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

Anshu Agarwal, Bishnu P. Nayak, and Kanury V. S. Rao¹

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)² concentrate in the periphery, adjacent to the red pulp, of the periarteriolar 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^{low} subpopulation of B cells was capable of originating GCs (12). Recent studies also question the criticality of AFC formation as a prerequisite 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 antiarsenate 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

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² Abbreviations used in this paper: AFC, antibody-forming cells; PALS, periarteriolar lymphoid sheath; GC, germinal center; FDC, follicular dendritic cells; HRPO, horseradish peroxidase; PNA, peanut agglutinin; ELISPOT, enzyme-linked immunospot; sIg, surface immunoglobulin.



FIGURE 1. Model peptide immunogens PS1CT3 and G32CT3. *A* shows the amino acid sequence of the parent peptide PS1CT3 and its analogue G32CT3 in the single letter code for amino acids (for details see text). In peptide G32CT3, the proline residue at position 5 has been substituted with a glycine residue. The two glycine-residue spacer at position 16 and 17 has been underlined. *B* depicts the peptide Tet-PS1, a biotinylated peptide containing the PS1 sequence in a tetrameric configuration. The double bond between the cysteines is meant to indicate a disulfide bond (see *Materials and Methods*). In the corresponding analogue Tet-G32, the proline residue within the PS1 sequence (indicated by a star) was replaced with glycine. Note that each molecule contains four copies of the B epitope sequence and two units of biotin.

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 cell 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 C₁₈ column (15 μm; δPAk, 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 t-butoxycarbonyl functionality and with F-moc at the α-amino group. After selective F-moc deprotection, a second lysine was coupled, but both the amino groups were derivatized with F-moc. F-moc deprotection of this lysine then yielded two reactive amino functionalities on which either the PS1 or the G32 sequence was sequentially built using the standard procedures. After completion of synthesis the amino-terminus was acetylated, and subsequent cleavage from solid support yielded a peptide containing two copies of either the PS1 or the G32 sequence. Air oxidation resulted in dimerization through disulfide bond formation at the carboxyl-terminal cysteines to generate a single molecule with four copies of the epitope sequence. Subsequent biotinylation with NHS-LC-biotin (Pierce, Rockford, IL) at the ε-amino group of the penultimate lysine yielded the peptides Tet-PS1 and Tet-G32 (Fig. 1*B*). The remaining procedure was as described above.

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 panning 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 5×10^6 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 (1×10^7 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 non-specific binding with a 1/1 (v/v) solution of 3% BSA (in PBS) and mouse nonimmune 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 50 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

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 $\mu\text{g/ml}$ of peptide/well in 100 μl of PBS (pH 7.2) at 37°C for 3.5 h. Subsequently, they were blocked with 300 $\mu\text{l/well}$ 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 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 titer value. Twofold higher concentrations of antiserum 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 $\mu\text{l/well}$. 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 $\mu\text{g/ml}$ solution of either peptide PS1CT3 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. Splenocytes from mice immunized with either peptide PS1CT3 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 1×10^7 , 1×10^6 , and 1×10^5 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 $\times 10$ magnification.

Results

Peptides PS1CT3 and G32CT3

The model peptide, PS1CT3, that we used in our studies is shown in Fig. 1A. This peptide represents a chimeric sequence in which residues 1–15 (segment PS1) 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 PS1CT3 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 PS1CT3 includes Ab specificities that cumulatively recognized the entire PS1 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 V_H and V_L 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 PS1CT3 and G32CT3 yielded comparable levels of early

primary IgM Abs, the anti-G32CT3 response was aborted, 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 PS1CT3 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 sIg 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 PS1CT3, proved to be of too low an affinity to permit successful cross-over (19).

From our description above, it is obvious that peptides PS1CT3 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 PS1CT3 and G32CT3 have previously been shown to represent T-dependent Ags, even at the level of early primary IgM Ab induction (17).

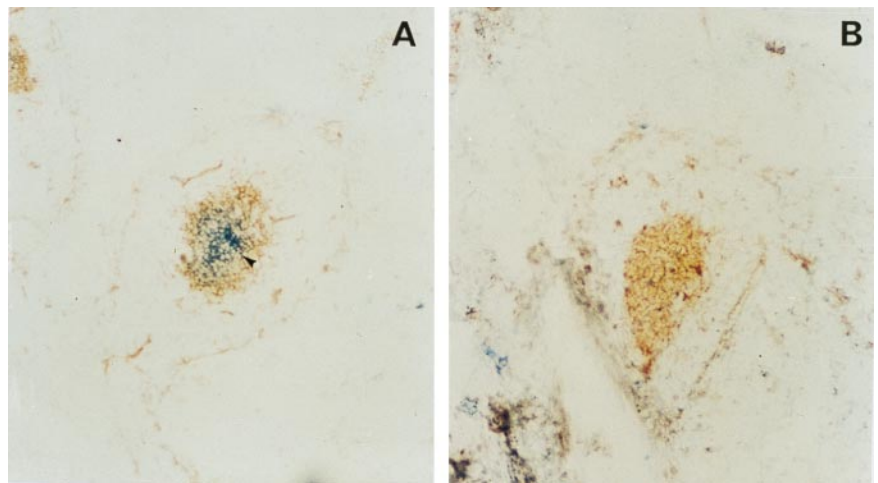
Immunization with peptide G32CT3 elicits a poor GC response

To evaluate any differences in GC responses against the two analogue peptides, parallel groups of BALB/c mice were immunized with peptide PS1CT3 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 tetrameric 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 PS1CT3. The kinetics of GC formation against PS1CT3 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 PS1CT3, 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-primed 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 PS1CT3. 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

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 $\times 100$ magnification of a representative spleen section, double stained for Ag-specific (Tet-PS1) and PNA⁺ 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.



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

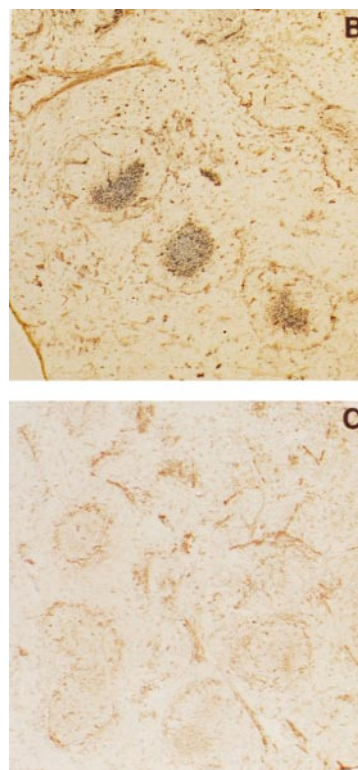
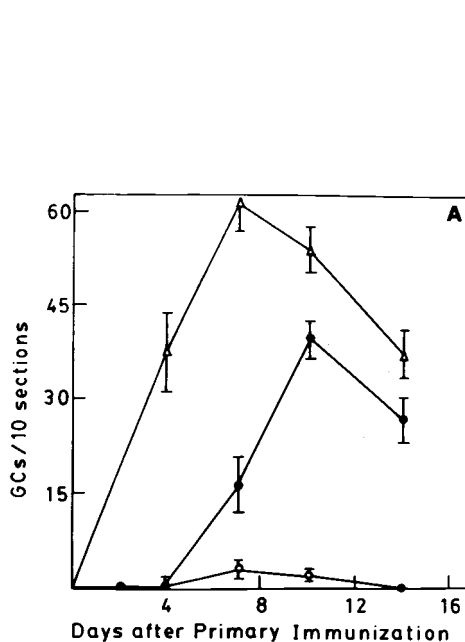
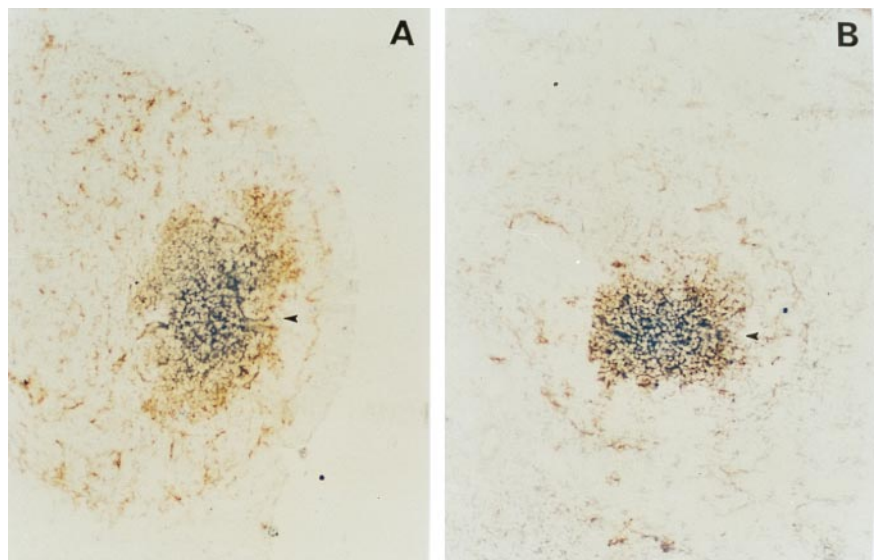


FIGURE 3. Ag-dependent modulation of GC responses. For the data in *A*, groups of four mice each, immunized with either peptide PS1CT3 (●) or G32CT3 (○), were sacrificed at the indicated time points, spleens were removed, and sections were processed for the immunohistochemical detection peptide-specific 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 $\mu\text{g}/\text{mouse}$ in CFA, base of tail, day -7) followed by immunization (day 0) with peptide G32CT3 (Δ). At least 30 dispersed sections were examined per spleen, and the data in *A* are presented as the number of GC (\pm 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, $\times 25$), 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.

FIGURE 4. Both IgM- and IgG-constituted immune complexes seed GCs. Immune complexes of peptide PS1CT3 with day 7 polyclonal Ab preparations of either anti-PS1CT3 IgM (A) or anti-PS1CT3 IgG (B) were separately included in *in vivo* GC reconstitution experiments along with CT3-primed enriched T cells and PS1CT3-primed enriched B cells as described in *Materials and Methods*. The figure shows the presence of Ag-specific GCs in splenic sections derived 10 days later from both groups. A description of the control groups is included, and the cumulative results are presented in Table I.



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

Table I. Immune complexes with either IgM or IgG Ab are equally supportive of germinal center formation^a

Group	Immune Complexes with	Mouse No.	GCs/10 Sections
1	IgM	1	39
		2	46
		3	34
		4	36
		5	32
2	IgG	1	38
		2	76
		3	39
		4	54
		5	42

^a 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.

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-primed B cells, whereas an active GC reaction was clearly obtained in the group receiving PS1CT3-primed 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.

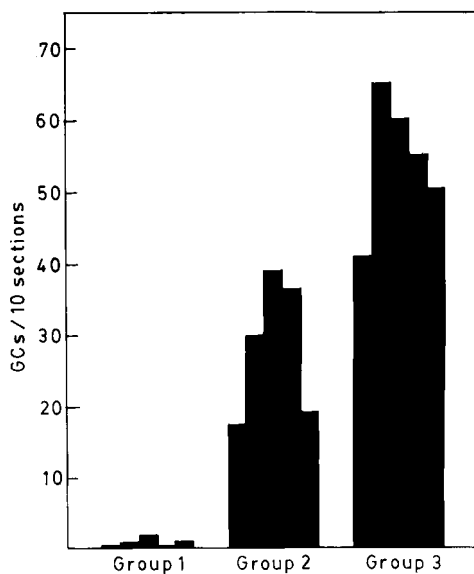


FIGURE 5. Differences in the extent of GC formation is independent of any qualitative difference in T cell priming. Groups of five irradiated mice each were given T cells enriched from mice primed with peptide CT3 7 days earlier and immune complexes generated from day 7 Ig from mice immunized with peptide G32CT3 (group 1) or PS1CT3 (group 2) or from mice preprimed (on day -7) with peptide CT3 before immunization (on 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 PS1CT3 (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 primary immunization 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 PS1CT3 or G32CT3 at 2 days postimmunization (Table II). Such a trend was maintained at all subsequent time points tested, where the frequency of anti-

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

Immunogen	Days After Immunization	Spots/ 1×10^7 Cells		
		Expt. 1.	Expt. 2	Expt. 3.
PS1CT3	2	6.2 ± 2.4	6.4 ± 1.3	9.0 ± 3.8
G32CT3	2	5.4 ± 2.7	4.0 ± 2.3	3.2 ± 1.2
PS1CT3	4	88 ± 16	94 ± 22	104 ± 28
G32CT3	4	64 ± 08	68 ± 12	62 ± 14
PS1CT3	6	85 ± 12	113 ± 22	110 ± 19
G32CT3	6	63 ± 06	86 ± 07	84 ± 11

^a Groups of four mice each were either immunized with peptide PS1CT3, G32CT3 or first preprimed with peptide CT3 followed by immunization with peptide G32CT3. At indicated times, spleens were removed, and peptide-specific Ab producing B cells were determined in an ELISPOT assay as described in *Materials and Methods*. Values for each experiment provided are the mean of those for individual mice in each group where each determination was performed in quintuplicate.

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

Group	No. of Primed B Cells Transferred.	GCs/10 Sections at	
		Day 6	Day 10
1	None	< 1	< 1
2	3×10^5	21 ± 8	37 ± 6
3	3×10^6	44 ± 9	58 ± 13
4	3×10^7	51 ± 14	73 ± 9

^a 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 I. Data shown are from one of two experiments, and values are the mean (\pm SD) of numbers for the four individual mice within each group.

G32CT3 Ab-producing B cells was always within twofold that of Ab against peptide PS1CT3 (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 difference in the frequency of Ag-activated B cells generated by immunization with either peptide PS1CT3 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 $45 \pm 8/1 \times 10^7$ cells (mean \pm 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 PS1CT3 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, PS1CT3- and G32CT3-binding cells. These preparations were then individually diluted with preimmune B cells to achieve a final concentration of Ag-specific B cells of 4.1×10^4 cells (i.e., at least 40-fold greater than that obtained for PS1CT3-immunized mice on

day 4; see Table II) before use in GC reconstitution experiments (see *Materials and Methods*). Immunohistochemical 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 IgM by competitive inhibition ELISA revealed a pronounced difference, with anti-G32CT3 IgM 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-

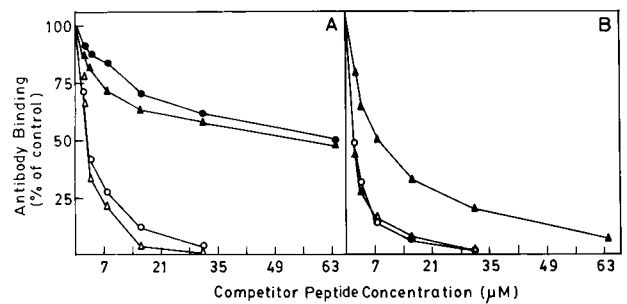


FIGURE 6. Relative avidities of day 7 polyclonal IgM (A) and IgG (B) responses to peptides PS1CT3 and G32CT3. Day 7 antisera from individual mice immunized with either peptide PS1CT3 (○) or peptide G32CT3 (●) were fractionated for IgM and IgG Ab preparations (see *Materials and Methods*). Two additional 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 (Δ) or day 7 anti-G32CT3 (Δ) 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 antiserum sample. Finally, peptide G32CT3 does not elicit an IgG response unless the mice are first preprimed with peptide CT3 (19).

bilities we next monitored the relative avidities of CT3-preprimed, anti-G32CT3 IgM and IgG as a function of time (Fig. 7). The earliest time point studied (day 4) was that when the IgG levels were high enough to obtain reliable data. As shown in Fig. 7, the distinction between anti-G32CT3 IgM and IgG affinities was evident very early, even at the stage when significant IgG Ab levels

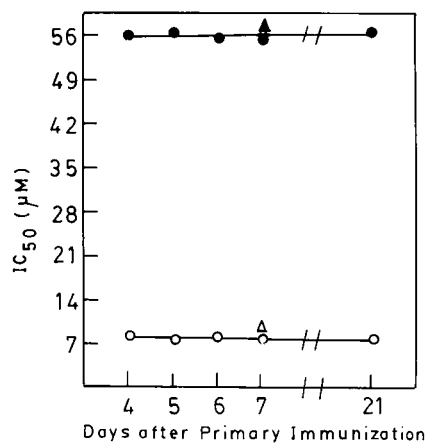


FIGURE 7. A time-course analysis of anti-G32CT3 IgM and IgG Abs in CT3-preprimed mice. Sera from groups of 10 mice each that were CT3 preprimed (on day -7) and then immunized with peptide G32CT3 (day 0) were collected at the indicated times, pooled within a group, and resolved for the IgM (●) and IgG (○) fractions. The IC_{50} 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 IC_{50} values, were determined for the day 7 IgM (Δ) and IgG (Δ) anti-G32CT3 fractions. These latter results are from one of two separate experiments.

first become detectable. Thereafter, there was no further increment in the difference, which remained invariant up to day 21 postimmunization (Fig. 7).

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)acetyl (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 causatory 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 analysis 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 sIg 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 sIgG⁺ 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 Klinman 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 sIg 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.

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