described for Fig. 2 were also included here. The results are representative of four independent experiments. B and C show representative staining for Ag-specific GCs (magnification ×25), as described in Materials and Methods, for spleen sections obtained on day 10 from mice immunized with either peptide PS1CT3 (B) or peptide G32CT3 (C). While GCs that stain intensely for Ag are evident in sections from PS1CT3-immunized mice, they are absent in those from G32CT3-immunized mice.
polyclonal antiserum purified for either IgM or IgG Abs (see Materials and Methods). Subsequently, the irradiated hosts also received enriched B cells from mice immunized 2 days earlier with peptide PS1CT3. Ten days later, the spleens were removed from the recipients, and sections were stained for detection of PS1CT3-specific GCs with peptide Tet-PS1. A representative staining is shown in Fig. 4, and the cumulative results are given in Table I. As indicated in Table I, comparable numbers of GCs were obtained regardless of whether the immune complexes supplied were constituted with the IgG or IgM isotype of Abs. Thus, at least for the present system, immune complexes generated with both IgG and IgM Abs appear to be equally proficient at supporting GC formation. We also extend these results to suggest that the absence of an IgM to IgG class switch in an anti-G32CT3 response is unlikely to be the cause of the poor anti-G32CT3 GC reaction that follows.

Differences in GC-initiating abilities are not due to qualitative differences in T cell priming

Although our earlier results indicated that peptides PS1CT3 and G32CT3 prime T cells equally well in vivo, it remained possible that qualitative distinctions in the nature of T cells primed (e.g., distribution of fine specificities, TCR repertoire, etc.) could account for the observed differences in the extent of GC formation reported here. To verify this, we again resorted to the in vivo GC reconstitution protocol, where enriched B cells from either PS1CT3- or G32CT3-immunized mice were transferred into irradiated hosts followed by enriched T cells derived from mice primed with peptide CT3. Thus, since both groups here received an identical primed T cell population, the influence of T cells can be expected to be normalized. For these experiments the immune complexes used were prepared from day 7 Ig obtained against the respective Ags. In addition to these two groups, a third group was included in which B cells transferred were from CT3-preprimed, G32CT3-immunized mice.

As evident in Fig. 5, no significant GC formation could be detected in spleens from mice transferred with G32CT3-preprimed B cells, whereas an active GC reaction was clearly obtained in the group receiving PS1CT3-preprimed B cells. Thus, the possibility of qualitative differences in the primary anti-PS1CT3 and anti-G32CT3 T cell responses as a cause of the observed differences in the magnitude of GC responses can be ruled out. Interestingly, however, G32CT3-specific B cells from CT3-preprimed mice proved to be fully competent at reconstituting GCs in irradiated hosts (Fig. 5). We take this latter result to suggest that the positive effect, in terms of competency for GC formation, of the availability of an increased frequency of Ag-activated T cells during the initial stages of a B cell response is exerted by somehow influencing, at a presently unknown level, the nature of anti-G32CT3 B cells that are generated.

Differences in GC-forming abilities are not due to differences in the frequency of early activated Ag-specific B cells

The observed differences in Figs. 3 and 5 could also be accounted for by invoking differences in the frequency of Ag-specific B cells that are initially activated. CT3 prepriming could then function by increasing such a frequency (or repertoire) by making available an excess of T cell help. The experiment shown in Fig. 5 employed Ag-activated B cells only 2 days postimmunization with peptide G32CT3. Thus, if such a probability were indeed operative, it would have to represent a very early event that occurs either with or shortly after Ag-induced activation from the preimmune B cell pool.

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**Table 1. Immune complexes with either IgM or IgG Ab are equally supportive of germinal center formation**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immune Complexes with</th>
<th>Mouse No.</th>
<th>GCs/10 Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IgM</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>IgG</td>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>42</td>
</tr>
</tbody>
</table>

*GC reconstitution experiments in individual groups of five irradiated mice each were performed using PS1CT3-immune complexes generated with day 7 polyclonal antiserum of either the IgM or IgG isotype as described in Materials and Methods. Spleens were then removed 10 days later, and the resulting sections were stained for the detection of PS1CT3-specific GCs (Materials and Methods). A minimum of 30 sections, spread longitudinally across the spleen, were examined per spleen. Data are expressed as the number of GCs averaged over 10 sections. Control groups included irradiated mice which received either naive B cells, naive T cells, or enriched B cells from CFA-mock immunized mice. No GCs could be detected in any of the latter three groups. Results presented here are from one of three independent experiments.*
FIGURE 5. Differences in the extent of GC formation is independent of any qualitative difference in T cell priming. Groups of five irradiated mice each were given T cells enriched from mice primed with peptide CT3 7 days earlier and immune complexes generated from day 7 Ig from mice immunized with peptide G32CT3 (group 1) or PSICT3 (group 2) or from mice preprimed (on day −7) with peptide CT3 before immunization (day 0) with peptide G32CT3 (group 3). Twenty-four hours later the mice also received enriched B cells from an independent cohort of mice immunized 2 days earlier either with peptide G32CT3 (group 1) or peptide PSICT3 (group 2). Mice in group 3 received enriched B cells from CT3-preprimed and G32CT3-immunized mice. Ten days later the spleens were removed for detection and enumeration, in sections, of Ag-specific GCs as described in Fig. 2. For groups 1 and 3, peptide Tet-G32 was used for Ag-specific staining, whereas peptide Tet-PS1 was employed for group 2.

We therefore sought, by ELISPOT assays, to compare time-dependent progression of frequencies of Ab-producing B cells after priming with the corresponding peptide. The earliest time point taken was at 2 days postimmunization and was followed up to day 6, at which time the GC reaction was initiated (see Fig. 3). The results of these experiments are summarized in Table II. Although low in number, no significant differences in the number of peptide-reactive spots could be detected in splenocytes of mice immunized with either peptide PSICT3 or G32CT3 at 2 days postimmunization (Table II). Such a trend was maintained at all subsequent time points tested, where the frequency of anti-G32CT3 Ab-producing B cells was always within twofold that of Ab against peptide PSICT3 (Table II). These results are consistent with our prior observations that the early primary IgM responses induced by these two Ags are not distinctly different (19). Further, the data in Table II indicate that the observed difference in the extent of GC formation is unlikely to have resulted from differences in the frequency of Ag-activated B cells generated by immunization with either peptide PSICT3 or peptide G32CT3.

To further clarify the influence of Ag-activated B cell frequency, we again performed GC reconstitution experiments, but with enriched B cells from CT3-preprimed mice that had been immunized 2 days earlier with peptide G32CT3. In initial experiments we first established, by an ELISPOT assay, that the frequency of anti-peptide Ab-producing B cells in such a population was 45 ± 8/1 × 10^7 cells (mean ± SD of three independent experiments). Varying numbers of enriched B cells from CT3-preprimed, G32CT3-immunized mice were transferred into irradiated recipients along with the other components described in Fig. 5. Peptide G32CT3-specific GCs were then enumerated in sections derived from spleens removed either 6 or 10 days later, and the results are shown in Table III. Only marginal differences (within twofold on day 10) in the extent of GC formation were observed between the various groups (Table III), suggesting the absence of a significant correlation between the frequency of initially induced Ag-activated B cells and the number of resulting GCs. It must be noted here that based on estimates from Table II, the lowest number of enriched B cells used in Table III would be expected to correspond, in terms of G32CT3-activated B cells on day 2, to frequencies in the range of that anticipated for cells from CT3-unprimed, G32CT3-immunized mice. On the other hand, the highest numbers represent those contained in CT3-preprimed, G32CT3-immunized mice. Thus, it would appear that poor GC formation in G32CT3-immunized mice is not a consequence of limiting frequencies of the early Ag-activated B cells produced.

To further rule out an influence of B cell frequency, purified B cells from mice immunized 4 days earlier with either peptide PSICT3 or G32CT3 were enriched for the Ag-specific subset by panning against plates coated with the homologous peptide, as described previously (16). A subsequent FACs analysis, after staining with either Tet-PS1 or Tet-G32, determined that these enriched B cell populations contained 3.8 and 2%, respectively, PSICT3- and G32CT3-binding cells. These preparations were then individually diluted with premimmune B cells to achieve a final concentration of Ag-specific B cells of 4.1 × 10^6 cells (i.e., at least 40-fold greater than that obtained for PSICT3-immunized mice on

### Table II. Estimation of Ab-producing B cells in the early immune response to the peptides

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Days After Immunization</th>
<th>Spots/10^7 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>PSICT3</td>
<td>2</td>
<td>6.2 ± 2.4</td>
</tr>
<tr>
<td>G32CT3</td>
<td>2</td>
<td>5.4 ± 2.7</td>
</tr>
</tbody>
</table>
| PSICT3    | 4                       | 88 ± 16relevant to the work described here. The protocol followed here was as described in Materials and Methods with the exception that varying numbers, as indicated, of enriched B cells from CT3-preprimed and G32CT3-immunized mice were taken. Though the number of primed B cells varied between groups, the total number of enriched B cells transferred, however, was always kept constant (3 × 10^7) by making up the difference with enriched B cells from naive mice. Thus, group 1 in this table received cells that were completely derived from naive mice (3 × 10^7). The number of sections screened and data presentation is as described for Table 1. Data shown are from one of two experiments, and values are the mean (±SD) of numbers for the four individual mice within each group.

### Table III. Frequency of Ag-activated B cells does not significantly affect the efficiency of GC formation

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Primed B Cells Transferred</th>
<th>GC Cells Sections at Day 6</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>2</td>
<td>3 × 10^7</td>
<td>21 ± 8</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>3 × 10^7</td>
<td>44 ± 9</td>
<td>58 ± 13</td>
</tr>
<tr>
<td>4</td>
<td>3 × 10^7</td>
<td>51 ± 14</td>
<td>73 ± 9</td>
</tr>
</tbody>
</table>

The data in Table II indicate that the observed difference in the extent of GC formation is unlikely to have resulted from differences in the frequency of Ag-activated B cells generated by immunization with either peptide PSICT3 or peptide G32CT3.

To further clarify the influence of Ag-activated B cell frequency, we again performed GC reconstitution experiments, but with enriched B cells from CT3-preprimed mice that had been immunized 2 days earlier with peptide G32CT3. In initial experiments we first established, by an ELISPOT assay, that the frequency of anti-peptide Ab-producing B cells in such a population was 45 ± 8/1 × 10^7 cells (mean ± SD of three independent experiments). Varying numbers of enriched B cells from CT3-preprimed, G32CT3-immunized mice were transferred into irradiated recipients along with the other components described in Fig. 5. Peptide G32CT3-specific GCs were then enumerated in sections derived from spleens removed either 6 or 10 days later, and the results are shown in Table III. Only marginal differences (within twofold on day 10) in the extent of GC formation were observed between the various groups (Table III), suggesting the absence of a significant correlation between the frequency of initially induced Ag-activated B cells and the number of resulting GCs. It must be noted here that based on estimates from Table II, the lowest number of enriched B cells used in Table III would be expected to correspond, in terms of G32CT3-activated B cells on day 2, to frequencies in the range of that anticipated for cells from CT3-unprimed, G32CT3-immunized mice. On the other hand, the highest numbers represent those contained in CT3-preprimed, G32CT3-immunized mice. Thus, it would appear that poor GC formation in G32CT3-immunized mice is not a consequence of limiting frequencies of the early Ag-activated B cells produced.

To further rule out an influence of B cell frequency, purified B cells from mice immunized 4 days earlier with either peptide PSICT3 or G32CT3 were enriched for the Ag-specific subset by panning against plates coated with the homologous peptide, as described previously (16). A subsequent FACs analysis, after staining with either Tet-PS1 or Tet-G32, determined that these enriched B cell populations contained 3.8 and 2%, respectively, PSICT3- and G32CT3-binding cells. These preparations were then individually diluted with premimmune B cells to achieve a final concentration of Ag-specific B cells of 4.1 × 10^6 cells (i.e., at least 40-fold greater than that obtained for PSICT3-immunized mice on
day 4; see Table I) before use in GC reconstitution experiments (see Materials and Methods). Immunohistological staining of splenic sections derived from such hosts 10 days later yielded 64 ± 9 and 3 ± 2 GCs/10 sections for PS1CT3- and G32CT3-activated B cells, respectively. Thus, these results along with those described above categorically rule out quantitative differences in B cells activated against the two Ags as the causative factor underlying the observed differences in the extent of GC formation in PS1CT3- and G32CT3-immunized mice. In this connection, earlier studies have demonstrated that the available frequency of Ag-activated immunocytes does not significantly influence either the extent of GC formation (27) or the eventual output in an immune response (28).

In separate GC reconstitution experiments, we found that immune complexes with day 7 anti-G32CT3 Ig from CT3-preprimed mice were unable to initiate GC formation in irradiated mice supplied with G32CT3-activated enriched B cells from mice not preprimed with peptide CT3 (<5 GCs/10 sections). In contrast, immune complexes with day 7 anti-G32CT3 Ig from CT3-unprimed mice were fully capable of reconstituting GCs from enriched B cells derived from CT3-preprimed, G32CT3-immunized mice (between 30–55 GCs/10 sections in a total of five mice). These results, by ruling out an influence of the nature of early anti-G32CT3 Ig, further reinforce that it is the qualitative difference in anti-G32CT3 B cells generated in the presence or the absence of CT3 prepriming that accounts for the observed differences in GC formation.

Analysis of early primary serum Abs to peptides PS1CT3 and G32CT3

We have shown here that it is the immediate to early events following Ag exposure that determine the eventual ability to generate GCs. Further, our results imply that the discriminatory influence is exerted at the level of the Ag-activated B cells that are produced. Given the extremely low frequency of Ab-producing B cells obtained within the first 2 days of peptide G32CT3 immunization, it was not conceivable for us to probe for differences at that level. We therefore examined the early primary serum Ab responses under the various conditions as the next best alternative.

A comparison of the relative avidities of anti-PS1CT3 and anti-G32CT3 day 7 IgG by competitive inhibition ELISA revealed a pronounced difference, with anti-G32CT3 IgG displaying a greater than 25-fold lower avidity than that against peptide PS1CT3 (Fig. 6A). Prepriming with peptide CT3 had no significant influence on the IgM response to either peptide (Fig. 6A). Interestingly though, a comparison of the relative avidity of day 7 IgG proved informative (Fig. 6B). While the relative avidity of anti-PS1CT3 day 7 IgG either with or without CT3-prepriming was comparable to that obtained for the corresponding IgM, the anti-G32CT3 IgG from CT3-preprimed mice was markedly enhanced over the day 7 anti-G32CT3 IgM (Fig. 6, A and B). The latter result suggests that CT3-induced IgM to IgG Ab switch in response to G32CT3 immunization was also accompanied by an improvement in Ab avidity. We note here that peptide G32CT3 does not induce an IgG Ab response in the absence of CT3 prepriming (19).

The enhanced avidity of day 7 anti-G32CT3 IgG on CT3 prepriming could result from one of two possibilities. First, it may represent an outcome of affinity maturation processes within GCs initiated early in response to prepriming with peptide CT3 (Fig. 3). Alternatively, it may serve as an indicator for either recruitment or persistence and amplification of a minority population of G32CT3-specific B cells with a high enough affinity to permit the IgM to IgG class switch. Our earlier results have shown that a minimum affinity of early activated B cells for Ag is necessary to undergo an Ab isotype switch (19). To distinguish between these two possi-

**FIGURE 6.** Relative avidities of day 7 polyclonal IgM (A) and IgG (B) responses to peptides PS1CT3 and G32CT3. Day 7 antisera from individual mice immunized with either peptide PS1CT3 (○) or peptide G32CT3 (●) were fractionated for IgM and IgG Ab preparations (see Materials and Methods). Two additional groups were included in which the mice were first preprimed with peptide CT3 (on day −7) followed by immunization (on day 0) to yield either day 7 anti-PS1CT3 (△) or day 7 anti-G32CT3 (△) antisera. The relative avidities of the IgM (A) and IgG (B) fractions were determined by competitive inhibition ELISA with the indicated concentrations of the homologous immunogen as competitor (see Materials and Methods). Values are expressed in terms of Ab binding observed at a given inhibitor concentration as a function of that in the absence of any competitor after subtracting the background observed for preimmune serum. The data shown are representative of those obtained in a total of 10 mice for each set, immunized on three separate occasions in groups of three or four each. Further, the IgM and IgG results given for each immunization set were derived for fractions from the same antisera sample. Finally, peptide G32CT3 does not elicit an IgG response unless the mice are first preprimed with peptide CT3 (19).

**FIGURE 7.** A time-course analysis of anti-G32CT3 IgM and IgG Abs in CT3-preprimed mice. Sera from groups of 10 mice each that were CT3 preprimed (on day −7) and then immunized with peptide G32CT3 (day 0) were collected at the indicated times, pooled within a group, and resolved for the IgM (○) and IgG (●) fractions. The IC50 values were determined for each time point by competitive inhibition ELISA as described in Fig. 6. The data shown are representative of four independent experiments. A parallel group of CT3-preprimed, G32CT3-immunized mice was simultaneously treated with mAb GL1 as previously described (28), and relative affinities, as IC50 values, were determined for the day 7 IgM (△) and IgG (△) anti-G32CT3 fractions. These latter results are from one of two separate experiments.
The early differences in anti-G32CT3 IgM vs IgG avidities with no further enhancement with time suggested that these differences were unlikely to have resulted from affinity maturation processes within GCs. Indeed, the absence of an improvement in IgG Ab affinity with time is not particularly surprising given our recent observations that primary IgG responses to polypeptide Ags do not display a significant improvement in affinity over time (P. Nakra and K. V. S. Rao, unpublished observations). To further verify that the observed avidity differences are not a consequence of the GC reaction, we analyzed day 7 sera from CT3-preprimed, G32CT3-immunized mice that had also been administered mAb GL1, an Ab specific for the costimulatory molecule B7-2. Treatment with mAb GL1 has been previously shown to abrogate GC formation in a primary immune response to the hapten (4-hydroxy-3-nitrophenyl)lacetyl (29). In the present case we first ascertained that administra­tion of previously suggested (29) doses of mAb GL1 completely inhibited detectable G32CT3-specific GC formation in CT3-preprimed mice (data not shown). A determination of day 7 anti-G32CT3 IgM and IgG avidities from such mice yielded values comparable to those in mAb GL1-un­treated mice (Fig. 7). These results unequivocally confirm that the observed difference in avidity of early primary anti-G32CT3 IgM and IgG Abs originates from events that are independent of and precede GC formation.

**Discussion**

While an exhaustive analysis of processes that occur within active GCs has been conducted by several groups (30–42), events that regulate its genesis are, at best, sparsely understood. It is now widely accepted that the primary site of resting B cell activation upon first exposure to a T-dependent Ag occurs in the T cell-rich areas of the PALS (43). The products resulting from this initiation are then thought to locally differentiate into AFC foci in the periphery of the PALS and also seed adjacent GCs in the lymphoid follicles (1–10). While prior formation of foci has generally been considered a prerequisite for GC formation, more recent data have questioned whether it is obligatory (15). A marked difference in clonal diversity of cells that seed GCs compared with that of B cells activated in the foci is also a common characteristic of the early primary humoral response to T-dependent Ags (1–10). These differences strongly suggest that not all B cells activated in the PALS are capable of supporting GC initiation. Consequently, an understanding of the regulatory processes that guide such a selection and of the cellular properties that define GC-seeding competency assumes importance.

Our earlier studies with peptide PS1CT3 and its single amino acid-substituted analogues (17) fortuitously provided us with a comparative system to study Ag-dependent modulation of GC responses. Of particular interest was the analogous peptide G32CT3, which, although as proficient as the parent peptide PS1CT3 at priming T cells, elicited a poor B cell response (19). Thus, it seemed possible to explore the role of Ag-activated B cells in directing the formation of GCs. The potential utility of our system was indeed realized in early experiments that revealed that while peptide PS1CT3 was capable of initiating a robust GC reaction, those generated in response to peptide G32CT3 immunization were both drastically curtailed in number and transient in appearance. Their previously demonstrated equipotency at in vivo T cell priming (19) led us to suspect, prima facie, that these differences probably arise out of quantitative/qualitative distinctions at the level of either B cells activated by the respective Ags or the early Abs produced against them. This inference could be further substantiated in experiments that also ruled out any qualitative distinctions in the nature of T cells primed by the two Ags as the causative factor.

There exists some degree of equivocality in the literature about the relative facility of immune complexes constituted by IgM and IgG isotypes of Abs in supporting initiation of a GC. Although early studies suggested a selectivity for IgG immune complexes (28), more recent experiments have demonstrated the presence of IgM-constituted immune complexes on FDCs within active GCs (1). In our case, at least with respect to peptide PS1CT3, immune complexes with polyclonal day 7 anti-PS1CT3 preparations of both IgG and IgM yielded comparable numbers of GCs. In addition to implying the absence of a marked preference for either Ab isotype, we extend these observations to infer that the poor anti-G32CT3 GC response was unlikely to have been due to the absence of a μ to γ class switch in the early primary anti-G32CT3 humoral response. Furthermore, we were unable to explain the differing extents of GC formation on the basis of differences in the frequency of early Ag-activated B cells generated in response to the two Ags.

The poor GC response to peptide G32CT3 immunization could easily be overcome by the simple expedient of pre­priming the mice with peptide CT3. As described previously (19), CT3 prepriming functions by increasing the available pool of Ag-activated T cells. Thus, generation of a productive anti-G32CT3 GC response required additional T cell help over and above that sufficient for that against peptide PS1CT3. In addition to confirming prior results that quantitative differences in T cell help can modulate early Ag-activated B cell behavior (14, 44), these findings point toward the conclusion that the level of T cell help required to initiate GC formation is Ag dependent.

Pre­priming with peptide CT3 has been previously shown to qualitatively influence early anti-G32CT3 Ab production by inducing an IgM to IgG isotype switch (19). However, the results described here rule out a role for the nature of the early primary Ab in deciding the facility of GC induction. Immune complexes with anti-G32CT3 Ig from CT3-preprimed mice were unable to support GC formation from G32CT3-activated B cells in mice not pre­primed with peptide CT3. Conversely, immune complexes with anti-G32CT3 Ig obtained from CT3-unprimed mice were competent at supporting GC formation with B cells from CT3-preprimed and G32CT3-immunized mice. Collectively these results clearly indicate that differences in G32CT3-activated B cells from CT3-primed vs unprimed mice were responsible for the observed variations in GC-forming efficiency between the two groups. Furthermore, our observations that the positive effect of CT3 pre­priming is abrogated when given 2 days after immunizing with peptide G32CT3 reveals that these distinctions appear early, either concomitant with Ag exposure or immediately thereafter.

Although the cumulative results from our in vivo GC reconstitution experiments pointed toward a qualitative deficiency in B cells activated on G32CT3 immunization, we were unable to characterize the nature of this deficiency. The very low frequency of early anti-G32CT3-activated B cells precluded any direct analysis. We were therefore forced to adopt an empirical approach in which we examined early primary serum Ab responses under the various conditions. It was expected that such an approach could provide an indirect insight into the nature of the B cells from which they originate, at least from the standpoint of Ag binding characteristics. Considering that it is also likely to reflect the status of Ig receptors on B cells that produced them, the pronounced difference
in the relative avidities of the early primary IgM response to peptides PS1CT3 and G32CT3 was particularly intriguing. It suggested the existence of an Ag affinity threshold that impedes recruitment of G32CT3-activated B cells from CT3-unprimed mice within GCs. While prior CT3 priming was found to have no influence on the avidities of anti-PS1CT3 and anti-G32CT3 IgMs, a surprising selectivity was observed for the early anti-G32CT3 IgG that resulted. The observed enhancement in the avidity of anti-G32CT3 IgG over that of the corresponding IgM Abs was in contrast to that observed for peptide PS1CT3, for which no significant difference in avidity could be detected between the two isotype populations. Although a discriminatory role for early primary Ab in deciding the efficiency of GC formation was earlier ruled out, the presence of a novel higher affinity Ab component in the CT3-preprimed anti-G32CT3 response is also likely to be indicative of the presence of a high affinity subset in the corresponding Ag-activated B cell pool. Such a subset is in all probability either absent in the G32CT3-immunized but not CT3-primed group or present in numbers too low to be detectable at the level of serum Ab. Our earlier studies have demonstrated the need for a minimum affinity of early Ag-activated B cells for Ag to be able to undergo the IgM to IgG class switch (19). The present data on comparative avidities of early anti-G32CT3 IgM and IgG, generated in CT3-preprimed mice, are therefore diagnostic of the facilitation of either the existence or the persistence, followed by expansion, of a higher affinity B cell subset under conditions of CT3 prepriming. Furthermore, results from a time-course comparison of IgM vs IgG relative avidities and from mAb GL1-treated mice clearly indicate that the appearance of the higher affinity subset precedes GC formation and, therefore, may be linked to its etiology. In this connection we have recently observed that G32CT3-activated B cells from CT3-preprimed mice in in vivo reconstitution experiments yielded only a marginal GC response when depleted of slgG+ B cells by exhaustive panning against anti-mouse IgG (A. Agarwal and K. V. S. Rao, unpublished observations). This further suggests a causal correlation between the presence of a high affinity anti-G32CT3 B cell subset and the efficiency of GC formation.

The presence of a higher affinity subset in CT3-preprimed, G32CT3-immunized mice could result from one of two alternate possibilities. First, it may represent a minority component of the total pool of G32CT3-activated B cells. In such a situation their subsequent elimination, under normal circumstances, is probably in an environment of intense interclonal competition for a limiting pool of T cell help (17, 19). Consequently, CT3 prepriming may ensure their survival and subsequent expansion by making available an increased (perhaps nonlimiting) pool of activated Th cells. An alternate explanation may be sought in the possibility that the higher affinity Ab-producing cells represent derivatives of a distinct lineage that Klinskman and colleagues have earlier proposed to be the exclusive GC-populating subset (11–13). If the latter explanation eventually proves to be true, it is then presently unclear why the G32CT3-induced subpopulation requires an increased proportion of T cell help compared with that for the anti-PS1CT3 B cells for either its induction or sustenance.

It is clear, therefore, that CT3 prepriming exerts a qualitative influence on the composition of early anti-G32CT3 B cells that are either generated or permitted to survive, with its resultant effect on the serum Ab profile. However, we have previously ruled out a role for Ab at the level of either average affinity or isotype composition. Consequently, we infer that the primary determinant of GC-seeding ability in the CT3-primed vs unprimed G32CT3-immunized mice must reside in qualitative differences in the early Ag-induced B cells generated in the two cases.

The differential Th cell thresholds required for promoting GC formation from early anti-PS1CT3- and anti-G32CT3-activated B cells argues for a dynamic balance that is regulated by the range of B cell affinities that is initially produced on first encounter with Ag. Although the determinants of B cell affinities invoked by an Ag remain elusive, we have previously proposed that thermodynamic considerations, defined by the chemical composition of an epitope, may apply (19). In addition, the relationship between the affinity for Ag and the GC-seeding capacity of Ag-activated B cells needs clarification. However, in light of the available related information in the literature, it is possible to offer a speculative working hypothesis. Studies from both our laboratory (20) and others (45) have indicated that the Ag-binding characteristics of slg receptors directly relates to the ability of a given B cell to recruit T cell help. Although alternate explanations may ultimately prevail, at the simplest level, affinity for Ag may dictate the extent of Ag uptake and, as a result, the avidity of a cognate B cell-T cell interaction by defining the density of ligand eventually presented. Variability in T cell recruitment ability may also be expected to lead to differences in the extent of activation of the B cell, particularly with respect to cell surface expression of accessory molecules such as LFA-1, ICAM-1, B7, CD40L, etc., whose presence has been shown to be critical for B cells to seed GCs (1, 28, 46–50).

In summary, our results support the idea that qualitative differences in B cell priming can lead to Ag-dependent differences in the extent of GC formation. While confirming earlier suggestions of intrinsic differences in the nature of B cell clones that participate in AFC and GC responses (15), they extend these findings by identifying the operation of an affinity threshold as the discriminatory criterion. In addition to emphasizing a role for Ag-activated B cells, our data minimize the influence of qualitative differences in the Ab that constitutes immune complexes deposited on FDCs to seed the GC reaction. It will be of interest to determine the generality of our findings in other systems. Further, the mechanism by which affinity for Ag influences the GC-forming competency and characteristics of B cells that successfully seed GCs remains to be explored.

References


