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General Discussion

Zouali: One of the issues concerning reproduction of the human repertoire is whether it is really possible to generate the human antibody repertoire in bacteria. Is it possible to generate pathogen-specific antibodies, for example? There are a number of natural strategies that are used by the immune system, and there are a number of problems in our experimental strategies. How are we going to achieve somatic mutations? How are we going to resolve the problem of the CDR3 diversity? We know that in a human, as opposed to a mouse, much of the diversity, at least 10^6 different specificies, is generated in the CDR3.

McCafferty: Can we generate human repertoires in bacteria? The answer is very obviously yes. We can combine human heavy and light chains, express them on phage, select low-affinity binders, and, by reasonably simple procedures, derive higher-affinity binders. Perhaps part of your question is whether the phage system can do the same things as the immune system. Our feeling is that we do not necessarily have to follow the immune system in the way that it derives high-affinity antibodies. For example, once we have derived a moderate affinity antibody and we want to improve the affinity, we could sequence many different binders, try to identify the hot spots involved in the binding interaction, and then randomize and change those nucleotides. The phage system can then be used to select the good binders. We are not religiously duplicating exactly what the immune system does.

Stollar: There has been discussion in the corridors about the size of the repertoire and the probability that one can generate any kind of antibody that one wants. People have tried to calculate the number of possibilities. I do not know if that is an accessible number. What are the thoughts on the probability of being able to generate (1) any specificity you want, and (2) any specificity with catalytic activity? Are we starting with a reasonable possibility of success in either of these goals, or are we hoping to be very, very lucky?

McCafferty: As far as the specificity is concerned, there is evidence that one can generate antibodies with at least μM K_d from naive libraries. The immune system starts off with a repertoire of around 10^7 antibodies and can generate binders to most antigens it has not specially deselected against. Various mathematical considerations suggest that it is possible, from a library of 10^7 clones, to generate antibodies with μM K_d that can

bind to 95% of the antigens to which you might be exposed. The general question of how to get much higher affinities applied both to phage-derived antibodies and the immune-system-derived antibodies. Our experience from the immune system is that one gets n*M* K_d binders relatively easily and, on occasion, beyond that. Perhaps the same type of numbers and molecular constraints will apply to phage-derived antibodies. The other consideration, of course, is whether you actually need much higher affinities than nanomolar for most applications.

Paul: I think it is important to figure out exactly what we are talking about. There is a repertoire, a potential number of antibodies that can be formed against all possible antigens that we encounter, and then there is a repertoire representing the number of antibodies that can be formed against a single epitope. It is crucial to get a sense of what that number is. There have been many calculations concerning the total repertoire, but there is relatively lesser information on the number of antibodies that can be formed against a single epitope.

Foote: I have a kind of perverse opinion on this subject. When I first learned about antibodies, I remember my professor going through the calculation that there are a hundred residues in an antibody variable region, there can be 20 amino acids in each place, and there would be 20^{100} possible specificities, but in working with the oxazolone system, I was struck by the lack of diversity we saw in the oxazolone-specific repertoire. When you thought in terms of primary structure, there were a number of different V-gene groups represented in the antibody response. There were the canonical genes, which were overrepresented. We saw them again and again with slight variations. We saw a lot of other V-genes used in combination with one of the canonical genes. For all this diversity and the 20^{100} possible sequences, there are basically just two modes of binding of the hapten. There is the binding mode observed early in the response with canonical-type antibodies and antibodies containing combinations of one canonical chain with another noncanonical chain. Superficially, there were a lot of sequence differences in this noncanonical chain. However, basically, the same key residues were present in the same positions in each case. So, the same geometry was being created, regardless of what part of the light- or heavy-chain loci the V-genes were coming from. Later in the response, we saw dominance of a different type of binding site that did not have any homology in the CDRs to the canonical forms. There were just two ways of binding oxazolone, not a big diversity.

McCafferty: The immune system of mouse and humans has been tailored over millions of years to do a particular job. With the phage display approach, it is possible that we can unleash ourselves from the constraints that evolution has imposed on the immune system. Hoogenboom and Winter (*1*) have displayed a repertoire made of 49 different heavy-chain germline V genes with a randomized synthetic CDR3 of five or

eight residues. With this semisynthetic library, they have generated 20 different oxazolone binding clones, which used five different V genes with an eight-residue CDR3 in every case. Each V gene had different CDR sequences. Within a given group using the same V gene, there were common sequence elements in the CDR3 that obviously complemented that particular V gene to generate a binder. This is an example where a wider primary response was generated in a synthetic repertoire than in a natural repertoire.

Stollar: Antibodies to DNA and RNA occur in the sera of patients with systemic lupus erythematosus (SLE), a prototypic autoimmune disease (*2–4*). High serum levels of antibodies to dsB-DNA are particularly diagnostic, and are often associated with flares of systemic clinical disease and lupus nephritis; some of the antibodies contribute to the pathogenesis of the disease. Lupus also occurs in certain strains of mice, with production of antibodies that resemble those from human patients. The association of antibodies to DNA with autoimmunity has been a strong motivation for analysis of how these antibodies interact with DNA, what stimuli may evoke their production, and what immunoglobulin genes encode them.

We have prepared a modified pGEMEX as a vector for production of single-chain Ig Fv (scFv) in *E. coli*. By swapping V gene segments and by directed mutagenesis with this vector, we have studied structural requirements for DNA binding and/or idiotype expression of three kinds of anti-DNA antibodies (*5,6*).

The plasmic vector pIg16 contains an insert that is expressed under the control of the T7 RNA polymerase promotor. Following the T7 promotor and a ribosome binding site, the insert contains: a processing and secretion signal sequence of the alkaline phosphatase gene; the $V_HD_HJ_H$ segment; a sequence for a linker peptide $(GGGGS)_3$; the V_LJ_L segment; a sequence for an $(His)_5$ peptide; a segment coding for a 58 amino acid domain of staphylococcal protein A; and a termination signal. In BL21 host cells, activation of the T7 RNA polymerase promotor is induced by IPTG. Bacteria transfected with pIg16 and induced with IPTG secrete soluble single-chain Fv (scFv) molecules into the medium. The scFv is purified from medium by affinity chromatography with an IgG-Sepharose column that binds to the staphylococcal protein A domain of the scFv. The scFv has binding properties similar to the parent molecule and is recognized by anti-idiotype antibodies made against the parent molecule. Expression in this system has been applied to three kinds of anti-DNA antibodies.

1. Antibody Z22 is a C57/Bl mouse monoclonal antibody induced by immunization with z-DNA. The scFv with H- and L-chain V regions of Z22 binds z-DNA with the same specificity and nearly the same affinity as the parent Fab fragment. An oligonucleotide extension

method was used to swap the Z22 V region segments with those of different antibodies and to introduce directed mutations. CDR3 regions of both the H and L chain V regions of antibody Z22 are required for optimal z-DNA binding. Small changes in structure can lead to appearance of autoantibody-like DNA binding.

2. Antibody 2C10 is an MRL-lpr mouse monoclonal autoantibody to native DNA. It binds poly(dA-dT), but not poly(dG-dC). Ultraviolet irradiation of an immune complex with antibody 2C10 cross-links oligonucleotides to H chain, but not to L chain. Bacterial expression of various H- and L-chain combinations tests the proposal that indicates that the H chain of 2C10 is dominant for determining reactivity with DNA.
3. Antibody 18/2 is a human IgM autoantibody to ssDNA. It cross-reacts with phospholipids and bacterial and platelet cell surfaces and, therefore, has properties of "natural autoantibodies." It also bears an idiotype that occurs on disease-associated autoantibodies to DNA. The scFv of 18/2 H and L chain V region is recognized by both polyclonal serum anti-idiotype antibody and a monoclonal anti-idiotype antibody.

Rodkey: Are you pretty sure that your heavy chain, the 2C10 heavy chain, is soluble and is a monomer, and that it is not aggregates of the heavy chain that binds the antigen?

Stollar: All of these recombinant products that went on to the gel-filtration column were soluble. The heavy chain behaved as a monomer on gel filtration.

Paul: To what extent are the anti-DNA antibodies toxic to the host cells, for example, by binding host DNA?

Stollar: That is an interesting question. The F_v that has both the heavy and the light chain of the 2C10 gives the lowest yield of the constructs that we have tried. We wondered whether some of the anti-DNA antibody is actually binding to DNA within the bacterial cell. We have examined that by lysing the cells on a membrane that traps the bacterial DNA and testing for F_v absorbed to the DNA. There was no F_v present on the membrane above background. So, I do not think that there is specific toxicity owing to anti-DNA antibody activity. The presence of this antibody, as with any other bacterially expressed recombinant protein, could be toxic, however, in other ways.

Deyev: What is the level of expression of the single-chain antibodies in the bacterial cells?

Stollar: The best is about 3–4 mg/L. The average is probably about 0.5 mg/L.

Deyev: How big a problem is removal of the signal peptide from the single-chain antibody?

Stollar: We have done an N-terminal analysis of material that we have blotted, and found that it has been processed and has the right amino terminus. The signal peptide appears to have been removed.

Deyev: Is solubility of your single-chain antibodies a problem?

Stollar: The antibodies remain soluble, and we purify them from the medium. Without either the protein A or the signal peptide in the construct, we can get an enormous amount of inclusion bodies, but we have the usual problem with that approach and get the usual low yield of refolded protein. The full construct produces material that is soluble to begin with and remains soluble.

Deyev: The V_L domain can express a very hydrophobic region. Can this hydrophobic region be the reason for the change in specificity of your heavy chain combined with different light chains?

Stollar: Possibly. In the one case of the combination of heavy and light chains of the autoantibody where we do not have as good an activity as expected, there may be incorrect folding.

Green: About your finding that the heavy chain can bind nearly as well as the intact single-chain antibody, I was wondering if you could summarize the overall experience about the frequency of this phenomenon.

Stollar: In some ways, what we find is consistent with a lot of experience. The CDR3 of the heavy chain is very diverse and very important in defining the antibody contact site. There are other examples of binding by heavy chain alone. Greg Winter found binding of lysozyme by V_H (*7*). In old experiments when people took an antibody and separated the heavy chain and the light chain, there was usually a bit of residual activity in the heavy chain and in the light chain. The binding affinity with the heavy chain alone in our case is not the same as the intact Fab. It is lower, which is consistent with the general experience. Most certainly there are many anti-DNA antibodies in which both heavy chain and light chain are involved in binding. The one known crystal structure of an anti-DNA antibody with bound trinucleotide uses both heavy and light chains for the binding, and all six CDRs are involved (*8*).

Green: My understanding of the old work of separating the heavy and light chains mechanically was that it is difficult to get pure heavy and pure light chains, and dimerization can also occur.

Stollar: We do know from gel filtration that expressed recombinant F_vs are not large aggregates.

Green: When we sequenced six catalytic antibodies, we found that three had essentially identical light chains and the other three had another—a different identical light chain. Then, a new family of antibodies against a somewhat different hapten had that same old light chain. Yet, the catalytic activity of the antibodies was different. This appears to be a very significant restriction, in that we may not encounter the diversity that one would expect. Introducing some randomization technique to increase the diversity may be very important. Perhaps this lack of diversity is not a problem in producing binding activity. From the point of view of catalysis, where you need presumably some variety of functional groups, this is a very serious restriction.

Stollar: The mathematics on the theoretical number of V, D, and J segments that are available leaves out the enormous biological editing done by the immune system, which discards an enormous number of combinations. Reintroduction of diversity, for instance, by mutagenesis of semisynthetic libraries of V genes through in vitro manipulations may, therefore, be important.

Green: We have discussed several biological variants of antibodies, the phage display antibodies, single-chain F_v, and simple light chain with catalytic activity. If one is interested in catalysis, one should allow one's imagination to roam even wider. The antibodies turn out to be attractive reagents because of their incredible ligand binding activity. We could think, for example, of introducing artificial amino acids, cofators, or functional groups in the antibody combining site. What we want to do is make and break chemical bonds.

Stollar: That idea was introduced from the Schultz lab in at least one example (*9*). Why has that not developed further? Why are there not other examples of introducing reactive cofactors that take advantage of the binding specificity of antibodies?

Thomas: Many of us are interested in introducing cofactors and coenzymes into antibody sites. Antibodies can be looked at as apoenzymes. Obviously the problem will be that big conformational changes can occur when the coenzyme is introduced into the antibody.

Hansen: We do not really know how catalysis works. Site-directed mutagenesis studies on enzymes have led to lots of surprises. The few mechanistic studies that have been done on catalytic antibodies have also yielded surprising results, in that the mechanisms used by some catalytic antibodies are very different from what is predicted on the basis of the design of the hapten used to generate that antibody. *De novo* catalyst design by physical organic chemistry methods has been extremely difficult. One has to be quite confident to dive into an antibody-combining site and start adding cofactors in order to get a good catalyst. At the moment, people are happy to just take what the immune system gives.

Paul: I would like to reframe the issue, both in terms of the cofactor placement as well as placement of chemically reactive residues in the correct orientation in the antibody-combining site. If you construct a minimal model of an antibody-combining site based on the assumption that a given number of residues are sufficient to achieve catalysis, for instance, the residues of the catalytic triad of serine proteases, histidine, aspartate, and serine, what is the probability of generating a correctly oriented constellation that can permit catalysis?

Webster: It would be practical, certainly, to do the computational side of this problem by saturation mutagenesis of the antibody-combining sites to generate all the possible orientations. Of course, you may do better using phage display or just standard hybridoma techniques to estimate the frequencies practically.

Stollar: To extend that little further, would you than say that the selection or pragmatic sort of approach is going to be more productive than a predictive or modeling approach in general?

Webster: I do not know. I do not really have enough experience with the phage-display or hybridoma techniques. Certainly, I think this is an experiment worth trying by computational methods. We could generate those models initially for a limited number of potential catalytic sites based on known mechanisms of actions of enzymes, assuming that a certain number of residues, say two, three, even four, are necessary for catalysis. These models can then be tested in the laboratory to see whether we did create the correct geometry. This is a tractable issue from a computational point of view.

Hansen: Triose phosphate isomerase has a single active site base, a glutamate. If the glutamate is changed to an aspartate, the base moves < 1 Á, but the catalytic activity drops by 1000-fold. Since in the catalytic antibody world, a 1000-fold change in k_{cat}/k_{uncat} is an impressive number, it seems that tinkering with the active site is going to be hard (*10*).

McCafferty: Sudhir, you asked a question about forming a catalytic triad you describe as a constellation. I have often heard Allen Fersht say that the chance of doing this is one over the number of atoms in the universe.

Paul: How did enzymes evolve? By that token, proteins should not possess catalytic activity at all.

McCafferty: The probabilities of achieving catalysis without imposing some sort of selection pressure are likely to be low. In response to the comment on modeling vs selection, I would think that these are not competing technologies. One can benefit from the other. Modeling may give us an approximation to put us in the right ball-park range, and selection can then pick one of the many billions of combinations that can arise.

Zouali: The work of Mark Greene has shown that it is possible to model successfully the combining sites. He was able to mimic the CDR3 loop of antibodies to CD4 by simple organic radicals (*11*). Moreover, these organic reagents also neutralize the HIV virus infection in vitro. Another advantage of these organic reagents over antibodies is their longer half-life.

Webster: It is quite possible that modeling have many advantages over the practical side in the sense that we can actually mimic somatic mutation quite easily by computer, possibly more easily than by current practical technologies.

Paul: There are, of course, also some practical problems in bacterial expression systems for antibodies. Are we satisfied with expression levels, secretion levels, and folding of antibodies in these systems?

Rodkey: Glycosylation is another problem.

Stollar: To what extent do you anticipate that lack of glycosylation in bacterial systems is a problem in the function of the antigen binding domain?

Rodkey: None for the antigen binding domain. There are other functions of antibodies residing in the F_c for which the correct glycosylation is absolutely required.

McCafferty: The simple answer is; if glycosylation is required for specificity isolated in bacteria, then the gene should be cloned and used in a mammalian expression vector.

Rodkey: Glycosylation is achieved by a series of enzymes for building correct structures.

Stollar: If glycosylation is important for the function under study, you should produce the antibody in a eukaryotic cell.

Deyev: Appropriate glycosylation is obtained in mammalian cells, as well as yeast cells. The yield of the antibodies in these hosts is poor, of course, compared to bacterial cells. A recent paper by Borrebaeck's group (*12*) shows that chaperonin proteins help in correct folding of single-chain antibodies in bacteria. This should be a very promising approach.

Paul: I believe that the work you are citing reports that packaging of phage particles is improved by GroE, resulting in a 200-fold increase in titers of the particles. I am not sure if the folding of the antibody *per se* is influenced by the chaperonin.

McCafferty: A 200-fold increase of titer in a sense implies 200 times more particles. This issue was not directly addressed in the paper. Is there an increase in the infectivity of the phage particles, for example, by better folding of the gene III protein? If you grow phage in TG1 cells and measure the number of actual particles and the number of infectious particles, only a few percent of the particles are infectious.

Green: One can get 50 mg of monoclonal antibody from 5 or 10 mL ascites. Can the bacterial systems yield comparable quantities of F_v or Fab fragments?

Stollar: The scale-up does not always go as well as small-scale production. Expression often looks good in 200-mL bacterial cultures. In larger cultures, for example, a 2-L culture, expression is not 10-fold greater. The scale-up requires appropriate control of nutrient, aeration, and everything else involved in optimal expression. Certainly, however, one can obtain 20 mg of the recombinant protein. This amount may take a while to build up, but it is within range of the technology.

Foote: Genentech has described a pBR-based Fab expression vector grown in a high-density fermentor using a tetracycline selection marker, because ampicillin resistance cannot be selected in liqiud medium. The lower copy number in this vector seems to keep the bugs in better shape, and g/L yields were obtained (*13*). For making full antibodies, CellTech has a vector containing a CMV promoter that seems to work very well based on glutamine synthetase selection. They report 500 IgG/L yields in a fermentor. I would say that the problems of expression of soluble, active antibodies have been solved for the kind of purposes we are interested in at this stage (*14*).

Paul: Is it practical to screen for catalysis directly using bacterial expression systems or phage display systems.

Green: We have established a system that allows facile and direct screening of hybridoma supernatants for catalysis. In principle, that system can be extended to screening of recombinant antibodies.

Stollar: One of the very powerful possibilities is genetic selection systems in vivo, in which only the surviving organisms express catalytic activity. We are beginning to set up such a system in our lab. One published proposal of this type of selection was based on a cleavage reaction that generated biotin, which served as a growth factor (*15*).

Hansen: The experience with selection of enzymes in such systems has often been that the bugs start doing things you do not quite expect.

McCafferty: The question of direct selection is very important in relation to catalytic antibodies, and the inability to do this is a definite weakness in the current catalytic antibody field. This notion of making antibodies that bind transition-state analogs has been around for a number of years. First, it has to be said that the activities of the antibodies are low. Second, we are assuming that we know how to synthesize chemicals that look like transition states, and then we are hoping that antibodies that bind these chemicals will be catalytic. The phage-display system will certainly allow derivation of lots and lots of binders that can be manipulated relatively easily. It may even allow selection for particular modes of binding and conformations of antibodies. Having done all that, we then look to see if the antibodies are catalytic. So, we remain dependent on the assumption that binding to the simulated transition state will confer catalytic activity of the antibody. Once an antibody of weak or moderate activity has been isolated, how do you go about improving its activity—by making antibodies that bind more and more strongly to the transition analog state? Will we succeed in selecting better and better enzymes by this approach, or will we throw away a lot of good catalysts simply because they did not bind the analog very well? I would consider direct catalytic selection a major requirement for advances in the catalytic antibody field.

Gabibov: The major problem in screening directly for the catalytic activity of antibodies is the presence of background conventional enzymes with high levels of activity.

Polanovsky: One general difference between the interactions of an antibody and an enzyme with substrates may be the level of flexibility in these systems. The active site of antibodies may be very rigid, and high-affinity binding of ligand is necessary for the biological activity of antibodies, but in enzymes, the system is flexible, and the affinity of enzymes for substrate is not so high. It may be necessary to think about the balance between rigidity and flexibility of the system when designing catalytic antibodies.

Stollar: There are certainly examples of flexibility in combining sites of

antibodies, but your argument suggests that maybe high affinity should not be the goal, but rather a moderate affinity in the range of the concentration of the substrate.

Stewart: It is clear that we need flexibility in enzyme-combining sites to achieve maximum fit. With respect to the peptide bundle we have worked with, the free ends of the chains that bear the active site residues are perhaps even too flexible. This is suggested by the circular dichroism data showing that there is less than complete helix formation. The interdigitating hydrophobic side chains near the carboxyl end and the covalent links should give quite good stability to that part, but if we have only 75% helix, that suggests that there is tremendous fraying at the N terminus. This may be the reason we have such low efficiency. I believe that in our case we need to increase the stability around the active site.

Foote: A lot of people in the field of protein structure seem to be really attracted to the idea of flexibility, but if your mission is to make a catalytic antibody based on rational hapten design, you may want to avoid flexibility. It is hard enough to predict one conformation, let alone two or three conformations, only some of which might be active. Flexibility may only increase the unpredictability by which the antibody works. I believe that there is some uncertainty as to the role of conformational change in the mechanism of enzyme activity. The two lobes of the lysozyme, for example, tend to fold around the polysaccharide substrate, whereas an enzyme with an absolutely simple substrate, such as superoxide dismutase, displays little or no conformational change following substrate binding. Is it the general case that larger, complex enzyme–substrate interactions display a significant degree of flexibility and conformational changes in the active site?

Campbell: In the case of lipases, it has just been shown that there is significant movement, and this enzyme uses a simple substrate.

Hansen: It is hard to draw a general correlation. A loop in triosephosphate isomerase, which uses a very small substrate, shows movement (*16*). It is especially difficult to draw a correlation because it is never clear whether these motions are important for pure catalytic enhancement or discrimination among substrates. In the case of triosephosphate isomerase, it is believed that the loop moves to exclude water from an active site and prevents an alternative reaction from occurring. Generalizations about the role of conformational changes are very difficult, just as generalizations about which CDRs are important in binding by antibodies are difficult. Depending on which particular system you are looking at, you might draw a different conclusion.

Foote: Antibody–antigen interactions generally follow the mechanism: $Ab + Ag \rightleftharpoons Ag \cdot Ab$. The affinity or equilibrium constant K_{eq} (k_{on}/k_{off}) is considered the biologically relevant physical parameter, inasmuch as "maturation" of the immune response following antigen challenge yields antibodies of progressively higher affinity. We examined the

assumption of affinity pre-eminence by measuring the kinetics of hapten binding in a library of antioxazolone (Ox) antibodies made at different stages after immunization. One V-gene family showed mediocre affinity, yet was prominent late in the response. This group proved to have diffusion-limited k_{on} constants. This implies that on-rate, in addition to affinity, may control lymphocyte selection during the immune response. We also examined the assumption that antigen recognition proceeds by the simple mechanism above. Several anti-Ox antibodies showed complex binding kinetics, combining a unimolecular isomerization of the antibody and a bimolecular association with hapten. In this mechanism, two different forms of an antibody coexist, and show differential binding to antigen, much like Monod-Wymann-Changeux model for allostery.

Paul: Have you modeled your antibodies?

Foote: No. One weakness is that we know nothing about what is causing this phenomenon at a structural level. I have called it a conformational equilibrium, but I really do not know the nature of the chemical change.

Paul: Are the k_{on} values important in natural selection of enzymes, or are the k_{off} values more important?

Tramontano: The optimal enzyme would work at the diffusion limit, which is 10^8 or $10^9 M^{-1}\ s^{-1}$. Whether that occurs because of k_{on} or k_{off} is irrelvant. The enzyme will optimize both steps.

Hansen: One is the microscopic reverse of the other. Depending on which way you look at the catalytic event, they are the same step.

Paul: In the canonical group of antibodies, was their selection for k_{on} as the response developed?

Foote: There was not a very big increase in k_{on} as a function of time in the canonical group. There was a shift to the completely different antibody structure, though. In that way, the overall on-rate of the antibody repertoire increased.

McCafferty: Professor Poljak reports changes in conformation with the lysozyme binding antibody. Has anyone done the sort of kinetic analysis you have done with your antibody in that system.

Foote: No. A number of crystallography groups report changes in structure in the liganded and unliganded forms of antibody. Detailed kinetics have not been done in any of these cases. Ian Wilson, for example, has published several structures of peptide–antibody complexes that show a conformational change, but he has called this an induced fit. There is no kinetic evidence for an induced mechanism in these cases (*17*).

Tramontano: Do all of your antibodies showing the kinetic effect arise at a certain time in the immune response?

Foote: Yes, these antibodies were all secondary and tertiary response antibodies. They had a rather high affinity. If they had appeared in the 7-d early response only, one might rationalize a dead-end phenomenon

that the immune system would discard, but in fact, the kinetic effect is preserved. The antibodies in those groups are very highly selected and mutated.

Paul: Is the conformational isomerism being selected for?

Foote: We have no idea if it is being selected for. We have no idea what meaning this has for the immune system. We are just observant.

Gololobov: You have analyzed monoclonal antibodies from the early stage and the late stage of immunization, and there is some evolution in this process for the rate constants. Is that correct?

Foote: For the canonical antibody, there is a definite increase in on-rate. Even though the overall on-rate was low, if you were to draw a line through the value for the initial antibody, almost all of the subsequent canonical antibodies had a faster on-rate. The on-rate increased up to the apparently natural limit, which is $10^7 M^{-1}\,s^{-1}$ for that type of binding site.

Hansen: Do the kinetic data suggest that the measured K_d or K_a will vary with concentration of either antibody or antigen?

Foote: I have no evidence of K_d varying with ligand or antibody concentration.

Polanovsky: Are both active sites involved in the cooperative effect in immunoglobulins?

Foote: The two different binding sites on the IgGs that I studied did not seem to interact at all. I inferred that there was conformational isomerization of each site from kinetics.

Svedas: One of the magic features of enzymes is their ability to use binding energy for catalysis, inferred from substrate specificity studies. Similar studies on catalysis by antibodies using a range of related substrates will be useful in determining whether there is a correlation between the catalytic activity and the binding energy.

Paul: Where do we stand today with the different technologies that are available to find and to generate catalytic antibodies? Immunization with transition-state analogs is perhaps the most classical aspect of the field. Does immunization with these analogs consistently produce catalytic antibodies? Is the reaction mechanism consistent with hapten design?

Tramontano: It is clear that simple models based on transition-state theory are not able to predict the levels of activity and the efficiencies of catalytic antibodies that have been found. The efficiencies can be even much greater than what is predicted. Indeed, studies have begun to emerge showing that reaction mechanisms can be much more sophisticated than predicted based on a single transition-state model. These are encouraging findings in many ways and mean that we need to refine the models. It is worth speculating about what proteins are able to do, based simply on molecular recognition aspects, and what other qualities of antibody-combining sites might be propitious to catalytic activity.

Hansen: We have to realize that we are really talking about differences of a few kcal/mol. If we see a rate acceleration of 100-fold and another of 10,000-fold, the overall difference in transition-state stabilization in those two cases is approx 3 kcal/mol at room temperature. That is a very small number. The fact that we cannot predict efficiencies seems to be equivalent to saying that we still do not have the tools to predict small differences in stabilization.

Thomas: An antibody NPN43C9 has been shown to hydrolyze a series of closely related aromatic esters and an amide at rates of up to 10^6 above the background reaction (*18*). Using a combination of computer modeling, site-directed mutagenesis, and binding studies, we have investigated the catalytic mechanism and partially mapped the antigen binding site of this antibody. The difficulty with our model is in CDRH1, the incorrect position of a histine residue. This is an unexpected difficulty in that CDRH1 is a short canonical loop, unlike CDRH3, which is always a problematic CDR. There is actually something other than just transition-state stabilization going on here. An acyl-antibody intermediate is found, suggesting a mechanism entirely different from what the immunizing hapten was designed to elicit. One of the questions concerns why a his residue is found in that position in the first place, because it is not actually neutralizing anything on the hapten. Unless the his is actually in linkage with a close-by arginine, which could form a salt bridge and neutralize the RO phosphonate group, I cannot see why a his is generated in that position. Tetranitromethane and many other amino acid-modifying reagents do not affect the activity. There is a large space above the his in the heavy chain where the hapten binds. The substrate seems still to fit in after the modifications, and the antibody remains catalytically active.

Green: You mentioned that the antibody binds a lot of *p*-nitroaniline or *p*-nitrophenol. I was wondering if any of your results shed some light onto why that binding takes place.

Thomas: We have tried to alleviate that problem by making the binding pocket for the *p*-nitrophenol bigger by mutagenesis, but the mutants have generally not been active. We substituted his for tyr-97 to achieve a general base mechanism rather than a nucleophilic mechanism, and this mutant was inactive. Various other mutants also tended to be inactive.

Paul: Is a crystal structure available?

Thomas: Attempts have been made to crystalize the Fab fragment and the single chain. These attempts have been unsuccessful, unfortunately.

Paul: Could you comment on the difficulty of hydrolysis of the unsubstituted amide described by Dr. Williams with that of the amide against which your antibody is directed?

Thomas: Yes, Dr. Williams' work looks very exciting. Ours is an incredibly active amide. There is not really very much of a comparison, to be quite

honest. The background reaction for our substrate is fast. This has been one of the problems in our mutagenesis work. Because the background reaction is very rapid, if we lose 10^2- or 10^3-fold activity, we basically lose the ability to study the effect of mutation. We do not have very much room to play with in making the mutants.

Green: The basic paradigm for obtaining catalytic antibodies is based on the Pauling postulate that a system displays catalytic activity when the energy of the rate-determining transition state is lowered, relative to reactant and to product. Jencks stated that antibodies raised against a stable stereochemical analog of the transition state may be catalytic. Since 1980 we and others have been studying hydrolysis reactions promoted by monoclonal antibodies. We now have in hand two families of esterolytic antibodies: (1) one was raised against a *p*-nitrophenyl phosphonate hapten; six catalytic antibodies have been characterized and studied by biochemical methods (kinetic parameters, amino acid residue labeling, and affinity labeling experiments), sequencing, and modeling, and (2) the second family was raised against a benzyl phosphonate hapten and has provided superior catalysts for the hydrolysis of the corresponding ester, with high selectivity for the substrate, rate enhancement of $> 2.6 \times 10^5$, and > 1000 turnovers. The second group of catalytic antibodies was obtained using a simple and sensitive direct screening method (catELISA) (*19*), and antibodies having this degree of catalytic power may now be considered for practical applications.

Zouali: You have subdivided your panel of catalytic antibodies into two groups. How many mice and how many fusions were used to derive these antibodies?

Green: These were two fusions. In the first fusion, we obtained four catalytic antibodies. In the second fusion, we obtained two. Group 1 and group 2 antibodies were derived from the different fusions. We have developed a screening technique that has allowed us to select directly for catalytic antibodies. If we were to perform a sufficiently large number of fusions, I am sure that we could generate hundreds of catalytic antibodies that would catalyze this hydrolysis reaction.

Paul: Using unactivated immunogens, Kohen et al. derived ester-hydrolyzing antibodies nearly 15 years ago (*20*). In studies described here and in literature on the activity of antitransition-state analog antibodies, the essential control of immunization with a ground state has not been done, to my knowledge.

Green: In one of the papers you are referring to, we raised monoclonal antibodies to an amide, and we found that some of those antibodies hydrolyzed the corresponding ester, dinitrophenol esters, and coumarin esters. The substrates are very active esters, and their hydrolysis is relatively easy. When we immunized against an amide, we thought we might obtain ester hydrolysis. However, the antibodies did not hydrolyze a more stable benzyl ester. I think the probability of an immunization with a ground-state amide resulting in antibodies capable of hydrolyzing

relatively stable alkyl esters is very small. It is true this may be the only case where a ground-state analog was used as the immunizing hapten.

Stollar: In considering the transition-state model, what happens if a reaction is complex and has two or three different transition states in sequential reactions? Are there reactions beyond the capability of designing a catalytic antibody?

Hansen: My sense is that antibodies will, in fact, catalyze multistep reactions, but at this point, I have no idea how to design a hapten that will elicit an antibody capable of doing so. I still believe that there will be no fundamental difference between antibodies and enzymes, the flexibility issues notwithstanding. In fact, even in the case of enzymes, it is very difficult to understand what the protein is doing to bind and interact with a variety of different transition states and a variety of different ground states along a complicated reaction pathway.

Tramontano: I think it is always worthwhile to try to at least predict the trends, even though we are off by factors of a 1000 or a 100. For example, we could try to assess strucutre by measuring the K_i of a variety of transition-state analog structures and thereby try to figure out if the structure of the hapten used to elicit the antibody is close to the transition state.

Green: I will take an optimistic view. I think we have really been unimaginative in the design of transition-state analogs. We have essentially copied structures that have appeared in the literature or have been efficient inhibitors of enzymes. Many of these are probably not true transition-state analogs. The idea of being able to do a multistep reaction is very appealing. Actually, Colin Suckling from Strathclyde has described an antibody-catalyzed Diels-Adler reaction in which he also observed an esterase reaction. Don Hilvert felt that maybe there were some problems in that chemistry. I am positive that it would be possible to design a catalytic antibody that would catalyze simultaneously more than one chemical reaction, for example, Diels-Adler plus hydrolysis. Furthermore, we might be able to do things with antibodies that have not been possible with enzymes. The surface of the antibody making contact with antigen is about 700–900 square Å. We exploit a very small proportion of that area in binding of small-substrate molecules. In the structure solved by Marcel Kinosov recently, there are only two loops of antibody that are playing a role in the binding of hapten. There are other loops that remain available. We could imagine a reaction taking place at one site, then moving over to a second site, maybe a third site, and maybe even a fourth site.

Paul: Let me ask the question many from outside the transition-state analog field are asking. Is there real hope that simple shape complementarity, the design approach that you are using, will produce catalysis of energetically demanding reactions? Neil Thomas cited the unexpected chemistry involved in the antibody that he works with, and there are other published examples. Are you going to be dependent on random

chemistry being generated purely because of affinity maturation, or will it be possible to design the chemical reactivity?

Tramontano: Surely, antibodies have all the correct elements to express enzyme activity. It is reasonable to say we might be able to do very much better by re-evaluating the way we design haptens. Indeed the results that Paul, Gabibov, and Friboulet are presenting show that they are indeed remarkable activities that we would never have expected. It is clear that the paradigms of hapten design need to be reconstructed.

Green: I believe that it will be possible to develop haptens that generate antibodies capable of catalyzing difficult reactions, like peptide hydrolysis.

Tramontano: The question can be reframed the way Dave Stollar asked it. How can you achieve catalysis of multistep reactions based on recognition of a single structure by an antibody?

Green: A hypothetical example is an antibody that would catalyze a Diels-Adler reaction, giving a product that is hydrolyzed by the same antibody. This is based on the assumption of more sophisticated hapten design permitting both reactions to be catalyzed by the antibody. Once you have designed an antibody against a hapten, it certainly is valuable to look at a variety of compounds as substrates. Concerning predictability, the *p*-nitrobenzylester-hydrolyzing antibody shows exquisite specificity that is perfectly predicted on the basis of the hapten design. If you take off the nitro group, there is no hydrolysis. If you change the glutaryl group to an acetyl group, the k_{cat}/K_m value falls by two orders of magnitude.

Paul: The only real way I can see of developing antibodies capable of catalyzing multistep reactions in vivo is to bring to bear some selective pressure that confers an advantage to the system if it produces the antibody.

Gabibov: I like very much your optimism, but we have to be more realistic. At this stage, I cannot imagine how you can produce catalytic antibodies that use complicated mechanisms based on immunization with haptens. Even if you assume a single-step mechanism, we do not have good transition-state analogs for many reactions, like nucleic acid cleavage. Perhaps there is a good future for transition-state immunization to obtain reagents that may be valuable for organic chemistry, like the Diels-Alder reaction, but I think rapid progress in the overall field, for example, medical applications, will not come from this type of approach. There is a fundamental question that has to be answered. Why do we have to mimic enzymes that may have evolved in their own unique way in nature? I think that the field of catalytic antibodies should focus on the specific features of antibody molecules and the immune system.

Paul: The original stimulus for formation of catalytic autoantibodies is not known. It is clear that the characteristics of these autoantibodies are very different from the type of antibodies that have been designed to

mimic enzymes. The autoantibodies catalyze difficult reactions, they are efficient, and curiously, they seem to hydrolyze multiple bonds in the same substrate.

Gabibov: Multiple cleavages could be the result of heterogeneity. The second possibility is that the antibody active site is converted to a different conformation, with the substrate working like a template. Also, we have to prove that distinct chemical groups in the antibody are involved in the catalytic events, for example, by using some site-specific chemical modification of the antibodies.

Paul: Microscopic flexibility in the antibody active site is the best way to explain cleavage at multiple bonds.

Gololobov: Many biomolecules can exist in more than one stable conformation. When antibodies raised against one conformation interact with another related conformation of the substrate, an imprecise fit may let more than one bond be broken. There is also the point of view presented by Hansen's activation of the ground state, in which activation of more than one bond can happen.

Paul: Since heterogeneity is an important issue, it is reasonable to consider how the use of monoclonal and polyclonal antibodies can influence the data. Monoclonal antibody preparations, contrary to the term, are often contaminated with other mouse antibodies when purified from ascites and with bovine antibodies when purified from tissue culture. This type of contamination can range from 10–50% of total antibody. Specific antibodies of interest in polyclonal antibody preparations can be very rare. In some of our patients, the anti-VIP antibody constitutes only 0.001% of the total IgG. The polyclonal antibodies can be purified by substrate-specific affinity chromatography, but these preparations may still contain multiple antibodies directed against the substrate. The heterogeneity may influence the experimentally determined values of kinetic constants.

Foote: There are known ways of dealing with contamination. Monoclonal antibodies purified by affinity chromatography generally contain 80–90% specific antibody.

Paul: In most catalytic antibody papers, a one-step protein-G purification with low-pH elution is used. Removal of unrelated antibodies is generally not done.

Gabibov: The value of k_{cat} will be influenced by presence of other antibodies, but the K_m value is a characteristic of the catalyst in the preparation. The K_d and K_m values for the nuclease antibodies suggest very high specificity. The low catalytic efficiency can be the result of high binding activity.

Gololobov: I do not know that that is true. The K_m of the anti-DNA antibodies is lower than DNAseI, but it is similar to the K_m of *Eco*RI. The rate constant of the antibody and this restriction enzyme are also similar.

Paul: Antibodies displaying very high affinity for substrate may be fated

to be noncatalytic. The major attraction of antibodies as potential catalysts is their high level of specificity for unique structural features of the substrate. Yet, this very feature of antibodies, the ability to bind ligands with high affinity, may impart rigidity to the active site and limit bond breakage, bond formation, and product release. Enzymes generally have K_m in the m*M* to m*M* range and, relatively speaking, may display poor substrate specificity.

Hansen: The binding of enzymes for their transition states has been calculated to be very strong, approaching K_a values of 10^{20} M^{-1} (*21*). Careful attention must be paid to the difference between substrate binding and transition-state binding in these systems. Generalizations are very tricky.

Paul: The question really concerns the relationship between substrate specificity and turnover number of a catalytic antibody. Substrate specificity is manifested primarily in recognition of the ground state, not transition states. Transition-state binding by the antibody is certainly stronger, but ground-state binding must also be fairly strong if good specificity is to be achieved. In this case, the K_m, rather than the k_{cat}/K_m, is the proper indicator of specificity.

Fastrez: I think I would disagree that specificity should be in terms of binding of the ground state, because for many enzymes, the binding energy is used for catalysis. For example, even in the case of simple enzymes like chymotrypsin, when more specific substrates are used, there is no increase in the binding constant, but the catalytic constant is increased. If we look more generally at how to design enzyme activities, there are three points of view. The first one is a thermodynamic point of view. If the transition-state analog approach is applied, we may generate at best catalysts whose activity will be determined by the difference between the binding constants for the substrate and the transition-state analog. Any activity larger than that might simply be the result of chance occurrence and contribution of catalytic residues as observed with antibodies raised against substrates (*20*) or the VIP antibodies (*22*). From a kinetic point of view, even if it is assumed that we have very good transition-state analogs and that they elicit the best catalytic antibodies, we still have the question of flexibility. In fact, there is substantial evidence for flexibility in enzymes, for example, dehydrogenases (*23*), enzymes responsible for phosphate transfer from ATP-like adenylate kinase (*24*), and tyrosine tRNA synthetase (*25*). This is a very relevant consideration, especially if we can select for flexibility. The question remains whether antibodies have the sufficient structural flexibility to work as catalysts. From the structural point of view, if we take the example of chymotrypsin, we should be reminded that the oxyanion hole is also important for catalysis, because it generates a change in geometry and a difference in binding energy

between the substrate and the transition state. The situation is more complex than just organizing the geometry of the three essential residues of the active site.

Tramontano: The thread going through this discussion is basically a structural one—that we can successfully model catalytic antibodies and understand their function on the basis of structure. There have recently been some strides in designing small molecules that are good ligand binders. It may be possible eventually to predict binding functions based on structure. On the other hand, organic chemists have been relatively unsuccessful in generating catalysts from small molecules. However, achieving chemical catalysis by rational design may be far more difficult, since precise orientations and small molecular movements can cause great differences in catalytic activity. I think it may be profitable to start thinking about mimicking catalyst selection and natural evolution of enzymes. Perhaps the structural hypotheses of catalysis that we are using today are intrinsically limited.

Foote: Concerning improved design of transition-state analogs, I think we have seen that we have come up against some limits. There is the limit imposed by the chemistry, the limit of stability, and difficulty of synthesis of the substrate analog. Second, several people have reported at this meeting that the same type of molecules are found over and over again in multiple isolations of antibodies. There is a somewhat limited repertoire. The end game in this field is that once we have an antibody with catalytic activity, ways will have to be found to improve the activity. One way is the genetic approach. John McCafferty described phage antibodies, selection methods for obtaining improved affinity, and so on. Molecular modeling can give us ideas of what changes to make. Concerning the type of antibody that should be used as the starting molecule, kinetics are very pertinent. There is a general recognition that the maximum catalytic efficiency is a k_{cat}/K_m value of around $10^8 M^{-1}\ s^{-1}$, which is the diffusion-limited rate. When I looked at binding of the oxazolone hapten, there was quite a broad range of on-rates, from 10^6–$10^7 M^{-1}\ s^{-1}$. If you start with an antibody with the wrong geometry, you may lock yourself into a low maximum rate. In the oxazolone antibodies, there were basically two geometries. The frequent one, with a very deep hapten binding pocket, requires diffusion of the hapten down a channel until it reaches an area where it can form hydrogen bonds and make a more permanent association. This geometry had a slow on-rate, 10^6–$10^7 M^{-1}\ s^{-1}$, and this on-rate was not improvable by mutation. The second antibody geometry we encountered had longer CDRs and an external binding site composed of a shallow depression on the surface, according to molecular modeling. This type of geometry had the diffusion-limited on-rate $10^8 M^{-1}\ s^{-1}$. If you wanted to design a catalyst, you might be better off starting with a fast-type antibody, in

which some chemistry can go on in the shallow binding site that is solvent accessible, rather than one that may bind hapen tightly, but is at the bottom of a pit.

Paul: All of us were excited about catalytic anti-idiotypic antibodies and the potential use of the technology. Dr. Friboulet, would you like to comment on the limitations of this approach?

Friboulet: I think the main limitation in our system is the specificity of the reaction we want to catalyze. When we induce the production of structural copy of the enzyme active site, we lose at each round a part of the information that is contained in the active site. If we take the example of acetylcholinesterase, it is a very fast enzyme, but with a very deep pocket in it for the substrate. The substrate has to go into this 20-Å-deep pocket. The enzyme has solved the problem by inducing an electrostatic field, with aromatic residues and charged residues along the cavity. It is difficult to imagine that the first antibody we raise is going to mimic all the properties of the enzyme. We think amino acids of the catalytic triad are present in the antibody, so we may have an antibody that functions like the enzyme. We have observed relaxed specificity to substrates and inhibitors in the antibody compared to the enzyme. The relaxed specificity may be useful for applications that require hydrolysis of a wide spectrum of substrates. In general, our approach may be promising because we are observing a good catalytic efficiency.

Paul: What is the expectation concerning the frequency with which catalytic antibodies arise? Is one peptide-hydrolyzing catalyst from four binders outrageous?

Williams: You should expect success.

Hansen: It is very hard to say whether you should see one in three when you immunize with a peptide and one in 30 when you immunize with a phosphonate transition-state analog, and so forth. There is very little systematic work that has been done using a related series of haptens as immunogens. A recent example, however, of this type of study is a paper by Janda et al. (*26*).

Tramontano: I just want to mention that a frequently asked and important question relating to antibodies to transition-state analogs is whether the control has been done to immunize with the substrate and see if the same proportion of catalytic antibodies are obtained.

Green: I think the answer depends on the antigen that you are using, the experimental conditions, and the kind of reaction that you are trying to catalyze. For a difficult reaction like peptide hydrolysis, I am sure that people think it outrageous, to use your word, when you obtain one hydrolyzing antibody out of four. That kind of number is outrageous compared to what we observed, something like nine ester-hydrolyzing antibodies out of about 960.

Paul: Do you think our findings reflect some unique property of VIP?

Green: I think we have talked a lot about this. I think that the antibody is doing something else here. Maybe the antibody is controlling a conformation of the VIP so that some sort of intramolecular chemistry becomes possible. Of all the antibodies we raised against a substrate analog, an amide, none were catalytic for the analogous ester. I think the probability of randomly picking up a catalyst is very, very small. It is easier for me to relate your data on VIP to some different kind of mechanism, rather than an enzyme-like or catalytic antibody-like mechanism.

Tramontano: I do not think it is fair to say that. It is not completely random in that Paul has observed binding, and he is first selecting for binding activity. The question is whether screening for binding of a substrate vs binding of a transition-state analog will give significantly different results. Moreover, VIP is a fairly complex molecule with 28 amino acids that can present many different conformations, and a polypeptide antigen cannot be compared directly to a hapten.

Paul: I think that is a fair evaluation of where we stand; that expectations are not clear and we cannot predict what to expect. The reaction could be unique to VIP, although there is no *a priori* reason to think so. It is true that there is no other example of peptide cleavage by an antibody, so this type of thinking cannot be ruled out at this stage. The second comment I have relates to immunization with the ground state and the issue of why the light chains are more active than Fab. Stabilization of the ground state would certainly make the reaction more difficult because the transition state must be stabilized even more than otherwise necessary. Light chains bind VIP with lower affinity than Fab. Presumably, that is the reason for the increased activity. We find that light chains have increased K_m, suggestive of decreased affinity for the ground state, and increased k_{cat}, showing increased turnover.

Zouali: Maybe the differences that you have in the frequencies have something to do with the way you set the threshold of positives and negatives when you select the antibodies.

Paul: It has been stated that a large proportion of the published work on catalytic antibodies may be artifactual because of contamination with high-activity conventional enzymes, no matter whether the antibodies are raised against activated or charged analogs, whether they are naturally occurring, or whether they are monoclonal or polyclonal. What are the standards for classification of a hypothetical catalyst as an antibody? It is essential, I think, to demonstrate purification of the antibody to constant specific activity. Similarly, the specific activity across the width of the protein peak obtained by resolutive chromatography should be constant and the activity should display the correct molecular mass. Another criterion that has been cited as being indica-

tive of an antibody is inhibition of the activity by the immunizing hapten. In this case, however, the hapten could also inhibit the activity of the contaminant enzyme.

Green: If titration of the activity with the immunizing hapten shows that there is a one-to-one correlation, this is an excellent argument that the activity is an antibody activity.

Paul: So, a quantitative argument is required. It is not sufficient simply to show efficient inhibition and low K_i values. What about the reactivity with substrate homologs—is that an acceptable criterion?

Tramontano: Reactivity with substrate homologs cannot be used as a criterion because of the possible unexpected specificities. It would be counterproductive to try to rationalize the data in the context of substrate homologs.

Paul: Enzyme inhibitors? Dr. Friboulet has used DFP profitably to inhibit the antibody activity irreversibly. Dr. Williams found no inhibition with enzyme inhibitors and felt this to be an argument against a contaminant.

Hansen: When your antibody activity is not inhibited by a classic protease inhibitor, you say, "Ah, there is no contaminating enzyme present." Suddenly, when the activity is inhibited, you say, "Ah, my antibody is acting like an enzyme."

Green: That is the advantage of this type of an experiment.

Gabibov: If you work with human subjects, it is good to observe different specific activities and kinetic constants from different subjecs.

Tramontano: I think if it can be shown that the antibody can be purified to constant specific activity, and then you apply all of the criteria that you stated here and whatever else might be available to characterize the activity, that is basically it.

Gabibov: For example, in the case of very fast enzymes, the calculations concerning possible contamination must be based on the catalytic activity of that particular enzyme. I do not think that you can deal with the problem of contamination more easily if you work with monoclonal antibodies compared to polyclonal antibodies. Each problem has to be resolved in context of enzyme we want to mimic.

Stollar: It is appropriate at this stage to consider the goals of catalytic antibody research. I sat down for dinner the other evening with Dr. Leuenberger, an enzymologist participating in the large Biocatalysis conference, and the questions that came were: Why are you doing this work? What would you do if you had the catalytic antibody you wanted? Will the antibodies be better than other processes and reagents that are available now?

Hansen: It still is very hard for organic chemists to design molecules that will selectively bind to other molecules. One can do that with very small target molecules, but designing synthetic organic molecules capable of selectively binding complex structures, like peptides, is nearly impossible at this time. Antibodies give you that for free. The

challenge is to engineer in an efficient catalytic activity, which we all agree is difficult. The final product will be a new catalyst that has unsurpassed specificity. I think that is what catalytic antibody research offers that conventional approaches do not.

Leuenberger: I do not know a lot about catalytic antibodies. My task is to use biotransformations to produce preparative amounts, kilogram or even ton amounts, of certain building blocks for the synthesis of optically active molecules. Our usual problem is to introduce chirality into a molecule, and what we do is to screen microorganisms that are able to introduce a chirality either by a resolution process or by a direct introduction of a new chiral center. What does the new technology of catalytic antibodies have to contribute that enzymes do no have? Will it be possible in the future to substitute enzymatic processes by catalytic antibodies? Will such catalyic antibodies be as stable as enzymes? Will they be as flexible as enzymes? For example, many enzymes catatlyze certain reaction types with a broad spectrum of substrates. If we are looking for an enzyme to catalyze a certain reaction type, we know where we have to look. In case of catalytic antibodies, as far as I understand, you would generate the specific catalyst for the specific reaction.

Tramontano: I think that catalytic antibodies are excellent starting points for designing industrial products. We ought to consider every possible notion to try to improve them rather than start from scratch. Dave Hansen is perfectly right in saying antibodies provide ligand binding for free. What we have shown in the last 5 or 6 yr is that you also obtain a certain level of catalytic activity.

Green: In catalytic antibodies, we have just discovered a beautiful island, and we do not fully know what is on it. I think all of the points that you make could be answered. For example, you mentioned chirality. I remember way before organic chemists were first able to achieve 98 or 99% chiral discrimination, Landsteiner in 1928 used rabbit polyclonal antibodies to demonstrate 99 to 1 discrimination for phenylglycine and tartaric acid derivatives. We now have in hand, for example, effective ester-hydrolyzing antibodies. You can imagine all kinds of reactions that would be very hard to accomplish using enzymes, but antibodies could catalyze well, e.g., hydrolysis of diastereomeric chiral compounds. There are biological compounds, endotoxin, for example, that are good targets for antibody-catalyzed hydrolysis. There is no way today for clearance of this compound, which is produced by gram-negative bacteria and results in life-threatening septic shock. It seems to me, however, that a lot of basic research will be necessary before we can cure cancer, AIDS, or dandruff with catalytic antibodies. Questions such as those of flexibility and what functional groups are best suited to achieve catalysis must be answered.

Fastrez: For medical applications, the exquisite specificity of the catalytic antibodies will be needed. For organic chemistry uses, such as synthesis

of sophisticated organic compound, and so on, it may not always be necessary to make catalytic antibodies, but enzymology research could benefit from incorporating some aspects of the philosophy of catalytic antibodies. For instance, consider the 20 amino acids or so that line the substrate binding activity, the equivalent of the CDRs. These residues can be changed at will in creating libraries. Nature appears to have already done that in the case of enzymes. There are examples of enzymes highly related to one another, with levels of sequence similarity of the order of 30%, that catalyze completely different reactions. One example is mandalate racemase (*24*). Another example is the α-β-hydrolase fold (*27*), in which apparently unrelated reactions are catalyzed on the same protein fold by relatively small changes in the equivalent of the CDRs (*28*).

Stollar: We all are motivated by the basic questions of mechanism, and that is certainly a very strong basis for proceeding forward. Some of the funding sources, however, might be more application-oriented.

Zouali: As an outsider to this field of catalytic antibodies, I would like to give some thoughts I had between a few glasses of Vodka and maybe raise some questions. I see this meeting as having attempted to bridge the basic immunology of antibodies and their biochemical properties. We heard convincing data on peptide cleavage by Paul's group. We have heard some interesting data on DNA-hydrolyzing antibodies by Gabibov's group. I was also quite convinced by the data on the hydrolysis of an unsubstituted amide by Dr. Williams. Dr. Friboulet's work showing that an anti-idiotypic antibody can mimic the active site of acetylcholinesterase is a unique observation. One should not believe that this type of mimicry is easy. Scott Rodkey will agree with this: reagents produced by this phenomenon of idiotypic mimicry are really quite unpredictable. I also had a number of surprises during this meeting. One of them was to discover that antibodies to DNA can catalyze RNA hydrolysis, I, like many other people, still do not understand how this works. Another surprise was Paul's work showing that the activity of the light chain of an antibody was higher than that of the corresponding Fab. I think we should keep all the possibilities open, and I am thinking of a very exciting recent result showing that the combining site of an antibody can be mimicked not only by a single-chain F_v, but also by the CDRH3 of this antibody, and that this CDRH3 is able to exert a neutralizing activity in vitro. Also remarkable are the observations of catalytic antibodies without immunization. Dr. Paul has shown us that in normal sera, we can find anti-VIP activity, and Dr. Gabibov has also noticed that normal sera have hydrolyzing activity—a small activity level, but it is there. Pierre Grabar (*29*) proposed over 20 years ago that one possible role of natural antibodies present in normal human sera is to lyse and clear the byproducts of metabolism. If you can go on and demonstrate that natural catalytic antibodies are physiologically relevant, this will be very important. The observation of Dr. McCafferty is

also relevant, because he has shown that from a library of a normal subject, you can isolate natural antibodies to haptens. There are, of course, a number of questions. One concern is the target of the catalytic antibodies, for example, in the case of Dr. Gabibov's work. It is difficult to understand what is really being seen by these catalytic antibodies. Dr. Tramontano has raised the important question of determining whether there is a correlation between affinity maturation and the evolution of the catalytic activity. Dr. Williams has a unique system to study this question, because by immunizing and hyperimmunizing his animals, he could derive antibodies at different stages of the immune response and then test their catalytic activity. I also see a problem with the frequency of the catalytic antibodies. Dr. Friboulet found that 1 out of 13 has a catalytic activity, Dr. Paul found only 1 out of 4, and Dr. Williams, only 1 out of 68. We should find ways to increase these frequencies, and I was thinking that maybe utilizing molecules like GM-CSF could help.

Gabibov: Dr. Zouali's general immunological view was very interesting to me. It is an interesting hypothesis that natural catalytic antibodies have some functional role and that these antibodies are part of the immunological repertoire. I do not think that VIP and DNA hydrolysis by antibodies are unique events. It will be most exciting to find or raise natural catalytic antibodies to other physiologically active compounds, especially molecules involved in specific diseases. This type of work can make this field more interesting for other scientists, immunologists, and physicians.

Zouali: Polyclonal immunoglobulins are used to treat some patients with autoimmune diseases. This is done by preparing a pool of plasma from many thousands of different people, purifying the IgG, and then injecting the purified IgG into patients with autoimmune diseases. This procedure is known to work in a number of patients. Some of you have shown that catalytic activity is present in normal human immunoglobulins. I would suggest that you look for catalytic activities against a panel of autoantigens in these pooled polyclonal IgG preparations. No one really knows how this therapy works.

Tramontano: One of the first papers showing chemical activity by antibodies was from the Israeli group on hydrolysis of steroid esters (*30*). This paper also proposed that catalytic antibodies could be useful reagents for diagnostics using new kinds of configurations in the assays. Another area where catalytic antibodies could find an immediate application is in the development of biosensors. Many of us, of course, are aware that there is a huge potential for catalytic antibodies in therapy, particularly if one could make chimeric molecules containing catalytic antibodies coupled to conventional antibodies against biomolecules that are involved in the pathogenesis of diseases. Clearly, there is a lot of potential for these antibodies, and there is quite a lot to be done.

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