Tech News

Antibodies 2.0

In March 2011, the US Food and Drug Administration approved a new drug for the treatment of systemic lupus erythematosus. Developed by Human Genome Sciences and GlaxoSmithKline, Benlysta targets a protein called BLyS and is, as *Nature News* pointed out, the first new drug for Lupus “in half a century.”

Benlysta's generic name, belimumab, bears the stem *-mab*, identifying it as a monoclonal antibody similar to Herceptin (trastuzumab), Rituxan (rituximab), and Remicade (infliximab). But Benlysta is different from those other therapeutics: Its variable region was created not by immunizing a laboratory animal, but in a test tube.

Benlysta was born via a process called phage display. Antibody phage display and related in vitro technologies such as yeast display and ribosome display use vast, more or less random libraries of immunoglobulin variable regions to identify molecular binders to specific antigens. Developed in 1990 and 1991 by groups at the MRC Center for Protein Engineering in Cambridge, UK, The Scripps Research Institute in La Jolla, Calif., and in Heidelberg, Germany, the technique has for two decades been used mostly to drive therapeutics development. Now, though, loosening intellectual property entanglements are freeing the antibody development community to embrace the technology as never before. European and American funding agencies have established projects to build high-throughput pipelines for the creation of in vitro antibodies, and plans are in the works to develop antibodies for the entire human proteome.

As Stefan Dübel of the Technische Universität Braunschweig, a member of the Heidelberg team, succinctly puts it, “They are now absolutely ready for prime time.”

Phage Display 101

Phage display is a technique so simple in concept, it's genius. According to John McCafferty, Director of Research at the University of Cambridge and lead author on the first phage display article from the Cambridge team in 1990, the general idea is to establish a screening system in which each antibody in the screening library ferries around its own genetic code.

“That's really what display technologies are all about,” he says, “this joining a gene and the product of that gene in a way where you can use the binding properties [of the antibody] to help you get to the gene.”

The inspiration came from a paper by George Smith at the University of Missouri, Columbia. At the time, McCafferty and his colleagues in Greg Winter's lab were interested in human antibody therapeutics, but the process was long — generating a mouse monoclonal, which takes months in itself, was bad enough, but then “humanizing” it piece by laborious piece was painfully slow. In 1988, Smith showed he could modify filamentous bacterial viruses, or bacteriophage, with a piece of the beta-galactosidase gene, which would then be displayed on the virus coat as a fusion with a minor coat protein. He used antibodies to beta-galactosidase in a “biopanning” experiment

to recover beta-galactosidase-expressing phage, and speculated the approach could be used more generally to clone genes for which the protein was on hand but not the DNA sequence.

“We looked at that and thought, wouldn't it be cool if you could turn that all the way around, [and] have the antibody on the phage and have the antibody recognizing targets?” recalls McCafferty.

So, he did. First, McCafferty demonstrated a single lysozyme-targeting antibody could be expressed on the phage surface. But it wasn't any standard immunoglobulin like those found in serum; it was a kind of ‘Franken-body’, a chimeric molecule in which the variable light chain and variable heavy chain portions of the standard immunoglobulin were tied together using an artificial linker. These constructs, called “single-chain Fvs,” or scFvs, are easier to manipulate and express in bacteria than intact immunoglobulins, and work just as well: In the presence of a million-fold excess of non-specific phage, the team was able to pull out viruses specific for lysozyme. (An alternative antibody format for display technologies is the Fab fragment, one arm of an intact Y-shaped immunoglobulin molecule.)

The following year, James Marks, with McCafferty, Winter, and colleagues, took the study to its logical conclusion, replacing the anti-lysozyme fragments with a random library of human antibody pieces from which binders to any antigen could, in theory, be pulled, and the modern phage display process was born.

Yeast surface display. The minimal binding subunit of the full IgG, or a single-chain antibody is expressed in yeast as a fusion to the yeast mating agglutinin Aga2p. Self assembly with mating agglutinin Aga1p yields about 50-100k copies of surface displayed scFv per yeast cell, with each cell expressing a single antibody clone. Credit: Eric Shusta

The Power of Display

In vitro strategies are not exclusively driven by phage display; other options include ribosome display, in which an antibody-encoding mRNA is tethered via a ribosome to the antibody it encodes, and yeast display, which expresses antibody libraries on the surface of yeast.

Typically, yeast libraries are less diverse than phage libraries, a consequence of the number of transformants the organism can produce. Yet, according to Eric Shusta, associate professor of chemical and biological engineering at the University of Wisconsin, who uses yeast display, the strategy does have its advantages.

Yeast display experiments are scored via flow cytometry, by mixing the yeast population with fluorescently labeled antigens. As a result, the approach quantitatively scores every clone, whereas phage display screens often rely on qualitative or semi-quantitative validation of smaller numbers of clones, typically by ELISA. In addition, because a flow cytometer can be tuned to select only, say, the top 5% of binders in a population, experiments can be designed to identify not just binders in general, but the highest quality binders — those that can bind antigen at low concentrations. As a result, some researchers have begun blending the two techniques, using the larger size phage libraries for initial panning rounds, then yeast display for more efficient screens.

“What’s really nice is the ability to do quantitative discrimination of antibody binding parameters directly in line with the screen,” says Shusta, who has developed an approach in his own lab to probe his yeast display libraries with detergent-solubilized cell extracts in order to find membrane protein binders, such as components of the blood-brain barrier that typically challenge phage-based strategies.

Of course, whichever strategy is used, all in vitro display methods offer advantages over traditional antibody methods. Polyclonal antibody preparations are easy enough to make, but the resulting sera are ill-defined, and more significantly, finite: Once that antibody preparation is gone, it’s gone; the only way to make more is to inject another animal, which may or may not provide a serum of equivalent quality.

Monoclonal antibodies, generated through hybridoma technology, are renewable. But the process is time consuming and laborious, taking months to complete. In contrast, in vitro screening can be done in a week or so, whittling libraries containing as many as 100 billion virus particles down to a handful of candidate binders.

Yet in vitro display technologies aren’t simply more convenient than hybridoma technology — they can yield antibodies that cannot be generated any other way, such as antibodies to proteins that are exceptionally well conserved between mouse and man.

In vitro display also enables selection strategies that would be difficult to pull off in immunized animals. For instance, according to Karen Colwill, a staff scientist at the Samuel Lunenfeld Research Institute, researchers could use phage display to generate antibodies specific for a protein in its complexed state, but not its uncomplexed one, a screen that would be nearly impossible via immunization because once the complex is injected into the animal, it would be processed into peptides and fall apart.

Another advantage: the coupling of antibody and gene simplifies downstream genetic manipulations, such as applying protein tags, reassembling intact immunoglobulin genes, and “affinity maturation” — shuffling the genetic deck to boost an antibody’s binding kinetics. In one such study, Dübel and his team tuned an scFv antibody to MUC1, a protein overexpressed in breast and ovarian tumors, by amplifying the gene using “error-prone PCR.” By sifting the resulting libraries through additional rounds of selection, the researchers created an antibody whose affinity was more than 500-times stronger than what they started with.

Finally, in this age of low-cost synthetic genes, in vitro approaches offer one additional advantage: the ability to distribute sequences rather than physical antibody preparations. “We can send it out in digital form,” says Susanne Gräslund of the Structural Genomics

Consortium. “People could synthesize the antibodies themselves in their own labs.”

What’s in your Library?

Current phage display libraries may contain on the order of 10^{10} different antibodies. Yet, designed to recapitulate the human immune system, even at that scale they still are but a shadow of one. Many researchers strive to create a single all-encompassing library for every target but, according to Jaume Pons, chief scientific officer at antibody therapeutics firm Rinat (part of Pfizer), the human immune system essentially builds a new library every time it encounters a new antigen. “B cells have short lives,” Pons says. “So over your life you’re creating new libraries all the time.”

That’s the strategy Pons’ team mimics for its in-house therapeutics development. One common phage display library design is that used by McCafferty, who took the variable heavy and light chain genes from 50 individuals and randomized them in an scFv library. Pons’ team, too, started with “naïve” libraries representing some 650 patients. But by deep-sequencing the library before and after each panning stage with different antigens, they have learned how the library morphs during selection with different classes of molecules, information like optimal heavy-light chain pairings and “complementary determining region” (CDR) configurations that they then plug back into their next-generation libraries.

Dev Sidhu, associate professor of molecular genetics at the University of Toronto, takes a different tack. Antibodies, Sidhu explains, are not all alike biochemically — they have different stabil-

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ities, reactivities, and expression levels. That can make certain high-throughput applications like antibody arrays, “a nightmare,” because each molecule flexes to the beat of its own thermodynamic drummer. “You cannot really work with them rapidly in parallel.”

So Sidhu, who previously worked at therapeutic antibody pharmaceutical company Genentech, has developed an alternative strategy for library generation: using a single, highly stable and expressed antigen framework and randomizing it with degenerate sequences in the variable CDRs.

Sidhu’s recently published “library D” has produced “several hundred” antibodies many with nanomolar affinities, he says, and the next-generation library, currently in development, has yielded “hundreds more.” Yet Sidhu concedes the process of library optimization is “boring,” focusing on minutiae like the precise distribution of amino acids that can be present at any given position, and the length of each CDR. He compares the work to tweaking a Ferrari; “You know 10 years later it will be much better, but you can’t say this was the big change that made it better.”

Sequencing for Antibodies

When it comes to finally identifying the right antibody, it’s all about the screening. Whether via phage display or hybridomas, antibody selection strategies all require building large libraries of potential binders and then screening for binders. But there is another way. In 2010 Sai Reddy was a postdoc with George Georgiou at the University of Texas, Austin. Georgiou’s lab develops monoclonal antibodies. But for this study, rather than slogging through the drudgery of immunizing animals, fusing hybridomas, and cellular cloning, Reddy and Georgiou decided to take advantage of a well-known feature of the mammalian immune system. “Right after you challenge that mouse with an immunization, they will be producing more antigen-specific antibodies than any other kind of antibody,” Reddy notes.

They injected pairs of animals with any of three separate test antigens, waited a week, collected terminally differentiated B cells called plasma cells, and sequenced their mRNAs, actually counting the different variable region genes. Some 100 million base pairs were sequenced at a cost of about \$10,000, says Reddy, now an assistant professor at the University of Colorado, Boulder. Some sequences represented a huge fraction of the total expressed immune repertoire — up to 10% in some cases. When they then synthesized these

Antibodies are not all alike, says Dev Sidhu. His strategy: Keep the backbone constant and make subtle changes to the variable regions. Credit: Dev Sidhu

most abundant genes and tested them, nearly 80% recognized the antigen.

It took less than a month, Reddy says, from animal sacrifice to antigen-specific antibodies, a significant time savings when compared to six months or more for the traditional screening route. And an industrial lab, he speculates, could probably pull it off in about two weeks.

Toward a Proteome-Scale Binder Resource

As fast as Reddy’s approach might be, it still isn’t nearly fast enough to tackle the proteome en masse. Many researchers study well characterized proteins for which high quality affinity reagents already exist. Yet the majority of human proteins have no available reagents at all. In a *Nature* editorial earlier this year, Aled Edwards of the University of Toronto, and colleagues surveyed the biomedical literature to show that research is skewed towards those genes for which antibodies or chemical inhibitors exist, leaving the bulk of genes understudied.

“Much of the work that has emerged from exploring the human genome over the past ten years lies fallow,” Edwards wrote. “Challenges notwithstanding, making protein-based research tools readily available must be a major objective in the decade to come.”

Work is already progressing on this front. The latest version (version 8) of Mathias Uhlen’s Human Protein Atlas (proteinatlas.org), for instance, includes some 14,500 antibodies covering more than 11,250 human genes. But those are polyclonals, a non-renewable resource.

This past July Colwill, Gräslund, and a collection of researchers calling themselves the “Renewable Protein Binder Working

Group,” published a study in which they challenged researchers to generate antibodies to 20 soluble SH2 domain proteins. The report, entitled “A roadmap to generate renewable protein binders to the human proteome,” details how five groups, including Sidhu’s, Dübel’s, and McCafferty’s, produced some 1,040 antibodies, 340 of them unique.

Put through their paces via surface plasmon resonance, immunoblot assays, immunofluorescence, and ultimately immunoprecipitation, these antibodies fared well, according to Colwill, with both hybridoma and display-based approaches producing high quality reagents. “They all had reasonable successes,” Colwill says, “there wasn’t a clear winner.”

At the same time, though, it’s obvious a proteome-scale resource requires in vitro technology, says Dübel, who delivered 362 antibodies of his own in about two months; if nothing else, making 100,000 monoclonals would require millions of animals, he says.

The SGC, says Gräslund, has already begun building on this pilot study with a new effort to develop in vitro antibodies to some 300 epigenetics-related proteins. And proteome-scale efforts are in the works, both in the US and Europe. EU-sponsored programs like the AffinityProteome and Affinomics, Dübel says, are already working to produce a “couple hundred to a couple thousand proteins.” This past December, the NIH issued RFA-RM-10-017, a \$20 million call for applications for “centers that can produce renewable, high quality affinity reagents against all human transcription factors.” From there, the proteome itself is just a matter of scale.

Ultimately, says Dübel, researchers may be able to sift through a whole-proteome antibody catalog, representing not just every one of the 25,000 or so human genes, but alternative splice variants, modified forms, and so on. With existing technology, he estimates the job could be completed for perhaps \$50 million to \$100 million.

“The method is there,” he says. “You just have to pay for it.”

Written by Jeffrey M. Perkel.

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