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## B Cell Responses to a Peptide Epitope. VIII. Immune Complex-Mediated Regulation of Memory B Cell Generation Within Germinal Centers

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# B Cell Responses to a Peptide Epitope. VIII. Immune Complex-Mediated Regulation of Memory B Cell Generation Within Germinal Centers<sup>1</sup>

Bishnu P. Nayak, Anshu Agarwal, Pooja Nakra, and Kanury V. S. Rao<sup>2</sup>

Using an *in vivo* reconstitution assay, we examine here the role of immune complexes in both formation of germinal centers (GC) and processes that occur subsequently within. The presence of Ag, as immune complexes, was found not to constitute a limiting requirement for the initiation of GC formation. No detrimental effect either on numbers or sizes of the resulting GC was observed when Ag-containing immune complexes were omitted during reconstitution. Thus, both recruitment and proliferation of Ag-activated B cells within GC appear not to be limited by Ag concentrations. In contrast, the presence of immune complexes was observed to be obligatory for the generation of Ag-specific memory B cells. This optimally required immune complexes to be constituted by IgG-class Abs with epitope specificities that were homologous to those of the GC B cells. The GC reaction was also found to be characterized by an enhancement of Ab specificity for the homologous epitope. Although some improvement in specificity was noted in recall responses from immune complex-deficient GC, the presence of appropriate immune complexes served to further optimize the outcome. Here again, isotype and epitope-specificity of the Ab constituent in immune complexes proved to be important. *The Journal of Immunology*, 1999, 163: 1371–1381.

Extensive studies over the last decade or so have unraveled much of the processes related to induction and maturation of T cell-dependent B cell responses. It is now clear that the primary site of B cell activation is in the T cell-rich extrafollicular sites. Soon thereafter, foci of specific Ab-producing B cells (AFCs)<sup>3</sup> concentrate in the periphery, in the neighborhood of the red pulp, of the periarteriolar lymphoid sheath (1). In 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 plasma cells are initiated (2–4).

Although the overall scheme has been documented with a variety of Ag systems, there are several aspects that continue to remain enigmatic. One of these is the constituents that are required to initiate a GC reaction. Early studies suggested that the formation of GCs was facilitated by the trapping of immune complexes within the network of follicular dendritic cells (FDCs) and B cells in the lymphoid follicle (5, 6). Such an inference has been derived from observations that immunization with Ag-specific immune complexes, as opposed to Ag alone, results in a more rapid induction of GCs (7, 8). Interestingly, the rate of somatic mutation was also found to be higher in such cases, resulting in secondary AFCs secreting higher-affinity Ab when compared with those from mice primed with Ag alone (7, 9). Finally, the rate of memory B cell

development was also enhanced in immune complex-immunized mice, leading to increased anamnestic responses (7). However, contrary to the inferences derived from such studies, there are other reports in the literature that document that the appearance of GCs precedes observation of detectable levels of FDC-bound immune complexes (10). Although it is possible that these latter results simply reflect limitations in the sensitivity of detection of immune complexes, nevertheless they suggest that deposition of at least significant levels of immune complexes onto FDCs are not a prerequisite to the initiation of GCs.

While the molecular regulators of somatic mutation within GCs continue to be an area of active interest (11), resolution is also awaited of the processes that occur subsequent to the generation of B cells bearing mutated sIg variant receptors. It is generally accepted that somatic mutation within GCs is followed by positive selection for those B cell variants with an increased affinity for Ag (12–14). The drive for positive selection is thought to be centered around Ag, which, in GCs, is available either in the form of FDC-bound immune complexes or iccosomes (see Ref. 15). A variety of mechanisms have been proposed to rationalize Ag access by GC B cells, a critical determinant of positive selection. These include competitive displacement of Ag from immune complexes (see Ref. 16), uptake as iccosomes (see Ref. 1), or proteolytic cleavage of Ag-Fab complexes from FDC-bound immune complexes (17). An interesting caveat has recently been provided by Manser and co-workers (18). They have elegantly demonstrated that, although affinity maturation was operative, specificity proved to be the overriding consideration that guided positive selection (18). Thus in an anti-arsenate response, GC B cells were found to be selected on the basis of increased specificity for homologous Ag, thereby reducing the probability of cross-reactivity with self-Ags (18).

From the discussion above, it is clear that there continue to remain gaps in our knowledge of processes that govern induction and maturation of T cell-dependent B cell responses. This is especially true of finer details of the GC reaction. In an earlier report, we had demonstrated that the GC seeding competency of an Ag-activated B cell is established early, soon after Ag exposure, and

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<sup>3</sup> Abbreviations used in this paper: AFC, Ab-secreting cell; GC, germinal center; FDC, follicular dendritic cell; PNA, peanut lectin agglutinin; HEL, hen egg lysozyme.

that it correlates with affinity for Ag (19). For these studies, we had employed an *in vivo* GC reconstitution protocol that involved adoptive transfer of Ag-primed B and T cells, along with appropriate immune complexes into irradiated mice. Using the same protocol, we, in the present report, have examined the influence of immune complexes on the GC reaction. We show that, although nonlimiting for initiating a GC reaction, the presence of immune complexes was nevertheless critical for the enhancement of Ag-specific B cell memory. Further, this activity was restricted to only those immune complexes in which the constituent Abs shared a common epitope specificity with the GC B cells, with an optimum requirement for IgG isotype of Abs. Finally, while enhancement of Ab specificity within GCs was initiated in the absence of detectable levels of immune complexes, the presence of appropriate immune complexes served to maximize the outcome.

## Materials and Methods

### Materials

HRP-labeled secondary Abs were obtained from Sigma (St. Louis, MO). Coated magnetic beads for panning B and T cells (Dynabeads, Mouse pan B and Mouse pan T) were purchased from DYNAL (Oslo, Norway). Derivatized amino acids for peptide synthesis were purchased from Novabiochem (Laufelfingen, Switzerland). Biotinylated peanut lectin agglutinin (PNA), streptavidin-alkaline phosphatase, and streptavidin-HRP were obtained from Vector Laboratories (Burlingame, CA), and anti-B7.2 (clone GL1) was obtained from PharMingen (San Diego, CA). Noncleavable kits for multiple peptide synthesis were purchased from Chiron Mimotopes (Victoria, Australia).

### Peptide synthesis

Peptides PS1CT3, CT3, and the Tyr<sup>33</sup>-substituted analogue of peptide PS1CT3 were synthesized on a Milligen 9050 synthesizer (Millipore, Bedford, MA) using F-moc chemistry (20). For peptide-specific staining of GC B cells, the tetrameric, biotinylated peptide was also synthesized by the F-moc chemistry, but using the strategy described earlier (19). Crude peptides were purified to >95% purity by reverse phase HPLC on a C-18 column (15  $\mu$ m;  $\delta$ PAK, Waters, Milford, MA; 19  $\times$  300 mm) using an aqueous gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The identity of all peptides were ascertained both by amino acid analysis and mass spectrometry.

Overlapping and analogue hexapeptide panels were synthesized by the method of Geysen (21) on noncleavable multipin kits following the standard protocol recommended by the manufacturer. After completion of synthesis, all peptides were routinely acetylated at the amino terminus with a 50/5/1 (v/v/v) mixture of dimethylformamide, acetic anhydride, and triethylamine.

### Radioiodination

Either 2 mg of peptide PS1CT3 or 1 mg each of mAbs PC287 and PC7bM were iodinated with Na<sup>125</sup>I in the presence of Iodobeads (Pierce, Rockford, IL) in 400  $\mu$ l of 10 mM phosphate buffer, pH, 7.2. The reaction time was 10 min at 4°C. After this, the solution was collected and the free iodine removed first by gel filtration followed by exhaustive dialysis against PBS. Sp. act. of iodination was calculated from the known molecular mass of peptide (4,029 Da) and taking an approximate molecular mass of 150,000 Da for the monomer Ig unit.

### 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, primary immunizations were generally given *i.p.* at a dose of 50  $\mu$ g/mouse as an emulsion in CFA. For polyclonal sera, mice were bled from the retroorbital plexus and, if necessary, sera within a group pooled. For CT3 priming, mice were immunized with 50  $\mu$ g/mouse of a CFA emulsion of peptide CT3 (at base of tail) followed by a booster seven days later. Lymph nodes were removed at 3 days (day 10) after the boost.

### Ab preparations and generation of immune complexes

Primary anti-PS1CT3 antisera either from GL1-treated or untreated mice was collected at day 7 after a primary immunization with peptide PS1CT3. Normally derived secondary anti-PS1CT3 IgG was obtained as described previously (22). Briefly, BALB/c mice were immunized with peptide

PS1CT3 as described above. Three weeks later, spleens were removed and splenocytes transferred into irradiated (550 rad) hosts. This was followed by a challenge with soluble peptide (50  $\mu$ g/mouse in PBS, *i.v.*) 16 h later (day 0), and sera was collected on day 5. Before analysis of polyclonal preparations, sera were resolved for the IgG components by passing over a protein G-Sepharose column (Pharmacia, Uppsala, Sweden). While the flow-through consisted of IgM, bound Abs were eluted with glycine-HCl buffer, pH 2.7. The eluate was immediately neutralized, concentrated, and then dialyzed against PBS before use. Where necessary, IgG fractions were further purified for peptide-specific Abs by incubating with a PS1CT3-Sepharose affinity column (overnight at 4°C). After the requisite washes, bound Ab was eluted in glycine-HCl buffer and processed as described above.

Monoclonal Ab preparations used here have been described earlier (23) and were obtained from hybridoma culture supernatants and concentrated as required. For the preparation of immune complexes, either day 7 primary polyclonal anti-PS1CT3 IgG (from GL1-untreated mice) or the individual mAb preparations were incubated with a 10-fold molar excess (based on the estimated number of specific binding sites) of peptide PS1CT3 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. With all mAbs used, that saturated immune complexes could be formed was first verified with the radioiodinated analogue of peptide PS1CT3.

### Enrichment of B and T cells from immunized mice and reconstitution of GCs *in vivo*

Either splenocytes from PS1CT3-immunized mice (for B cells) or inguinal lymph node cells from CT3-primed mice (for T cells) were first depleted of RBCs followed by adherent cells as described earlier (19). For enriched B cells, the corresponding splenocyte suspension was depleted of T cells by two rounds of treatment with excess anti-Thy1.2-coated magnetic beads (DYNAL). On the other hand, for enriched T cells, lymph node cells were similarly deprived of B cells by using anti-B220-coated magnetic beads (DYNAL). The cell purity thus obtained was between 90–95% as determined by a FACS analysis.

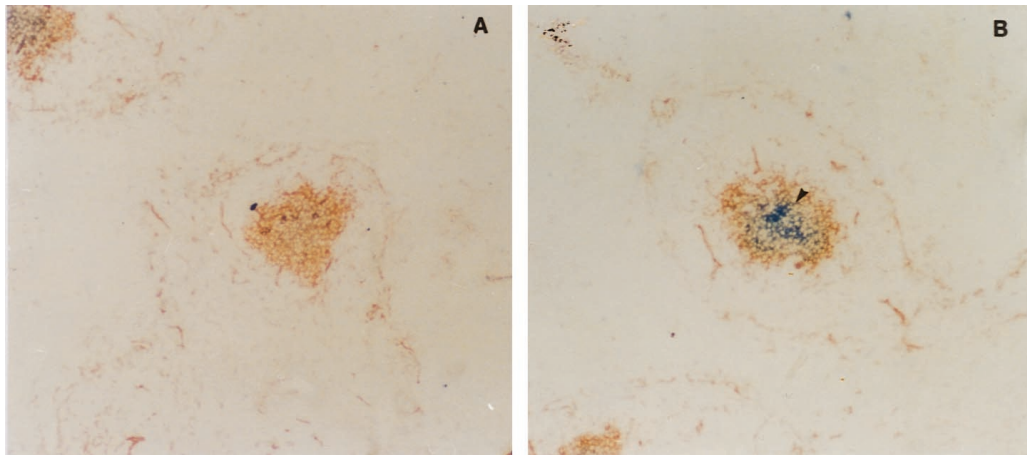
For reconstitution of GCs, the protocol employed was described before (19). Briefly, a total volume of 200  $\mu$ l containing immune complexes of 1  $\mu$ g Ab and  $5 \times 10^6$  enriched T cells from CT3-immunized mice were transferred (*i.v.*) into irradiated (550 rad) BALB/c mice. Twenty-four hours later, these mice also received (*i.v.*) enriched B cells ( $1 \times 10^7$  in 200  $\mu$ l/mouse) derived from splenocytes of mice immunized 2 days earlier with peptide PS1CT3. For enumeration of GCs, spleens were removed 10 days after B cell transfer and sections prepared for immunohistochemical detection of Ag-specific GCs. For quantitation of memory responses, spleens were removed 3 wk after B cell transfer, and the resulting splenocytes were transferred into freshly irradiated (550 rad) recipients. Sixteen hours after this transfer, the hosts were challenged with soluble peptide PS1CT3 (50  $\mu$ g/mouse in PBS, *i.v.*). Blood was collected 5 days later, and the sera was purified for the IgG fraction as described above.

### Immunohistochemical staining of Ag-specific GCs

Immunohistochemical staining of Ag-specific GCs was performed as described previously (19). Briefly, 6- $\mu$ m thick sections of frozen spleens were thaw mounted onto glass slides and fixed in ice-cold acetone. Endogenous peroxidase activity was quenched with 0.1% phenylhydrazine, and sections were blocked for nonspecific binding with a 1:1 proportion of a 3% BSA solution and mouse nonimmune serum. Slides were then incubated for 90 min with 20  $\mu$ g/ml of PNA-biotin in HEPES, pH 7.5, washed, and then followed with a second incubation with streptavidin-HRP (5  $\mu$ g/ml in PBS, 45 min). Both incubations were performed at 37°C. Staining for Ag-specific B cells was performed with the tetrameric peptide Tet-PS1 (19) at a concentration of 50  $\mu$ g/ml overnight at 4°C. After a wash, the slides were then treated with the recommended concentration of streptavidin-alkaline phosphate conjugate in PBS for 45 min at 37°C. Bound conjugates were then visualized in a sequential manner. The HRP conjugate was first detected by color development with the AEC staining kit (Vector Laboratories), where a red color for PNA<sup>+</sup> cells was obtained. Bound alkaline phosphatase was revealed with the blue staining kit (Vector Laboratories), which detected the presence of Ag-specific cells as a blue color.

### ELISAs

ELISA assays for determination of PS1CT3-specific Ab levels were as described earlier (22). Ab cross-reactivity with pin-bound hexapeptides was also evaluated by ELISA. For this, the protocol recommended by the



**FIGURE 1.** Immunohistochemical staining for Ag-specific GCs reconstituted *in vivo*. GCs were reconstituted either in the presence or absence of day 7 polyclonal primary anti-PS1CT3 IgG as described in *Materials and Methods*. *B*, Representative staining of an Ag-specific GC in splenic sections of irradiated hosts not provided with immune complexes is shown. The PNA<sup>+</sup> B cells are seen as red, whereas Ag-specific staining appears as blue. *A*, A GC devoid of Ag-specific B cells, obtained from splenic sections of a mouse mock-immunized with CFA is shown.

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

## Results

### *Ag-specific immune complexes do not constitute a limiting requirement for the initiation of a GC reaction*

The model Ag used for these experiments was a synthetic peptide PS1CT3 (sequence: HQLDPAFGANSTNPDGGDIEKKIAKMEK ASSVFENVNS). This has previously been shown to represent a T cell-dependent immunogen and is comprised of a well-characterized B (residues 1–15, segment PS1) and a T (residues 18–38, segment CT3) cell epitope (23). Separating the B and T cell epitopes is a spacer of two glycine residues (positions 16 and 17) that was included for reasons described (24). We have earlier shown that the murine IgM response to this peptide includes individual Ab specificities that collectively recognize the entire PS1 segment (23). However, subsequent class switch to IgG was found to be restricted to only those Ab subsets directed against a tetrapeptide epitope between positions 4 and 7 (sequence: DPAF) of the PS1 segment (23). This apparent monospecificity for the DPAF epitope was also shown to be retained in the secondary response (23).

In an earlier study (19), we had demonstrated that primary GCs could successfully be reconstituted in irradiated hosts upon co-transfer of Ag-primed T cells and Ag-containing immune complexes followed 24 h later by an additional transfer of enriched B cells from mice primed 2 days earlier with Ag. Therefore, this ability to regenerate GCs from its individual constituents provided us with a system to examine some of the processes that have been shown to occur during the GC reaction. In the present report, we have focussed on both the relevance and regulatory influences of Ag-constituted immune complexes.

Although Ag-comprised immune complexes represent a critical constituent of GCs, there are contradictory reports in the literature on whether they are obligatory for the initiation of a GC reaction. Thus while early reports suggested that GC formation is facilitated by the trapping of immune complexes within the FDC network (5,

6), Kroese et al. (10) have subsequently noted that the appearance of GCs precedes observation of detectable levels of FDC-bound immune complexes. Consequently, as a first step, we sought to re-examine this issue by reconstituting Ag-specific GCs either in the presence or absence of immune complexes containing Ag. The procedure employed was identical with that described earlier (Ref. 19, see also *Materials and Methods*). Enriched T cells from mice primed with a peptide representing the T cell epitope segment of peptide PS1CT3 (peptide CT3) were transferred into freshly irradiated mice either in the presence or absence of immune complexes between peptide PS1CT3 and the IgG fraction of primary day 7 polyclonal anti-PS1CT3 antisera from BALB/c mice. These irradiated hosts also received 24 h later enriched B cells from a separate cohort of mice that had been primed 2 days earlier with peptide PS1CT3. We have previously shown that, when immune complexes were included, this protocol supports Ag-specific GC formation and that it was completely dependent upon the presence of both Ag-activated B and T cells (19). Further, the number of specific GCs obtained were found to peak by day 10 following B cell transfer, after which the numbers declined to reach undetectable levels between day 16 and 18 (19).

In the present experiment, spleens were removed from the hosts at 10 days after B cell transfer, and the resulting sections were stained for the detection and enumeration of Ag-specific GCs (*Materials and Methods*). Interestingly, the presence of Ag-specific GCs could be detected even in mice not provided with immune complexes during adoptive transfer (Fig. 1). Indeed, as shown later, deprivation of externally supplied immune complexes had no effect on the magnitude of the GC response either in terms of number or size distribution.

It was possible that the GC response observed in the immune complex-deprived group may have resulted from residual levels of either free Ag or immune complexes occurring as contaminants in the B cell preparation. To verify this, we synthesized an additional analogue of peptide PS1CT3 where the Phe residue at position 33 was substituted with Tyr to permit labeling with radioactive iodine. After first ascertaining that this Tyr substitution did not alter immunogenicity of the peptide (data not shown), we radioiodinated the analogue peptide to a final sp. act. of  $9.7 \times 10^6$  Ci/ $\mu$ mol. Subsequently, individual BALB/c mice were immunized with the

Table I. *The magnitude of a GC response is immune complex independent<sup>a</sup>*

Group	GCs/10 Sections	Size		
		Small	Medium	Large
1	25 ± 6	8	78	14
2	26 ± 4	6	80	14
3	23 ± 5	10	75	15
4	32 ± 7	8	76	16

<sup>a</sup> As described in *Materials and Methods*, PS1CT3-specific GCs were reconstituted either in the absence (group 1) or presence of immune complexes generated with the mAbs PC7bM (group 2) or PC287 (group 3). Group 4 represents normal, nonirradiated mice that were immunized with peptide PS1CT3. Ten days after B cell transfer (or after immunization for group 4), the mice were sacrificed and GCs enumerated in spleen sections. At least 30 dispersed sections were analyzed per spleen. GC numbers are presented as an average (± SD) over 10 sections for five mice per group. For size distribution, a minimum of 50 peptide-specific GCs per group were analyzed for size and scored as either small, medium, or large on the basis of width in the largest dimension of <25, between 25–50, and >50 PNA<sup>+</sup> cell diameter, respectively. These data are presented as a percent of the total number of GCs examined per group.

radioactive peptide in a manner and dose identical with that employed for the parent peptide (*Materials and Methods*). Subsequently, enriched B cell preparations were derived from these mice and quantitated for radioactivity present per  $1 \times 10^8$  B cells as an estimate of the level of contaminating peptide transferred during reconstitutions. However, in such experiments, we were unable to detect the presence of any radioactivity above background levels in such preparations (data not shown). This was equally true of B cell preparations derived from all the three mice that had been immunized with labeled peptide. Thus, it would appear that the B cells employed in GC reconstitution experiments were devoid of contamination with at least detectable levels of peptide Ag. From the sp. act. of the radiolabeled peptide, we estimate a lower reliable limit of detection to be 5 fmol of peptide/ $10^7$  B cells (based on a cut off value of mean background cpm + 5 SD).

To further probe whether immune complexes are required for the initiation of a GC reaction, we also employed those constituted with anti-PS1CT3 mAbs that had been raised earlier (23). Two mAb preparations, mAb PC7bM and mAb PC287 (23), were initially employed. Although both mAbs have been shown to be directed against the immunodominant DPAF epitope within PS1CT3, mAb PC7bM was an IgM Ab, whereas PC287 was of the IgG isotype (23). Peptide PS1CT3-specific GCs were reconstituted in vivo either in the presence or absence of peptide containing immune complexes of either of these two mAbs. At 10 days after B cell transfer, spleens were removed for an analysis of the magnitude of the peptide-specific GC response. To facilitate a comparison with the native situation, a parallel group of nonirradiated BALB/c mice were immunized with peptide PS1CT3 and the GC response was analyzed 10 days later. The results obtained from such an experiment are presented in Table I. As is evident, the magnitude of the GC response, both in terms of numbers and size distribution, remained relatively invariant regardless of the presence or absence of externally supplied immune complexes (Table I). Further, it was also independent of the isotype of mAb used for the preparation of immune complexes. Finally, the magnitude of the GC response in irradiated-reconstituted mice was comparable to the primary GC response in healthy mice (Table I). Collectively, therefore, the data in Table I seem to suggest that both recruitment and proliferation of Ag-activated B cells was equally well facilitated under all of the variable conditions employed.

Although in our experiments with the radioactive peptide we were unable to detect Ag contamination in the B cell preparation

used for adoptive transfer, the formal possibility of the presence of Ag levels below detection limits cannot be definitively excluded. Nevertheless, the cumulative data strongly suggests that the Ag load within follicles does not constitute a limiting entity during GC formation. In addition, the comparable magnitude of the GC response obtained in irradiated-reconstituted mice and normal, immunized mice serves to further validate our reconstitution protocol.

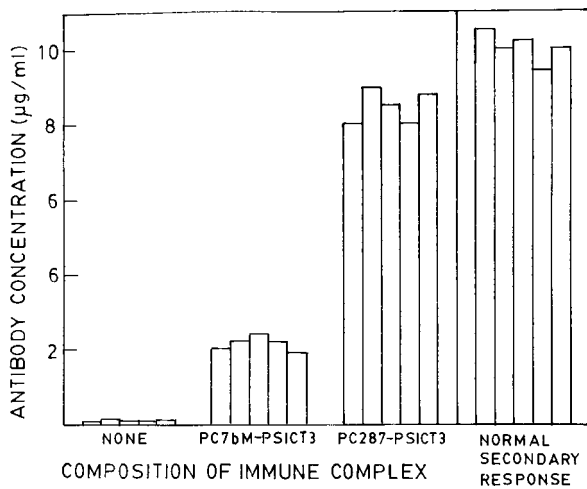
#### *Immune complexes influence generation of Ag-specific B cell memory*

A critical end product of the GC reaction is the generation of Ag-specific memory B cells. Although a variety of intercellular interactions between GC B cells, T cells, and FDCs have been implicated (1, 3, 17, 18), a direct role, if any, for Ag-containing immune complexes remains to be clarified. To obtain preliminary information on this, we sought to determine the extent of PS1CT3-specific memory B cells produced in the various groups described in Table I. To estimate memory B cell generation, we used the protocol established by Zinkernagel and coworkers (25), where splenocytes were transferred into freshly irradiated (550 rad) hosts and followed by a challenge with soluble Ag several hours later. Relative memory responses could then be quantitated as recall IgG titers measured within a few days of antigenic challenge. Although the early time point of measurement of recall IgG necessarily yields low titer values, it was demonstrated that it, nevertheless, accurately reflects differences in the frequency of responder cell populations (25). More recently, we have also validated the application of this procedure for the relative estimation of anti-PS1CT3 memory B cell responses (22).

Splenocytes from the various groups described in Table I were collected at 21 days after B cell transfer and transferred again into freshly irradiated hosts. Following this, anamnestic IgG titers were determined at 5 days after antigenic challenge as described in *Materials and Methods*. The results thus obtained are depicted in Fig. 2. A dominant, albeit selective, influence of incorporated immune complexes is clearly evident on the extent of Ag-specific B cell memory that is produced (Fig. 2). While the magnitude of the GC response was indistinguishable from the other groups, recall IgG responses were barely detectable in splenocytes from immune complex-deprived mice (Fig. 2). In contrast, significant recall IgG levels were obtained from immune complex-supplemented mice, although immune complex preparations with the IgG Ab proved markedly more effective than those with the IgM mAb (Fig. 2). Indeed, recall responses from mice provided with peptide-PC287 immune complexes were comparable with secondary responses from healthy, nonirradiated mice immunized with peptide PS1CT3 (Fig. 2).

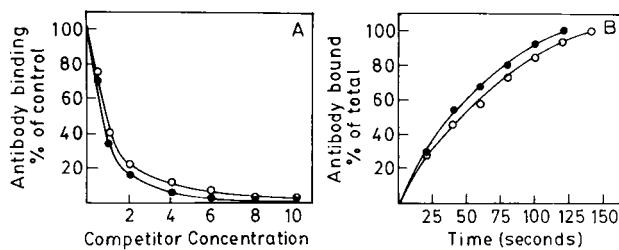
In addition to a quantitative parity, we also performed qualitative comparisons between the latter two groups described above. This was achieved by examining the relative avidity for peptide of the two IgG preparations by competitive inhibition ELISA, and also the kinetics of Ag binding in fluorescence quenching assays. The data from both of these experiments are shown in Fig. 3, where, as is evident, both preparations displayed similar Ag binding properties either in terms of avidity (Fig. 3A) or saturation rates (Fig. 3B). Thus, in conjunction with our earlier described characterizations, the data in Fig. 3 further substantiate that our reconstitution protocol generates GCs that are reminiscent of the native situation, at least in the context of the present system.

However, it remained to be established that, when provided externally, immune complexes do in fact localize within GCs. For this we employed immune complexes where either peptide or mAb was radioiodinated. A knowledge of the sp. act. of the radiolabeled



**FIGURE 2.** Immune complexes regulate production of Ag-specific memory B cells. GCs were reconstituted in irradiated mice from PS1CT3-primed B and CT3-primed T cells as described in *Materials and Methods*. Three weeks after B cell transfer, spleens were removed and the resulting splenocytes transferred into freshly irradiated hosts. These mice were subsequently challenged with soluble peptide, and the resulting anamnestic responses quantitated as peptide PS1CT3-specific titers as described in *Materials and Methods*. For normally derived secondary anti-PS1CT3 responses, BALB/c mice were immunized with a single dose of peptide PS1CT3. Twenty-one days later, the splenocytes were removed, transferred into irradiated recipients, and recall responses determined as described above. The x-axis identifies individual groups in terms of the immune complex that was provided. Data from individual mice are shown and are from one of four separate experiments.

constituents was also expected to permit estimation of the immune complexes actually resident within the GCs. Peptide PS1CT3-specific GCs were reconstituted in irradiated hosts either in the absence or presence radioactive immune complexes between the peptide and either mAb PC7bM or mAb PC287. Subsequently derived



**FIGURE 3.** Recall anti-PS1CT3 IgG from reconstituted GCs are comparable to normally derived secondary anti-PS1CT3 responses. Purified IgG fractions of recall responses from reconstituted GCs supplemented with mAb PC287-peptide PS1CT3 immune complexes (○) and normally derived secondary anti-PS1CT3 IgG (●) from Fig. 2 were compared for their relative avidities for peptide by competitive inhibition ELISA (A). In addition, these fractions were also purified for peptide-specific IgG over a peptide-affinity column for a measurement of Ag binding rates (B). For this, the preparations were diluted in PBS to a final concentration of 200 nM, to which was added 20 µl of PBS containing a 10-fold molar excess (based on estimated binding sites assuming bivalency per IgG molecule) of peptide PS1CT3. The resultant binding interaction, under pseudo-first order conditions, was monitored as time-dependent quenching of tryptophan fluorescence in a Shimadzu RF-1501 spectrofluorometer (Shimadzu Corporation, Tokyo, Japan) until saturation was achieved. Excitation was at 280 nm and emission was recorded at 335 nm. Data shows the extent of saturation as a function of time. Data in both panels are from one of two independent experiments.

**Table II.** Radiolabeled immune complexes localize within GCs<sup>a</sup>

Immune Complex	Constituent (in fmol)/20 µm Section	
	Peptide	mAb
PS1CT3-PC287	5.1 ± 1.3	3.3 ± 0.9
PS1CT3-PC7bM	3.9 ± 1.1	2.3 ± 0.7

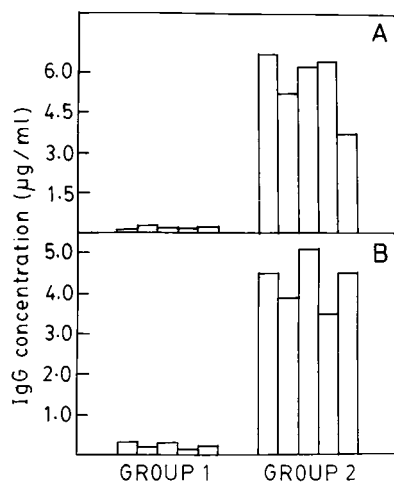
<sup>a</sup> PS1CT3-specific GCs were reconstituted in irradiated hosts either in the presence or absence of immune complexes between peptide PS1CT3 and anti-PS1CT3 mAbs as described in *Materials and Methods*. Here, either radiolabeled peptide or radiolabeled mAb was used, and the two mAbs employed were PC7bM and PC287. At 7 days after B cell transfer, spleens were removed and 20-µm sections were obtained for the immunohistochemical localization of GCs as described for Fig. 1. These GCs were excised as previously described (45) and pooled in groups of between 10 and 20 for determination of incorporated radioactivity. Values given are the mean obtained for three individual mice within each group, where a minimum of three pools were measured. Molar quantities were calculated from the known specific activity of iodination of the individual components which were as follows: PS1CT3,  $9.7 \times 10^6$  Ci/µmol; mAb PC287,  $6.1 \times 10^8$  Ci/µmol; mAb PC7bM,  $3.2 \times 10^8$  Ci/µmol of monomer. For the IgM mAb PC7bM, molar estimates were calculated on the basis of a monomeric unit.

splenic sections were immunohistochemically stained for detection of Ag-specific GCs before their excision and determination of radioactive content. The results from such an experiment are shown in Table II. As is evident, at least some of the externally provided immune complexes do localize within Ag-specific GCs. Further, the extent of localization appears to be comparable regardless of whether the immune complexes contained the IgM or IgG isotype of Ab. Finally, the stoichiometry of peptide to Ig monomer obtained was close to the theoretically expected value of 2:1 (Table II).

Another notional possibility that could account for the results in Fig. 3 is that Ag dissociating from the provided immune complexes could be captured by Abs being secreted by the activated B cells in the transferred population. To rule this out, we also examined the serum half-life of the radiolabeled peptide in irradiated BALB/c mice. Mice were immunized (i.v.) with 2.5 nmol of radiolabeled peptide and then periodically bled for the determination of radioactivity. These experiments yielded a short serum half-life for peptide of <4 h (data not shown). Thus, given the conditions of our protocol where limiting amounts of Ag (~14 pmol/mouse) in the form of immune complexes were given and that B cells were introduced 24 h after injection of immune complexes, the possibility of significant amounts of peptide exchange appears near negligible. This is further supported by the data in Table II where the stoichiometry of peptide to mAb within GCs was close to the expected value. Finally, injection of either soluble peptide (250 pmol/mouse) instead of preformed immune complexes, or immune complexes of peptide with the Fab of mAb PC287 also did not lead to any enhanced memory generation (data not shown).

Thus, in light of the observations above, we interpret the data in Fig. 2 to suggest that, within GCs, immune complexes regulate the extent of Ag-specific memory B cells that are produced. Further, this regulation is also modulated by the isotype of Abs that constitute the GC resident immune complexes.

To establish that the findings reported above were not restricted to peptide PS1CT3 but, rather, represents a general property of immune complexes in GCs, we chose two additional, well-characterized protein Ags hen egg lysozyme (HEL) and OVA. Irradiated hosts were reconstituted with Ag (i.e., either HEL or OVA)-primed T and B cells as earlier described either with or without appropriate immune complexes produced from day 7 primary polyclonal IgG. Anamnestic responses were subsequently determined and the results are presented in Fig. 4. It is unequivocally clear for both Ags that, while significant levels of memory IgG was



**FIGURE 4.** Immune complexes also enhance memory B cell generation against representative protein Ags. Irradiated mice were reconstituted with enriched B and enriched T cells from either HEL (A)- or OVA (B)-immunized mice, either with (group 2) or without (group 1) appropriate immune complexes generated with day 7 primary polyclonal antisera. Subsequent recall responses were generated and quantitated as described for Fig. 2A. Data from individual mice are shown.

obtained from immune complex-supplemented GCs, this was only marginal when immune complexes were excluded.

#### *Regulation of memory B cell generation by immune complexes is specificity-restricted*

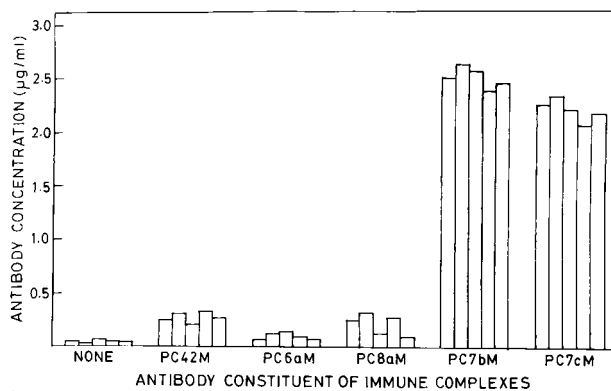
Our observations that generation of memory B cell responses is dependent upon the presence of immune complexes within GCs raised the intriguing question of whether the epitope fine-specificity of the constituent Abs has any regulatory influence. To examine this, we used additional anti-PS1CT3 mAbs, but directed against alternate determinants within the PS1 segment of peptide PS1CT3. Although, as depicted in Fig. 2, immune complexes with IgG class Abs proved superior in eliciting a memory response, we have shown earlier that the anti-PS1CT3 IgG response in BALB/c mice is exclusively directed at the DPAF epitope (23). This was also reflected at the level of mAbs that were obtained (23). In contrast, the early primary anti-PS1CT3 IgM response was shown to consist of heterogeneous epitope specificities where it was possible to obtain a variety of mAbs directed against a variety of determinants within the PS1 sequence (23). Consequently, for the purposes of the present study, we were limited to the use of anti-PS1CT3 IgM mAbs.

The IgM mAbs used have been described earlier, and their properties are summarized in Table III. Immune complexes were pre-

Table III. *Binding characteristics of anti-PS1CT3 IgM mAbs<sup>a</sup>*

IgM mAb	IC <sub>50</sub> for Peptide (µM)	Epitope Specificity
PC7bM	21.0	DPAF
PC7cM	4.0	DPAF
PC42M	1.5	NSTN
PC8aM	10.0	NSTN
PC6aM	32.0	FG

<sup>a</sup> The mAbs listed here are specific for the PS1 segment of peptide PS1CT3 and, as previously described (23), were obtained from a fusion with splenocytes from a mouse that had been immunized 4 days earlier with peptide PS1CT3. While their epitope specificities have been characterized earlier (23), relative affinities for peptide PS1CT3 were determined by competitive inhibition ELISA. IC<sub>50</sub> values given are the mean of two independent measurements.



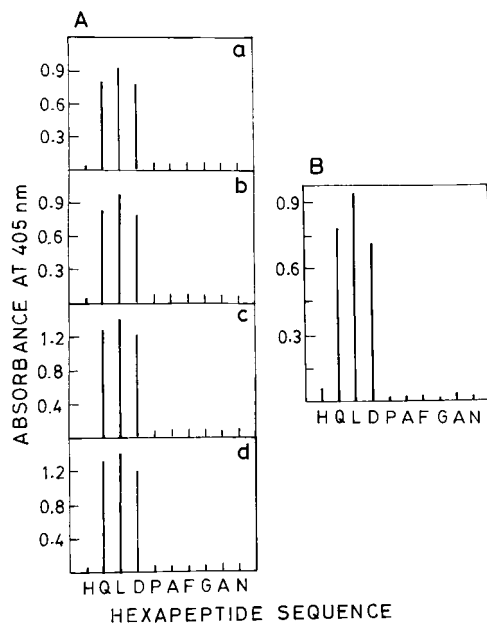
**FIGURE 5.** The memory enhancement activity of immune complexes is specificity restricted. Immune complexes were prepared between peptide PS1CT3 and each of the anti-peptide IgM mAbs listed in Table III. These were then independently included in PS1CT3-specific GC reconstitution protocols, followed by elicitation and estimation of recall IgG levels as described for Fig. 2. A control group was also included where immune complexes were omitted during the cell transfer. Data are from individual mice within each group and are representative of two independent experiments.

pared between peptide PS1CT3 and each of these mAbs and subsequently employed during in vivo GC reconstitution experiments. Anamnestic IgG responses that resulted from each of these groups were quantitated, and the results are given in Fig. 5. The notable feature of the data shown is that the quantum of PS1CT3-specific B cell memory generated was strictly dependent upon the epitope fine-specificity of the mAb that constituted the immune complex (Fig. 5). Thus, while immune complexes generated with DPAF-specific mAbs were relatively potent at inducing a recall response, those comprised by Abs directed against alternate determinants were only marginally stimulatory (Fig. 5).

To understand the basis of the preference for immune complexes composed of Abs of a unique specificity, we next analyzed the distribution of epitope specificities within the anti-PS1CT3 IgG recall responses in all the groups described in Fig. 5. For this, purified IgG fractions were screened for cross-reactivity against a panel of overlapping, single residue displaced hexapeptides that collectively spanned the PS1 segment of peptide PS1CT3. For comparative purposes, secondary IgG from normal PS1CT3-immunized mice was also included. Although results for only representative groups are shown in Fig. 6A, all sera tested yielded a unique reactivity profile restricted to three overlapping hexapeptides of sequence QLDPAF, LDPAFG, and DPAFGA. This, as we have proved earlier (23) is indicative of monospecificity for the DPAF epitope. Thus, the data in Fig. 6A reveal that, regardless of quantitative differences among the groups, epitope specificity of the recall IgG response remained invariant and directed against the DPAF epitope. It may be noted from Fig. 6A that the normally derived secondary IgG from nonirradiated mice also displays DPAF monospecificity. In addition to the results described here, we found that the recall responses from reconstituted GCs supplemented with mAb PC287-peptide immune complexes also displayed, as might be expected, DPAF monospecificity in epitope mapping studies (data not shown).

#### *Selection for DPAF monospecificity precedes the onset of a GC reaction*

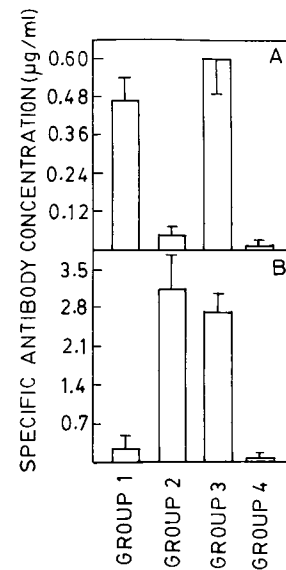
In a previous report, we had demonstrated that the early primary murine IgM response to peptide PS1CT3 was comprised of distinct



**FIGURE 6.** Epitope-specificity of recall (A) and early primary (B) anti-PS1CT3 IgG responses. Purified IgG fractions from recall antisera derived from the experiment in Fig. 5 were pooled within a group and screened for cross-reactivity against the overlapping PS1-derived hexapeptide panel as previously described by ELISA (Ref. 23, see also *Materials and Methods*). The x-axis identifies each hexapeptide as its amino-terminal residue. Background reactivity, which was subtracted, was determined by simultaneously evaluating cross-reactivity of each preparation with that against an irrelevant peptide of sequence AQGNSM. Data shown are from one of three experiments. Although all sera tested from Fig. 5 gave essentially identical profiles, only representatives are shown in A. These indicate groups where immune complexes were constituted with the following mAbs: *a*, no immune complex; *b*, mAb PC42 M; *c*, mAb PC7bM. For comparative purposes, the profile obtained for normally derived secondary IgG is also shown (*d*). B gives the results for day 7 IgG from PS1CT3-immunized mice that had also been simultaneously treated with mAb GL1 to inhibit GC formation (see *Results*).

epitope specificities (23). Interestingly, however, a subsequent class switch to the IgG isotype was accompanied by a stringent and exclusive selection for the anti-DPAF subset (23). Such DPAF monospecificity was also demonstrated to be retained in the secondary response (23). To verify the stage at which selection for DPAF monospecificity occurs, we examined sera from PS1CT3-immunized mice where GC formation had been inhibited by treatment with the anti-B7.2 mAb, GL1. Consistent with earlier reports (26), we have shown that multiple high-dose injections of GL1 also abrogates GC formation in PS1CT3-immunized mice, although the primary humoral response was only marginally affected (19). Both IgM and IgG fractions of day 7 sera from such mice were analyzed for distribution of epitope specificities by screening against the hexapeptide panel described in Fig. 6A. In these studies, the IgM anti-PS1CT3 response was found to retain the multispecific character described earlier for that from mice not treated with GL1 (not shown, but see Ref. 23). In contrast, the early IgG response displayed monospecificity for the DPAF epitope (Fig. 6B). This reactivity profile was identical with that described for both primary and secondary IgG from GL1-untreated mice (23).

That dominant selection for the DPAF epitope occurs even in the absence of any detectable GC formation strongly suggests that this event precedes GC formation. Presumably then, it is this se-



**FIGURE 7.** Specificity-restricted memory enhancement activity of immune complexes is also revealed with a hapten-carrier system. Initially, a large group of BALB/c mice were immunized with DNP-BSA (100 µg/mouse in CFA, i.p.) to obtain day 7 antisera. After resolution of the IgG fraction, a part of this preparation was passed over an affinity column coupled to DNP-keyhole limpet hemocyanin to separate the DNP-specific and BSA-specific IgG subsets. Subsequently, immune complexes with DNP-BSA were prepared with either anti-DNP IgG (group 1), anti-BSA IgG (group 2), or the total unfractionated IgG (group 3) and independently included during reconstitution of GCs with DNP-BSA-primed T and B cells as described in *Materials and Methods*. A separate control group was also included where no immune complexes were provided (group 4). Splenocytes from these hosts were then transferred, 21 days later, into freshly irradiated recipients and recall responses elicited by challenge with soluble DNP-BSA. DNP-specific IgG titers (A) were estimated by ELISA against DNP-Ficoll as coat Ag, whereas BSA-specific titers (B) were determined against native BSA. Values are mean titers ( $\pm$ SE) obtained from three individual mice within each group and the data represent one of two separate experiments.

lected subset that populates GCs. As a result, the requirement for immune complexes constituted by DPAF-specific Abs noted in Fig. 5 probably follows as a natural outcome of this selection.

To further verify the existence of specificity-restricted memory enhancement, we also selected, as an additional model immunogen, a hapten-carrier system, DNP-BSA, for study. Mice immunized with DNP-BSA were bled 7 days later and the sera purified over an affinity column for the DNP-specific and BSA-specific IgG subpopulations. Subsequently, immune complexes were generated between either of these fractions and DNP-BSA before utilization in GC reconstitution protocols with Ag-primed T and B cells. Both the resulting anti-DNP or anti-BSA anamnestic IgG responses were then determined, and the results are presented in Fig. 7. It is obvious from Fig. 7 that incorporation of immune complexes prepared from anti-DNP IgG results in specific enhancement of anti-DNP memory responses, but not that against the carrier BSA. On the other hand, the reverse was true when immune complexes with anti-BSA Abs were incorporated instead.

Thus, collectively the data in Figs. 5–7 clearly suggest that the B cell memory enhancement activity of immune complexes within GCs is epitope restricted by the epitope specificity of the Abs constituting the immune complexes.



Table IV. *Specificity maturation is enhanced by the presence of appropriate immune complexes<sup>a</sup>*

Analog Peptide	Relative Reactivity for Ab					
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
LEPGWG	+++	+/-	+	+	-	-
LDPSFG	+	-	-	-	-	-
LEPSYG	-	-	-	-	-	-
LDPGWG	+++++	+	++	+	-	-
LEPCYG	++	+	+	+	-	-
LEPSFG	-	-	-	-	-	-
LEPALG	-	-	-	-	-	-
LEPCLG	-	-	-	-	-	-
LEPSLG	-	-	-	-	-	-
LDPAWG	++	+	+	+	+/-	+/-
LDPAYG	-	-	-	-	-	-
LEPAYG	-	-	-	-	-	-
LEPAWG	++	+/-	+/-	+/-	-	-
LDPLFG	+++++	+	+	+	-	-
LDPCWG	+++	++	++	++	+/-	-
LEPLFG	+	-	-	-	-	-
LDPALG	-	-	-	-	-	-
LDPAVG	-	-	-	-	-	-
LEPALG	-	-	-	-	-	-
LEPAIG	-	-	-	-	-	-

<sup>a</sup> Purified IgG fractions from either primary or recall anti-PS1CT3 responses were screened for cross-reactivity against analogs of the PS1-derived hexapeptide LDPAFG (see text). For subtraction of background a nonspecific hexapeptide of sequence LVIMSG was used. Taking the reactivity of a given preparation with the parent peptide as 100%, relative reactivity with individual analogs was scored as follows: <25%, -; 25–50%, +/-; 50–100%, +; 100–200%, ++, 200–300%, +++; 300–400%, +++++; >400%, ++++++. Group 1 represents the IgG fraction of primary anti-PS1CT3 antisera from GL1-treated mice. Group 6 identifies normally derived secondary IgG obtained as described in Fig. 2. The remaining groups represent recall IgG from reconstituted GCs and differed in terms of the immune complex provided during GC reconstitution. These are as follows: group 2, no immune complex provided; group 3, peptide-mAb PC42M; group 4, peptide-mAb PC7bM; group 5, peptide-mAb PC287. Data presented is that from one of two independent experiments.

#### *Immune complexes optimize but do not drive specificity acquisition within GCs*

Although several studies have documented that GCs also represent the sites where affinity maturation of Abs occurs (reviewed in Refs. 27–29), we have shown previously that progression of a murine primary humoral response to peptide PS1CT3 is not characterized by an increase in Ab affinity (19, 30). As a result, the present system did not permit an analysis of the role of Ag-containing immune complexes in affinity maturation. However, Manser and coworkers have recently demonstrated that improvement in Ag-specificity of Ab is yet another hallmark of the GC reaction (18). Such a “specificity maturation” was postulated to be critical in ensuring memory responses with little to no cross-reactivity with self-Ags (18).

To evaluate for specificity maturation in the present system, we took advantage of the fact that both primary anti-PS1CT3 IgG from GL1-treated mice and recall IgG from GCs reconstituted under various conditions were exclusively directed against the DPAF epitope within PS1CT3. Therefore, we synthesized analogues of a hexapeptide representing residues 3–8 (sequence: LDPAFG) of peptide PS1CT3. In these analogues, individual residues within the DPAF epitope (D, A, and F) were variably substituted with alternate, chemically similar amino acid residues to generate a panel of analogue sequences. This panel was then used to screen for Ab cross-reactivity with recall IgG produced under the influence of a representative set of immune complexes. For a comparison with the specificity of anti-DPAF IgG before the onset of the GC reaction, the IgG fraction of sera from GL1-treated mice was also analyzed. Further, secondary IgG obtained under normal conditions from nonirradiated mice was included as a qualitative reference for the expected end product of a GC reaction.

Results obtained from the experiment described above are shown in Table IV. Primary anti-PS1CT3 IgG from GL1-treated mice displayed poor specificity where several of the analogues

proved more reactive than the parent peptide (Table IV). In contrast, normally derived secondary responses were markedly more Ag-specific, at least in the context of the panel tested (Table IV). These results clearly point to the fact that an improvement in Ab specificity is also a characteristic of murine humoral responses to peptide PS1CT3. Notably, some enhancement of Ab specificity could also be noted for recall IgG obtained from immune complex-depleted GCs (Table IV). Supplementation of GCs with immune complexes derived from non-DPAF-directed mAbs had no additional effect (Table IV). On the other hand, GCs containing immune complexes prepared from DPAF-specific mAbs yielded a marked improvement in Ab specificity, with again a greater efficacy for immune complexes generated from IgG mAb as opposed to IgM (Table IV). Indeed, the cross-reactivity profile for recall IgG from GCs supplemented with PC287-constituted immune complexes was almost identical with that obtained for normally derived secondary anti-PS1CT3 IgG (Table IV). Thus, it appears that, although an enhancement of epitope-specificity of Ab can occur in their absence, the presence of immune complexes constituted by Abs of the appropriate specificity and isotype is necessary to optimize the outcome.

## Discussion

Germinal centers represent histologically defined structures that support maturation and differentiation of Ag-activated B cells, principally in T cell-dependent humoral responses. A prerequisite to GC formation is the migration of Ag-activated B cells into splenic follicles, which, in turn, appears to be regulated by a variety of factors (31) that include an endogenous transcription machinery (32) and the presence of appropriate chemokine receptors on the cell surface (33). Subsequent to this, B cells undergo rapid proliferation, creating a secondary follicle, which then differentiates into a GC with defined light and dark zones (34, 35). It is within this complex microenvironment where somatic mutation is

initiated, followed by selection of a significant frequency of mutated clonotypes into the memory compartment (27, 36).

Although there have been earlier suggestions that prior deposition of Ag-containing immune complexes onto the FDC network within the primary follicle is also required to initiate a GC reaction, our own results presented here indicates that this does not constitute a limiting requirement. Adoptive transfer of only Ag-primed T and B cells, in the absence of any added immune complex, was sufficient to generate GCs in irradiated recipients. While we cannot definitively rule out the possibility that trace, undetectable amounts of either immune complexes or free Ag molecules were cotransferred with B cells, we note that the magnitude of the GC response in this group, both in terms of number and size distribution, was indistinguishable from those in groups provided with immune complexes. These observations suggest that both recruitment and proliferation of B cells within GCs are independent of the presence of at least significant levels of immune complexes. Such an inference would also be consistent with the findings of Kroese et al. (10). They had earlier demonstrated, using lethally irradiated and reconstituted rats, that the development of GCs precedes detection of FDC-bound immune complexes. However, the issue of whether kinetics of GC formation is influenced by immune complexes, a possibility that has been suggested earlier (37), was not addressed in this study.

While the initiation of a GC reaction was found not to require the prior presence of significant amounts of Ag in the form of immune complexes, subsequent generation of memory B cells was strictly immune complex dependent. Moreover, for peptide PS1CT3, the memory-enhancement activity was restricted to immune complexes constituted by Abs specific for the DPAF epitope and with an optimal requirement for IgG as opposed to the IgM isotype. In previous studies, we have shown that the early primary anti-PS1CT3 humoral response is comprised of a heterogeneous distribution of Ab fine-specificities that collectively span the entire PS1 sequence (23). However, subsequent class switch and maturation was restricted to only that subset of Ag-activated B cells that were directed against the DPAF epitope (23). Here, by employing the monoclonal anti-B7.2 Ab GL1 to inhibit GC development, we have extended these results to indicate that selection for DPAF monospecificity precedes initiation of the GC reaction. Therefore, it follows that seeding of GCs would also be expected to be restricted to this positively selected subset of anti-DPAF B cells. Such an inference is entirely in keeping with our findings that memory responses were invariantly DPAF-monospecific regardless of the nature of immune complexes employed in GC reconstitution. This was also the case when immune complexes were excluded. Consequently, the observed specificity-restricted memory enhancement activity of immune complexes strongly suggests that this is facilitated only when GC B cells encounter immune complexes constituted by Abs with an identical fine specificity for epitope. Such an inference was also supported by our results with DNP-BSA where, again, selective amplification of memory B cells with epitope specificities that were homologous to that of the Ab constituent in the provided immune complexes was observed. Further, the fact that the mAbs PC7bM and PC7cM Abs, whose derivatives are absent from the anti-PS1CT3 IgG repertoire (30), also displayed memory enhancement ability indicates that it is identity at the level of epitope specificity rather than clonality that dominates as the selection criterion.

Although our data implicate a role for immune complexes in determining the quantum of B cell memory that is generated in the course of a GC reaction, the precise stage at which this operates remains to be defined. It is possible that immune complexes function by increasing the repertoire of GC B cells that are inducted

into the memory differentiation pathway. In this connection, Cerny and coworkers have recently proposed that immune complexes in GCs lower the threshold of B cell activation (37). The rationale for such a proposal was based on the earlier demonstration that covalent binding of the complement protein C3 to Ig in immune complexes constitutes by being able to bridge B cell Ag receptor with the CD21/CD19/CD81 complex, a potent stimulatory signal for B cells (38). In this connection, it may be noted that complement receptors, expressed on the surface of FDCs, appear to play an important role in follicular trapping of immune complexes (1, 18, 39). While the majority of mAbs used in this study were of the IgM isotype, the only exception, mAb PC287, was of the IgG1 subclass (19). However, because the complement fixing abilities of these mAbs was not examined, we cannot presently confirm whether such a mechanism was indeed operative for the test system employed here.

An alternate explanation for the memory enhancement activity could derive from the possibility that immune complexes simply promote proliferative expansion of already differentiated memory B cells. A comparison of the memory B cell repertoires generated from GCs prepared in the absence or presence of the various immune complexes described here can be expected to shed some light in this regard. Nevertheless, it is also interesting to note that the magnitude of a GC response appears to have no direct bearing on the extent of Ag-specific B cell memory that is eventually produced. Thus, while the resulting anamnestic responses differed greatly depending upon the inclusion or exclusion of relevant immune complexes, the magnitude of the GC response was relatively invariant.

One factor that could potentially have interfered with our results was the possible presence of pre-existing natural anti-PS1CT3 Ab in irradiated recipients. However, we have earlier been unable to detect the presence of naturally occurring anti-PS1CT3 responses either at the level of serum Ig or Ab-producing B cells (19, 40). Further, injection of soluble peptide, along with the T cell fraction, also did not result in the generation of PS1CT3-specific B cell memory. In the event that functionally relevant natural Abs to peptide were existent, soluble peptide immunization would be expected to generate immune complexes *in vivo*, with the consequent promotion of memory responses. Finally, transfer of sera from nonimmune irradiated mice, either with or without a prior incubation with peptide PS1CT3, along with the T cell fraction also did not lead to any enhancement in subsequently evaluated memory responses (B.P.N., unpublished observations). Thus, these results collectively rule out a complicating role for naturally occurring serum Ig in irradiated recipients.

The process by which GC B cells acquire Ag from immune complexes is also an issue that remains to be examined. This is equally true of both events, leading to positive selection of mutated B cells and enhancement of the memory pool. However, several alternate mechanisms have been put forward in the literature. For example, it has been suggested that Ag capture by GC B cells could be mediated by competitive displacement from immune complexes presented either on intact FDCs or on the surface of iccosomes (e.g., see Ref. 16). Indeed, in support of such a possibility, our own recent results indicate that the memory enhancement activity of immune complexes is dependent on the ability of Ag to dissociate from the Ab (B.P.N., P.N., and K.V.S.R., manuscript in preparation). Thus, for example, an immune complex where peptide PS1CT3 was chemically cross-linked to mAb PC287 was unable to induce amplification of PS1CT3-specific memory B cell responses from reconstituted GCs (B.P.N., P.N., and K.V.S.R., manuscript in preparation). Nevertheless, an alternate mechanism may also lie in the direct endocytic uptake of

icosomes by B cells, followed by Ag processing and presentation (e.g., see Ref. 1). Finally, Leanderson and colleagues have envisioned positive selection within GCs to occur by the direct binding of B cells to Ag within immune complexes (17). Subsequent release of such bound B cells has been proposed to be mediated by proteolytic cleavage of surface Ig (17). In principle, any of the above mechanisms could explain positive selection of mutated GC B cells, particularly under conditions where the Ag supply is limiting. However our results, which reveal the importance of epitope-specificity of Abs constituting the immune complexes, suggests that the latter two mechanisms are unlikely to account for at least the memory enhancement activity of immune complexes within GCs. It is difficult to envisage specificity-restriction to be operative in the context of Ag nonspecific uptake of icosomes by endocytosis. Further, the observed requirement of homologous epitope specificities between the responder GC B cells and the Ab constituent of immune complexes also suggests that such B cells would be unable to directly bind immune complexes as the relevant epitope is expected to be masked by Ab binding. However, any definitive conclusion must await further analysis.

Our data also lend support to the notion of "specificity maturation" within GCs as proposed earlier by Manser and coworkers (18). Interestingly though, detectable levels of Ag supply in the form of immune complexes again appeared to be redundant for initiating this process. However, immune complexes of the appropriate specificity and isotype of Ab were required for further optimizing Ab specificity. These observations raise the possibility that enhancement of Ab specificity within GCs may occur in two stages: an Ag-independent step followed by an Ag-dependent one. Although this proposal remains to be verified, it is likely that the Ag-independent stage correlates with the recent demonstrations that engagement of GC B cells by soluble Ag (and, therefore, also cross-reactive self-Ags) leads to apoptosis (1, 41–43). This can be expected to result in a "filtering" of mutated GC B cells in favor of the less cross-reactive clonotypes. Subsequent optimization could represent an Ag-driven process where the requirement for surviving B cells to competitively displace Ag from immune complexes imposes an additional level of stringency.

In summary, results presented in this report suggest that, within GCs, immune complexes play a major role in enhancement of the memory B cell compartment. Further, this activity is specificity restricted, demanding an identity at the level of epitope fine specificity of GC B cells and the Abs that constitute the immune complex. Thus, in addition to clone autonomous Ab maturation within GCs (18, 44), memory B cells also appear to be primarily generated in a "specificity autonomous" fashion. These findings may have interesting implications for the relative extents of epitope-specific memory B cells produced in immune responses to multivalent Ags. Finally, an extension of our studies on the regulation of Ab specificity by immune complexes may also provide a further insight into processes that restrict generation of autoreactive B cells as a result of somatic mutations within GCs.

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## References

- Kosco-Vilbois, M. H., H. Zentgraf, J. Gerdes, and J.-H. Bonnefoy. 1997. To "B" or not to "B" a germinal center B cell? *Immunol. Today* 18:225.
- Kelsoe, G. 1996. Life and death in germinal centers (redux). *Immunity* 4:107.
- Liu, Y.-J., O. de Bouteiller, and I. Fugier-Vivier. 1997. Mechanism of selection and differentiation in germinal centers. *Curr. Opin. Immunol.* 9:256.
- Nossal, G. J. V. 1994. Differentiation of the secondary B lymphocyte repertoire: the germinal center reaction. *Immunol. Rev.* 137:173.
- Klaus, G. G. B., J. H. Humphrey, A. Kunkl, and D. W. Dongworth. 1980. The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. *Immunol. Rev.* 53:3.
- Mandel, T. E., R. P. Phipps, A. Abbot, and J. G. Tew. 1980. The follicular dendritic cell: long term antigen retention during immunity. *Immunol. Rev.* 53:29.
- Kunkl, A., and G. G. B. Klaus. 1981. The generation of memory cells. IV. Immunization with antigen-antibody complexes accelerates the development of B-memory cells, the formation of germinal centers and the maturation of antibody affinity in the second response. *Immunology* 43:371.
- Laissue, J., H. Cottier, M. W. Hess, and R. D. Stoner. 1971. Early and enhanced germinal center formation and antibody responses in mice after primary stimulation with antigen isologous antibody complexes as compared with antigen alone. *J. Immunol.* 107:822.
- Nie, X., S. Basu, and J. Cerny. 1997. Immunization with immune complexes alters the repertoire of antigen-reactive B cells in the germinal centers. *Eur. J. Immunol.* 27:3517.
- Kroese, F. G. M., H. S. Wubbena, and P. Nieuwenhuis. 1984. Germinal center formation and follicular antigen trapping in the spleen of lethally X-irradiated and reconstituted rats. *Immunology* 57:99.
- Storb, U. 1998. Progress in understanding the mechanism and consequences of somatic hypermutation. *Immunol. Rev.* 162:5.
- Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell* 67:1121.
- McHeyzer-Williams, M. G., M. J. McLean, P. Lalor, and G. J. V. Nossal. 1993. Antigen-driven B cell differentiation in vivo. *J. Exp. Med.* 173:295.
- Weiss, U., R. Zobebein, and K. Rajewsky. 1992. Accumulation of somatic mutants in the B cell compartment after primary immunization with a T cell-dependent antigen. *Eur. J. Immunol.* 22:511.
- Thorbecke, G. J., A. P. Armin, and V. K. Tsiagbe. 1994. Biology of germinal centers in lymphoid tissue. *FASEB J.* 8:832.
- Han, S., B. Zheng, Y. Takahashi, and G. Kelsoe. 1997. Distinctive characteristics of germinal center B cells. *Semin. Immunol.* 9:255.
- Andersson, K., J. Wrammert, and T. Leanderson. 1998. Affinity selection and repertoire shift: paradoxes as a consequence of somatic mutation? *Immunol. Rev.* 162:172.
- Manser, T., K. M. Tumas-Brundage, L. P. Casson, A. M. Giusti, S. Hande, E. Notides, and K. A. Vora. 1998. The role of antibody variable hypermutation and selection in the development of the memory B-cell compartment. *Immunol. Rev.* 162:182.
- Agarwal, A., B. P. Nayak, and K. V. S. Rao. 1998. B cell responses to a peptide epitope. VII. Antigen-dependent modulation of the germinal center reaction. *J. Immunol.* 161:5832.
- Atherton, E., and R. C. Sheppard. 1989. *Solid Phase Peptide Synthesis: A Practical Approach*. IRL Press, Oxford.
- Geysen, H. M., S. Rodda, T. J. Mason, G. Tribbick, and P. G. Schoofs. 1987. Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* 102:259.
- Agarwal, A., and K. V. S. Rao. 1997. B cell responses to a peptide epitope. III. Differential T helper thresholds in the recruitment of B cell fine specificities. *J. Immunol.* 159:1077.
- Agarwal, A., S. Sarkar, C. Nazabal, G. Balasundaran, and K. V. S. Rao. 1996. B cell responses to a peptide epitope. I. The cellular basis of restricted recognition. *J. Immunol.* 157:2779.
- Rao, K.V.S., and A. R. Nayak. 1990. Enhanced immunogenicity of a sequence derived from the hepatitis B virus surface antigen in a composite peptide that includes the immunostimulatory region from human interleukin-1. *Proc. Natl. Acad. Sci. USA* 87:5519.
- Bachman, M. F., T. M. Kundig, H. Hengartner, and R. M. Zinkernagel. 1994. Regulation of IgG antibody titers by the amount of persistent immune-complexed antigen. *Eur. J. Immunol.* 24:2567.
- Han, S., K. Hathcock, B. Zheng, T. B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interactions in germinal centers: role of CD40 ligand and B7-2 in established germinal centers. *J. Immunol.* 155:556.
- Berek, C. 1992. The development of B cells and the B cell repertoire in the microenvironment of the germinal center. *Immunol. Rev.* 126:5.
- MacLennan, I. C. M. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117.
- Nossal, G. J. V. 1994. The molecular and cellular basis of affinity maturation in the antibody response. *Cell* 68:1.
- Nayak, B. P., R. Tuteja, V. Manivel, R. P. Roy, R. A. Vishwakarma, and K. V. S. Rao. 1998. B cell responses to a peptide epitope. V. Kinetic regulation of repertoire discrimination and antibody optimization for epitope. *J. Immunol.* 161:3510.
- Tarlington, D. M. 1998. Germinal centers: form and function. *Curr. Opin. Immunol.* 10:245.
- Schubart, D. B., A. Rolink, M. H. Kosco-Vilbois, F. Botteri, and P. Mathias. 1996. B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal center formation. *Nature* 383:538.
- Forster, R., A. E. Mattis, E. Kremmar, E. Wolf, G. Brem, and M. Lipp. 1996.

- A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 87:1037.
34. MacLennan, I. C. M., and D. Gray. 1986. Antigen-driven selection of virgin and memory B cells. *Immunol. Rev.* 91:61.
  35. Nieuwenhuis, P., and D. Opstelten. 1984. Functional anatomy of germinal centers. *Am. J. Anat.* 170:421.
  36. Gray, D., and T. Leanderson. 1990. Expansion, selection and maintenance of memory B cell clones. *Curr. Top. Microbiol. Immunol.* 159:1.
  37. Song, H., X. Nie, S. Basu, and J. Cerny. 1998. Antibody feedback and somatic mutation in B cells: regulation of mutation by immune complexes. *Immunol. Rev.* 162:211.
  38. Dempsey, P.W., M. E. D. Allison, S. Akkarajv, C. C. Goodnow, and D. T. Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271:348.
  39. Vora, K. A., J. V. Ravetch, and T. Manser. 1997. Amplified follicular immune complex deposition in mice lacking the Fc receptor  $\gamma$ -chain does not alter maturation of the B cell response. *J. Immunol.* 159:2116.
  40. Tuteja R., A. Agarwal, L. Vijaykrishnan, B. P. Nayak, S. K. Gupta, V. Kumar, and K. V. S. Rao. 1997. B cell responses to a peptide epitope. II. Multiple levels of selection during maturation of primary responses. *Immunol. Cell. Biol.* 75:245.
  41. Pulendran, B., G. Kannourakis, S. Nouri, K. G. C. Smith, and G. J. V. Nossal. 1995. Soluble antigen can cause enhanced apoptosis of germinal center B cells. *Nature* 375:331.
  42. Shokat, K. M., and C. C. Goodnow. 1995. Antigen-induced B-cell death and elimination during germinal center immune responses. *Nature* 375:334.
  43. Han, S., B. Zhen, J. P. Dal, and G. Kelsoe. 1995. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 182:1635.
  44. Vora, K. A., and T. Manser. 1995. Altering the antibody repertoire via transgene homologous recombination: evidence for global and clone-autonomous regulation of antigen-driven B cell differentiation. *J. Exp. Med.* 181:251.
  45. Zeigner, M., G. Steinhauser, and C. Berek. 1994. Development of antibody diversity in single germinal centers: selective expansion of high affinity variants. *Eur. J. Immunol.* 24:2393.