CHAPTER III

Immune complex mediated regulation of B cell generation within germinal centers
**INTRODUCTION**

Extensive studies over the last decade or so have unraveled much of the processes related to induction and maturation of T-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 antibody producing B cells (AFCs) concentrate in the periphery - in the neighborhood of the red pulp - of periarteriolar lymphoid sheath (PALS) (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 antigen systems there are, however, 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 the B cells in the lymphoid follicle (5,6). Such an inference has been derived from the observations that, immunization with antigen-specific immune complexes, as opposed to antigen alone, results in a more rapid induction of GCs (7,8). The rate of somatic mutations has also been found to be higher in such cases, thus resulting in secretion of high affinity antibodies by secondary AFCs compared to mice immunized with antigen alone (7,9). Finally, the rate of memory B cell development was also enhanced in immune complex-immunized mice leading to increase in anamnestic response (7). Contrary to the inferences derived from such studies, however, there are other reports in the literature, which document that the appearance of GCs precedes observation of detectable levels of FDC-bound immune complex (10). Although it is possible that these latter results simply reflect limitations in the sensitivity of detection of immune complexes they, nevertheless, 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 continues 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 believed that after somatic mutations in the variable region of the Ig gene of the GC B cells, follows the process of positive selection based on the affinity for the antigen (12-14). The process of positive selection is thought to be centered around antigen 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 antigen access by GC B cells - a critical determinant of positive selection. These include competitive displacement of antigen from immune complexes (see ref. 16), uptake as iccosomes (see ref. 1), or proteolytic cleavage of antigen-Fab complexes from FDC-bound immune complexes (17). An interesting caveat has recently been provided by Manser and coworkers (18). They have elegantly demonstrated that, although affinity maturation was operative, specificity proved to be the overriding consideration that guided positive selection (18).

From the above discussion, it is clear that there continue to remain gaps in our knowledge of processes that govern the induction and maturation of T-dependent B cell responses. This is especially true to the finer details of a GC reaction. In an earlier report it has been demonstrated that the GC seeding competency of an antigen-activated B cell is established early, soon after antigen exposure - and that it correlates with affinity for antigen (19). For these studies, an in vivo GC reconstitution protocol was employed that involves adoptive transfer of antigen-primed B and T cells, along with appropriate immune complexes into irradiated mice which has been reported previously from our
laboratory (19). Same protocol has been used here to examine the influence of immune complexes on the GC reaction. The results from this study suggests that although non-limiting for initiating a GC reaction, the presence of immune complexes was nevertheless critical for the enhancement of antigen-specific B cell memory. Further, this activity was restricted to only those immune complexes in which the constituent antibodies shared a common epitope specificity with the GC B cells, with an optimum requirement for IgG isotype of antibodies. Finally, while enhancement of antibody specificity within GCs was initiated in the absence of detectable levels of immune complexes, the presence of appropriate immune complexes - however - served to maximize the outcome.
RESULTS

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

The model antigen used for these experiments is a synthetic peptide PS1CT3 which has been discussed in chapter 1. Further earlier studies in the lab have demonstrated that primary GCs could successfully be reconstituted in irradiated hosts upon co-transfer of antigen-primed T cells and antigen-containing immune complexes (19). Twenty four hours later the same host is given an enriched population of B cells from mice primed with antigen 48hrs before. This ability to regenerate GCs from these individual constituents, therefore provided a system to examine processes of epitope driven induction, specificity maturation and their selection into memory compartment, which so far has been believed to occur during the GC reaction. The present report also focuses on both, the relevance and regulatory influences of antigen-constituted immune complexes.

Although antigen-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 reported that the appearance of GCs precedes observation of detectable levels of FDC-bound immune complexes. Consequently, as a first step, it was sought to re-examine this issue by reconstituting antigen-specific GCs either in the presence or absence of immune complexes containing antigen. The procedure employed has been described in material methods. The enriched T cell population 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 (IC). These ICs were prepared in vitro with peptide PS1CT3 and the IgG fraction of primary day 7 polyclonal anti-PS1CT3 antisera obtained from BALB/c mice. These irradiated hosts were given, twenty four hours later, an enriched B cell population from a separate cohort of mice that were primed with PS1CT3 48hrs before. The previous results in the laboratory has already shown that, when Ag-Ab immune complexes were included for an in vivo GC reconstitution, this induces formation of antigen-specific GC and has been shown to be completely dependent upon the presence of both antigen-activated B and T cells (19). Further, the number of specific GCs obtained were found to peak by day 10 following B cell transfer (19).

On the basis of this information, spleens were removed from the hosts day ten post B cell transfer and the resulting sections stained for the detection and enumeration of antigen-specific GCs (Materials and Methods). Interestingly, the presence of antigen-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 antigen or immune complexes occurring as contaminants in the B cell preparation. To verify this, an additional analogue of peptide PS1CT3 was synthesized 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, we radio-iodinated the analog peptide to a final specific activity of $9.7 \times 10^6$ Ci/μmole. Subsequently, individual BALB/c mice were immunized with the radioactive peptide in a manner and dose identical to 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. This was equally true for the 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 antigen. From the specific activity of the radiolabeled peptide, we estimate a lower reliable limit of detection to be $5 \text{ fmole}$ of peptide/$10^7$ B cells (based on a cut off value of mean background cpm + $5 \text{ S.D.}$).

To further probe whether immune complexes are required for the initiation of a GC reaction, previously raised anti-PS1CT3 mAbs were employed to prepare IC (23). Initially, two mAb preparations, mAb PC 7bM and mAb PC287 (23) were employed which differ in their isotype (PC7bM is of IgM isotype whereas PC287 belongs to IgG isotype) but map to the same epitope (segment DPAF) (23). Peptide PS1CT3-specific GCs were reconstituted in vivo either in the presence or absence of externally supplied immune complexes made from either of these two mAbs. On day ten, post 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, in parallel a group of non-irradiated BALB/c mice were immunized with peptide PS1CT3 and the GC response analyzed ten days later. The results obtained from such an experiment are presented in Table 1. As is evident, the magnitude of the GC response - both in terms of numbers and size distribution - remained relatively invariant regardless of presence or absence of externally supplied immune complexes (Table 1). 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 1). Collectively, the data in Table 1 seem to suggest that both recruitment and proliferation of antigen-activated B cells is facilitated equally well under all the variable conditions employed above.

Although, there was no detectable levels of antigen contamination in the B cell preparation used for adoptive transfer, the formal possibility of the presence of antigen levels below detection limits cannot be definitively excluded. Nevertheless, the cumulative data strongly suggests that the antigen 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 this reconstitution protocol.

**Immune complexes influence generation of antigen-specific B cell memory**

A critical end product of the GC reaction is the generation of antigen-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 antigen containing immune complexes remains to be clarified. To obtain preliminary information on this, the extent of PS1CT3-specific memory B response was determined in the various groups which has been described in Table 1. In order to estimate memory B cell generation the protocol established by Zinkernagel and co-workers was employed (25); where the splenocytes from these mice were transferred into freshly irradiated (550 rads) hosts and followed by a challenge with soluble antigen 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 this, nevertheless, accurately reflects differences in the frequency of responder cell populations (25).
Splenocytes from the various groups described in Table 1 were collected at twenty one days post B cell transfer and then transferred again into freshly irradiated hosts. Following this, anamnestic IgG titers were determined five days after a soluble antigen 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 antigen-specific B cell memory that is produced (Fig. 2). While the magnitude of the GC response was indistinguishable between the other groups but there was a marked difference at the memory IgG level. The recall response in terms of IgG level could barely be detected from mice that was not supplemented with any ICs, along with primed B and T cells. In contrast, all the groups which received the ICs showed a significant level of IgG titre but the group of mice that received ICs of IgG isotype antibody showed a marked enhancement in the IgG titre than the group that received ICs made with IgM mAb. Fig-2.

In addition to a quantitative parity, the qualitative comparisons of memory response was made between two groups; mice immunized with peptide PS1CT3 and the group where GC was reconstituted with IC of IgG isotype antibody. This was done by examining the relative avidity for peptide of the two IgG preparations by competitive inhibition ELISA, and also the kinetics of antigen binding by fluorescence quenching assays. The data from both of these experiments are shown in Fig. 3 where, it is evident that, both preparations displayed similar antigen binding properties either in terms of avidity (Fig. 3A) or saturation rates (Fig. 3B). Thus in conjunction with the earlier described characterizations, the data in Fig. 3, further substantiate that this 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 these in vitro prepared immune complexes are given intra-venously, they do in fact localize within GCs. For this radioiosination of peptide and/or antibody are used to prepare the ICs. The knowledge of the specific activity of the radiolabelled 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 presence or absence of radioactive immune complexes between the peptide and either mAb PC7bM or mAb PC287. Subsequently derived splenic sections were immunohistochemically stained for detection of antigen-specific GCs, prior to their excision and determination of radioactive content. The results from such an experiment are shown in Table 2. As is evident, at least some of the externally provided immune complexes do localize within antigen-specific GCs. Further, the extent of localization appears to be comparable regardless of whether the immune complexes contained the IgM or IgG isotype of antibody. Finally, the stoicchiometry of peptide to Ig monomer obtained was close to the theoretically expected value of 2:1 (Table 2).

Another notional possibility that could account for the results in Fig. 3 is that antigen dissociating from the provided immune complexes could be-captured by antibodies being secreted by the activated B cells in the transfered population. To rule this out, the serum half-life period of the radiolabelled peptide in irradiated BALB/c mice was examined. Mice were immunized (i.v) with 2.5 nmoles of radiolabelled peptide and then periodically bled for the determination of radioactivity. These experiments yielded a short serum half-life for peptide of less than 4 hrs. Thus given the conditions of this protocol where limiting amounts of antigen (~ 14 pmoles/ mouse) in the form of immune complexes were given and that B cells were introduced twenty four hours 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 2 where the stoicchiometry
of peptide to mAb within GCs was close to the expected value. Finally, injection of either soluble peptide (250 pmoles/mouse) instead of pre-formed immune complexes, or immune complexes of peptide with the Fab fragment of mAb PC287 also did not lead to any enhanced memory generation. From the above observations, the data in Fig. 2 can be interpreted as within GCs, immune complexes regulate the extent of antigen-specific memory B cells that are produced. Further, this regulation is also modulated by the isotype of antibodies that constitute the GC resident immune complexes.

To further strengthen the findings reported above, that this is not only restricted to peptide PS1CT3 but, rather, represents a general property of immune complexes in GCs, experiments were performed with two additional, well characterized protein antigens hen egg lysozyme (HEL) and Ovalbumin (Ova). Irradiated hosts were reconstituted with antigen specific GCs (HEL or Ova) 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 antigens that, while significant levels of memory IgG was 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**

Above observations; that, the 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 antibodies has any regulatory influence. In order to examine this, monoclonal antibodies raised to PS1CT3 but
mapping to alternate determinants other than DPAF within the PS1 segment of peptide PS1CT3 were used to prepare immune complexes.

The properties of IgM mAbs here are being described in Table 3. Immune complexes were prepared between peptide PS1CT3 and each of these mAbs. Subsequently these were employed for in vivo GC reconstitution. Anamnestic IgG responses that resulted from each of these groups were quantitated and the results are given in Fig. 5. The notable feature of this observation 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 antibodies directed against alternate determinants (other than DPAF domain) were only marginally stimulatory (Fig. 5).

To understand the basis of the preference for immune complexes composed of antibodies 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 crossreactivity 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 from only a representative group are shown in Fig. 6A, all sera tested yielded a unique reactivity profile restricted to three overlapping hexapeptide of sequence, QLDPAF, LDPAFG and DPAFGA. This, and the earlier report (chapter 1) is indicative of monospecificity for the DPAF epitope. Thus, the data in Fig. 6A reveals, regardless of quantitative differences among the groups, epitope specificity of the recall IgG response remained invariant and directed
exclusively against the DPAF epitope. It may be noted from Fig 6A that the normally derived secondary IgG from non-irradiated mice also displays DPAF-monospecificity.

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

In the previous chapter and previous reports from the laboratory, has demonstrated that, the early primary murine IgM response to peptide PS1CT3 is comprised of distinct 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 shown to be retained in the secondary response (23). To verify the stage at which selection for DPAF-monospecificity occurs, the sera from PS1CT3-immunized mice where GC formation had been inhibited by treatment with the anti-B7.2 mAb, GL1 was examined. Multiple high dose injections of GL1 abrogates GC formation in PS1CT3-immunized mice, although the primary humoral response was only marginally affected, which is very much consistent with earlier reports (26). Both IgM and IgG fractions of day 7 sera from such mice were analyzed for distribution of their epitope fine specificities by screening against a hexapeptide panel of peptides described in Fig. 6A. The early IgM anti-PS1CT3 response was found to retain the multispecific character mapping to the entire PS1 sequence which is consistent with the results from mice not treated with GL1 as shown in chapter 1. In contrast, the early IgG response displayed monospecificity for the DPAF epitope (Fig. 6B). This reactivity profile was identical to that described for both primary and secondary IgG from GL1-untreated mice (23).

Thus, this dominant selection for the DPAF epitope occurs even in the absence of any detectable GC formation suggesting that, this event precedes the initiation of a GC reaction. It is most likely that this selected subset might populate the B cell follicle
leading to a GC formation and the requirement for immune complexes constituted by DPAF-specific antibodies as shown in Fig. 5 probably follows as a natural outcome of this selection.

To further verify the existence of specificity-restricted memory enhancement, a hapten-carrier system - DNP-BSA was selected as an additional model immunogen for further study. Mice immunized with DNP-BSA were bled on day seven and the sera purified over an affinity column for the DNP-specific and BSA-specific IgG subpopulations. Subsequently, immune complexes were made in vitro between either of these fractions and DNP-BSA prior to their inclusion in GC reconstitution protocols with antigen-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 antibodies 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 antibodies constituting the immune complexes.

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

Although several studies have documented that GCs represent the sites where affinity maturation of antibodies occurs (reviewed in ref. 27-29), it has been shown in chapter 1 and previously (19) that progression of a murine primary humoral response to peptide PS1CT3 is not characterized by an increase in affinity for the antibodies (19).
As a result, the present system did not permit an analysis of the role of antigen containing immune complexes in affinity maturation. However, Manser and co-workers have recently demonstrated that improvement in antigen-specificity of antibody 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 crossreactivity with self antigens (18).

To evaluate for specificity maturation in this peptide antigen system, it was of an advantage as 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. Thus it was possible to synthesize analogues of a hexapeptide representing residues 3 to 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 in order to generate a panel of analog sequences. This panel was then used to screen for antibody crossreactivity with recall IgG produced under the influence of a representative set of immune complexes. For a comparison with the specificity of anti-DPAF IgG prior to the onset of the GC reaction, the IgG fraction of sera from GL1-treated mice was also included for analysis. Further, secondary IgG obtained under normal immunisation conditions from non-irradiated mice was included as a qualitative reference.

Results obtained from the experiment described above are shown in Table 4. Primary anti-PS1CT3 IgG from GL1-treated mice displayed poor specificity where several of the analogs proved more reactive than the parent peptide (Table 4). In contrast, normally derived secondary responses were markedly more antigen-specific, at least in the context of the panel tested (Table 4). These results clearly establish the fact that an improvement in antibody specificity is also a characteristic of murine humoral
responses to peptide PS1CT3. Some enhancement of antibody specificity was noticed for recall IgG obtained from mice where the GCs were reconstituted in the absence of IC. Reconstitution of GCs with immune complexes made from non-DPAF directed mAbs had no additional effect (Table 4). On the other hand, GCs containing immune complexes prepared from DPAF-specific mAbs yielded a marked improvement in antibody specificity, with again a greater efficacy for immune complexes generated from IgG mAb as opposed to IgM (Table 4). Indeed, the crossreactivity profile of recall IgG antibodies from GCs supplemented with PC287-constituted immune complexes was almost identical to that obtained for normally derived secondary anti-PS1CT3 IgG (Table 4). Thus it appears that, although an enhancement of epitope-specificity of antibody can occur in absence of ICs, the presence of immune complexes constituted by antibodies 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 antigen-activated B cells, principally in T-dependent humoral responses. A prerequisite to GC formation is the migration of antigen 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 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 germinal center 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 antigen-containing immune complexes onto the FDC network within the primary follicle is also required to initiate a GC reaction, the results presented here indicates that this does not constitute a limiting requirement. Adoptive transfer of only antigen-primed T and B cells, in the absence of any added immune complex, was sufficient to generate GCs in irradiated recipients. While it cannot be ruled out that the possibility of trace or undetectable amounts of either immune complexes or free antigen molecules were transferred along the B cells, but 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 immune complexes at least to a significant level. 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 which has been suggested earlier (37) - was not addressed in this study.

While the initiation of a GC reaction, as found, does not require the prior presence of significant amounts of antigen in the form of immune complexes, subsequent generation of memory B cells was - however - strictly immune complex-dependent. Moreover, for peptide PS1CT3, the memory-enhancement activity was restricted to immune complexes constituted by antibodies specific for the DPAF epitope and, with an optimal requirement for IgG as opposed to the IgM isotype. In previous studies it has been shown that the early primary anti-PS1CT3 humoral response is comprised of a heterogeneous distribution of antibody fine-specificities that collectively span the entire PS1 sequence (23). However, subsequent class switch and maturation was restricted to only that subset of antigen-activated B cells that were directed against the DPAF epitope (23). Here, by preventing the initiation of a GC reaction with the administration of anti-B7.2 antibodies (GL1), it has been shown that selection for DPAF-monospecificity precedes initiation of the GC reaction. It, therefore, suggests 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 the previous 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 antibodies 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 antibody constituent in the provided immune complexes was
observed. Further, the fact that the monoclonal antibodies PC7bM and PC7cM-antibodies 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 these 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 but 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 co-workers 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 BCR with the CD21/CD19/CD81 complex, a potent stimulatory signal for B cells (38). 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 antigen-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 - however - relatively invariant.

The process by which GC B cells acquire antigen 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. Several alternate mechanisms have, however, been put forward in the literature. For example, it has been suggested that antigen 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 (eg. see ref. 16). Indeed, in support of such a possibility, recent results in the laboratory indicate that the memory enhancement activity of immune complexes is dependent on the ability of antigen to dissociate from the antibody (Nayak, B.P., Nakra, P and Rao, K.V.S., manuscript in preparation). Here, the equimolar of antigen and antibody (PC287) was allowed to form immune complexes in vitro and the peptide was chemically crosslinked to the antibody in its binding state. When such ICs were used to reconstitute GCs, and on further soluble antigen challenge unable to induce any detectable amount of memory response in comparison to the cases where the GC was reconstituted with normal ICs (PS1CT3-PC287mAb). Nevertheless, an alternate mechanism may also lie in the direct endocytic uptake of iccosomes by B cells, followed by antigen processing and presentation (eg. see ref. 1). Finally, Leanderson and colleagues have envisioned positive selection within GCs to occur by the direct binding of B cells to antigen 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 antigen supply is limiting. However the results here highlight the importance of epitope-specificity of antibodies constituting the immune complexes, thereby suggesting 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 antigen non-specific uptake of iccosomes by endocytosis. And for the amplification of epitope specific B cells, must require the engagement of B cell receptor specific for the epitope and the antigen held onto the antibody on ICs. But such B cells would be unable to directly bind immune
complexes as the relevant epitope is expected to be masked by antibody binding. However, any definitive conclusion must await further analysis.

The data presented here also lend support to the notion of 'specificity maturation' within GCs as proposed earlier by Manser and co-workers (18). Interestingly though, detectable levels of antigen supply in the form of immune complexes again appeared to be redundant for initiating this process. Immune complexes of the appropriate specificity and isotype of antibody, however, were required for further optimizing antibody specificity. These observations raise the possibility that enhancement of antibody specificity within GCs may occur in two stages - an antigen independent step, followed by an antigen dependent one. Although this proposal remains to be verified, it is likely that the antigen independent stage correlates with the recent demonstrations that engagement of GC B cells by soluble antigen (and, therefore, also crossreactive self antigens) leads to apoptosis (1, 39-41). This can be expected to result in a 'filtering' of mutated GC B cells in favor of the less crossreactive clonotypes. Subsequent optimization could represent an antigen-driven process where the requirement for surviving B cells to competitively displace antigen 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 antibodies that constitute the immune complex. Thus, in addition to clone autonomous antibody maturation within GCs (18,41), 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
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Finally, an extension of this study on the regulation of antibody 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.
Table 1. The magnitude of a GC response is immune complex independent. 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 monoclonal antibodies PC7bM (group 2), or PC287 (group 3). Group 4 represents normal, non-irradiated mice that were immunized with peptide PS1CT3.

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<th>Group</th>
<th>GCs/10 Sections</th>
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GC numbers are presented as an average (± S.D.) over ten sections for five mice per group.

For size distribution, a minimum of fifty 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 a germinal center in terms of PNA⁺ cell number. <25: Small, between 25-50: Medium and >50: Large.

These data are presented as a percent of the total number of GCs examined per group.
Table 2. Radiolabeled immune complexes localize within GCs.

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 were used to prepare immune complexes and the two mAbs employed are PC7bM and PC287. On day seven post B cell transfer, spleens were removed and 20μm cryosections were made for the immunohistochemical localization of GCs. These GCs were excised and pooled in groups of 10 to 20 in number for determination of radioactivity incorporation.

<table>
<thead>
<tr>
<th>Immune Complex</th>
<th>Constituent (in fmoles)/20 μm section</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide</td>
</tr>
<tr>
<td>PS1CT3-PC287</td>
<td>5.1 ± 1.3</td>
</tr>
<tr>
<td>PS1CT3-PC7bM</td>
<td>3.9 ± 1.1</td>
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</tbody>
</table>

Values presented here are mean of values obtained from three individual mice within each group and a minimum of three pools were measured.

Molar quantities were calculated from the known specific activity of iodination of the individual components which are as follows: PS1CT3, 9.7 x 10⁶ Ci/μmole; mAb PC287, 6.1 x 10⁸ Ci/μmole; mAb PC7bM, 3.2 x 10⁸ Ci/μmole of monomer.

For the IgM mAb PC7bM, molar estimates were calculated on the basis of a monomeric unit.
Table 3. Binding characteristics of anti-PS1CT3 IgM mAbs.
The mAbs listed here are specific for the PS1 segment of peptide PS1CT3 and were obtained from the fusion of splenocytes from a mouse that was immunized with peptide PS1CT3 four days prior to the day of fusion. While their epitope specificities have been characterized earlier (23), relative affinities for peptide PS1CT3 were determined by competitive inhibition ELISA.

<table>
<thead>
<tr>
<th>IgM mAb</th>
<th>IC$_{50}$ for peptide (µM)</th>
<th>Epitope specificity</th>
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</thead>
<tbody>
<tr>
<td>PC7bM</td>
<td>21.0</td>
<td>DPAF</td>
</tr>
<tr>
<td>PC7cM</td>
<td>4.0</td>
<td>DPAF</td>
</tr>
<tr>
<td>PC42M</td>
<td>1.5</td>
<td>NSTN</td>
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<tr>
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<td>NSTN</td>
</tr>
<tr>
<td>PC6aM</td>
<td>32.0</td>
<td>FG</td>
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</table>

IC$_{50}$ values given are the mean of two independent measurements.
Table 4. Specificity maturation is enhanced by the presence of appropriate immune complexes. Purified IgG fractions from either primary or recall anti-PS1CT3 responses were screened for crossreactivity against analogues 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 analogue 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 response from GL1-treated mice. Group-6 represents normal secondary IgG response as described in Fig. 2. The remaining groups represent recall IgG response from mice reconstituted with GCs and differed in terms of the constituents of immune complexes that was provided during GC reconstitution. These are as follows: Group-2: no immune complex; Group-3: peptide-mAb PC42M; Group-4: peptide-mAb PC7bM; Group-5: peptide-mAb PC287.

<table>
<thead>
<tr>
<th>Analog peptide</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
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<td>+/-</td>
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<tr>
<td>LDPSFG</td>
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</table>

Data presented is that from one of two independent experiments.
Figure 1. Immunohistochemical staining for antigen specific GCs reconstituted in vivo. GCs were reconstituted either in the presence or absence of ICs made from day 7 polyclonal primary anti-PS1CT3 IgG as described in Materials and Methods. Panel B depicts the representative staining of an antigen-specific GC in splenic sections of irradiated hosts not provided with immune complexes. The PNA+ B cells are seen as red, whereas antigen-specific staining appears as blue. Panel A shows a GC devoid of antigen-specific B cells, obtained from splenic sections of a mouse mock-immunized with CFA.
Figure 2. Immune complexes regulate production of antigen-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. The X-axis identifies individual groups in terms of the immune complex that was provided. Data from individual mice are shown, and is a representative of one of the four separate experiments.
Figure 3. Comparison of recall anti-PS1CT3 IgG from mice where GCs were reconstituted with PS1CT3-pc287mAb ICs and secondary response from normally immunized mice. Panel A: relative avidities for peptide by competitive inhibition ELISA. Panel B: measurement of antigen binding rates. The purified IgG fractions of recall responses from reconstituted GCs supplemented with mAb PC287-peptide PS1CT3 immune complexes (●), and normally derived secondary anti-PS1CT3 IgG (○). Data presented here is a representative of two independent experiments.
Figure 4. Memory B cell responses against representative protein antigens in presence or absence of immune complexes. GCs were reconstituted with enriched B and enriched T cells from mice immunized with; Panel A: HEL; Panel B: Ovalbumin. Group 1: in absence of immune complexes; Group 2: in presence of immune complexes. Data from individual mice are shown and is a representative of four independent experiments.
Figure 5. Anamnestic IgG responses from mice where GCs were reconstituted with antibodies of IgM isotype differing in their specificity. Immune complexes were prepared between peptide PS1CT3 and each of the anti-peptide IgM mAbs listed in Table 3. 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.
Figure 6. Epitope-specificity of recall (panel A) and early primary (panel 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. **Panel A:** immune complexes were constituted with the following mAbs; (a) no immune complex; (b) mAb PC42M; (c) mAb PC7bM. For comparative purposes, the profile obtained for normally derived secondary IgG is also shown (d). **Panel B:** day 7 IgG from GL1 treated PS1CT3-immunized mice. Data shown here is a representative of three independent experiments.
Figure 7. Specificity-restricted memory enhancement property of immune complexes with a hapten-carrier system. Reconstitution of GC in presence of ICs prepared with antigen DNP-BSA and antibody of IgG isotype having specificity for DNP alone (Group 1); BSA alone (Group 2); total IgG against DNP-BSA (Group 3); and control having no IC (Group 4). Panel A: DNP-specific anamnestic responses. Panel B: BSA specific anamnestic responses. Values are mean titers (±S.E.) obtained from three individual mice within each group and the data represent one of the two independent experiments.
REFERENCES


