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Superantibodies

Synergy of Innate and Acquired Immunity

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Abstract

The antibody molecule possesses a number of so-called unconventional binding sites in the variable domain that are expressed and function independently from the antigen-binding site. These sites are encoded in the germline, predominantly in framework residues. By this definition, these sites function as part of the innate immunity, and are not subject to antigen-driven mutation and maturation. In this article, we focus on the evidence for the function and utility of the self-binding domain. The self-binding or autophilic domain has been discovered on murine germline-encoded antibodies from the S107/T15 Vh family. Autophilic antibodies form self-complexes after attaching to targets, but remain monomeric in solution. A peptide has been identified that confers self-binding if chemically attached to antibodies. Because this modification enhances the overall avidity of antibodies for target binding, therapeutic and diagnostic antibodies can be biotechnologically improved.

The concept of superantibodies is introduced here to describe the unique coexistence and synergism of acquired immunity with innate immunity via antigen-specific and unconventional functional domains. As not every antibody qualifies as a superantibody, biotechnology engineering can produce superantibodies with superior targeting and therapeutic properties.

Index Entries: Antibody; variable domain; unconventional binding site; autophilic binding; superantibody.

The Antibody Molecule

The elucidation of the amino-acid sequence of immunoglobulin has made a major contribution to the understanding of protein structures. Pioneering work by the laboratories of Edelman *(1)*, Porter *(2)*, Hilschmann *(3)*, and Putnam *(4)* on the sequence analysis of myeloma and Bence–Jones proteins has developed the concept that proteins can be divided into structural domains. These domain-sequence regions were discovered through the striking sequence similarity within the variable and constant antibody regions, and by the ability of enzymes to cleave the immunoglobulin into

distinct fragments with different biological properties. Subsequently, the model developed by sequence data was confirmed with crystallographic data on antibody fragments and complete molecules. From these studies, our current knowledge of the structure and function of antibodies has emerged.

The antibody molecule consists of polypeptide chains of two different lengths—the heavy and light chain—which are linked by disulfide bonds. Together they form a tetrameric structure. Each chain is divided into a variable and constant domain. The variable domain sequence differs from antibody to antibody, and determines the antigen specificity. The constant domain is subdivided into three or four domains, which mediate general biological effector functions, such as complement fixation, phagocytosis, or transport across cellular membranes. The variable domain of both chains can be further subdivided into so-called complementarity-determining regions (CDR) and framework regions (FR) *(5)*. In the three-dimensional immunoglobulin model *(6)* the CDR appear as loops protruding from the compact beta-sheet structure made up from the framework sequence. There are four FR and three CDR regions in each chain. The CDRs form the binding site for antigens and are involved in multiple molecular contacts with antigen. The antigen specificity resides in the sequence of CDR that are unique and different in each antibody. Similarities and structural homologies among FR sequences reveal the heritage of variable-domain genes from so-called V-gene families. The size of these gene families varies among species from 2–100 genes.

The Nonacquired V-Domain Functions

From the structural organization of the antibody molecule and particularly the variable domains, the genetic history, or evolution, can be deduced. Variability and adaptation for antigen-binding evolves by a process of mutation and selection during the lifetime. On the other hand, biological functions common to groups of antibodies are inherited and have evolved over evolutionary time. Figure 1 schematically depicts the relationship between the genetic origin and the different parts of the V domain. It is important to distinguish the V-domain-associated biological functions from functions residing in the constant Fc domain. Furthermore, as will be explained later, the V-domain innate-immune functions and sites are optional for the antibody molecule, meaning that some antibodies do not contain an innate immune site or function.

Example of Innate V-domain Function: Autophilic Antibodies

Several innate biological antibody functions located in the V domains have been discovered. The best-understood is the binding site for protein A, which is expressed by a majority of Vh3 family structures in the V domain *(7)*. Binding of certain bacterial proteins to B cells via the V-domain site

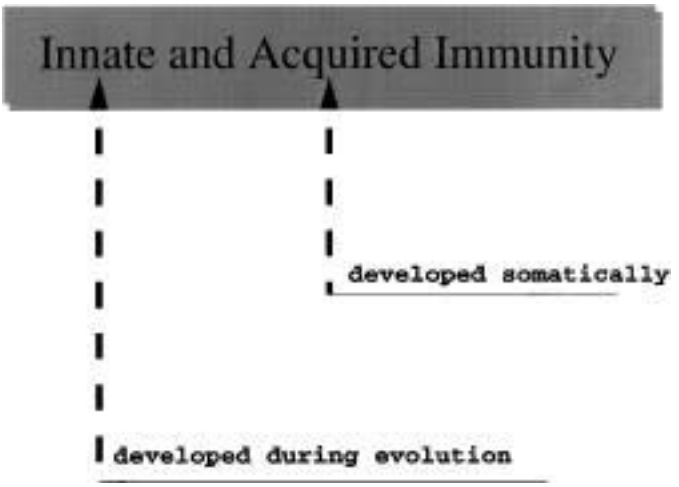

Fig. 1. Evolutionary origin of innate and acquired immunity. Innate immunity mediated by the antibody molecule is not subject to mutational selection during the immune response, while acquired immunity improves during the immune process by mechanism of mutation and clonal selection.

can stimulate B cells to divide *(8)*. These proteins have been called B-cell superantigens, analogous to T-cell superantigens. The stimulation is unrelated to antigen specificity, and does not involve the classical antigen-contacting CDR loops.

Other so-called unconventional binding sites in the V domain are the nucleotide-binding site *(9)* and the self-binding site *(10)*. The most interesting activity sometimes associated with antigen binding is catalytic activity *(11)*. In Table I, these sites and functions are summarized.

Besides V-domain sites with a specified single biological function, other marker sites located in the framework regions have a much broader and more complex biological meaning. These are the so-called shared or public idiotypic binding sites recognized by cross-reactive antiidiotypic antibodies. These public idiotypes are typically expressed on antibodies found in autoimmune diseases or chronic infections. Their biological function is poorly defined: they have been proposed to be involved in network regulation of the immune response *(12)*.

The main focus of this article is self-binding or autophilic antibodies as they occur naturally or as engineered antibodies.

The phenomenon of self-binding antibodies involving variable-domain regions was discovered with a monoclonal murine antibody from the S107/T15 Vh family *(13)*. This type of self-binding is different from Fc–Fc mediated aggregation seen with antibodies from certain isotypes *(14)*.

The structural requirement for the observed self-binding was delineated by immunochemical and structural studies *(15)*. Fab fragments—but not free H or L chains of T15—were potent inhibitors of self-binding

Table 1
Unconventional (Innate) Immune Sites

Site	Location	Function	Expression
Protein A Binding Site	FR1 and CDR3 Vh	B-cell stimulator	Vh3 IgM
Nucleotide Binding Site	Fr1-V1 and FR4-Vh	unknown	all Igs
Catalytic site	invariant residues in CDR1 V1	proteolysis	rare on light-chain dimers
Shared Idiotype	framework residues	network regulation	autoantibodies/ antimicrobial
Antophilic Binding Site	Fr2-V1 and FR4-Vh	induces self-binding	rare anticarbohydrate

antibodies, indicating that intact V-domain structures are required for self-binding.

Self-binding is highly dependent on the polymeric Ig structures *(13)*, as monomeric T15 only shows marginal self-binding, dimeric T15 more binding, and pentameric IgM 11E7 the strongest self-binding potential at a molar comparison.

The sequences of S107 and MOPC 603—both self-binding antibodies with different strengths—were examined for hydropathic complementarities. Two regions in the Vh domain revealed strong hydropathic complementarities: Vh50-60 was complementary to Vh63-74 *(10)*. Differences in the hydropathic scores of T15 and M603 complementarities are in agreement with the differences in self-binding strength. A 24-mer peptide was synthesized to cover the sequence Vh50-73, and tested as inhibitor of self-binding. Both self-binding of T15 and M603 were inhibited by this peptide at the micromole range and not by control peptides. The sequence region Vh50-60 covers the CDR2, while the complementary sequence 63-74 is in FR3.

The autophilic site is present on antibodies with different specificity *(16)* and by this property is not subject to antigen-driven selection. This independent association of the antigen-binding site and the self-binding domain strongly suggests an innate origin of the self-binding locus. In support of this is the germline nature of the S107/T15 autophilic antibody family. The C-terminal residues of the peptide are in the framework, while the N-terminal portion of the self-binding sequence region is clearly CDR territory. Thus, an adaptation of the CDR via antigen-driven selection for the conserved framework must take place to produce the inverse hydropathy relation of the N- and C-terminal parts of the self-binding region. This situation serves as an example of how innate and adaptive immunity work together in a coevolutionary process (*see* Fig. 2).

The biological significance and advantage of autophilic antibodies could explain the evolution of the self-binding site in the germline. Antibodies with autophilic properties are directed against nonprotein antigen,

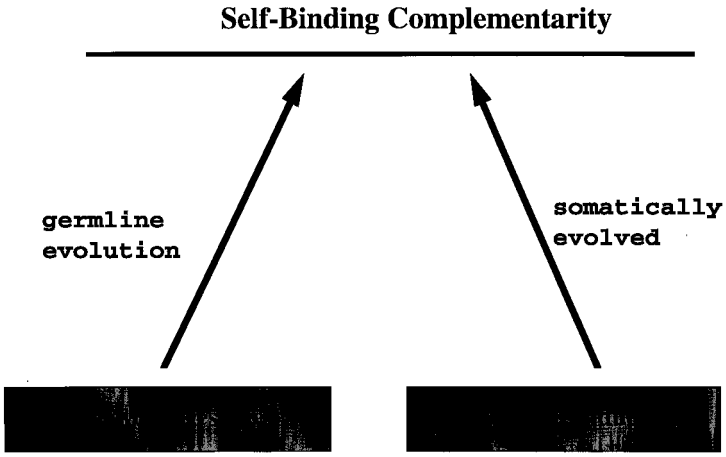

Fig. 2. Evolutionary and structural components of the self-binding domain. The self-binding site consists of two sequence regions localized in the framework and CDR regions. The coevolution of these regions via germline evolution and somatic evolution is indicated.

the S107/T15 against C-polysaccharide *(17)*, and the R36 against a ganglioside *(18)*. For these antigens, T-help cannot be generated.

Therefore, antigen-driven maturation for these T-independent responses cannot take place. Anticarbohydrate antibodies are typically IgM, which can compensate for low antigen-binding affinity by the increase of the overall avidity through the pentameric IgM molecule. If antibodies against T-independent antigens of pathogens invading mucosal surfaces are needed, polymeric IgA is produced.

To compensate for low antigen-binding of monomeric IgAs, the self-binding locus was "invented" by nature. Experiments by Claflin and colleagues *(19)* show that autophilic IgA antibodies with the self-binding property are superior to IgAs that are not autophilic. These authors demonstrated the biological advantage of the autophilic S107/T15 antiphosphorylcholine antibody. The self-binding antibody is several times more potent in protecting immune-deficient mice against infection with pneumococcal pneumonia than non-self-binding antibodies with identical antigen specificity and similar affinity. This observation was confirmed using T15 transgenic mice *(20)*.

It is also interesting to note that the antigen receptor of the autophilic S107/T15 structure on B cells may be exquisitely sensitive to stimulatory signals during the development of the immune system, and that this could explain the dominant expression of the T15 idiotype in certain strains of mice *(21)*.

Engineering Autophilic Antibodies

Taking a page from nature, we tested the hypothesis that attaching an autophilic peptide to antibodies would generate a self-binding anti-

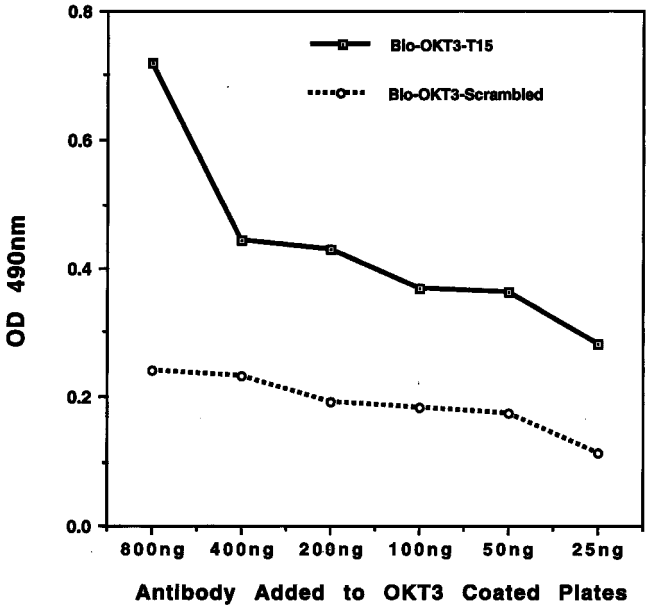

Fig. 3. Self-binding of MoAb OKT3. The autophilic 24-mer peptide is affinity-crosslinked to OKT3. ELISA plates are coated with OKT-24-mer (200 μg/well) and increasing amounts of biotinylated OKT3-scrambled peptide or OKT3-autophilic-24-mer were added. ELISA is developed with avidin-HRP.

body with enhanced antigen-binding avidity. We selected affinity-based crosslinking techniques over nonspecific chemical-linking methods. For antibody chemical crosslinking, two methods are available: 1) attaching proteins or peptides to a modified carbohydrate moiety in the Fc part using periodate *(22)* and 2) photoaffinity insertion of azido compounds *(9)*. We used both methods to attach the 24-mer autophilic peptide derived from the CDR2-Fr3 region of T15 antibody to a monoclonal antibody OKT3. As seen in Fig. 3, peptide-crosslinked OKT3 exhibits self-binding in ELISA.

OKT3 crosslinked with the 24-mer T15 Vh peptide or a scrambled peptide were biotinylated and added to ELISA plate coated with OKT3-T15Vh. Only the OKT3-T15 bound to the coated plates, while the OKT3-scrambled did not. It has not yet been proven that the autophilic-engineered OKT3 binds with enhanced avidity to its target cells.

Therapeutic Application of Autophilic Antibodies

Passive immunotherapy with MAbs has evolved as the most regulatory advanced and therapeutic effective approach developed by the biotechnology industry today. IDEC's Rituxan and Genetech's Herceptin are the prominent examples of the potential of biotechnology in the field of cancer drugs. In a recent survey of biotechnology drugs under development, 90 of 350 new medicines are MAbs *(23)*. The majority of the monoclonal drug

candidates are developed for the cancer field. While these biotechnological achievements with MAbs are impressive with their proven clinical efficacy, there is room for improvement. Drawbacks in passive immunotherapy include the large amount of antibodies often required to be infused, making these drugs expensive, and explaining the inability of antibodies to penetrate large tumor masses.

The autophilic modification of antibodies presented here will address these issues of therapeutic antibodies, and can render them more effective as antitumor drugs. Since scFv and Fab fragment can be photoaffinity-crosslinked with the autophilic peptide (unpublished data), it will be possible to target large tumors using autophilic fragments with improved vascular penetration. This prediction is based on a unique property of autophilic antibodies. As shown earlier, the autophilic T15 antibody does not form physical complexes in solution.

The advantages and improvements of autophilic antibody therapy of tumors are several:

1. The overall avidity of autophilic antibodies is higher than of conventional antibodies. The effect of autophilic molecule polymerization can best be understood by comparing the IgG dimer with the IgM pentamer, whereby the IgM can bind with higher avidity then the IgG. This predicts that autophilic antibodies are more efficient in targeting than conventional antibodies.
2. For therapy of tumors that are sensitive to negative growth signals, such as B-cell lymphoma, dimerizing antibodies against the BCR induces hyper-crosslinking in the absence of a second signal. This in turn enhances growth-inhibition and apoptosis *(24)*. Thus autophilic-induced hyper-crosslinking increases therapeutic efficacy.
3. Because of better targeting with autophilic antibodies and enhanced penetration with autophilic fragments, less of the antibody would be needed to achieve equal or better therapeutic results when compared to immunotherapy using conventional antibodies.

In Fig. 4, a hypothetical model is shown to visualize the way autophilic antibodies could attach a greater number of antibodies than conventional antibodies. This model schematically depicts the binding of a conventional antibody and self-binding autophilic antibody to tumor antigens on a tumor cell. The self-binding domain allows the attachment of additional antibodies to antibodies already bound to the tumor. This creates a lattice of tumor-bound antibodies. It is interesting to note that self-binding does not occur in solution, but only after the antibody has attached to the surface. This implies that the layering antibodies require the interaction of the self-binding domain and contact to the antigens on cells or to the plastic surface of a plate. Three kinds of binding forces for the autophilic antibody are shown: 1) binding to tumor antigen via the antigen-binding site; 2) Self-binding between the homophilic domains of two antibodies,

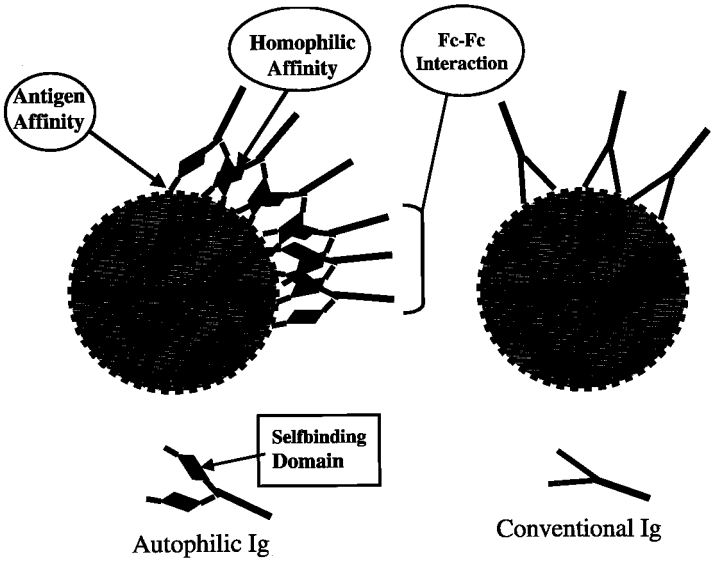

Fig. 4. Schematic representation of how conventional and homophilic antibodies bind to tumor-cell target.

and 3) greater Fc–Fc interaction because of the higher density of antibodies in the layer covering the tumor.

The Concept of Superantibodies

From the discussion of the relationship between innate and acquired immunity, we have developed the term "superantibody" *(25)*. This term was coined in analogy with the term "superantigen" to signify a special property not typically expressed by antibodies. An antibody becomes a superantibody when features of the innate and acquired immunity meet and work together. In Fig. 5, the relationship between innate and acquired immunity as it creates a superantibody is shown.

Not every antibody is a superantibody. Superantibodies belong to a rare and elite class. Superantibodies may play a minor role in the immune defense against targets for which T-help is not available (T-independent antigens), and maturation of the antibody binding site cannot take place.

The concept of superantibodies is introduced here to describe the unique coexistence and synergism of the acquired immunity with the innate immunity via antigen-specific and unconventional functional domains. As not every antibody qualifies as a superantibody, biotechnological engineering can produce superantibodies with superior targeting and therapeutic properties.

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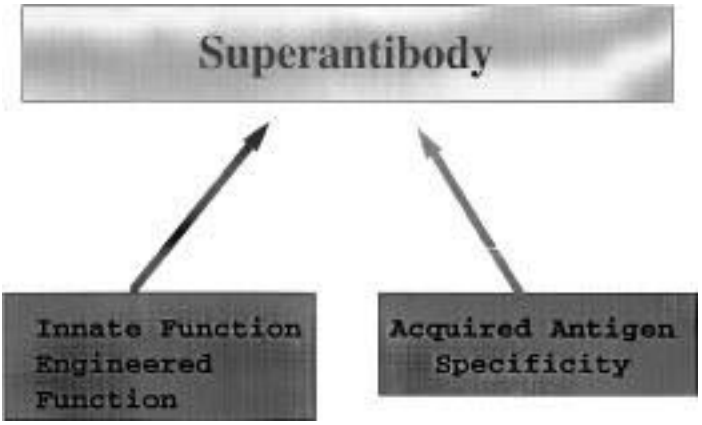

Fig. 5. Relationship of innate and acquired immune functions in superantibodies. Coevolution and synergism of both immune functions produce superantibodies.

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Discussion:

Schowen: Do you think the killing effect that you observed is the induction of apoptosis? Let me ask a second question—is the catalytic activity linked to the induction of apoptosis?

Kohler: To answer your first question: yes, we believe it is apoptosis. As a matter of fact, there are studies by others showing that incomplete signaling compromises survival of B-cell tumor cells in vivo. Apoptosis has not been previously shown, but our data suggest that the apoptotic finding is significant. To answer your second question: we have no evidence about the relationship between catalysis and apoptosis, but it is possible.

Gololobov: What is the mechanism of apoptosis?

Kohler: We envisioned that the presence of multiple ligands on cell surfaces basically creates a lattice of multiple antibodies, providing stronger crosslinking than if just a single antibody is attached. We looked by fluorescence and we saw enhanced patching and internalization, which occurs within minutes when we use the homophilic engineered antibody compared to the naked antibody. I think it is a very effective crosslinker. It's basically the same difference as in IgG and IgM. We are creating something multivalent, like an IgM, or even higher than IgM.

Zouali: Do you view superantibodies as precursors of antibodies to pathogens, or do you think the superantibodies represent a different repertoire within the immune system?

Kohler: I think they add to the repertoire of the immune system. If you look at the S107 antibody which binds phosphatidylcholine—which is actually the first example of a homophilic type of antibody—Betty Diamond made a mutant changing the specificity from phosphatidylcholine to double-stranded DNA—the mutant preserved the homophilic activity. So now you have actually created another level of the repertoire. You can have antibodies with homophilic activity of different specificity. Basically, this is what we are doing artificially by engineering. We can take any antibody and make it homophilic. We don't touch the antigen specificity. We don' touch the CDRs, which is actually the beauty of the system. Yes, it's another level of the repertoire.

Zouali: I like the term "superantibody." It sounds like a television advertisement. We may ourselves have generated a super IVIG preparation, which is a mixture of IgG from 20,000 people. We purified the IVIG on a column of a specific autoantigen and we got what we call super IVIG, with unusual biological activity at a high-molecular-weight range. So it seems like there may be some innate or natural superantibodies in the preparation.

Kohler: Exactly. As I tried to point out, nature has produced these special type of antibodies—superantibodies—and your high molecular fraction may be a reflection of this activity.

Zouali: We are surprised that the percentage was about 0.2% of total immunoglobulins.

Kohler: That low? We have actually made IVIG from the homophilic antibodies. It works very nicely.

Unidentified: I wondered whether you create an immunogenic site in the process of generating your superantibody.

Kohler: Yes, we have thought about this. But as we take these peptides from naturally occurring proteins like the complement, there is no autoimmune reaction. The engineered superantibodies are self, perfect self. The antibody itself is part of the S107 Vh family. As the antibody is an innate structure, we believe we don't create *de novo* new antigens. We have not found any antibodies against these peptides.

Unidentified: I understand you are taking from self, but in the process of engineering, you might create neoantigens.

Kohler: That's possible, yes.

Tribbic: In creating these superantibodies, have you considered whether the antibodies themselves were complement-fixing antibodies? Would that make a difference in the antibodies that you would direct this construct to?

Kohler: We don't know whether complement fixation would be affected. However, our intended effects are downstream of complement fixation.

Green: Would you consider polyspecific antibodies as superantibodies? Are the terms overlapping?

Kohler: Polyreactive antibodies could become another member of the family, but that's not my area.

Paul: Heinz, inverse hydropathy seems to work in the model antibody you showed. However, the concept is somewhat controversial. Can you comment on the generality with which one can apply the concept to design peptides that might bind other peptides of known sequence? My second point deals with an unrelated issue. I think it would be useful to come up with quantitative values of the binding affinities when we want to make biologically meaningful conclusions. Everything binds everything else. Binding, however, is often an irrelevant and nonmeaningful phenomenon. So give us a sense of the binding affinity of ligands for various superantibody sites.

Kohler: A mouthful of questions. Let me address the second question first. I think the term superantibody should be applied when you can actually identify and improve the biological activity of a conventional antibody. The criterion then depends, in part, on what the antibody is meant to target. Is it a soluble ligand? Is it a receptor? If you give a signal to a receptor, this might have different biological effects: B stimulation; suppression; enhancement. In regard to superantibody affinity, it is certainly possible to get quan-

titative data. But the binding itself would not qualify for membership in this exclusive club. You must have the biological activity, either in a negative or in a positive form. The inverse hydropathy matter is controversial, but it has worked twice for us. I know it hasn't worked for some other people. I think it can be used to design peptides with homophilic properties.

Paul: The original inverse hydropathy concept was the two strands of DNA code for the ligand and the receptor. If true, that would be a revolution in design of ligands and receptors.

Kohler: Yes, I think you should organize another conference just on this question.