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Diversification and Selection Mechanisms for the Production of Protein Repertoires

Lessons from the Immune System

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Abstract

The physiological mechanism for producing antigen-specific antibodies is based on a two-phase neo-Darwinian process: the first phase consists of diversity generation (formation of the repertoire), and the second phase is antigen-mediated selection. In this article, we consider how the natural immunoglobulin gene-diversification processes can be exploited both in vivo and in vitro in order to allow the generation of novel antibody (and heterologous protein) repertoires.

Index Entries: Rerarrangement; somatic hypermutation; antibody; immunoglobulin; transgenic mouse.

Introduction

Antibodies are produced in serum in response to antigen challenge. The natural process of specific antibody production is essentially Darwinian, comprising a cyclical alternation of diversification and selection. Thus, segmental rearrangement of the immunoglobulin V, D, and J gene segments leads to the creation of a primary repertoire of B lymphocytes, which carry a wide range of antigen specificities. Diversity at this stage is effected by both combinatorial mechanisms (caused by the diversity of

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potential assortments of immunoglobulin heavy and light chains as well as of V, D, and J gene segments) as well as by junctional mechanisms. Junctional diversity is achieved through nontemplated nucleotide insertion and deletion at the borders of the juxtaposed immunoglobulin V, D, and J gene segments. Antigen selects B cells from this primary repertoire that express a cognate antibody.

This primary repertoire is generally not large enough to contain high-affinity binders for all antigens, and so the immune system uses a multistage diversification/selection strategy in order to fashion high-affinity antibodies. The B cells from the primary repertoire that express an antibody that binds weakly to the antigen are selected by the antigen and then undergo a process of clonal expansion. During this clonal expansion, a hypermutation process occurs in which nucleotide substitutions are targeted to a small segment of DNA that includes the functionally rearranged immunoglobulin V-gene segments. This diversification process creates a secondary repertoire, and all its members derive from B cells that have been initially selected out of the primary repertoire on the basis of their (possibly weak) antigen binding. The mutations occasionally create an antibody with improved antigen binding, and such clones from the secondary B-cell repertoire are selected by antigen. Indeed, in the germinal centers where this clonal expansion takes place, hypermutation and antigen selection appear to occur in concert. This presumably allows multiple rounds of diversification and antigen selection—a cyclical process well-suited to this stepwise enrichment of high-affinity antibodies *(1)*.

It is interesting to consider that—apart from using two distinct strategies of antibody diversification (gene rearrangement in the primary repertoire; somatic hypermutation for the secondary repertoire)—this stepwise process of affinity maturation implies different stringencies of affinity selection at different stages (*see* Fig. 1). Thus, in selecting the initial antigen-binding specificities from the primary repertoire, it is necessary for the immune system to distinguish a capacity for antigen binding in the relatively low-affinity range. If the affinity threshold for selection is set too high, holes in the immune repertoire will presumably occur; if this threshold is set too low, problems of autoimmunity can be anticipated. In contrast, the problem of selecting high-affinity antibodies during the germinal-center reaction, the challenge must be to discriminate the excellent binders from those that simply bind the antigen very well. Therefore, selection occurs in a higher affinity range.

Results and Discussion

Production of Human Antibodies

The natural immune system has evolved in order to produce antigen-specific antibodies, and seems to do the job pretty well. The object of antibody engineering, however, is to produce antibodies with different attributes than those that can be readily isolated naturally. For example,

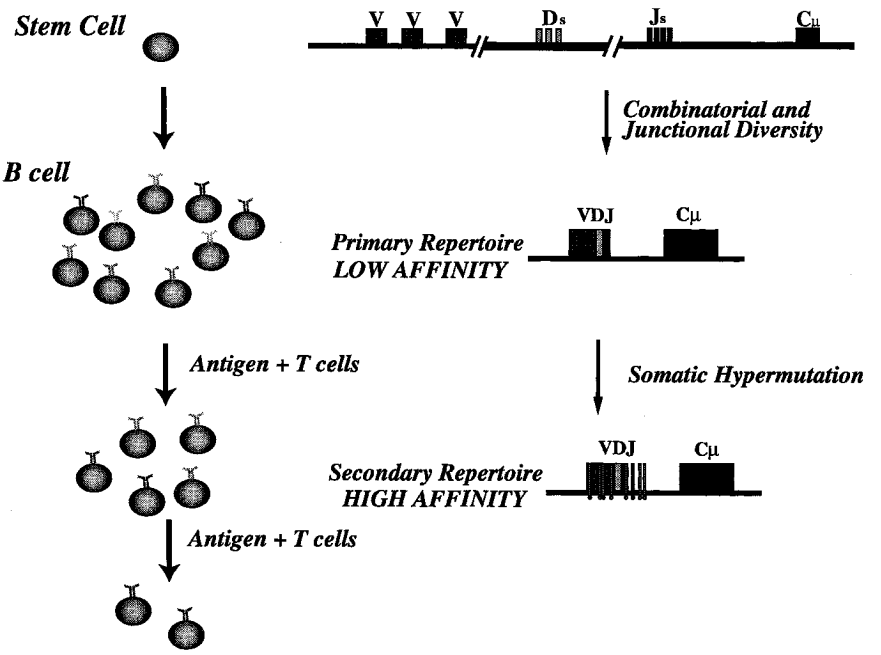

Fig. 1. The in vivo strategy for making high-affinity antibodies. There are two distinct stages of repertoire generation. The primary antibody repertoire is formed by gene rearrangement. Interaction with antigen (which can occur in the low-affinity range) leads—with T-cell help—to B-cell expansion and the initiation of somatic hypermutation—the second stage of repertoire generation. Antigen selection of specificities from this secondary repertoire occurs in the high-affinity range.

since the development of mouse monoclonal antibodies, there has been interest in isolating human antigen-specific monoclonal antibodies because of their potential application for human therapy. While many strategies can be employed in order to produce large amounts of the monoclonal antibodies expressed by individual human B cells, the isolation of human B cells that express a high-affinity antibody for a particular antigen can often be a difficult and challenging undertaking. Appropriate in vivo immunization is often not practicable, and in vitro selection of high-affinity binders without prior immunization is often feasible.

Case-by-Case Humanization

Major success in producing antigen-specific human monoclonal antibodies was achieved using case-by-case humanization of mouse monoclonal antibodies. Thus, in the initial exemplifications *(2–4)*, the rearranged V domains of antigen-specific mouse monoclonal antibodies were linked to human-constant regions, thereby creating chimeric antibodies that were largely of human origin but which retained the antigen specificity conferred by the mouse V region. The subsequent development of CDR-grafting strategies enabled the creation of antibodies that contain even less

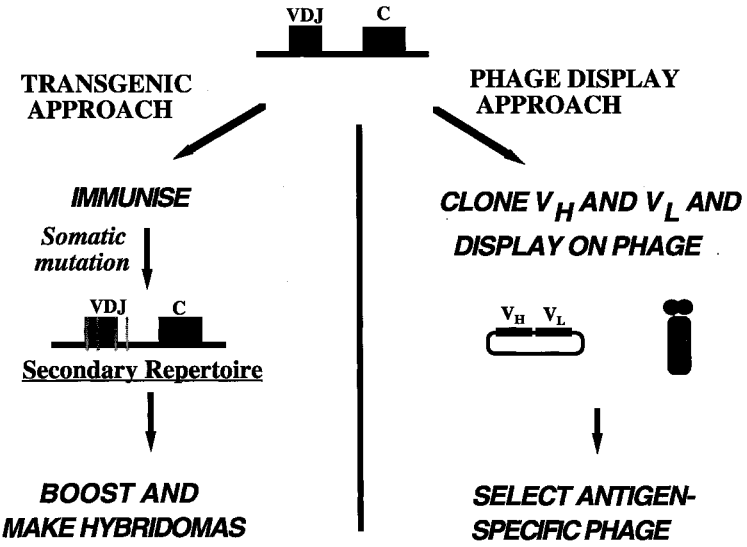

Fig. 2. Comparison of transgenic and phage display approaches to the preparation of high-affinity monoclonal antibodies. In the examples shown, both exploit a primary human antibody repertoire generated in vivo (in humans in the case of phage display; in transgenic mice carrying human immunoglobulin transloci in the transgenic approach). High-affinity specificites in the transgenic approach are isolated following traditional immunization and hybridoma preparation from the translocus mice. In the phage display approach, high-affinity specificites are selected in vitro from a phage library established by cloning and scrambling the V_H and V_L genes of peripheral blood lymphocytes.

nonhuman sequences, with both the antibody-constant regions and V-gene framework of human origin, and only the V-region CDRs (which are usually largely responsible for conferring antigen specificity) of mouse origin *(5)*.

Artificial Antibody Repertoires

While such case-by-case humanization of mouse monoclonal antibodies has been largely successful in making designer human monoclonal antibodies, it is a difficult undertaking. Therefore, over the last 10 years, much attention has been given to the possibility of creating repertoires of human monoclonal antibodies from which antigen is used to select cognate specificities *(6)*. To circumvent the problem that antigen selection against a wide range of specificities cannot be performed in vivo in man, two distinct major approaches have been followed (*see* Fig. 2).

The Phage Display Approach

In the first approach, phage display technology is used to exhibit a wide range of antigen-binding specificities obtained by PCR amplification of the expressed immunoglobulin V genes from human peripheral blood

lymphocytes. In vitro antigen selection is then used to isolate phage display a cognate specificity. Although multiple rounds of antigen selection can be performed, in most instances of this approach, there is only one round of immunoglobulin gene diversification. This diversification will have occurred in vivo and is used to provide the original V-gene repertoire that was sampled in the creation of the phage libraries. So a good antigen binder will only be obtained if it was originally present in the scrambled V_H/V_L repertoire of peripheral blood lymphocytes. In order to compensate for the lack of iterative cycling of diversification and selection, the phage display approach instead exploits an artificially large initial repertoire. By engineering such artificially large repertoires in vitro (e.g., by scrambling V_H and V_L combination or by using in vitro DNA synthesis to introduce additional V-gene diversity, for instance, in CDR3) it has been possible to create phage libraries with larger repertoires than those routinely available in the in vivo primary repertoire *(7,8)*.

While multiple rounds of diversification are not a routine part of the phage antibody strategy, it is of course not necessary for diversification to be restricted to a single round. It is now possible up to selecting an initial binder to engineer additional diversity. For example, having selected a good V_H, it is possible to combine it with a whole library of the V_Ls and screen from a secondary library to obtain affinity maturation *(7,8)*. However, such cycling of diversification and selection are not an integral feature of the phage selection strategy.

The Transgenic Approach

The alternative repertoire approach to human monoclonal antibody isolation has been provided by translocus mice *(9)*. Such mice carry human immunoglobulin gene loci in their germline configuration, introduced into the mouse germline. The immunoglobulin V, D, and J gene segments of the human heavy- and light-chain loci can then undergo productive rearrangement in mouse lymphocytes (in an analogous manner to that of their mouse counterparts), thus yielding a repertoire of mouse lymphocytes that express human antibodies. Indeed, if the mice in question also carry targeted gene disruptions of their own endogenous immunoglobulin gene loci, the result is a mouse strain whose B cells are typical except for the fact that instead of displaying mouse immunoglobulins on their surface, they display human immunoglobulins *(10)*. Such transgenic mouse strains can be challenged with a range of antigens, and will go on to produce human (instead of mouse) antibodies in response to the antigen challenge.

This strategy involves introducing relatively large chunks of DNA into the mouse germline so that the mice are capable of making a wide diversity of V-D-J integrations. The loci must also be large enough to include all the necessary *cis*-acting DNA elements responsible for hypermutation recruitment, to allow efficient diversification for production of the secondary B-cell repertoire. Success has been achieved in this endeavor, and

several mouse strains are available that can be used for the production of high-affinity human antibodies following immunization *(11,12)*.

The advantage of this transgenic approach is that it mimics the natural process of antibody production. Thus it allows the in vivo strategy of cycling the diversification and selection phases to be exploited. It is also useful that, once the effort has been made to create suitable translocus mouse strains, conventional immunization and hybridoma technology can be used to isolate the desired antibodies. Conversely, phage display technology may offer the advantage of greater speed, may prove easier for the parallel isolation of a multitude of specificities, and also avoids the use of animals. To combine the best of both systems, a modified phage display type approach should be developed—but one in which repeated iterative rounds of diversification in vitro can readily be carried out, thus allowing a strategy of alternation of diversification and selection to facilitate affinity maturation.

Use of Hypermutating Cell Lines

A major barrier to performing an alternating cyclical strategy of diversification/selection in vitro (in an attempt to parody the in vivo diversification selection process) is largely confounded by the lack of availability of cell lines that have been shown to perform localized immunoglobulin gene hypermutation during culture. Recently, however, we have identified a human (lymphoma cell line) that mutates its immunoglobulin V genes constitutively during in vitro culture. This localized hypermutation shows all the classic features of in vivo antibody hypermutation—a very high mutation frequency (around 10^{-4} per basepair per generation), preferential targeting to the immunoglobulin V domain, a preference for nucleotide substitutions as opposed to other mutations, a transition bias, preferential mutational targeting to characteristic hotspots, etc. *(13)*. The main difference is that hypermutation in this lymphoma line shows a greater preference for targeting for G and C residues than in vivo hypermutation in humans. Such a cell line could therefore prove very useful for creating diversified antibody repertoires in vitro and coupling this diversification to selection.

For example, the nucleotide substitutions introduced by the hypermutation process into the lymphoma's expressed immunoglobulin V-gene domains occasionally leads to V-gene inactivation (e.g., through stop codon creation). This generates lymphoma variants that lack surface immunoglobulin expression. Such variants can be readily purified by flow cytometry (*see* Fig. 3). Thus hypermutation of the expressed V domain that occurs during in vitro culture leads to phenotypically selectable alterations in the expressed antibody. Cell lines of this type could therefore prove useful for the in vitro selection of high-affinity antibodies. Clearly, the approach is also capable of further extension.

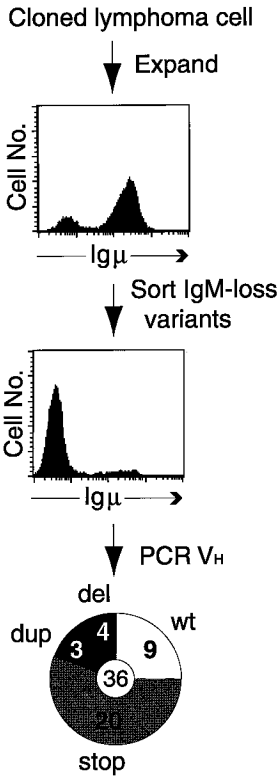

Fig. 3. Constitutive hypermutation in a clonal lymphoma line allows isolation of variants expressing mutated antibodies exhibiting desired characteristics. The figure shows how a clonal lymphoma line can be expanded in culture and give rise to IgM-loss variants that can be purified by flow cytometry; these variants contain a range of intactivating mutations (dup, duplications; del, deletions; stop, stop codons; wt, wild type V_H, unmutated) within the V-gene segment. The approach can be extrapolated to other immunoglobulin mutations.

Using Immunoglobulin Gene-Diversification Strategies to Create Heterologous Repertoires

Immunoglobulin gene rearrangement is catalyzed by the RAG1 and RAG2 proteins: the gene segments that constitute the DNA substrate need to be in accessible chromatin and be flanked by short (heptamer and nonamer) signal sequences that do not become included within the final, rearranged immunoglobulin VDJ gene segment. This rearrangement process therefore readily lends itself to catalyzing rearrangements of artificial DNA substrates that have been engineered to contain the necessary short rearrangement signal sequences. Artificial repertoires based on non-immunoglobulin gene elements could therefore easily be assembled by introducing a suitably constructed DNA minilocus substrate into a trans-

genic mouse or a progenitor lymphoid cell line that performs V-(D)-J integration.

Similarly, transgenic experiments have revealed that the somatic hypermutation mechanism (normally responsible for production of the secondary antibody repertoire) can be targeted to heterologous sequences using suitably constructed transgenes, and thereby create mutated repertoires derived from any desired starting sequence *(14,15)*. Thus, there may well be an exciting future in exploiting the antibody diversification mechanisms (both rearrangement and hypermutation) either in vivo or in cell-lines in order to generate selectable repertoires based on nonimmunoglobulin structures.

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Discussion

Paul: Perhaps it is technologically reasonable to compare in vivo models of somatic hypermutation to phage display methods. But phage display cannot impose the poorly understood selection pressures responsible for affinity maturation in vivo. Understanding the mechanisms of somatic hypermutation and linking this penomenon to B-cell clonal maturation is one of the major barriers in immunology, and it is somewhat surprising that this area is not more intensely studied. My question is: In tumor cell lines with hypermutable antibody genes, survival of the cells is unlinked to antigen binding. Is it then possible to figure out which signals are required

by the B-cell receptor to achieve clonal expansion (i.e., the signals linking the survival of the cells with the antigen-binding event.)

Neuberger: I think the cell lines might be quite good for looking at the mechanisms of the somatic hypermutation per se. I think we cannot look at the molecular basis of antigen selection in this cell-line model. That is not done by the B cell on its own. T-cell help is required, and there are many other available systems to look at that phenomenon. Several studies have been done in which we take a lysozyme-specific B-cell or B-cell transfectant and mix it with T cells, and we try to understand the molecular basis of the selection. In the case of the tumor cells, we don't even know the antigen. It is better to answer your question in the whole mouse because the T cells are available.

Paul: But an in vitro model would be enormously helpful. Can we go back to a progenitor cell that might have intact responses to various selection pressures?

Neuberger: To me the great question with selection is: there are two stages at which selection occurs from the repertoire in the mouse. There is the primary repertoire. These immature B cells are sitting around, and you probably select from an initial antigen-binding affinity of less than 10^5. Therefore, a very clever and controlled selection process is needed to select B cells over the background. On the other hand, how the immune system says that a 10^{10} affinity is better than a 10^9 is an unanswered question, and may involve competing processes. One process could be signaling through the BCR and crosslinking the receptors. Whether internalization and trafficking of the antigen—or how long the antigen stays bound to the internalized receptor—play any role in the development of high affinity is also unanswered. Another major factor in the selection for high affinity in the germinal center is the way antigen presentation occurs. I don't think we know actually—even on that basic level—how these processes control selection.

Kohler: What significance do you place on receptor editing and secondary VDJ joining? Have you observed these in your culture system or in your animal model?

Neuberger: For those of you who haven't followed the field of receptor editing, this phenomenon is studied in mice which carry a single immunoglobulin specificity—i.e., all the B cells are specific for a single antigen. The mice begin to hate this state of affairs. The immune system is evolved to diversify, and it does anything in its power to diversify its repertoire. So, by putting a single immunoglobulin locus in the mice, you discover all sorts of processes that might be normal processes that you never knew about before. Receptor editing is the way the mice get rid of the original VDJ sequence so as to try and get another one. From the data I have seen from the transgenic mouse models, I am pretty convinced that a lot of receptor editing goes on in the primary repertoire. For example, in the

bone marrow, you do see VDJ joining, which generates weakly autoreactive specificity, and then that specificity is edited out. This is not just some weird artifact of transgenic mice. The other question was whether receptor editing happens in a germinal center, where the binder is selected and where receptor editing might be a way to start the selection from scratch. Whether that process is going on physiologically in a major way is totally open. It could just be a transgene artifact. To answer your question about the cell-line model, receptor editing does not happen in these cells. The cells hardly express the RAG recombinase gene.

Kohler: But Kelsoe has observed receptor editing in germinal centers.

Neuberger: Yes, but the experiment is done by imposing a transgenome, which still leaves open the question of physiological occurrence.

Kohler: Do you think a special signal is needed to get receptor editing going?

Neuberger: When you start making a monoclonal mouse, there will be all sorts of processes that can happen. I am not questioning whether it can't happen—I'm questioning whether it happens. If receptor editing makes a major contribution to the repertoire, you'd expect to find two B cells in the same person with related V_Hs, but that can have either kappa or lambda partners. I have not seen evidence to that effect. I am still skeptical as to whether receptor editing makes a major contribution.

Fitzgerald: I was curious that you emphasized the difference between the transgenic system and phage system. On the issue of continuous diversification and selection in the mouse, given that continuous rounds of mutation and reselection of phage particles are possible, it would seem the phage system is a good mimic.

Neuberger: The phage display system is not a generic or easy strategy. You clone, then you diversify, and then you select, and it's laborious. Of course, you can do that again and again and you can use sophisticated tricks to make diversified CDRs. Clearly, the phage system is useful to make human antibodies of value. There is no question you can get great binders out of phage display.

Paul: Phage display, unfortunately, does not account for selection factors other than antigen binding. On the other hand, transgene technology is not easy, either.