Regulation of T-dependent humoral responses: The alternate paradigm

Kanury V. S. Rao

Immunology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India

Extensive work has been done on understanding processes that govern both initiation and progression of T-dependent humoral responses. However, the majority, if not all, of these have employed – as model antigens – small, rigid and structurally simple molecules providing a minimal surface area for contact by either antibody or the B cell antigen receptor. In contrast to this, the physiologically relevant antigens normally experienced by B cells (e.g. in the course of an infection) are much larger molecules with a great deal of structural complexity and diversity. An important, but unanswered, question which arises from this is whether the nature of antigen impinges in any way on qualitative aspects of a humoral response. The present article discusses our results using synthetic peptides as model antigens. As shown, such models provide an added level of resolution, thereby permitting delineation of several novel mechanisms.

THE adaptive immune system is characterized by two distinct but, nevertheless, interrelated activities – recognition and response. While the overall recognition capacity is a cumulative property of the diverse antigen receptors expressed on the surface of B and T lymphocytes, response is clonally derived from those lymphocytes bearing the appropriate antigen receptors. The recognition potential of adaptive immunity is amply exemplified by the preimmune B cell repertoire which, as is now well known, is endowed with the collective ability to recognize a virtually limitless array of molecular species – encompassing wide-ranging size, structural and even, chemical heterogeneity[1,2]. Interestingly though, this recognition capacity is frequently restrained by factors that govern induction of the response. Thus, at the first level, the ability to elicit a B cell response depends upon the chemical complexity of the antigen. Small, structurally simple molecules are generally incapable of producing B cell responses on their own. In addition, the qualitative nature of the response is also determined by the nature of antigen. For example, generation of memory B cell responses requires the antigen to either be proteinaceous in nature, or at least be intimately associated with a protein component. In other words, the versatility of recognition is always fine-tuned by disciplinarian response thresholds. Although this principle is self-evident it, as we shall see later, constitutes an all-pervading feature during the various stages of a humoral response – with important implications for both efficacy and specificity of its effector functions.

Cumulative studies, performed over the last two decades, from a variety of laboratories have greatly facilitated our understanding of processes involved in the induction and maturation of T-dependent antigen-specific humoral responses[3–20]. These studies now permit delineation of an overall scheme which can be summarized as follows: Antigen-dependent activation of 'naive', resting B cells occurs following a specific interaction between antigen and those cells bearing the appropriate sIg receptor. Accumulated evidence suggests that this interaction first occurs in the T cell-rich extrafollicular sites of secondary lymphoid organs (Figure 1). This, with exceptions, leads to the appearance of specific antibody producing B cells (AFCs) which concentrate in the periphery – adjacent to the red pulp – of the periarteriolar lymphoid sheath (PALS) (Figure 1). Antigen-activated B cells in the foci differentiate to produce unmutated Ig over the next ten to twelve days, before undergoing apoptosis and eventually disappearing. The low affinity Ig that is produced in this period forms complexes with circulating antigen and, subsequently, becomes sequestered on the surface of Fc and complement receptor-bearing follicular dendritic cells (FDCs) residing in primary B cell follicles (Figure 2).

Within days of detection of the first foci, proliferating B cells can also be observed in the primary follicles. These gradually develop into large germinal centres (GCs), reaching a maximal size in the second week after immunization. This increase, both in size and number of GCs, is accompanied by a concomitant decline in PALS foci. Although it is widely believed that B cells in both the foci and GCs derive from a common precursor there is, however, conflicting evidence in the literature which suggests that B cells in the AFC and GC compartments may represent distinct lineages[21–23].

Within GCs, at least two distinct regions – the dark and light zones – can readily be distinguished. The dark zone consists of proliferating B cells, or centroblasts, where they undergo somatic mutation of Ig variable region genes. The mutated progeny come out of cell cycle, express mutated antigen receptors and, subsequently, are thought to undergo affinity maturation. This latter process

e-mail: kanury@icgeb.res.in

is driven by antigen, which is available as immune complexes (ICs) on the surface of FDCs (Figure 2). Those antigen-specific mutant B cells with increased affinity and specificity are positively selected to differentiate into either antibody secreting plasma cells or memory B cells.

While the sequence of events that succeed activation of antigen-specific resting B cells has been extensively validated, current focus is on resolving issues of mechanistic detail. Of particular interest are the individual steps that comprise the GC reaction, and include a delineation of the molecular regulators of somatic hypermutation, clonal selection and differentiation of antigen-activated B cells into the memory compartment. It must, however, be kept in mind that the majority – if not all – of the above studies have been conducted using various haptens as model antigens. The commonly employed ones have been rigid, structurally simple molecules providing a minimal surface area of contact for the corresponding paratope on either antibody or B cell sIg receptor. In contrast to this, the physiologically relevant antigens normally experienced by B cells (e.g. in the course of an infection) are much larger molecules with a great deal of structural complexity and diversity. An important question that, therefore, arises is whether the nature of antigen impinges in any way on qualitative aspects of a humoral response. Indeed, at the outset, it can be anticipated that simultaneous recognition of multiple determinants presented by complex antigens may necessitate adjunctory levels of modulation, beyond those elucidated with hapten models.

Selective and hierarchical recognition: The partisan traits of humoral immunity

An empirical dogma that has pervaded down the years is that the entire accessible surface of any protein constitutes an antigenic continuum[24]. This notion is a logical inference that derives from our joint realizations of the plasticity of the preimmune B cell repertoire, and the fact that the B cells generally recognize protein antigens in their native form[1,2]. Indeed, such an assumption has spurred the generation of a variety of algorithms aimed at *ab initio* identification of B cell antigenic determinants on polypeptides, based on predicted surface accessibility of their segments.

Although the above proposal has not been subject to a rigorous verification, experiments with a limited set of polypeptide antigens have, however, revealed that this does not necessarily constitute a universally applicable principle. On the contrary, these studies have brought into focus the biased character of a T-dependent humoral response by demonstrating that only a fraction of the

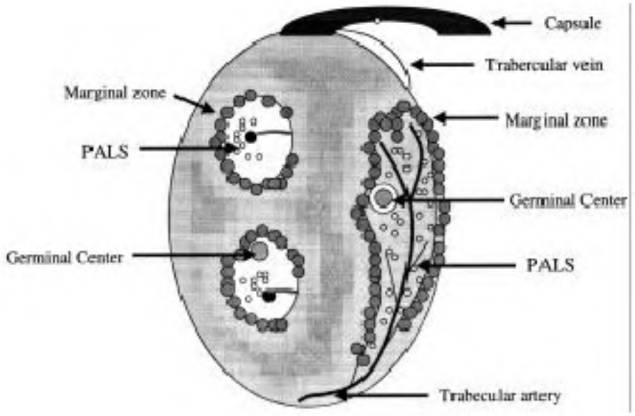

Figure 1. Illustrative cross-section of the spleen. The splenic artery enters the spleen at its hilum and immediately branches into smaller 'trabecular' arteries which, in turn, branch into even smaller 'central' arteries. The central arteries course through into the splenic parenchyma, eventually branching into 'penicilliary' arterioles. Investing the central arteries and penicilliary arterioles is a concentric cuff of lymphocytes, the great majority of which are T cells. The cuff, termed as periarteriolar lymphoid sheath (PALS), constitute the T cell zone of the spleen. Periodically, usually at arterial branch points, B-lineage lymphoid follicles – either primary or secondary – appear as outgrowths of the PALS. Surrounding the primary follicle or mantle zone of the secondary follicle is the marginal zone. The marginal zone consists of both B and T cells and is thought to be the site in which splenic B and T cells initially encounter blood-borne antigen. Indeed the marginal zone appears to be the primary site of entry of both B and T lymphocytes into the white pulp. Unlike other secondary lymphoid organs the spleen lacks the high endothelial venule (HEV), but it is thought that the specialized marginal zone sinuses (which are supplied by smaller arterial branches of the central arteries) serve a similar function.

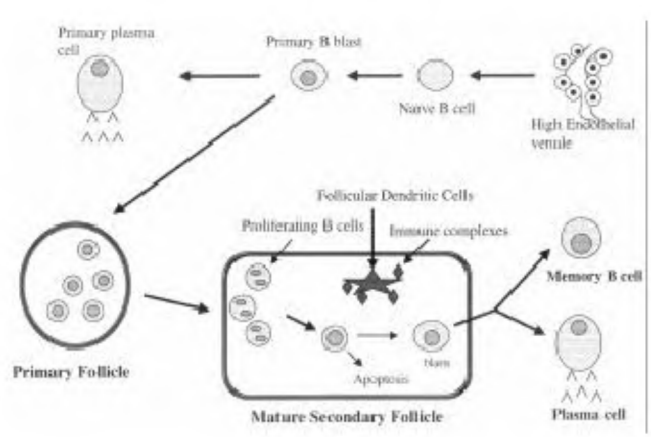

Figure 2. B cell maturation in secondary lymphoid tissue. Naive B cells enter the T cell zones of lymph nodes via the high endothelial venule (HEV). In the presence of antigen and primed T cells, specific B cells are activated and the majority of these differentiate into primary plasma cells secreting antibodies of the early primary response. These antibodies are genetically unmodified (low affinity) and of the IgM isotype. A subset of these early activated B cells, however, migrates to and colonizes primary follicles where it begins to proliferate and form germinal centres (GCs). Within GCs, proliferating B cells are subject to a mutation and selection process which is designed to increase the affinity and functional efficiency of the memory immunoglobulin repertoire. These latter processes appear to be governed by the antigen present as immune complexes on the surface of follicular dendritic cells (FDCs). This second phase eventually gives rise to both (i) relatively long-lived plasma cells that produce high affinity (genetically modified) and high titer, predominantly non-IgM (usually IgG, with some IgE) antibodies and (ii) memory B cells capable of differentiating into similar plasma cells upon subsequent exposure to antigen.

anticipated epitopes are actually recognized in the course of an antibody response[25–33]. In addition to epitope-selectivity, another facet was also revealed which was typified by the observation of quantitatively unequal levels of antibodies produced against even the limited subset of epitopes that are recognized[34]. These dual phenomena of 'selective' and 'hierarchical' immunodominance would, therefore, appear to implicate additional levels of regulation in terms of both recognition and subsequent amplification of immune responses. Consequently, T-dependent humoral responses to multideterminant antigens are likely to be more involved than those against haptens.

Immune selectivity: The two levels of enforcement

The phenomenon of biased recognition of epitopes appeared, to us, to provide a useful context within which to examine immune regulation. The model antigens employed were specifically designed polypeptides obtained either through recombinant DNA[35], or synthetic peptide[36] methodologies. An examination of murine humoral responses to such antigens revealed several interesting features. The early primary IgM response, detected within a few days of immunization, was found to be constituted by individual antibody specificities directed against a multitude of determinants encoded within the antigen sequence[36,37]. In contrast, however, the earliest IgG response that could be detected displayed a markedly restricted epitope-specificity, usually focusing on a unique epitope of antigen[36,37].

An analysis of clonal heterogeneity of antibodies directed against such immmunodominant epitopes in the early primary and secondary stages of the response revealed the existence of yet another level of selection. Hybridomas generated from the early IgM stage displayed a great deal of heterogeneity, both with respect to gene segment utilization and CDR3 composition of the immunoglobulin heavy chain. This suggested that a diverse range of antibody paratope phenotypes was readily induced against individual epitopes. In contrast to this, epitope-specific IgG hybridomas derived from a secondary response were severely restricted in terms of repertoire heterogeneity[38].

The above preliminary studies highlighted two important features that provided the framework for future investigations. First, restricted epitope recognition in a humoral response was an acquired property that followed initial activation of preimmune B cells by antigen. Consequently, it was likely to derive from immunological, rather than structural considerations. The second feature was that, in addition to epitope specificities, selection was also experienced at the level of epitope-specific B cell repertoires that were retained. Thus, for multideterminant antigens, optimization of a humoral response is initiated by a dual selection for the appropriate subset of epitopes, as well as a restricted spectrum from the epitope-specific B cell repertoire pool.

Epitope selection: A question of thresholds

A multiplicity of co-operative mechanisms were found to regulate epitope selection, all of which were brought into play soon after preimmune B cell activation, but prior to the initiation of the GC reaction. Selection was demonstrated to result from competitive processes that were enforced between the multitude of early antigen-activated B cells[36]. Interestingly though, contrary to expectations, the driving force was found not to emanate from a rapidly declining antigen supply. Rather, it was the limiting pool of antigen-primed T helper cells within the first few days of immunization that necessitated a competition for survival[36]. The criterion for success was described by the affinity of the sIg receptor on individual clonotypes for their corresponding epitope on antigen[36]. Affinity for epitope, in turn, was implicated in defining the ability of these clonotypes to recruit T cell help – presumably by modulating their efficacy as antigen presenting cells (APCs)[36]. In the absence of a parity in frequencies of antigen-primed B and T cells, successful engagement of the T cell subset by high affinity clonotypes denied timely access for the lower antigen-affinity B cells, thereby resulting in their elimination.

From a functional perspective, the primary humoral response can be broadly distinguished into three discrete stages. The first is activation of resting B cells into IgM producers. Two related events that follow from this are, antibody isotype switch and differentiation of selected subsets into memory B cells. In view of our findings that the ability to recruit T cell help – in a competitive milieu – constituted the principal selection criterion, we asked whether the extent of Th cell involvement in each of these individual stages was comparable. This was addressed by an adoptive transfer protocol using graded levels of antigen-primed T cells along with naive B cells, and then following the extent to which a humoral response progressed upon subsequent antigenic stimulation. Results from such experiments revealed that both activation of preimmune B cells into IgM producers, and differentiation – at the later stage – into memory B cells proceeded well in the presence of limiting numbers of activated T cells[39]. However, an IgM to IgG isotype switch required at least an order of magnitude higher frequency of T cells. The increased requirement for antigen-primed T cells during antibody class switch has also been reported by other laboratories[40,41]. Thus, based on these findings, we had proposed that induction and progression of a primary T-dependent humoral responses is comprised of a single rate limiting step – in terms of Th cell requirements – which also coincided with the step involving antibody isotype switch[37]. Interestingly, when we analysed for distribution

of antibody specificities, we found that it was at this step where selection for restricted epitope specificity also took place[37]. While the heterogeneity of the primary IgM antibodies remained unchanged over their life span, the earliest IgG detected displayed the restricted epitope profile characteristic of both late primary and secondary IgG[37]. Further, it could be demonstrated that this restriction was a result of the earlier described competitive processes which favour higher affinity B cells, and is driven by the limiting pool of antigen-primed Th cells available at that time[37]. Of particular interest is our more recent finding that this affinity threshold also operates to 'filter' antigen-activated B cells that are permitted to seed GCs[42,43]. Consequently, this affinity threshold also exercises its impact on the secondary response by determining the spectrum of epitope-specific B cells which will eventually differentiate into the memory subset.

Our identification of an affinity threshold which limits the repertoire of early antigen-activated B cells recruited within GCs, complements well the recent findings of Lahvis and Cerny[44]. They have shown that induction of the GC phenotype on B cells, as measured by up-regulation of cell surface PNA-receptors, was far more demanding in terms of Th cell requirement relative to induction of activation markers such as B7-2 and I-A (ref. 44). As already indicated earlier, the efficiency of T cell recruitment by individual, activated clonotypes is determined by the affinity of its sIg receptor for antigen/epitope, although the precise molecular aspects of this mechanism are presently unclear. Consequently, the existence of an affinity threshold may be explained as a minimal T-dependent prerequisite for induction of those cellular properties required for either homing to follicles or proliferation within.

An important feature underscored by our studies on model peptide antigens was that affinity-dependent selection occurs prior to the initiation of a GC reaction. Indeed we have shown that, at least for the peptides studied, there was no further improvement in affinity of antigen-specific antibodies obtained in the secondary response. These results pose an apparent discrepancy with observations on various haptens, where affinity maturation is thought to principally occur by way of selection from mutated B cells generated in GCs. Although, as recent data suggest, affinity maturation processes may continue well beyond the tenure of the GC reaction it was, nevertheless, dependent upon generation of mutant B cells within GCs[45]. It is possible, as discussed later, that this discrepancy may be related to the complexity of antigen.

That the variable epitope-specific affinities produced in an early primary response to a multideterminant antigen constitute the basis for selection poses, in its own right, an intriguing question. Keeping in mind the plasticity of the preimmune B cell repertoire, what then are the factors which mediate production of antibodies of non-identical affinity to the various epitopes. This issue became particularly relevant when, in the course of our studies, we were able to successively rule out many of the predictable influences such as mouse H-2 haplotype, surface accessibility, positional effects and secondary structural propensities of epitope. This elimination of all obvious correlates consequently left, by default, the possibility that the relative immunogenicity of a B cell epitope on a multideterminant antigen may represent an inherent property of its chemical composition. In principle, such an inference was not altogether improbable, considering that the amino acid constitution of an epitope is expected to influence the energetics of its complexation with either antibody or B cell sIg receptor. This, in turn, would characterize the affinity of binding, where the dissociation constant (K_d) relates to the Gibbs free energy of complexation (ΔG) as, $\Delta G = RT \ln K_d$.

Recent results from our laboratory point towards a distinct correlation between the amino acid composition of an epitope and avidity of the primary response elicited against it, in the context of multideterminant antigen (manuscript in preparation). Some degree of variability was, however, introduced depending upon the availability of residue chain for interaction with paratope. Interestingly, an analysis for the extent to which each of the individual, naturally occurring amino acid residues contributed towards defining the immunogenicity of a given epitope within a multideterminant antigen, identified a clear correlation with the potential free energy that these diverse residue side chains could provide in a binding interaction.

Antibody optimization and memory B cell differentiation in GCs

In similarity with prior observations for haptens, progression of humoral responses to peptide antigens was also found to be characterized by improved antibody specificity for epitope/antigen. This occurred by way of somatic mutation followed by selection in GCs and was reflected in terms of optimized interface complementarity during paratope (of antibody or sIg receptor) and epitope interactions. Although enhancement of antigen-specificity of antibodies – as a consequence of the GC reaction – has been known for some time, the notion that 'specificity maturation' may constitute the overriding selection criterion over 'affinity maturation' has recently been proposed by Manser and co-workers[46]. This cardinal prescription for optimized specificity is readily understood in light of the need to constantly ensure that both memory B cells and secondary antibodies generated possess minimal to no cross-reactivity with self antigens. Although mechanisms ensuring deletion of autoreactive B cells become transiently activated in GCs, their efficacy is always restricted to the spectrum of antigens and the concentrations in which they are available in the environment of GCs. Indeed, in general, the effectiveness of all antigen-driven

B cell deletion mechanisms, either during ontogeny or subsequent 'windows of tolerance susceptibility', can never exceed beyond that prescribed by the immediate antigenic milieu. In contrast, achievement of an idealized specificity for the inducing antigen/epitope constitutes, by virtue of ignorance, a generic but comprehensive strategy to ensure minimal self-reactivity. Notably, this would not be limited by the antigenic environment at that particular location and time, but would also certify ignorance of antigens exposed to memory B cells during recirculation.

An important aspect to be borne in mind, when considering the question of specificity, is that very high antibody affinities for antigen do not harbour well for fidelity. The thermodynamic component of such interactions is so high that modification of a few non-covalent interface interactions is still permissive of binding albeit, usually, with lower affinities. This is also true of low affinity interactions where the poor enthalpic contributions, usually reflective of some inadequacy during interface association, also epitomizes low fidelity. As a consequence, the affinity throughput of a GC reaction is likely to be restricted to within limits of a window of optimal affinity ranges. That such parameters in fact regulate the outcome of a GC reaction is strongly supported by the results of Manser and co-workers[46]. Although very high affinities of anti-Ars antibodies could be generated by random *in vitro* mutagenesis of a germline precursor this, however, also led to a lowering of antigen specificity[46]. It is presumably for this reason that such mutations were found not to be selected for, in the course of an anti-Ars immune response[46]. Indeed only those affinity-improving mutations, which also concomitantly improved antigen-specificity were selected, although the increase in affinity was well below the maximal limits that could be achieved[46].

The existence of several novel principles could be discerned upon our examination of GC responses to peptide antigens. The first, as already described, was the identification of an affinity threshold that restricts early antigen-activated B cells permitted to seed GCs. It is pertinent to reiterate our earlier observations that this selection on the basis of minimum affinity also translates, for multideterminant antigens, into a restricted subset of epitope specificities. As a result, this early screening also qualitatively characterizes the nature of the secondary response. Following selective recruitment of antigen-activated B cells within GCs, our recent data demonstrate that further optimization – by the process of somatic mutation followed by selection – is directed primarily in favour of increased on-rates of antigen binding, with no significant consequence on binding affinity[47]. Although improved binding on-rates have also been noted by Foote and Milstein[48] in immune responses to the hapten phOx this, however, was not independent of affinity maturation. It is again possible that these differences, as discussed later, derive from differences in the nature of antigen studied.

Both the probable mechanism and implications of a kinetic control of the GC reaction could be deciphered by our subsequent study of the role of antigen-containing immune complexes (ICs) in GCs. This analysis was facilitated by our development of a procedure wherein adoptive transfer of antigen-primed T and B cells, in addition to appropriate ICs, reconstituted primary antigen-specific GCs in irradiated mice[42]. Notably, with this protocol, we were able to demonstrate that development of GCs was independent of the prior presence of at least significant levels of ICs within follicles. However, their presence was determined to be obligatory for differentiation of GC B cells into the memory subset[43]. At one level, these results highlighted the regulatory independence of these two processes. In other words, the extent of GC development had no direct bearing on the proportion of memory B cells that are produced as a result[43].

An intriguing feature of memory B cell differentiation in GCs was that it occurred in a 'specificity autonomous' fashion. That is, GC B cells were found to differentiate only upon encounter with ICs constituted by antibodies sharing an epitope-specificity identical with that of the sIg receptor on the cells[43]. Further, in addition to promoting differentiation, the presence of appropriate ICs was also found to be critical for optimizing antigen-specificity of the resulting secondary response[43]. An indication of the mechanistic basis for these findings was provided by our later observations that memory differentiation in GCs occurs by way of antigen exchange between ICs and B cells. Of particular note was the fact that this process was entirely regulated by the rate at which GC B cells acquire antigen from ICs (unpublished results). Reduced on-rates of B cell-antigen association were non-permissive. These studies provided us with an important clue as to how enhanced antigen-specificity could be achieved as a result of the GC reaction. The relationship between binding on-rates and antigen-fidelity was clarified in experiments utilizing analogues of the model peptide which bore the identical epitope, but in an altered conformation. This perturbation in the conformational degrees of freedom of epitope lowered the on-rates of binding of these analogues to antibodies generated against the parent peptide, although the affinity of binding remained unaltered. Interestingly, this reduction in binding rates was sufficient to render the analogue, in the context of ICs, completely inactive at promoting memory differentiation of peptide-specific GC B cells. Thus, the remarkably high degree of fidelity that a kinetically controlled process ensures is exemplified by these findings, wherein even perturbations in conformation of epitope can be discriminated. At a functional level, this differentiation threshold also imparts a discriminatory capacity to the memory B cells which result. Thus, in contrast to the parent antigen, analogues with poorer binding on-rates were unable to effectively recruit the parent peptide-specific memory B cells into a secondary response *in vivo*.

An insight into the regulatory mechanisms implicated in the kinetic control of memory B cell differentiation and activation is also provided by related results from our laboratory. With the same peptide model, we have demonstrated that the kinetics of antigen recognition by activated B cells directly determines the extent and quality of T cell help that it can recruit[49,50]. An obvious explanation for such an effect could be that enhanced antigen uptake also leads to increased density of MHC class II-associated ligand presented for TCR (T cell antigen receptor) engagement. However, our recent experiments implicate a more direct effect. They suggest that the kinetics of antigen-BCR (B cell antigen receptor) association differentially modulates intracellular signal transduction pathways leading, eventually, to altered expression of costimulatory molecules on the B cell surface[51]. Nevertheless, regardless of the mechanism, the rapidity of cognate Th cell (T helper cell) engagement is known to constitute a crucial rescue signal for GC B cells which, otherwise, are notably apoptosis-prone[52]. It is, therefore, not unexpected that the kinetic control of B cell selection/differentiation in GCs also constitutes a fidelity-threshold which, eventually, contributes towards increased antigen-specificity of the memory B cells produced. We have recently suggested that enhancement of B cell specificity for antigen in GCs occurs in two distinct stages[43]. The first represents an antigen-driven step where, as described by other groups[53,54], binding to soluble antigen – including self antigen – leads to deletion of GC B cells. The second stage could well involve 'purification by ignorance' where those mutant clonotypes with decreased on-rates of antigen recruitment from ICs are eliminated by virtue of their inability to access T cell help in a timely manner.

That it is the kinetics of antigen binding which modulates reactivation of memory B cells could readily be ascertained with analogues of the parent peptide possessing altered binding rates to IgG responses elicited by the parent peptide. As already indicated earlier, analogues with decreased on-rates of binding also served as poor recall antigens. However, analogues with significantly higher on-rates, relative to the parent peptide, proved to be more potent than the parent peptide at eliciting secondary IgG responses from parent peptide-specific memory B cells *in vivo*. At this stage, an interesting dichotomy became apparent. While memory B cell reactivation was stringently controlled, the secondary IgG elicited from these cells was relatively less discriminatory in that it bound all analogues – regardless of on-rate differences – with near identical affinities. In other words, immune response thresholds appear to be more tightly regulated than immune recognition thresholds.

An accumulated perspective

At least for peptide antigens, an overall scheme that directs maturation of a primary humoral response emerges, which is somewhat distinct from that delineated for hapten antigens. In the former case, response maturation seems to occur in two sequential, stages – presumably at distinct sites within secondary lymphoid organs. The initial exposure to a T-dependent antigen leads, as a result of low thresholds, to activation of diverse B cell clonotypes which are heterogenous with respect to both antibody repertoires and epitope-specificities. Soon thereafter, competitive processes are enforced which restrict both the repertoire and epitope-specificity of B cells retained. The impetus for this process is provided by the limiting frequency of antigen-activated Th cells in the early stages of the response, where the criterion for success is defined by the affinity of individual clonotypes for the corresponding epitope on antigen. An important facet of the early primary response to T-dependent peptide antigens is that variability in antibody affinity to individual epitopes is intrinsically defined by both the amino acid composition of epitope and availability of its residue chains for interaction with antibody/sIg receptor paratope. The absence of interface complementarity during epitope–paratope interaction at this stage enhances the side chain dependent variability – in terms of enthalpic contributions – towards antibody or sIg receptor binding. Non-identity in affinity of antibody responses to the various epitopes is a direct consequence of this.

The affinity-driven selection process which operates in the early primary response also impacts, by defining the spectrum of epitopes to be recognized, on the later stages. This is effected by ensuring that only those antigen-activated B cells selected at this stage are allowed to seed GCs. Within GCs, the second phase of antibody or, more precisely, B cell sIg receptor optimization is initiated. The cumulative processes of somatic hypermutation, clonal selection and memory differentiation result in the production of antigen-specific memory B cells with an improvement in the mutually interrelated properties of specificity, paratope complementarity and antigen binding on-rates. While the relationship between specificity and interface complementarity in a binding interaction is widely acknowledged, it is the added imposition of an association rate threshold that reinforces the discriminatory capacity by providing the biochemical distinction which eventually leads to either response or death by ignorance. Thus, in addition to active antigen-driven deletion mechanisms within GCs, this sequential perfection of an epitope–paratope fit, first in terms of binding affinity and then followed by that for association rates, is also likely to contribute in a significant way towards ensuring that the eventual B cell products are devoid of self-reactivity. The singular role of rates of antigen binding as the principal determinant of functional specificity, is underscored by our own results which document that it provides for the ability to discriminate between even minor perturbations in the conformational flexibility of epitope.

An important but perhaps, in retrospect, self-evident principle that our studies also highlight is the fact that an immune response is more tightly regulated relative to immune recognition. Thus in an early primary response, the initial recruitment of diverse B cell repertoires directed against a plethora of antigenic determinants is subsequently restrained by the enforcement of selection pressures. Similarly, in the secondary response, while memory B cell reactivation demands high fidelity, the antibody products of these cells are at least less discriminatory in terms of epitope binding. This principle may perhaps have an important bearing on the study of autoimmune responses as it draws attention to the functional distinction between antigen cross-reactivities observed, and the etiological agent responsible for the induction of such a response.

Distinctions between anti-hapten and anti-polypeptide responses: A matter of structural complexity?

Although no direct comparisons have been made, it is now becoming increasingly evident that there are qualitative distinctions between the nature of humoral responses to haptens, and those against the structurally more complex antigens. While the overall principles remain equally applicable, the difference lies in the extent of time-dependent improvement in the quality of antibodies that are initially recruited from the germline repertoire. Anti-hapten responses display a concomitant increase in equilibrium and kinetic binding properties, both of which are achieved within GCs. In contrast, although limited in scope, humoral responses to protein antigens show no increase in antigen-affinity between the early primary and later stages. This distinction was first noted by Zinkernagel and co-workers[55] in their study of murine immune responses to the vesicular stomatitis virus. Indeed in this case, no increase in either affinity or antigen binding on-rates could be observed in monoclonal antibodies derived from the primary, secondary and tertiary responses. In a similar vein, the avidity of a murine anti-HEL response was found to remain relatively constant even after multiple immunizations. However, the kinetic features of antigen binding were not examined. Finally, as described here, murine humoral responses to peptide antigens display kinetic, but not affinity maturation.

Our own observations that, at least for multideterminant antigens, selection of germline antibodies/B cell sIg receptors with optimal affinity occurs very early in the primary response offers a possible clue towards resolving these differences. In this connection, interclonal competition has also been noted in the pre-GC phase of anti-hapten responses – although the mechanisms have not been looked into. Thus, the extent of paratope optimization required can be expected to depend upon the quality of output of this selection process. It must be remembered here that, as discussed earlier, the fidelity requirement inherently imposes an upper limit on functionally achievable antigen affinities. Therefore, if one accepts the precept that the end goal of the adaptive immune system is to produce an immune response with maximal bio-efficacy, but with no compromise on fidelity, this objective translates into a physico-chemical property of antigen binding optimized for both equilibrium affinity and association rates. The functional requirement for bio-efficacy, in turn, also prescribes a need for minimum stability of the complex with antigen. As a consequence, the twin controls on affinity and stability of epitope–paratope complex will, therefore, result in defining an upper limit for the allowed on-rate of this binding interaction for a given epitope–paratope pair. This becomes obvious from the relationship between these three parameters as defined by the equation, $K_a = k_{on}/k_{off}$; where K_a is the affinity constant, and k_{on} and k_{off} represent the association and dissociation rates, respectively. The extent of qualitative improvement that is achievable in GCs must, therefore, be viewed in this context. In instances where the germline response yields antibodies optimized for both parameters, no further improvement can be expected as a result of the GC reaction.

The discussion above, therefore, leads to a pertinent question. What are the circumstances which define whether the preimmune B cell response will lead to production of antibodies pre-optimized for binding to the cognate epitope? A possible answer may lie in the chemical complexity of the antigen. The existence of such a possibility is indeed supported by the above extreme examples of responses to haptens at one end – which require both affinity and kinetic maturation – to viruses at the other, where both are absent. In this connection, peptide antigens may be considered as intermediate examples – both in terms of structural complexity and immune regulation – where antibody responses to it undergo kinetic, but not affinity maturation.

How then does the chemical complexity of antigen influence the quality of germline antibody responses? This can readily be explained in terms of the overall surface area and structural heterogeneity of determinants that are provided by antigen for immune recognition. In cases where both these parameters are large, one could expect a concomitant increase in the probability of generating an idealized fit, against at least some of the epitopes, even at the level of preimmune B cell recognition. Conventional hapten antigens are smaller than the average surface area encompassed within an antibody paratope. Therefore, the probability of generating optimal paratope fits from the preimmune repertoire would be low to negligible – thus explaining the low affinity primary responses normally observed against these antigens. On the other hand, multimeric protein complexes such as those present on viral surfaces, provide ample scope for elicitation of idealized germline antibody responses against at least some of the

epitopes available. It is this corresponding clonotype subset that would be selected – against a background of a multitude of lower affinities – by mechanisms described earlier, for recruitment within GCs. Depending then upon the degree of perfection of fit, somatic mutation and subsequent clonal selection processes will result either in no further optimization or an improvement at the level of affinity, on-rates, or both.

The probability factor, as discussed above, becomes particularly relevant if one considers the fact that – in contrast to the enormous genetic diversity of the germline antibody repertoire – the structural repertoire is severely limited. Thus, for example, an analysis of three hundred and eighty-one antibody sequences has revealed that 87% of these can be accommodated within as few as ten canonical structural classes. This restricted structural diversity of the antibody repertoire can only be expected to enforce dependence of the probability of obtaining a good fit on the size, chemical and structural heterogeneity that the antigen provides.

Thus, although the qualitative nature of a T-dependent humoral response varies according to the antigen system that is used, it is possible – by combining simple physicochemical rules with our knowledge of the demands imposed upon an immune response – to synthesize these within a common framework of an overall guiding principle. The limited number of protein antigens examined from the standpoint of response maturation, however, restricts our above proposal to level of a working hypothesis. Additional data from a variety of structurally more complex T-dependent antigens will be required to establish its veracity.

1. Paige, C. J. and Wu, G. E., *FASEB J.*, 1989, **3**, 818–824.
2. Kofler, R., Geley, S., Kohler, H. and Helmberg, A., *Immunol. Rev.*, 1992, **128**, 5–21.
3. Stenzel-Poore, M. P., Bruderer, U. and Rittenberg, M. P., *Immunol. Rev.*, 1988, **105**, 113–136.
4. Makela, O. and Kaartinen, M., *Immunol. Rev.*, 1988, **105**, 85–104.
5. Berek, C. and Milstein, C., *Immunol. Rev.*, 1988, **105**, 5–21.
6. Jacob, J., Ramtin, K. and Kelsoe, G., *J. Exp. Med.*, 1991, **173**, 1165–1175.
7. Nossal, G. J. V., *Cell*, 1992, **68**, 1–4.
8. Lalor, P. A., Nossal, G. J. V., Sanderson, R. D. and McHeyzer-Williams, M. G., *Eur. J. Immunol.*, 1992, **22**, 3001–3011.
9. Liu, Y. L., Johnson, G. D., Gordon, J. and MacLennan, I. C. M., *Immunol. Today*, 1992, **13**, 17–21.
10. Leanderson, T., Kallenberg, E. and Gray, D., *Immunol. Rev.*, 1992, **126**, 47–61.
11. Jacob, J., Przylepa, J., Miller, C. and Kelsoe, G., *J. Exp. Med.*, 1993, **178**, 1293–1307.
12. Kuppers, R., Zhao, M., Hansman, M. and Rajewsky, K., *EMBO J.*, 1993, **12**, 4955–4967.
13. McHeyzer-Williams, M. G., McLean, M. J., Lalor, P. and Nossal, G. J. V., *J. Exp. Med.*, 1993, **178**, 295–307.
14. Zeigner, M., Steinhauser, G. and Berek, C., *Eur. J. Immunol.*, 1994, **24**, 2393–2400.
15. Nossal, G. J. V., *Immunol. Rev.*, 1994, **137**, 173–184.
16. Kelsoe, G., *Immunol. Today*, 1995, **16**, 324–329.
17. Storb, U., *Immunol. Rev.*, 1998, **162**, 324–329.
18. Neuberger, M. S., Ehrenstein, M. R., Kilx, N., Jolly, C., Yelamos, J., Rada, C. and Milstein, C., *Immunol. Rev.*, 1998, **162**, 107–116.
19. Blanden, R. V., Rothenfluh, H. S., Zylstra, P., Weiler, G. F. and Steele, E. J., *Immunol. Rev.*, 1998, **162**, 117–132.
20. Klein, U., Goosens, T., Fisher, M., Kanzler, H., Braeuninger, A., Rajewski, K. and Kuppers, M., *Immunol. Rev.*, 1998, **162**, 261–280.
21. Linton, P. J., Decker, D. J. and Klinman, N. R., *Cell*, 1989, **59**, 1049–1059.
22. Linton, P. J., Lo, D., Lai, L., Thorbecke, G. J. and Kilinman, N. R., *Eur. J. Immunol.*, 1992, **22**, 1293–1297.
23. Decker, D. J., Linton, P. J., Zaharevitz, S., Biery, M., Gingeras, T. R. and Klinman, N. R., *Immunity*, 1995, **2**, 195–203.
24. Benjamin, C. J. *et al.*, *Annu. Rev. Immunol.*, 1984, **2**, 67–101.
25. Berzofsky, J. A., Richman, L. K. and Killon, D. J., *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 4046–4050.
26. Herzenberg, L. A. and Tokuhisha, T., *J. Exp. Med.*, 1982, **155**, 1730–1737.
27. Wicker, L. S., Benjamin, C. D., Miller, A. and Sercarz, E. E., *Eur. J. Immunol.*, 1984, **14**, 447–453.
28. Sadegh-Nasseri, S., Kipp, D. E., Taylor, B. A., Miller, A. and Sercarz, E. E., *Immunogenetics*, 1984, **20**, 535–540.
29. Sadegh-Nasseri, S., Dessi, V. and Sercarz, E. E., *Eur. J. Immunol.*, 1986, **16**, 486–492.
30. Manca, F., Fenoglio, F., Kunkl, A., Cambiaggi, C., Pira, L. and Celada, F., *Immunol. Today*, 1988, **9**, 300–305.
31. Gajewski, T. F., Joyce, J. and Fitch, F., *J. Immunol.*, 1989, **143**, 15–22.
32. Kunkl, A., Fenoglio, D., Manca, F., Pira, G., Cambiaggi, C., Strom, R. and Celada, F., *Int. Immunol.*, 1992, **4**, 627–636.
33. Scheerlinck, J. Y., DeLeys, R., Saman, E., Brys, L., Geldhoff, A. and Bactselier, P. D., *Mol. Immunol.*, 1993, **30**, 733–739.
34. Vijayakrishnan, L., Kumar, V., Agrewala, J., Mishra, G. C. and Rao, K. V. S., *J. Immunol.*, 1994, **153**, 1613–1625.
35. Kumar, V., Bansal, V. J., Rao, K. V. S. and Jameel, S., *Gene*, 1992, **110**, 137–144.
36. Agarwal, A., Sarkar, S., Nazabal, C., Balasundaram, G. and Rao, K. V. S., *J. Immunol.*, 1996, **157**, 2779–2788.
37. Kumar, A., Kumar, V., Shukla, G. and Rao, K. V. S., *Vaccine*, 1994, **12**, 259–266.
38. Tuteja, R., Agarwal, A., Vijaakrishnan, L., Nayak, B. P., Gupta, S. K., Kumar, V. and Rao, K. V. S., *Immunol. Cell. Biol.*, 1997, **75**, 245–252.
39. Agarwal, A. and Rao, K. V. S., *J. Immunol.*, 1997, **159**, 1077–1085.
40. Stedra, J. and Cerny, J., *J. Immunol.*, 1994, **152**, 1718–1726.
41. Kelsoe, G., *Adv. Immunol.*, 1995, **60**, 267–288.
42. Agarwal, A., Nayak, B. P. and Rao, K. V. S., *J. Immunol.*, 1998, **161**, 5832–5841.
43. Nayak, B. P., Agarwal, A., Nakra, P. and Rao, K. V. S., *J. Immunol.*, 1999, **163**, 1371–1381.
44. Lahvis, G. P. and Cerny, J., *J. Immunol.*, 1997, **157**, 1783–1793.
45. Takahashi, Y., Dutta, P. R., Cerasoli, D. M. and Kelsoe, G., *J. Exp. Med.*, 1998, **187**, 885–895.
46. Manser, T., Tumas-Brundage, K. M., Cassom, L. P., Giusti, A. M., Hande, S., Notides, E. and Vora, K. A., *Immunol. Rev.*, 1998, **162**, 182–196.
47. Nayak, B. P., Tuteja, R., Manivel, V., Roy, R. P., Vishwakarma, R. A. and Rao, K. V. S., *J. Immunol.*, 1998, **161**, 3510–3519.
48. Foote, J. and Milstein, C., *Nature*, 1991, **352**, 530–534.
49. Vijayakrishnan, L., Sarkar, S., Roy, R. P. and Rao, K. V. S., *J. Immunol.*, 1997, **159**, 1809–1819.
50. Vijayakrishnan, L., Manivel, V. and Rao, K. V. S., *J. Immunol.*, 1998, **161**, 4661–4670.
51. Vijayakrishnan, L., Natarajan, K., Manivel, V., Raisuddin, S. and Rao, K. V. S., *J. Immunol.*, 2000, **164**, 5605–5614.

52. Kosco-Vilbois, M. H., Zentgraf, H., Gerdes, J. and Bonnefoy, J. H., *Immunol. Today*, 1997, **18**, 225–230.
53. Pulendran, B., Kannourakis, S., Nouri, R., Smith, K. G. C. and Nossal, G. J. V., *Nature*, 1995, **375**, 331–334.
54. Shokat, K. M. and Goodnow, C. C., *Nature*, 1995, **375**, 334–337.
55. Roost, H. P., Bachmann, M. F., Haag, A., Karlinke, U., Pliska, V., Hengartner, H. and Zinkernagel, H. M., *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 1257–1261.

ACKNOWLEDGEMENTS. The work done in our laboratory is the collective output of co-workers too numerous to list, but whose names will be found in the list of references. I am indebted to them for their contributions. In addition, many of our own ideas have crystallized through discussions with colleagues like Dr Dinakar Salunke, Dr Satyajit Rath (both from the National Institute of Immunology) and Dr G. C. Mishra (National Centre for Cell Science). My thanks are also due to them.

Received 19 December 2000; accepted 4 January 2001

Trends in basic immunology research – 2001 and beyond

Dipankar Nandi* and Apurva Sarin#

*Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India
#National Centre of Biological Sciences, GKVK Campus, Bangalore 560 065, India

The immune system is primarily involved in protection against pathogens and opportunistic organisms. Similar to a nation's defence organization, the immune system involves different components. This diversity allows the immune system to defend against different types of attacks by microbes. The past century has witnessed tremendous progress in understanding the components and mechanisms involved in the immune response. This article attempts to highlight areas of active research in basic immunology in the coming years.

THE mechanisms by which vertebrates have devised survival strategies to protect themselves from pathogens and opportunistic organisms constitute the subject of immunology and the past century has witnessed tremendous strides in the growth and establishment of this field[1,2]. This transition from an 'esoteric' science of undefined factors and mechanisms to 'mainstream' science has been due to the development of defined reagents: monoclonal antibodies, cDNAs and the use of genetically modified mice harbouring targeted mutations in immune function-related genes. Although it is hazardous to predict the future, an attempt is made here to list some of growing areas of immunology in the coming years (Box 1). The knowledge obtained in these basic immunological pursuits finds applications in several other areas of applied immunology and disease pathogenesis; however, not all aspects have been addressed in this article due to limitations of space.

The two main arms of the immune response are innate and adaptive (Figure 1). The innate arm is evolutionarily conserved, acts early and constitutes the first line of defence. The adaptive arm is evolutionarily recent and the immune response is relatively delayed (compared to the innate system), to generate a specific response towards a particular antigen. This specificity is conferred by antigen-specific receptors on B cells and T cells. The past few decades have witnessed tremendous progress in under-

Box 1. Broad areas of active research in basic immunology

1. Studying the components and mechanisms involved in the innate immune response. Understanding the mechanisms by which the innate response modulates the adaptive immune response.
2. Events leading to activation, proliferation, death and homeostasis in lymphocytes (T cells, B cells and NK cells).
3. Mediators and mechanisms involved in the interaction of lymphoid and non-lymphoid cells under normal, pathogenic and aberrant (e.g. autoimmune, allergic, etc.) conditions.
4. Characterization of cells and mechanisms involved in immunological memory.
5. Genomics and the immune response: Genes involved in immune defects/modulation of immune responses and the role of genetic polymorphisms in the immune response, disease resistance and susceptibility.

*For correspondence. (e-mail: nandi@biochem.iisc.ernet.in)