CHAPTER 1

INTRODUCTION
The adaptive immune response is mediated by antibodies, which serve to recognize foreign antigens and protect against pathogens. The naïve primary immune repertoire includes a vast array of B-lymphocytes possessing germline antibody receptors. These receptors can respond to any incoming antigen without having been previously exposed to it. Recognition of incoming antigen requires the generation of vast array of BCR combinations in the naïve B cell repertoire as the potential antigenic space is inestimable. Explanation for the diversity of the antibody specificities have mostly been analyzed through genetic recombination of germline VDJ gene segments (Tonegawa, 1983). This diversity is generated at multiple levels. Combinatorial diversity arises from the use of different combinations of V, D and J segments. Superimposed on this is junctional diversity, created by the imprecise joining of the gene segments during V(D)J recombination and N terminal addition of nucleotides by terminal deoxynucleotide transferases (Komori et al., 1993). Finally, lgs are heterodimers of rearranged receptor genes, with both subunits contributing to Ag recognition, thus creating an additional layer of combinatorial diversity.

The physicochemical basis of complementarity in charge and shape that results in the specificity of molecular recognition has been a subject of intense scrutiny. This phenomenon of degenerate recognition specificity of the primary antibody response has been a focus of attention in the context of affinity maturation in T-dependent humoral immune response (Manivel et al., 2002). Enigmatically, however, the number of functionally active antibody genes generated as a result of permutation and combination of VDJ gene segments during the process of somatic recombination in an individual are finite when compared with the potential antigen encountered which is practically infinite (Cook and Tomlinson, 1995). Therefore additional mechanisms are likely to exist to recognize the practically infinite
antigenic distribution by using a limited antibody repertoire.

Logically, such potential mechanisms would allow a given germline antigen receptor to engage multiple antigens, for this conformational flexibility of antigen binding site (paratope) of germline antibody has been proposed as the possible mechanism (James et al., 2003; Manivel et al., 2000; Wedemayer et al., 1997). However conformational flexibility or in other words flexibility of antigen combining site in germline antibodies does not represent a continuum of conformations, meaning it has certain pre-existing discrete conformations possible (James et al., 2003). Considering this fact, the role or contribution of this flexibility cannot account for the discrepancy between possible primary antibody repertoire and potential antigens encountered. There have to be some other mechanisms to generate diversity in primary immune repertoire besides the known mechanisms. On the basis of thermodynamic data and molecular dynamics (MD) simulations paratope has been proposed to be pliable (Manivel et al., 2000; Thorpe and Brooks, 2007; Zimmermann et al., 2006). With the intention of crystallographically proving the flexibility in the paratope of 36-65 mAb, Sethi et al. had done structural analysis of this Fab with phage-display derived dodecapeptides. While attempting to image alternative ways of expansion of primary antibody repertoire, he discovered a mechanism of diversity generation in primary immune repertoire, which involved differential binding of epitopes within a given paratope conformation of germline antibody (Sethi et al., 2006). This observation clearly proves that this can also be a mechanism for generation of diversity in the primary immune repertoire, because here a single conformation of paratope was alone responsible for binding of diverse independent dodecapeptides.
This thesis presents an initiative to illustrate the processes which add diversity in primary antibody repertoire. While, the mechanism proposed by Sethi et al is very attractive, but in order to generalize it would require further investigation. Moreover there can be other mechanisms which add diversity in primary immune repertoire. So to answer these questions, we have done crystallographic analysis of previously studied germline mAb Fab with more diverse set of dodecapeptides, which were also selected by bio-panning a random phage library. The crystallographic analysis of Fab of another germline mAb BBE6.12H3 in the antigen free state as well as its complexes with dodecapeptides was done. These peptides were again selected by following the same method of bio-panning a random phage library against this mAb. We have also done structural analysis of Fabs of these germline mAbs with two dodecapeptides which bind both monoclonal Abs with comparable affinities to address the question of convergence of Ab specificity at structural level. This analysis will address two very fundamental questions. 1) What happens when pathogen changes its antigenic surface in order to evade immune system? 2) Spatial and temporal presence of circulating or resident germline receptors in the secondary lymphoid organs, further limits possible repertoire, which is generated as a consequence of somatic recombination event. This structural analysis of convergence of mAbs specificities will give insights on how primary immune system economizes the available receptors in recognizing infinite antigenic space.

The systems used as a probe for our studies involves immunologically well characterized antibodies derived from the major idiotypic response to the hapten p-azophenylarsonate and (4-hydroxy-3-nitrophenyl)-acetyl in A/J and C57BL/6 mice strains, respectively. Both these antibodies are from the primary immune response.
against the hapten, hence has not undergone any somatic hypermutation. The germline status of these antibodies was further confirmed by their sequence analysis.

Thus, the work presented in this thesis involves the use of two germline mAb Fabs and a series of dodecapeptides, which were selected by bio-panning a random phage library against these two primary mAbs. These peptide antigens have diverse amino acid compositions. Out of the total peptides chosen, two were shown to cross-react with both the antibodies. Germline mAbs BBE6.12H3 and 36-65 were obtained by raising ascites in mice from previously generated hybridomas and were purified from the same. The mAbs were subjected to a controlled papain digestion to generate Fab fragments, which were subsequently used for crystallization. All the peptides used in the study were made, purified and characterized in house.

This thesis has six chapters in all, including this introduction. In chapter 2, review of literature, the relevant aspects of the current understanding of germline antibody structure and function along with the proposed role of degeneracy in enhancement of the cognitive repertoire are detailed. Additional subsections are devoted to the description of the current knowledge on antibody structure and the part of the immune system relevant to antibodies and affinity maturation. Chapter 3, materials and methods, deals with the techniques, equipment and reagents used for the accomplishment of the studies undertaken. The bases for usage of the technique are followed by description of the actual implementation relevant to the study. Chapter 4 and 5, detail the results of the crystallographic studies carried out. Description of the structures is supported both graphically with relevant representations of the determined structures and with tabulation of analyzed parameters. The results are critically discussed in chapter 6. Comparison of the determined structures yielded some very interesting observations providing novel
clues for generation of diversity in the primary immune receptors. The underlying mechanisms responsible for polyspecific recognition potential of germline antibodies were deciphered. The structural details of convergence of two heterologous germline mAbs in binding flexible peptide antigens were also elucidated. Together, these antibody-antigen structures provide snapshots into how the immune system uses the germline antibodies to generate multiple possible specificities that allow for differential recognition of epitopes by adopting various mechanisms. This study also reveals how unrelated mAb structurally adept for recognition of flexible antigen. Moreover structural investigations that snapshots Ab and Ag flexibility would be interesting not only from a biophysical perspective but should also contribute to an understanding of molecular recognition.