Structural Immunology: Mechanisms of Antigen Recognition

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Abstract | The mechanisms of antigen recognition by the immune system have been extensively addressed during past two decades. Ingeniously designed X-ray crystallographic experiments have provided great insights with regard to antigenic specificity and self/non-self discrimination, the hallmark of the adaptive immune system. These studies have also helped shed light on the mechanistic aspects of the immune recognition potential and the molecular events necessary for a successful immune response. Our laboratory has made important contributions in understanding the physiological processes associated with self-nonself discrimination in terms of physico-chemical principles of molecular interactions. Crystallographic approaches were employed in analyzing how immune system reacts when encountered with the antigens that keep changing shape. We have addressed the breakdown in specificity of immune recognition by providing molecular insights into the functional mimicry. The conundrum of a limited antibody repertoire faced with an infinite range of antigens was also addressed demonstrating that while the pluripotency of germline antibodies can be expressed through an ensemble of conformational states, each one of these states was itself capable of recognizing a wide range of antigens through varied juxtapositions.

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Germline antibodies: Antibodies belonging to the primary immune response which have not seen any antigen *a priori*.

1. Introduction

Specificity of interaction is central to molecular recognition in biological systems. Any physiological event results from molecular interaction involving different proteins binding with high specificity. One of the ideal systems for investigating molecular recognition processes involving wide diversity and high specificity is the immune system. The ability of the cellular and humoral arms of it to recognize an infinite repertoire of distinct antigens makes it fascinating physiological phenomenon for investigations at structural level.

Since the identification of antibodies at the end of the 19th century, scientists have sought to explain the finer details of the antigen–antibody interaction[1–3]. Extensive genetic and biochemical studies have led to better understanding of the basis of self/non-self discrimination by the antibodies and identified determinants of antigenic specificity[4,5]. Application of X-ray crystallography in immunology has come a long way — from being a tool to image molecular structures of the proteins associated with the immune system to addressing the mechanisms themselves by suitably designing the experimental systems. These studies have been particularly powerful when combined with other physico-chemical approaches and have facilitated emergence of a clearer picture of the molecular events associated with a successful immune response.

2. Antigen–antibody recognition

Our understanding of the nature of antigen–antibody interactions has been furthered by analyses of more than 855 crystal structures of

Hapten: A small molecule which can elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one which also does not elicit an immune response by itself.

Paratope: The part of an antibody that recognizes the epitope.

Epitope: The part of a macromolecule that is specifically recognized by antibodies, B cells, or T cells of the adaptive immune system.

various antibodies. These include 575 structures of antibody in complex with diverse antigens including proteins, peptides, nucleic acids and carbohydrates. Additionally, 73 antibody-hapten structures have been determined (Research Collaboratory for Structural Bioinformatics, 2007) which shed light on the mechanistic details of antibody interaction with small ligands, both naturally occurring and synthetic.

Antibodies are Y shaped molecules comprising of two heavy and two light chains of 50 and 25 kDa respectively that are joined by disulphide linkages. Each antibody has 2 antigen combining sites that are formed by the variable domains of the heavy and the light chains. The 3 stretches in the variable domains of the heavy and light chains that show greater variability, and therefore determine the specificity, are known as the Complementarity Determining Regions or CDRs. Structurally these regions are in the form of loops present between β strands of the immunoglobulin folds of the variable domains of the two chains and form a continuous surface— the paratope— that determines the diversity in the antigen binding specificity. Whereas higher variability both in length and sequence is seen in the case of CDR H3, a small number of main chain conformations or canonical structures are known for CDR H1, H2, L1, L2 and L3[6,7]. The central region of the antibody paratope, comprising predominantly of the C terminal part of CDR L1, the first and sometimes the middle portion of CDR L2, the whole of CDR L3, H1 and H3 and N terminal and middle of CDR H2, are usually found to be involved in the interaction with ligands in antigen–antibody complexes along with, in some cases, residues from the framework regions[8,9]. The shape of the antigen combining site formed by the CDR loops can vary from resembling a small crevice or a wedge shaped cavity for haptens[10] to a broad and flat surface for binding to a proteinaceous antigen[8]. The complementarity between an antibody paratope and its cognate antigen is achieved in terms of both shape and electrostatics utilizing extensive network of Van der Waals and hydrophobic interactions and hydrogen bonds and few salt bridges[8,9]. Many aromatic residues are believed to be involved in the contact, whereas the involvement of small apolar aliphatics is rare[8]. Additionally, water molecules have been shown to act as bridges that increase the complimentarity between the antibody paratope and its cognate antigen[11]. Conformational changes in both antibody and antigen have been observed to facilitate binding of not only the cognate antigen but also expand the recognition repertoire of the antibody[12]. The surface area of the antibody paratope that gets buried on antigen binding varies from around 150 Å in case of antibody binding to phosphocholine to 850 Å in case of binding to lysozyme[8].

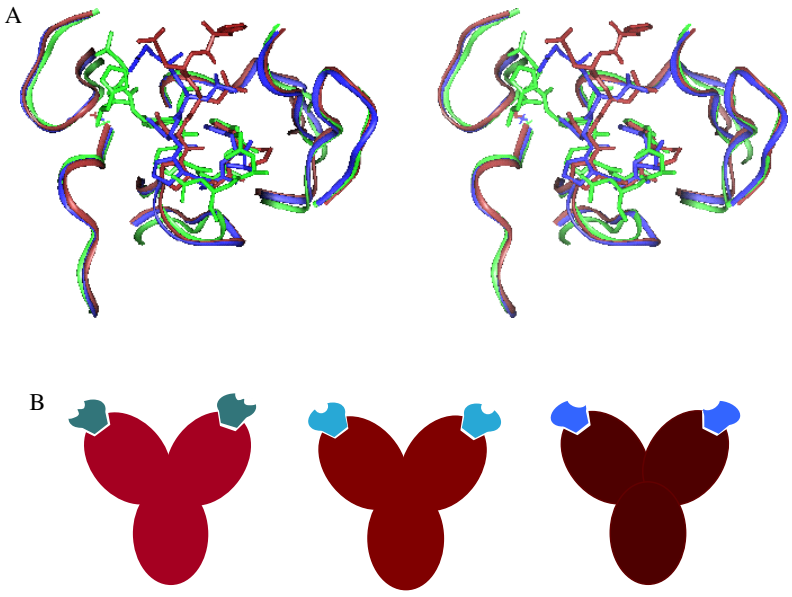
The fact that the number of potential antigens far exceeds that of the lymphocytes in an individual has long constituted an intriguing puzzle. Virtually any chemical entity, ranging from the simplest of molecules to the most complex of macromolecular structures, can serve as antigens to the B cells that are a part of the humoral immune response. Over the years, our laboratory has been working towards delineation of the molecular mechanisms involved in immune recognition. Here we discuss some of our major observations in the area giving finer details about the unique molecular processes that define antigen–antibody interactions.

3. Antibody response against flexible antigen

The primary function of the humoral immune response concerns recognition and neutralization of the foreign antigens by antibodies. Considering that each antigen may have a finite number of specific antibodies against it, it is interesting to know how the immune system would counter a flexible antigen. Would there be an antibody molecule to recognize each of the many different conformations of the antigen or would antibody molecules show pleuripotency with respect to the ever-changing antigen?

To investigate this scenario, a panel of murine monoclonal antibodies generated from the secondary response against a flexible peptide antigen was used. The antigen, PS1 (HQLDPAFGANSTNPD) was derived from the large envelope protein of the hepatitis B virus and includes a B-cell epitope[15]. The rationale behind using this peptide, as representative of an antigen having ever-changing shape, was that the peptide does not have a single definite conformation in solution, as observed from the proton-decoupled ^{13}C nuclear magnetic resonance and circular dichroism studies[15,29]. In other words, the peptide was considered appropriate model for flexible antigens. The crystal structures of three monoclonal antibodies, PC283, PC282 and PC287, in complex with PS1 were determined. Crystal structures of two of these antibodies, PC282 and PC287, were also determined at in their native antigen-free forms at high resolution[13,14]. All of these antibodies recognized a common four-residue stretch (DPAF) as an immunodominant epitope[15]. The Fab-peptide crystal structures provided a description of epitope-paratope interactions and conformational features of PS1 when bound to the three independent antibodies. In all the complexes, the major energetic

Figure 1: Antibody response against flexible antigen. Conformational economy of the antibody in recognizing the antigen that keeps changing shape. A. Stereoscopic view of superimposition of the 6 CDR loops of anti-PS1 antibody PC282, PC283 and PC287 in complex with the peptide demonstrating conformational convergence on binding an immunodominant epitope DPAF of PS1 peptide. B. Conformational convergence in different antibodies on binding to a flexible antigen. Different antibodies show similar binding site conformations when bound to an antigen that keeps changing shape.



Molecular mimicry: Functional equivalence of chemically dissimilar ligands.

contribution to antibody binding of PS1 came from interactions formed by the four residues, DPAF (Fig. 1A). The murine immune response against PS1 was found to progress such that, although the primary response is directed against diverse determinants, the antibodies in the secondary response are unanimous in their specificity for the stretch DPAF. This immunodominant nature of DPAF is in accordance with the observation that the same residues exhibit maximum interactions with the paratope in all the three structures. Another interesting observation was that the sequence differences in the CDR H3 and consequent differences in the polar interactions present in the unbound state lead to different conformations of this CDR in the three antibodies. However, upon binding to the epitope DPAF, the conformation of this CDR in the three antibodies is similar. There is an explicit shift from different to similar in case of CDR H3 conformation. Hence, it was evident that, on epitope stabilization, the CDR H3 converges to a common conformation.

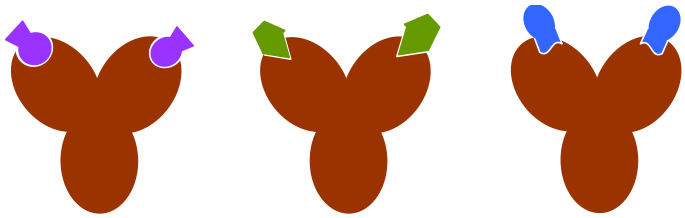
The conformational convergence observed in these studies leads to indicate that the immune system might encourage, against an individual antigen, a single effective binding mode for successful neutralization, resulting in similar conformations of CDRs of different antibodies bound to their cognate antigens (Fig. 1B) It appears that the immune system evolves high-affinity receptors only to a single conformation of the epitope even though linear peptides adopt multiple conformations in solution. Thus it can be concluded that structural convergence in epitope-paratope recognition may be designed for the optimization of immune response and therefore restricted conformational repertoire on antigen binding may be helpful in minimizing the probability of the generation of self-reactive antibodies, thus enhancing self-nonself resolution.

4. Breakdown in the antigenic discrimination

Even though immune mechanisms have evolved towards fine discrimination between molecules which may otherwise appear similar, the specificity of immune recognition is defined by the structural properties of the different antigens. Since molecular recognition is mediated by a common set of noncovalent interactions, it is possible to encounter systemic dysfunction due to unrelated molecules sharing common structural features. Such a breakdown in antigenic discrimination is manifested as molecular mimicry in the immune response. Extensive studies have been done to understand the mechanism(s) underlying the recognition of chemically dissimilar ligands by a common receptor using the mimicry between a peptide and a carbohydrate, as a model system. Concanavalin A, a lectin from *Canavalis ensiformis*, is known to bind the mannose-containing carbohydrates on the cell surface. In addition to the carbohydrate ligands, Con A also recognizes the peptide, DVFYPYPYASGS (12 mer;[16,17]).

To establish the structural basis of mannopyranoside-peptide mimicry, crystal structure of the 12mer peptide complexed with Con A was determined. The YPY motif of the peptide exhibited significant structural similarity in terms of shape and hydropathy features with the trimannoside ligand, but it was found that the peptide did not occupy previously characterized carbohydrate binding site of Con A, providing only indirect explanations for peptide-carbohydrate mimicry[18]. Carbohydrate-peptide mimicry was also analyzed in the context of the humoral immune response, wherein it was established, in terms of antibody cross-reactivity, and also observed during maturation of the immune response[19,20]. Immunization of mice with α-D-mannopyranoside

Figure 2: Degeneracy in antigen recognition due to flexibility in antibody paratope. A single antibody can bind to different antigens using flexibility in its binding site.



gave rise to antibody response that recognized the 12mer peptide. Correspondingly, the 12mer peptide also generated mannopyranoside cross-reacting polyclonal antibody response. Moreover the peptide was found to enhance the memory response against sugar[20]. Topological quasi-equivalence between the peptide and the carbohydrate moiety was implied in these observations. However, the precise molecular description of the functional mimicry between the otherwise chemically independent antigens as seen by the humoral response required the generation and characterization of monoclonal antibodies.

Monoclonal antibodies were raised against mannopyranoside and subsequently screened for the ability to recognize the mimicking peptide towards understanding the immunological basis of the peptide-carbohydrate mimicry at molecular level[21]. Monoclonal antibodies that were specific to the sugar as well as those that recognized peptide-carbohydrate mimicry were generated from the anti-mannopyranoside response. Nearly half among the 12 clones characterized exhibited binding to the carbohydrate-mimicking peptide. Three anti-mannopyranoside mAbs, two mimicry recognizing (2D10 and 1H11) and one non-crossreacting (1H7), were extensively characterized. The monoclonal antibodies recognizing molecular mimicry exhibited affinities for 12mer peptide and the mannopyranoside ligand well within the range of physiological relevance.

Kinetic analysis of antigen binding to the three anti-mannopyranoside monoclonal antibodies exhibited differences in terms of their association and dissociation phases of binding, suggesting possible implications at the structural level[21]. Relatively fast association and substantially slow dissociation rates of 1H7 in comparison with antibodies 2D10 and 1H11 could imply that 1H7 binding site may be predesigned for the carbohydrate antigen, while that of 2D10 and 1H11 might be required to undergo conformational changes while binding to the antigen. The conformational flexibility of mAb 2D10 was also manifested as increase in dissociation rates on increasing the temperature, thereby weakening the binding to the mannopyranoside ligand. Larger changes as a function of temperature during both association and dissociation phases of binding suggest a flexible/loose fit of the ligand in the antigen combining site in the case of antibody 2D10.

Thermodynamic analyses of antigen binding to the antibodies are also consistent with the structural interpretations based on the kinetic data. Changes as a function of temperature in the equilibrium-free energy were substantial for 2D10 in comparison with 1H7. Although binding to the mannopyranoside ligand was enthalpy driven for both antibodies, the entropy contributions were significantly different. In the case of 2D10, entropy changes during association were highly unfavorable, and that during dissociation was marginally favorable. In contrast, the entropy changes during both association as well as dissociation steps were unfavorable and significantly smaller in the case of mAb 1H7. The proposition that while the 1H7 may have a predefined fit for binding to the immunogen, the mimicry recognizing antibody 2D10 possesses conformational flexibility in the CDRs, is thus reinforced. Therefore, it can be inferred that monoclonal antibodies 2D10 and 1H11 adopt toward accommodating the sugar through the plasticity of interactions brought about by the flexibility of the combining site. This conformational adaptation of the flexible combining sites could also be the reason for the antibodies to recognize the mannopyranoside-mimicking 12mer peptide as well. Among the antibodies characterized, 1H7, which did not recognize mimicry, exhibited fine specificity akin to that observed in the case of polyclonal immune response. This antibody was also shown to be thermodynamically less flexible.

Thus, the kinetic and thermodynamic experiments enabled to infer that conformational flexibility in the antigen-combining site of an antibody may account for its ability to recognize multiple ligands and the extent of plasticity could be implicated with the corresponding binding specificity (Fig. 2). Plasticity could provide a wider binding repertoire, a necessary requirement for independent ligands to bind to a common site. Thus it was shown that molecular mimicry between the chemically unrelated ligands does not require complete structural equivalence; instead, the quasi-equivalence involving critical structural properties may adequately provide physiologically effective molecular mimicry, which is found to be a consequence of paratope flexibility.

5. Molecular mechanisms of antibody diversity

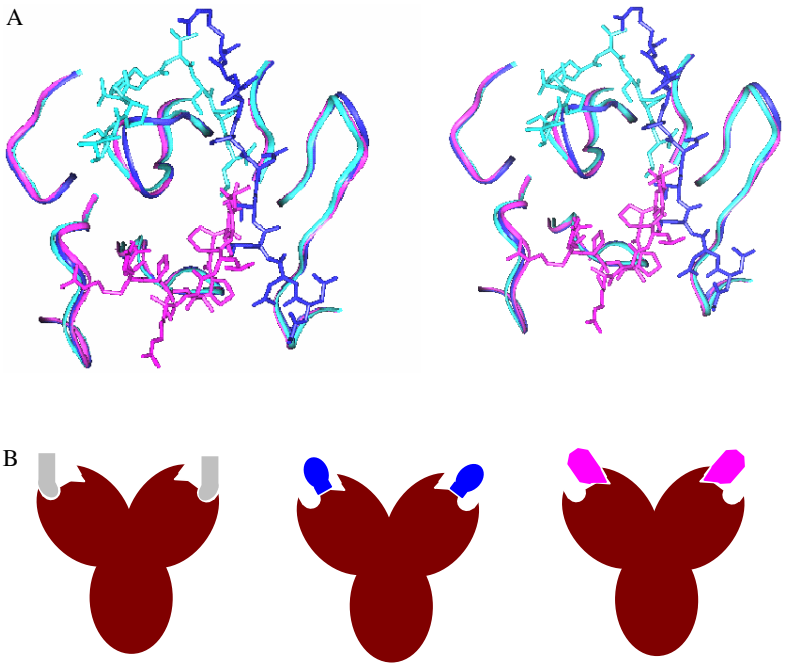
Generation of a sufficiently large and diverse antibody population that can counter the ever-expanding universe of potential antigens has been a puzzle for many years. The antigen recognition potential of adaptive immunity is generally thought to reside in the large repertoire of clonally expressed T and B cell antigen receptors. However, the fact that the number of potential antigens far exceeds that of the lymphocytes in an individual has long constituted an intriguing puzzle. This is particularly relevant for the B cell antigen receptor (BCR), which, unlike the T cell receptor (TCR), is not restricted to recognition of peptidic antigens alone. Rather, virtually any chemical entity, ranging from the simplest of molecules to the most complex of macromolecular structures, can serve as antigenic determinants for B cells. If one adds to this the fact that even relatively simple molecules can provide a multiplicity of recognition sites, the latent paradox of a finite receptor repertoire coping with a potentially infinite epitope repertoire becomes self-evident. So how do primary (germline) antibodies, which have not seen any antigen *a priori*, specifically address diverse antigens? Though the genetically designed machinery of V(D)J recombination for generating diverse antibody receptors on the B cell surface and subsequent clonal selection, solves the puzzle partially, still there is discrepancy in the number of germ line antibodies that can be made through genetic recombination, which is limited to $\sim 10^8$, and the antigens that need to be recognized, which potentially are infinite. Studies on the structural basis of antibody diversity[22–25] gave us newer insights in the mechanism of generation of diversity by flexibility in the germline antibody in addition to the somatic hypermutation and V(D)J recombination.

To address this problem further, binding characteristics of antibodies derived from early primary and secondary responses were studied thermodynamically for a set of antigens[26]. In this experiment, the affinity of these antibodies were measured at 25 °C and 30 °C, and it was found that while the affinity of the germline antibodies decreased with an increase in temperature, it remains almost constant for the mature antibodies, thus demonstrating flexibility in the germline antibodies. Therefore it was hypothesized that flexibility of the germline paratope serves the dual purpose of high affinity and specificity. Thus, by simply manipulating conformational flexibility of the antigen-combining site, the transition from one extreme of a degenerate recognition capability, to the other of a highly specific effector response is readily achieved.

However, structural investigations to validate this hypothesis revealed a novel and fascinating molecular mechanism of expanding the repertoire of immune specificities. Towards analyses of how a germline antibody can bind a diverse set of antigens and the implications of the resultant crossreactivity, a germline monoclonal antibody 36-65, identified in the context of an immune response against the hapten p-azophenylarsonate (Ars), was used as a model system. Antibody 36-65 has an affinity for the hapten of 8.1mM (K_D), which showed a 40-fold decrease when the temperature was increased from 25 °C to 35 °C, indicating conformational flexibility in the antibody paratope. In contrast, the corresponding mature antibody 36-71 had an affinity of 0.06mM (K_D) for the hapten, which upon increasing the temperature to 35 °C, changed only marginally, suggesting relative rigidity in the affinity-matured antibody. Degeneracy in the recognition potential of antibody 36-65 was demonstrated by screening a random 12mer phage-displayed peptide library and selecting a series of independent peptides, which bind to germline antibody 36-65 with affinities comparable to that of Ars[27].

Crystallographic studies involving germline antibody 36-65 in the antigen-free form and in complex with three distinct phage displayed screening derived dodecapeptides were carried out[28]. In all, four antigen-free antibody structures were determined, which revealed a degree of structural diversity consistent with the thermodynamic evidence of conformational flexibility of the germline antibody. No two molecules exhibited similar combining site topologies, with differences attributable to variations in both side chain and backbone conformation. However, the structure of 36-65 in complex with three independent 12-mer peptides, revealed, contrary to expectation, a single conformational state of the 36-65 binding site mediated specific recognition of all three peptide ligands (Fig. 3A). Moreover, this particular conformational state is distinct from any of the conformers observed for the free antibody. Just as surprisingly, the peptides bound to spatially separate subsites within the combining site, where they adopt entirely different conformations. These subsites show virtually no overlap; of ~25 antibody residues in contact with the peptide in one or the other complex, only two are shared by all three complexes. Notably, these two residues appear to help lock the 36-65 binding site into the single conformation found in the complex structures. This structural state may then be further stabilized through somatic mutations during affinity maturation, and the conformation-specific interactions built in within the antigen-combining site may well be utilized

Figure 3: A molecular mechanism of antibody diversity. Structural basis for generation of multiple recognition specificities by germline antibodies. A. Stereoscopic view of superimposition of the 6 CDR loops of germline antibody 36-65 bound to diverse dodecapeptides demonstrating alternate juxtaposition of different antigens in a common antibody paratope conformation. B. Differential ligand positioning on diverse antigen binding to a common antibody. Multispecificity is conferred by ligand binding to spatially distinct regions of an otherwise topologically identical binding site.



for modulating antibody specificity during affinity maturation.

Thus it was concluded that though the flexibility in the CDRs was structurally apparent, it was striking that a single paratope conformation recognized multiple independent epitopes such that the binding of dissimilar antigens represented autonomous epitope footprints on the antibody-combining site. This new mechanism was termed as "differential ligand positioning" and helped further in addressing the antibody diversity puzzle along with the mechanisms of genetic recombination and conformational flexibility (Fig. 3B).

6. Conclusions

This review addresses three fundamental aspects of the antibody response. Generation of antibody diversity was explored through molecular mechanisms to explain how germline antibodies were endowed with degenerate recognition specificity. The structural basis of molecular mimicry in the immune response was analyzed addressing the mechanisms and consequences of breakdown in the antigenic discrimination. Likewise, the economy of the paratope conformational repertoire was addressed in terms of the binding modes of an antigen that keeps changing shape. The insights gained from these studies have certainly highlighted paradigm shift in our understanding of immune recognition. During early days, antigen–antibody recognition, like any other biological recognition processes, was thought to be defined in terms of complimentarity of shape and charge. The examples elucidated here highlight the complexity associated with molecular specificity associated with immune mechanisms.

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